PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

Ph.D. Thesis

MODULATION OF THE IMMUNE RESPONSE AND DISEASE INDUCED BY THE INFECTION WITH THE HUMAN RESPIRATORY SYNCYTIAL VIRUS BY HEME-OXIGENASE 1 ENZYME AND PANNEXIN 1 HEMICHANNEL. TWO NEW ENDOGENOUS REGULATORS OF IMMUNITY AGAINST THIS VIRUS.

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Thesis Co-Advisor: Dr. Claudia A. Riedel

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Thesis submitted to the Pontificia Universidad Católica de Chile in partial fulfillment of the requirements for the degree of Doctor of Biological Sciences with a major in Molecular Genetics and Microbiology

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By

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2018

ACKNOWLEDGEMENTS

First of all, I would like to thank at the most important people in my life: my mom and my lovely husband who always accompanied me, supported me, and believed in me.

I want to thank Dr. Alexis Kalergis, my thesis director, and Dr. Claudia Riedel, my co-advisor for all the opportunities given during my years as a Ph.D. student, and for being always available to discuss my work, listen to my ideas, and provide suggestions. I am also grateful to Dr. Claudia Riedel, my thesis co-director, for her contributions to my work and her good disposition to discuss ideas and experiments.

I want to thank all the people that I worked with in the laboratory, who have always been available to help me, for discussing experiments, and for laughing during lunch time. I would like a special thanks to the members of the laboratory that become my friends for being with me always and support me in the difficult time. Thanks Nata, Ires, Omi, Herni, Jorge and Karen. Special thanks to Miguel, for initiated this project and to Gisela and Roberto for helping me in almost every experiment. Also, I would like to thank Pablo, for supporting me in the experiments, for her ideas, contribution with suggestions for this thesis manuscript and her friendship.

This work would have not been possible without the financial support of: CONICYT, which awarded me a 4-year doctoral fellowship; VRI from Pontificia Universidad Católica de Chile, which helped with the financing of two trips to attend international and national meetings.

The work performed in this thesis received funding from the following grants: FONDECYT 1150862, and Millennium Institute on Immunology and Immunotherapy (P09/016-F).

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GLOSSARY

ARLI: Acute lower respiratory tract infection

BAL: Bronchoalveolar lavage

DCs: Dendritic cells

PRRs: Pattern recognition receptors

RLRs: Retinoic acid-inducible gene-I-(RIG-I-) like receptors

TLR: Toll like receptors

NLRs: Nucleotide-binding oligomerization domain-(NOD-) like receptors

CXCL10 : Chemokine [C-X-C motif] ligand 10

CXCL1 : Chemokine [C-X-C motif] ligand 1

CCL2 : Chemokine (C-C motif) ligand 2

CCL3 : Chemokine (C-C motif) ligand 3

CCL4 : Chemokine (C-C motif) ligand 4

CCL5 : Chemokine (C-C motif) ligand 5

IL-1β: Interleukin 1 beta

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-4: Interleukin 4

IL-13: Interleukin 13

IL-17: Interleukin 17

IFN- α: Interferon alpha

IFN-β: Interferon beta

IFN-γ: Interferon gamma

TNF-α: Tumor necrosis factor alpha

HEp-2: Human Epidermoid carcinoma strain #2

hRSV: Human Respiratory Syncytial Virus

IRF-3: Interferon Regulatory Factor 3

ISGs: Interferon-Stimulated Genes

HO-1: Heme Oxygenase 1

Panx1: Pannexin 1

mRNA: messenger RNA

PFU: Plaque-forming unit

pi: Post-infection

qRTPCR: quantitative Real Time Polymerase Chain Reaction

RNA: Ribonucleic Acid

RNase: Ribonuclease

Tregs: T regulatory cells

PMN: Polymorphonuclear

CoPP: Cobalt-protoporphyrin-IX

SnPP: Protoporphyrin IX dichloride

HO-1: Heme oxygenase-1

Nrf2: Nuclear factor (erythroid-derived 2)-like 2

Keap1: Kelch-like ECH-associated protein 1

Bach1: BTB Domain and CNC Homolog 1

ARE: Antioxidant responsive element

ROS: Reactive oxygen species

DENV : Dengue virus

Mø : monocyte/macrophages

LPS : Lipopolysaccharide

HCV : Hepatitis C virus

HBV : Hepatitis B virus

HIV : Human immunodeficiency virus

EBOV: Ebola virus

EV71: Enterovirus 71

RESUMEN

El sistema inmune es una compleja red de defensa y ataque contra diferentes amenazas foráneas o endógenas. Además de células y moléculas solubles que conforman esta red, existen moléculas de otros sistemas en el hospedero que pueden modular la respuesta inmune. En este estudio, hemos denominado a dichas moléculas como reguladores endógenos de la respuesta inmune y nos hemos enfocado en el efecto de la modulación de dos de estas moléculas en la severidad de la enfermedad inducida por la infección del Virus respiratorio sincicial humano (VRSh). Específicamente, hemos descrito los efectos de la modulación de los reguladores endógenos hemo-oxigenasa 1 (HO-1) y panexina-1 (Panx1).

HO-1 es una enzima inducible bajo condiciones de estrés y es responsable del catabolismo del grupo hemo, generando como subproductos; biliverdina, CO y Fe^{2+}. Se ha descrito que esta enzima posee una importante participación en la regulación de la inflamación y posee actividad antiviral contra diferentes tipos de virus incluyendo el virus de inmunodeficiencia humana (VIH), influenza y dengue, entre otros. En este trabajo, se planteó como hipótesis que la actividad enzimática de HO-1 es fundamental para prevenir la enfermedad inducida por VRSh. Para evaluar el rol de HO-1 en la modulación de la enfermedad inducida por VRSh, se utilizaron dos estrategias experimentales para promover la expresión de esta enzima. Se utilizó una inducción farmacológica, utilizando protoporfirina de cobalto (CoPP), y un sistema genético basado en la sobreexpresión exógena de HO-1 en un modelo de ratón transgénico. Nuestros resultados muestran que la inducción farmacológica de HO-1 mediada por CoPP o la expresión de HO-1 en el modelo transgénico protegen al hospedero de la enfermedad inducida por la infección por VRSh, disminuyendo

parámetros de la enfermedad tales como pérdida de peso corporal, carga viral, título viral e inflamación pulmonar.

La inducción de HO-1 promueve una disminución en la producción de citoquinas y quimioquinas pro-inflamatorias e incrementa los niveles de IL-10, generando un ambiente anti-inflamatorio en el pulmón. De forma concomitante, se produce un aumento en la expresión de interferon del tipo I (IFN del tipo 1) en los pulmones de los ratones que sobre-expresan HO-1, promoviendo un estado antiviral en las vías aéreas de los ratones infectados. Además, se realizaron ensayos *in vitro* en células A549 para evaluar el efecto de la inducción de HO-1 en la replicación de VRSh y producción de partículas virales infectivas, apoyando los resultados obtenidos *in vivo*.

Panx1 es un hemicanal expresado de manera ubicua en la membrana celular. Su principal función es mediar la liberación de ATP al medio extracelular, donde el ATP actúa como señal de daño para reclutar células inflamatorias como macrófagos y neutrófilos. Además, recientemente se ha descrito que Panx1 es requerido para la entrada de VIH en la célula hospedera, y el bloqueo de este hemicanal por fármacos como probenecid reducen la replicación de éste patógeno. En este trabajo se planteó como segunda hipótesis que Panx1 contribuye en el desarrollo de la enfermedad inducida por VRSh, facilitando propagación de VRSh. Nuestros resultados muestran que Panx1 es regulado positivamente luego de la infección y su actividad es promovida a tiempos tempranos posterior a la exposición de la célula hospedera ante el virus. En este contexto, el bloqueo de la actividad de Panx1 resulta en una inhibición de la propagación viral tanto *in vitro* como *in vivo*. Además, los ratones tratados con probenecid presentan menores signos de inflamación pulmonar. Por último, se observó que existe una menor secreción de IL-1β en el pulmón de los ratones tratados con probenecid que se correlaciona con una menor inflamación pulmonar.

El presente estudio demuestra por primera vez, que la infección causada por el VRSh es modulada tanto por la enzima HO-1 como por Panx1 y ambos reguladores endógenos del sistema inmune son excelentes blancos terapéuticos para el control de la infección del VRSh.

ABSTRACT

The immune system is a complex network of attack and defense against different foreign or endogenous threats. Besides immune cells and soluble molecules that are involved in the network interaction, other host molecules execute various functions in cellular processes and also could modulate the immune response. In this study, these molecules are referred as endogenous regulators of the immune response and here we evaluated the role of two endogenous regulators of the immune response on the severity of the disease induced by human respiratory syncytial virus (hRSV) infection.

Specifically, we described the effects of the modulation of the endogenous regulators heme oxygenase 1 (HO-1) and pannexin 1 (Panx1).

HO-1 is a stress-inducible enzyme that is responsible for heme catabolism, producing the byproducts biliverdin, CO, and Fe^{2+}. HO-1 has an essential role in the regulation of inflammation and exerts antiviral activities against a broad spectrum of viruses including human immunodeficiency virus (HIV), influenza, and dengue among others. In this study, our hypothesis was that the enzymatic activity of HO-1 is necessary to prevents the disease induced by hRSV. To evaluate the role of the HO-1 enzyme on the modulation of the disease induced by hRSV infection in mice, two experimental approaches were used to induce the expression of this protein. We used a pharmacological induction, using cobalt-protoporphyrin (CoPP), and a genetic system by the overexpression of HO-1 in a transgenic mouse model. Our results showed that both, pharmacological induction and the transgenic model, can protect the host of the disease induced by hRSV infection. In both models, mouse showed a decrease of the lung disease parameters such as reducing body weight loss, reducing viral load, viral titers and diminishing the lung inflammation, as evidenced by a

reduced neutrophil infiltration, a significant sign of the pulmonary disease caused by hRSV. On the other hand, HO-1 induction promotes a diminished pro-inflammatory cytokine and chemokine production and increases the levels of IL-10, generating an anti-inflammatory environment in the lungs. Concomitantly, an increase in interferon type I (IFN type I) expression is produced in the mice lungs that overexpress HO-1, promoting an antiviral state in the airways of infected mice. Also, *in vitro* assays in A549 cell were performed to evaluate the effect of HO-1 induction on hRSV replication and infective viral particle production, supporting the results obtained *in vivo*.

Furthermore, Panx1 is ubiquitously expressed in many mammalian tissues. Its primary function is release ATP to the extracellular medium when the ATP acts as a danger signal that recruits inflammatory cells such as macrophages and neutrophils. Besides, recently it has been described that Panx1 is necessary for HIV entry into the host cell. Also, the blocking of this hemichannel mediated by drugs as probenecid reduces the replication of this pathogen. In this study, we stated as a second hypothesis that Panx1 is required for viral propagation and development of the disease induced by hRSV, since is overexpressed after infection and its activity is promoted at early times after hRSV exposure. In this context, the blocking of Panx1 action results in the inhibition of viral propagation *in vitro* and *in vivo*. Also, the mice treated with the inhibitor showed fewer signs of lung inflammation. Finally, we observed a lower secretion of IL-1β in the lungs from mice treated with probenecid that is related to a decrease in lung inflammation.

The present study demonstrated for the very first time that the infection caused by hRSV is modulated by both the endogenous regulators HO-1 and Panx1 and both molecules are excellent therapeutic targets to control hRSV infection.

1. INTRODUCTION

The immune system is an interactive network of organs, cells and soluble factors that recognizes and destroys agents that could endanger the health of an individual. In a healthy individual, the body continually attempts to maintain homeostasis, a natural state of balance of all its organs and the nervous and circulatory systems (Parkin and Cohen, 2001). When this homeostasis fails by either trauma, pathogens, or during tumor development, the immune system responds to restore balance (Parkin and Cohen, 2001). The immune system can mobilize a response against invading pathogen, toxin, or allergen and can distinguish self from non-self antigens (Chaplin, 2010). The mammalian immune system can display two types of responses: the innate response and the adaptive response (**Figure 1**). Both kinds of responses are deeply interconnected. The innate immune system is the responsible for initiating the immune response and is the first to confront invading pathogens. Cells of the innate immune system, such as DCs, macrophages, and neutrophils, can recognize molecular patterns conserved by a vast number of pathogens, including LPS, peptidoglycan, flagellin, cytoplasmic DNA, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) (Parkin and Cohen, 2001). These molecules are known as pathogen-associated molecular patterns (PAMPs) and are recognized by specific receptors, such as Toll-like receptors (TLRs), lectins and proteins of the complement system (Takeda and Akira, 2001). Pathogens have evolved several evasion mechanisms to interfere with the function of cells innate immune response. For this reason, an efficient pathogen defensive is achieved by a combination of innate and adaptive immune responses. The adaptive immune response requires the activation of innate immunity and needs a longer time to develop (compared to innate immune responses). However, adaptive immunity has the advantage of being highly

specific and generates immunological memory. Adaptive responses are based primarily on the antigen-specific receptors expressed on the surface of T and B lymphocytes (Chaplin, 2010).

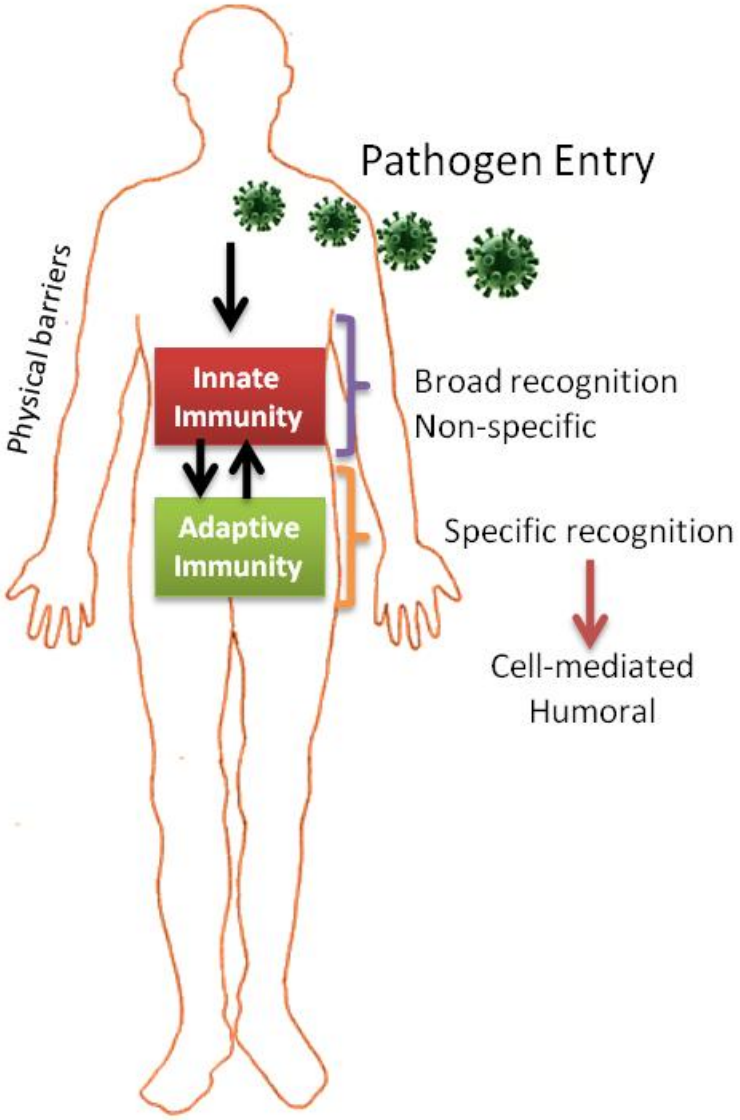

Figure 1. The interplay between innate and adaptive responses in host defense. Innate and adaptive immune responses work together to allow a full range of reactions of appropriate strength and specificity. In addition to their direct and broadly protective role, innate immune responses recruit adaptive leukocytes and contribute to their activation. Once activated, lymphocytes mount humoral and cell-mediated responses against pathogens that are highly specific. The production of cytokines stimulates both the innate and adaptive responses. Innate and adaptive immunity thus work synergistically to protect the host from all dangers (adapted from primer to the immune response, 2nd edition, Tak Mak Mary Saunders Bradley Jett).

1.1 Endogenous molecules and the immune system network

The immune system has evolved to develop a broad range of responses against pathogens while avoiding autoimmunity. The balance in the immune response is enabled by stimulatory and inhibitory signals that contribute to the regulation of the immune responses (Vigano et al., 2012). To maintain homeostasis, the termination of the immune response and the resolution of inflammation are important processes (Gorini et al., 2013). Pathogen infections can lead to an uncontrolled inflammatory response that can be harmful for the host. The immune cells respond to the pathogen but also can synthesize and utilize different endogenous molecules with a broad spectrum of function and activities not related with the immune response including receptors, synthesizing and metabolizing enzymes, transporters, etc. (Hasko and Cronstein, 2004). The presence of these endogenous molecules on immune cells potentially represents an unprecedented opportunity for the specific and selective modulation of the immune response. On this regard, several potential pharmacological targets could have the availability of directly and indirectly act as pharmacological agents, already in clinical use for several non-immune indications, and with a usually favorable therapeutic index to modulate the immune response.

In the present study, we investigated the role of two well-characterized endogenous regulators of the immune response, HO-1 and Panx1, in the context of the disease induced by hRSV.

1.2 Human Respiratory Syncytial Virus infection.

Human respiratory syncytial virus (hRSV) is the most important cause of acute lower respiratory tract infection (ALRI) in infants and young children worldwide (Bueno et al., 2008). Data from a recent meta-analysis showed that this pathogen causes up to 33.1 million of ALRI in children under age five each year, of which around 3.2 million of cases need hospital admission worldwide (Shi et al., 2017). Further, hRSV infection causes the death of 48.000- 74.500 children every year in developing countries (Nair et al., 2010;Shi et al., 2017). For these reasons, hRSV is considered a global health burden. In Chile, as worldwide, hRSV is the principal etiological agent, reaching up to 80% of hospitalized ALRI during the winter months (Avendano et al., 2003). The hospitalization rate of hRSV is 2–3% of infected children, with 0.1% of mortality (Avendano et al., 2003) (**Figure 2**).

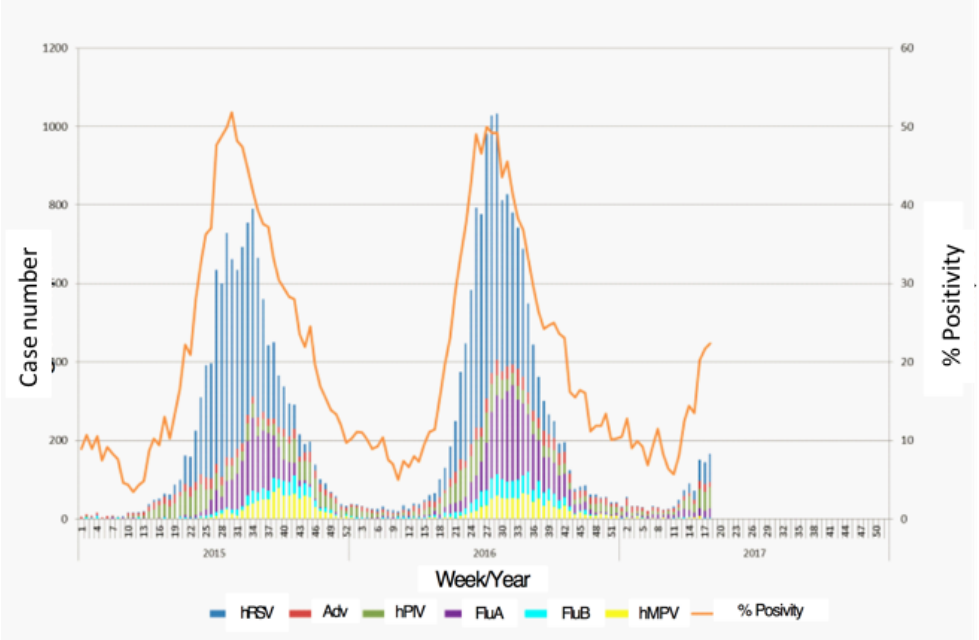

Figure 2. Respiratory virus infection rate per epidemiological week in Chile. Respiratory viruses case numbers per epidemiological week per infectious agent and positivity percentage of total samples analyzed from 2015 to at present week of 2017 in Chile. (Source: Public Health Institute, Chile.)

Human RSV is a highly contagious pathogen (Kulkarni et al., 2016). Estimations suggest that about 70% of infants below the first year of life are infected with this virus, while 100% of children under two-year-old have been infected at least once with hRSV (Collins et al., 2001). Infections in children and adults are recurrent during life, and protective immunity against the pathogen is ineffective, despite the production of antibodies after infection (Hashem and Hall, 2003).

Despite more than 60 years of intensive research on the hRSV pathogenesis, antiviral drugs and treatment against the virus are very limited, and no vaccine is currently available to induce long-term protection against this virus. The only approved therapeutic approaches are palivizumab (Shook and Lin, 2017), a humanized monoclonal antibody, that is available to protect against hRSV infection in high-risk infants, and ribavirin, which is rarely used due to toxicity concerns and questionable benefits (Shook and Lin, 2017). Thus, the study and design of new approaches to antiviral drugs and vaccines against hRSV are imperative to control the annual outbreaks of the virus and to decrease the high rate of infant hospitalization.

1.3 hRSV characteristics

Human RSV is a *Pneumoviridae* virus that belongs to the genus *Orthopneumovirus* (Afonso et al., 2016). Other members of the *Pneumoviridae* family include metapneumoviruses (Afonso et al., 2016). Human RSV is an enveloped non-segmented negative-sense single-stranded RNA virus. The viral particle consists of a helical nucleocapsid covered by a lipid membrane derived from the infected host cell (Hacking and Hull, 2002). The virion is pleomorphic, being either spherical or filamentous. Indeed, a recent study suggests that this can be the most predominant morphology of the virus (Hacking and

Hull, 2002). Human RSV genome has 15.2 Kb of length consisting of 10 genes encoding for 11 proteins, as there are two overlapping open reading frames, each of them encoding for an individual protein (M2-1 and M2-2) (Rodriguez and Ramilo, 2014). The lipid envelope contains three viral transmembrane glycoproteins: the attachment G protein, the fusion F protein, and the small hydrophobic SH protein. Underneath the envelope locates the matrix M protein, which is a non-glycosylated protein involved in the assembly of the viral particle (Batonick and Wertz, 2011). As part of the nucleocapsid, there are four proteins: nucleoprotein N, the phosphoprotein P, the transcription factor M2-1 and the polymerase L. Human RSV expresses two non-structural proteins, named NS1 and NS2, which inhibit the production of type I interferon activity by the host cell (Rodriguez and Ramilo, 2014). **(Figure 3)**

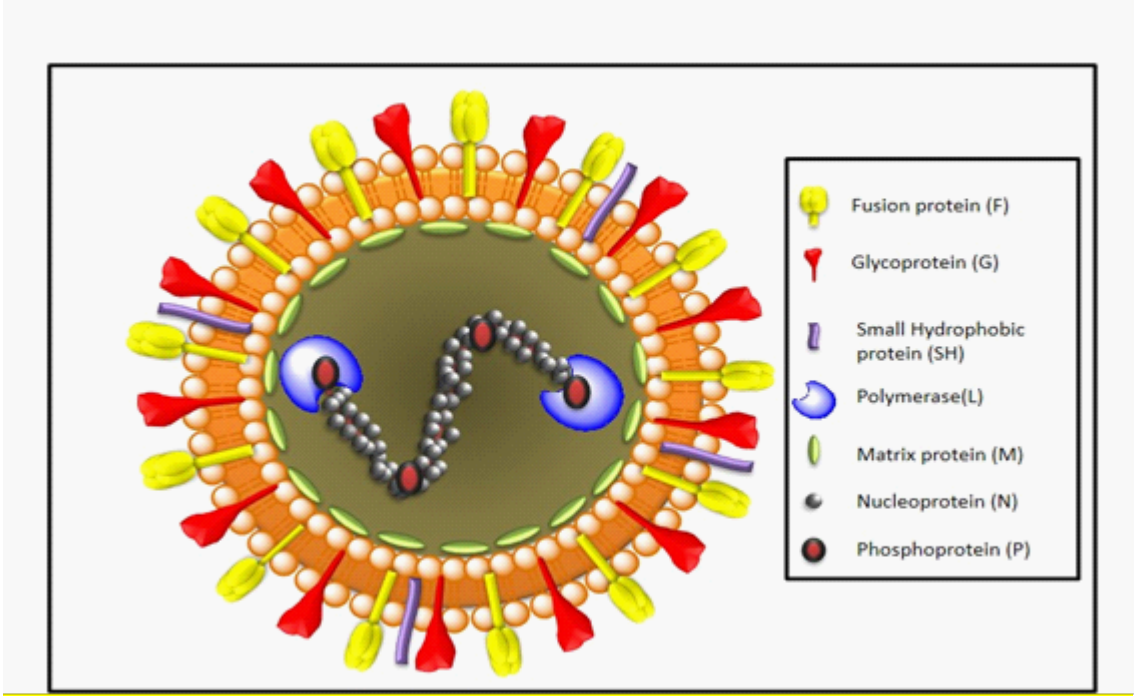

Figure 3. Schematic structure for hRSV. The image shows a schematic structure of hRSV and its structural proteins (Bohmwald et al., 2014).

1.4 Human RSV infective cycle

The transmission of hRSV requires direct contact with either infected patients or infected secretions. Small droplets containing hRSV can enter in the host through the nose, eyes and the upper respiratory tract, which deliver the virus to epithelial cells (Espinoza et al., 2014). Although the primary target of hRSV infection is airway epithelial cells, this virus also can infect other cell types, such as structural cells of the airway and immune cells (Lay et al., 2013). Human RSV infection in host cells begins with the attachment and entry of the virus through the activity of the G and F glycoproteins, respectively. The RNA of the virus enters the cells upon the fusion of the viral envelope with the cell plasma membrane (Collins et al., 2013) . Once inside the host cell, the transcription of viral genes and viral genome replication initiates, two processes those are essential for the infective cycle. While in vitro studies have shown that mRNA and proteins from the virus are detected inside the cell four to six hours after infection, expression peaks at 20 hours post infection (Collins et al., 2013). The transcription leading to mRNA synthesis and the replication of genomes for new viral particles are separated processes, which are modulated by the activity of the M2-2 protein (Collins et al., 2013).

The production and delivery of viral particles start after 12 hours post infection and persist up to 48 hours after viral entry (Collins et al., 2013) **(Figure 4)**. Human RSV infected cells show cytoplasmic inclusion bodies that contain viral RNA and proteins, including N, P, M2-1 and L (Lindquist et al., 2011). Inclusion bodies support the RNA synthesis, and recent studies showed that these structures could also sequester cellular signaling components to impair the cellular response to infection (Lindquist et al., 2011). The structural elements of hRSV are mobilized to the plasma membrane for the assembly and budding of viral particles

(Batonick and Wertz, 2011). The minimal molecular requirement for viral particle assembly includes F, M, N, and P proteins, in addition to the genome and antigenome (Lindquist et al., 2011). The budding of hRSV takes place at the apical membrane in polarized cells. The F protein goes to the apical membrane through the secretory pathway from the endoplasmic reticulum and Golgi, where is associated to lipid raft (Batonick and Wertz, 2011). The rest of the hRSV structural proteins and the RNA genome also traffic to the apical membrane from the cytoplasm and viral inclusion bodies (Ghildyal et al., 2009). The matrix protein is localized in the nucleus in early stages after infection but is mostly cytoplasmatic in late phases of infection (Ghildyal et al., 2009).

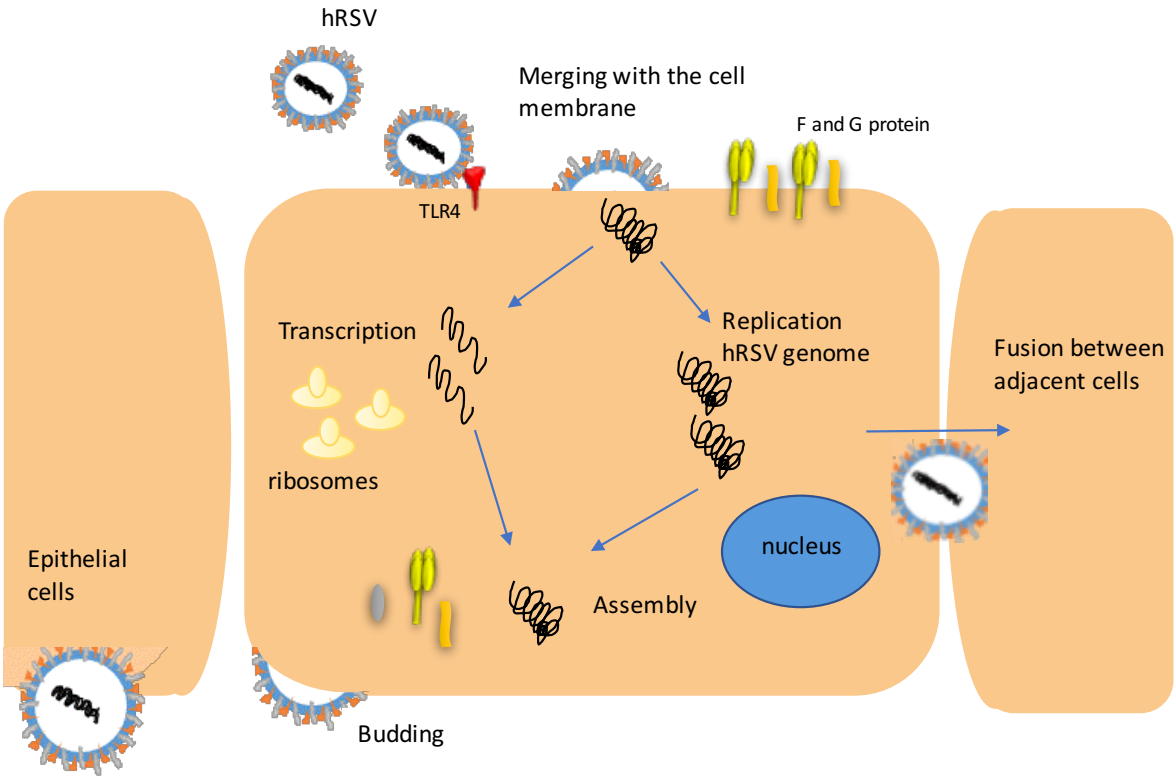

Figure 4. A schematic representation of hRSV lifecycle. Viral lifecycle of hRSV initiates with the attachment to the host cell membrane follow by the entry that occurs by the release of the viral genome into the cytoplasm. The negative-sense RNA genome undergoes primary transcription to produce mRNAs. The viral genome is replicated by the viral polymerase where antigenome intermediates are produced from genomic templates before the production of negative-sense genomes. Then, the assembly occurs and the release of new virus particles (Shahriari et al., 2016).

1.5 Immune Response elicited by hRSV

1.5.1 Innate immune response to hRSV

The innate immune response is crucial for early control of hRSV before the induction of the adaptive response. After infection, ciliated epithelial cells, alveolar macrophages and dendritic cells (DCs) display the innate antiviral response in the airways. Human RSV is recognized by host pattern recognition receptors (PRRs) on infected cells, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-(RIG-I-) like receptors (RLRs), and nucleotide-binding oligomerization domain-(NOD-) like receptors (NLRs) (Zeng et al., 2012). Signaling through these receptors lead to the secretion of several chemokines and pro-inflammatory cytokines. The most relevant are CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), RANTES, IL-1β, IL-6, IL-8, and TNF-α (McNamara et al., 2005;Yoon et al., 2007). The expression of these molecules induces the recruitment of innate cells such as monocytes, macrophages, neutrophils, dendritic cells and natural killer cells (Russell et al., 2017). Also, infected cells produce type I IFNs, IFN-α, and IFN-β, which promote an antiviral state in the airways by inducing transcription of many IFN-stimulated genes (ISGs) (Sen and Sarkar, 2007).

1.5.2 Adaptive immune response to hRSV.

The adaptive immune response elicited against hRSV is essential to promotes viral clearance as well as protection against subsequent hRSV infection. Resident lung DCs initiated the activation of the adaptive immune response, capturing viral antigens and migrating to lung draining lymph nodes and activating hRSV specific CD4 and CD8 T cells. CD8 T cells play a crucial role in viral clearance during the primary infection and recurrent reinfections (Cannon et al., 1987). CD4 T cells contribute to control hRSV infection through

their capacity to differentiate into multiple subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs). Th1 CD4 T cells promote viral clearance through their production of IFN-γ, while Th2 cells that produce IL-4, IL-5, and IL-13 are associated with more severe hRSV disease (Zeng et al., 2015). Th17 and the production of IL-17 are associated with mucus production and airway hyperreactivity. However, they may also contribute to viral clearance (Lukacs et al., 2010). Tregs cells also play an important role restricting lung inflammation during hRSV infection and coordinates the early recruitment of virus-specific CD8 T cells into the lung tissue and airway (Fulton et al., 2010). In addition to T cells, B cells are also essential for the adaptive immune response against to hRSV through the production of neutralizing antibodies specific to the F and G proteins. In humans, both serum IgG and mucosal IgA antibodies play important roles in protection against subsequent hRSV infection. However, both anti-RSV IgG and IgA titers wane rapidly over time in humans.

Neutralizing antibody titers were undetectable in 25-50% of young children, while over 75% of adults observed a four-fold reduction in neutralizing anti-RSV IgG titers within a year following natural hRSV infection (Murphy et al., 1986;Brandenburg et al., 1997). Therefore, the antibody response elicited against hRSV does not confer lifelong protection, contributing to the ability of hRSV to cause repeated infections.

1.6 Design of new approaches to control hRSV infection.

The design of therapeutic and prophylactic strategies against hRSV must consider the promotion of viral clearance but also limit the harmful effects of exacerbated inflammation caused by the immune response towards hRSV. As the inflammatory responses under hRSV infection are characterized by high levels of pro-inflammatory cytokines and the production of reactive oxygen species (ROS), the identification of regulatory mediators with the capacity to control or inhibit viral-induced both lung pathology and viral replication are crucial to the drug design against hRSV. In fact, recent studies have described that both anti-inflammatory and antioxidant mediators help to regulate lung immunopathology during hRSV infection (Castro et al., 2006;Loebbermann et al., 2012). Here, we will focus in determining the potential modulatory role of the immune response mediated by the endogenous molecules heme oxygenase-1 enzyme and pannexin 1 hemichannel during hRSV infection. Both molecules exert essential regulatory effects on the immune response and have been described as potential therapeutic targets during viral infections.

1.7 Heme oxygenases and heme degradation

Heme oxygenases are metabolic enzymes that catalyze the oxidative degradation of heme groups to produce carbon monoxide (CO), free iron (Fe^{2+}) and biliverdin, with the latter rapidly converted into bilirubin by biliverdin reductase (BVR) (Shan et al., 2006) (**Figure 5**). There are three isoenzymes of heme oxygenase: heme oxygenase-1 (HO-1) is an inducible form expressed ubiquitously in several tissues (Takahashi et al., 1999), HO-2 is a constitutively expressed form present in organs such as the brain, testes and the vascular system (Takahashi et al., 1999). Although an additional form, named HO-3 has been reported in rats, in humans had no enzymatic activity, suggesting that the Hmox3 gene is derived from HO-2 transcripts (Hayashi et al., 2004).

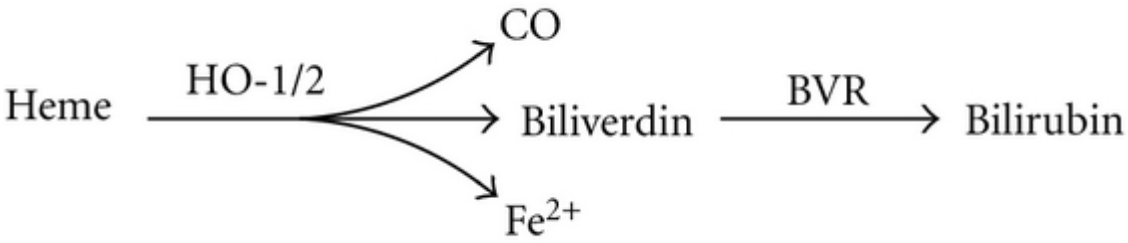

Figure 5. Heme group degradation reaction catalyzed by Heme-Oxygenases (HOs). Free heme is converted by HOs into free iron (Fe^{+2}), carbon monoxide (CO) and biliverdin. Immediately after production, biliverdin is transformed to bilirubin by the biliverdin reductase. Figure adapted from (Sikorski et al., 2004)

HO-1 is a 32-kDa protein with 288 residues and also is known as heat shock and stress response protein HSP32 (Rahman et al., 2013). The three-dimentional structure of HO-1 is predominantly α-helical with the heme sandwiched between the distal and proximal helices. A number of conserved glycines in the distal helix provide flexibility to accommodate substrate binding and product release (Rahman et al., 2013). Stressful conditions, promote an increase in HO-1 enzymatic activity due to the induction of its expression (Alam and Cook, 2003;Müllebner et al., 2015). Several stimuli lead to the up-regulation of HO-1

including cytokines, bacterial lipopolysaccharide, hypoxia, increased levels of the HO-1 substrate, heme and reactive oxygen/nitrogen species (Alam and Cook, 2003;Müllebner et al., 2015). HO-1 also mediates tissue protection, since its inhibition increased tissue damage, while tissues were protected when HO-1 was upregulated before an acute experimental injury (Kubulus et al., 2008). The cytoprotective properties of HO-1 are partially explained for the degradation of excessive free heme and to the generation of the heme degradation products CO and biliverdin, which can mimic HO-1-mediated effects (Müllebner et al., 2015). HO-1 plays a critical role in the modulation of cellular responses to injury and carries out essential biological functions as a cytoprotective enzyme (Ewing et al., 2005).

1.7.1 Regulation of HO-1 expression

The expression of HO-1 is mainly regulated at the transcriptional level by the Keap1/Nrf2 and the Bach-1/Maf systems (Alam and Cook, 2003). In a steady state, the expression of HO-1 is blocked by the repressor protein Bach1, which heterodimerizes with MAF to bind DNA, specifically at an antioxidant responsive element (ARE) in the in the Hmox1 gene promoter site (**Figure 6A**). At the same time, Nrf2 forms a complex with Keap1 in the cytoplasm, with Keap1 promoting the proteasomal degradation of Nrf2 (**Figure 6A**) (Alam and Cook, 2003).

Upon cellular stress, proinflammatory stimuli or stimulation with heme analogs, Nrf2 is released from Keap-1 and Nrf2 is not degraded. Then, the transcription factor is accumulated in the cytoplasm and phosphorylated by various protein kinases, which induce its translocation to the nucleus. In this process, the Bach1-MAF complex dissociates, and

free MAF now interacts with Nrf2 to form a transcriptionally active complex that promotes the transcription of *Hmox1* (**Figure 6B**) (Alam and Cook, 2003).

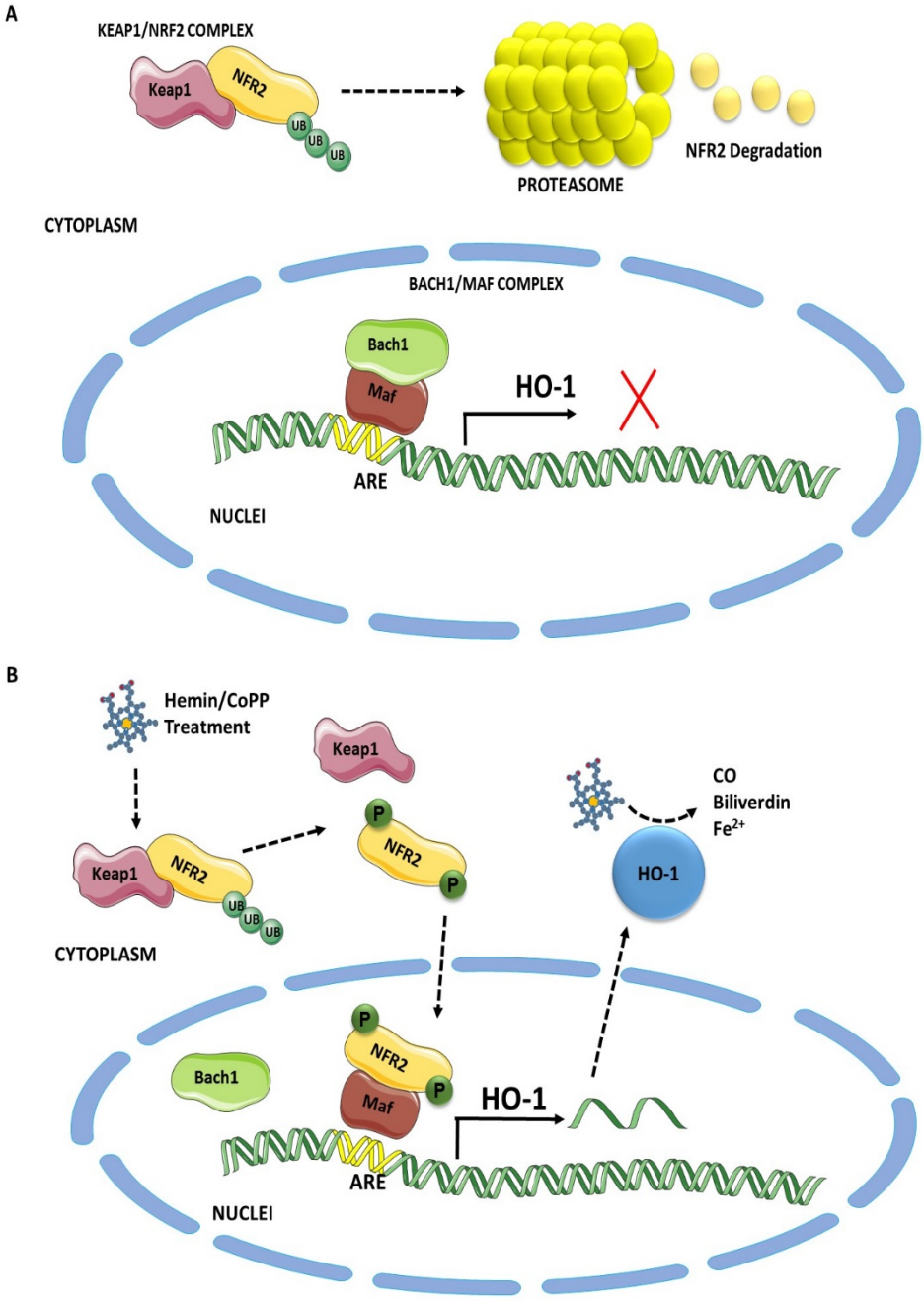

Figure 6. HO-1 expression is tightly regulated at the transcriptional level by the Keap1/Nrf2 and the Bach-1/Maf system. (**A**): In a steady state, *Hmox1* gene expression is suppressed by the binding of Nrf2 to Keap1 in the cytoplasm, which induces the proteaosomal degradation of Nrf2. In the nucleus, the binding of the complex Bach1/MAF to an antioxidant responsive element (ARE) in the *Hmox1* gene promotor site blocks the

transcription of HO-1. (**B**): Schemes for the induction of HO-1 mediated by heme analogs that produce the dissociation of Keap1 and Nrf2. Then Nrf2 is phosphorylated and translocated to the nucleus. The Bach1-MAF complex is dissociated, releasing MAF that interacts with Nrf2 promoting the transcription of *Hmox1*. Modified from (Espinoza et al.)

Analogous to the heme group, such as hemin and other metalloporphyrins, artificially induce the HO-1 expression (**Figure 6B**). The most characterized HO-1 inducer is cobalt-protoporphyrin-IX (CoPP), which promotes the upregulation of HO-1 gene expression (Shan et al., 2006). Allosteric HO-1 inhibitors can also induce upregulation of HO-1 expression, as occurs with tin protoporphyrin IX dichloride (SnPP) (Ewing et al., 2005). However, despite inducing HO-1 expression, SnPP irreversibly inhibits the activity of this enzyme (Marinissen et al., 2006). On other hand, CoPP treatment has also several side effects which often are not considered. CoPP administration can lead to a depletion of hepatic cytochrome P450 level. Furthermore, CoPP suppresses thyroid and testicular hormone concentrations in serum, affects copper metabolism, elevates plasma ceruloplasmin levels, and has many other side effects as the induction of the expression of cyclooxygenase-2 though an independent pathway of HO-1 induction (Schmidt, 2007;Lin et al., 2015).

1.7.2 Modulatory effects of HO-1 on the immune response

HO-1 has several modulatory effects on the immune system, suggesting that HO-1 is an interesting target for clinical research in infectious and autoimmune diseases, as well as for oncology, transplantation and obstetrics (Constantin et al., 2012;Dunn et al., 2014).

Deficiencies in HO-1 expression result in the development of a chronic inflammatory status supporting its participation in immune-regulation. The patients that suffer from HO-1 deficiencies present a pattern of common characteristics. These characteristics include increased counts in blood leukocytes, high amounts of Th1-type cytokines, including IL-1β, IL-6, IFN-γ and TNF-α, elevated serum levels of IgM, increased numbers of

polymorphonuclear (PMN) cells and monocyte/macrophages (Mø) in the spleen. Also, nonlymphoid tissues present high levels of PMN and Mø that cause oxidative tissue injury elicited by these cells (Soares et al., 2009;Riquelme et al., 2016).

The immunomodulatory capacity of HO-1 also is supported by several experimental models, such as acute lung inflammation induced by LPS in which HO-1 overexpression reduces the recruitment of polymorphonuclear leukocytes (PMN) to the lungs, decreasing the oxidative tissue damage (Konrad et al., 2016). Moreover, the induction of HO-1 inhibited DC activation and its immunogenicity (Chauveau et al., 2005), reducing cytokine production and the ability of prime naïve T cells (Pae et al., 2004).

1.7.3 Antiviral activity mediated by HO-1 induction

Recent studies have shown that HO-1 exerts essential antiviral properties. Specifically, overexpression of HO-1 attenuates infection by several viruses including ebola, influenza, enterovirus, hepatitis C virus (HCV), hepatitis B virus (HBV) and HIV (Espinoza et al.). The mechanisms behind the protective effect of HO-1 upon viral infection are not fully elucidated; the currently available data will be described in this section.

HO-1 induction elicited by the treatment with CoPP had a direct antiviral effect against HBV infection by suppressing viral replication and reducing liver damage in a mouse model of acute HBV infection (Sass et al., 2003;Protzer et al., 2007). The expression of HO-1 inhibits HBV replication directly in hepatocytes at a posttranscriptional step by reducing the stability of HBV core protein and thus blocking refill of nuclear HBV covalently closed circular (ccc) DNA (Protzer et al., 2007). Experiments using a small interfering RNA directed against *HO-1* showed that this effect depended on the expression level of this enzyme (Protzer et al., 2007).

Furthermore, overexpression or induction of HO-1 promoted the inhibition of HCV replication and decreased oxidative liver damage (Zhu et al., 2008). Enzymatic products of HO-1 also showed antiviral activity against HCV replication. Experiments performed in hepatoma cell lines incubated with biliverdin demonstrated that this compound could directly interfere with HCV replication by triggering the expression of antiviral interferons, such as interferon alpha2 and alpha17 (Lehmann et al., 2010). Also, biliverdin interferes with the activity of the nonstructural 3/4A protease of HCV, and HO-1 derived iron inhibits the function of the non-structural 5B (NS5B) RNA-dependent RNA polymerase of HCV (Fillebeen et al., 2005;Zhu et al., 2010).

The HO-1 activity also impairs the replication of HIV. The administration of hemin, an HO-1 inducer, inhibits the HIV infection of monocytes (Devadas and Dhawan, 2006). Protection was verified by achieving almost undetectable levels of viral RNA and cells free of HIV-1 p24 protein in a dose-dependent manner (Devadas and Dhawan, 2006). Also, HIV replication was significantly inhibited in humanized non-obese diabetic SCID mice treated with hemin (Devadas and Dhawan, 2006). Treatment with tin protoporphyrin IX dichloride (SnPP), a competitive inhibitor of HO-1 activity, attenuated the effect mentioned above, suggesting a fundamental role for HO-1 in the modulation of HIV infection (Devadas and Dhawan, 2006). Finally, bilirubin derived from HO-1 has been reported to inhibit the protease activity of HIV, which negatively affects virus replication (Liu et al., 2016).

The antiviral properties of HO-1 induction have also been reported for respiratory viruses, such as influenza virus. Overexpression of HO-1 in mice lungs reduced inflammatory cell infiltration into the lung and decreased apoptosis of respiratory epithelial cells in influenza-infected mice, suggesting that HO-1 expression prevents an exacerbated

immune response in the lungs and its subsequent damage (Hashiba et al., 2001). These observations are supported by the aggravated disease observed in HO-$1^{-/-}$ deficient mice infected with influenza, as compared to control mice (Hashiba et al., 2001;Cummins et al., 2012). Also, Rupestonic acid derivatives that promote HO-1 expression showed anti-influenza virus activity, which recently was attributed to the activation of type-I IFN expression, as well as the induction of ISGs, possibly in a HO-1 enzymatic activity-independent manner (Ma et al., 2016).

The antiviral properties of HO-1 have also been described for ebola virus (EBOV). Studies performed in cell culture showed that HO-1 induction suppresses EBOV infection (Hill-Batorski et al., 2013). The upregulation of HO-1, elicited by CoPP decreased EBOV replication in Vero cells and Huh 7,0 VP30 cells. CoPP treatment administrated both pre- and post-infection, considerably reduced EBOV replication in both cell lines, demonstrating that HO-1 presents antiviral properties against EBOV that is mediated by intracellular mechanisms (Hill-Batorski et al., 2013). Although CoPP-treatment showed impaired transcription/translation of EBOV gene products, the precise mechanism mediating the blockade of virus replication within these cells was not reported (Hill-Batorski et al., 2013).

Importantly, induction of HO-1 expression has beneficial effects on cells infected with viruses that trigger the secretion of reactive oxygen species and activation of NADPH oxidase, such as enterovirus 71 (EV71) (Tung et al., 2011). The overexpression of HO-1 causes a decrease of EV71-induced NADPH oxidase/ROS production and reduces the replication of this pathogen. This effect was abolished if cells were pretreated with zinc protoporphyrin IX, a HO-1 activity inhibitor (Tung et al., 2011). Similar results were obtained with the administration of CO. These findings suggest that HO-1 may induce several

cytoprotective and antiviral effects on cells infected with EV71, which could lead to reduced tissue damage and eventually disease resolution (Tung et al., 2011).

In addition, HO-1 exerts antiviral activity against dengue virus (DENV). Studies performed in Huh-7 cells infected with DENV and treated with CoPP or hemin showed an inhibition of DENV protein synthesis and RNA replication (Olagnier et al., 2014). These effects were blocked by the treatment with SnPP promoting DENV replication. The anti-DENV effect exerts by HO-1 was mediated by biliverdin that trigged the host antiviral IFN response by non-competitively inhibiting DENV NS2B/NS3 protease (Olagnier et al., 2014). In addition, it has been reported that HO-1 induction in ICR suckling mice infected with DENV delayed mortality induced by DENV infection. Furthermore, similar antiviral effects were obtained by the treatment with andrographolide (a plant-derived compound) that inhibited DENV both in vitro and in vivo by inducing HO-1 expression (Tseng et al., 2016).

Taking into consideration, the multifunctional properties of HO-1 described above, and the exacerbated inflammation and lung damage caused by hRSV. Here, we examined the potential antiviral and anti-inflammatory effects of HO-1 induction against hRSV infection. On this regard, in this study, we proposed to evaluate pannexin 1 as a second molecule to suggest potential targets to modulate the immune response elicited by hRSV. In the next section, we described the current knowledge about the role of pannexin 1 hemichannel on the regulation of the immune response and its involvement during viral infection

1.8 Pannexin 1: The immunomodulatory and viral promoter hemichannel

Pannexin family consist of three members, Panx1, 2, and 3 (**Figure 7**) (Baranova et al., 2004). Panx1 is ubiquitously expressed in many mammalian tissues (Ma et al., 2009;Yip

et al., 2009). Panx 2 expression was initially identified to be restricted to the central nervous system (CNS), however recent work has determined that Panx2 is readily expressed in several organs including eyes, lungs, and colon (Ray et al., 2006;Le Vasseur et al., 2014). Panx3 is localized in osteoblasts, synovial fibroblasts, and chondrocytes (Barbe et al., 2006). Structurally, all Panx(s) consist of a cytosolic N-terminal domain, four transmembrane domains with two extracellular loops and a cytosolic C-terminal domain (Boassa et al., 2007). Although their structure is similar to connexins (Cxs), Panxs and Cxs do not share sequence homology (Penuela et al., 2007). Human Panx1 is a 47.6 kDa protein that contains 426 amino acids (Penuela et al., 2013). In most tissues and cell types, pannexins form hemichannels that mediate regulated the exchange of second messenger molecules, such as adenosine triphosphate (ATP), between cytoplasm and the extracellular space (Makarenkova and Shestopalov, 2014). Under normal conditions, Panx channels are closed due to their high conductance (**Figure 8**). However, several laboratory conditions such as metabolic inhibition using antimycin A and iodoacetic acid, and chemotherapeutic drugs such as staurosporine, doxorubicin, and etoposide, trigger Panx1 channel opening (Velasquez et al., 2016). However, physiological and infectious conditions that induce activation of Panx1 channels are poorly explored (Velasquez et al., 2016).

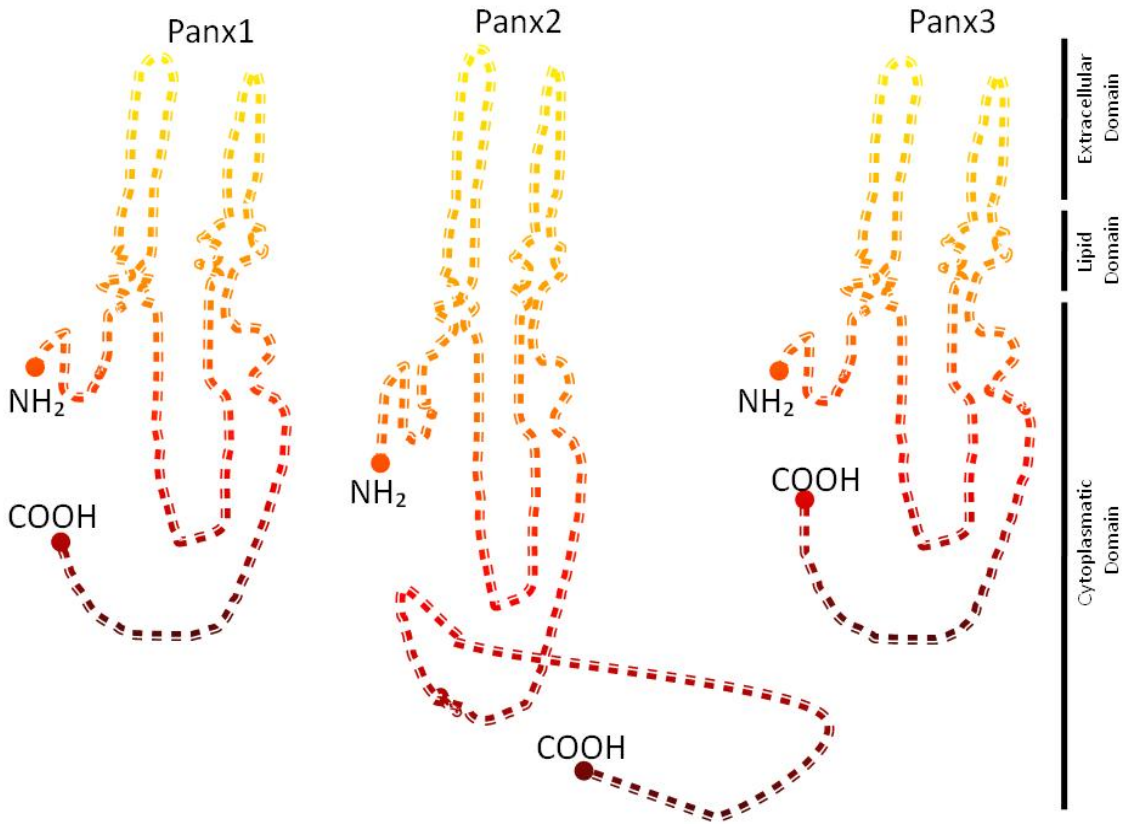

Figure 7. The pannexin family. The three members of the pannexin family are polytopic proteins with four transmembrane domains, cytoplasmic N- and C-termini of various lengths and two extracellular loops (Penuela et al., 2014).

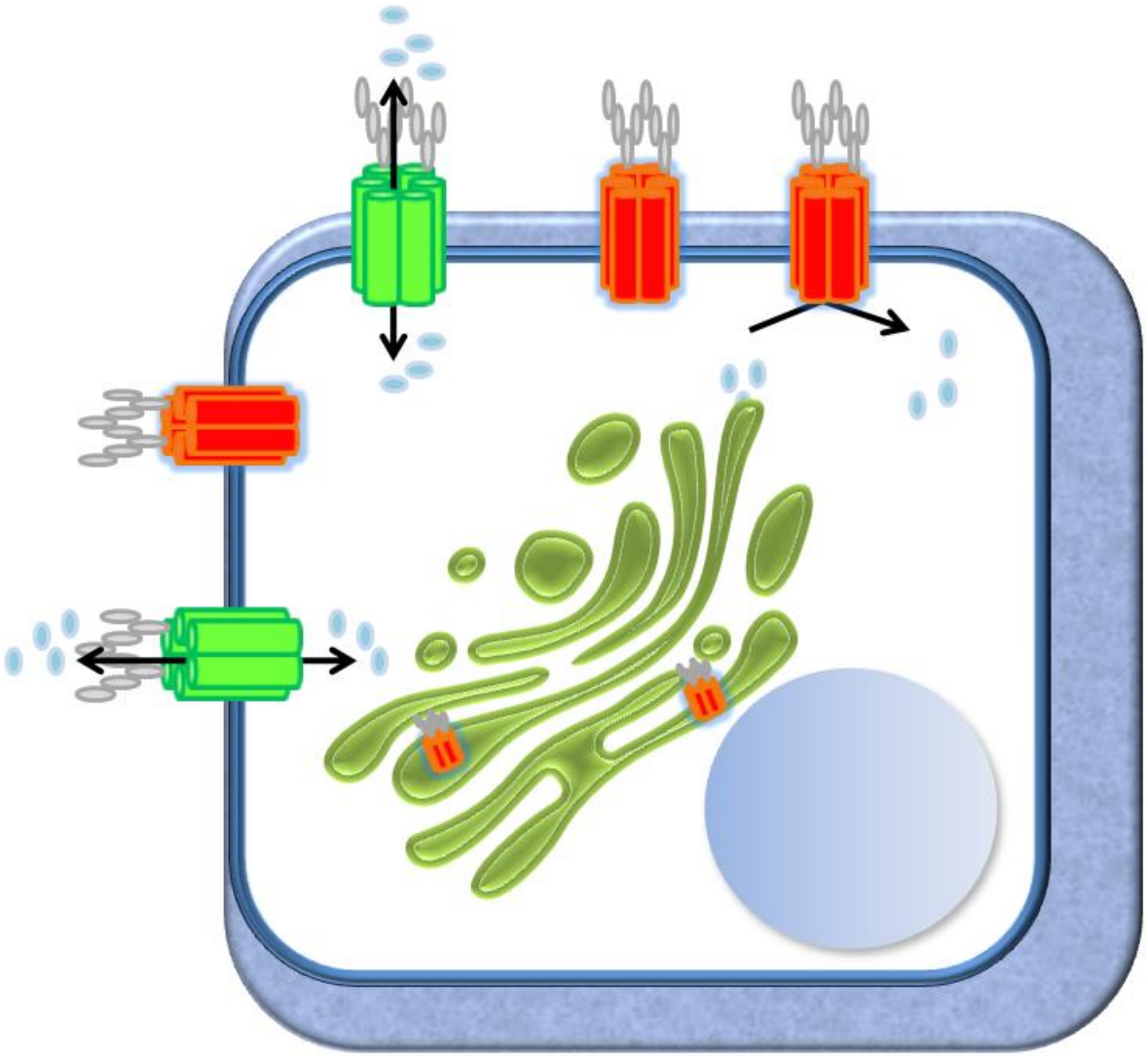

Figure 8. The life cycle of pannexins. Pannexins are co-translationally inserted into the endoplasmic reticulum, where are subjected to high-mannose glycosylation (small gray chains) and assemble into multimeric channels. Pannexins are transported to the cell surface via the classical endoplasmic reticulum–Golgi secretory pathway. Pannexin channels are closed to preserve the electrochemical gradient across the cell membrane (red channels), but can be open (green channels) and allow the passage of large molecules (<1 kDa) such as ATP and UTP (light blue circles; black arrows)(Penuela et al., 2014).

Extracellular ATP is an essential signaling molecule throughout the inflammatory pathway. ATP acts as a danger signal that causes activation of the inflammasome (Gombault et al., 2012), increase of immune cell infiltration (Corriden and Insel, 2012), and fine-tuning of several signaling cascades including those critical for the resolution of inflammation (Hill et al., 2010). Extracellular ATP is a ligand for several purinergic receptors which includes P2X1–7 (ATP-gated cation channels) and P2Y receptors 1,2,4,6,11–14 (G-protein coupled receptors; most are Gi or Gq coupled) (Junger, 2011). P2X receptors bind ATP exclusively as a ligand, while specific P2Y receptors have affinity for ATP as well as UTP, ADP, UDP or UDP-glucose (Junger, 2011).

Previously, the source of extracellular ATP was thought to be mainly from damaged and necrotic cells that release their intracellular contents (Vanlangenakker et al., 2008), or via vesicular release mechanisms (Lazarowski, 2012). However, reports showed that ATP could be released in a controlled manner through hemichannels, such as pannexins and connexins (Chekeni et al., 2010;Penuela et al., 2013).

1.8.1 Pannexin 1 and the inflammasome

NLRP3 inflammasome activation requires the signaling of extracellular ATP, leading to the assembly of scaffold components: the cytoplasmic receptor NLRP3, the adaptor protein ASC and the effector protein caspase-1 (Gombault et al., 2012). In fact, caspase-1 is activated through the action of the NLRP3 inflammasome. Also, caspase-1 activation is necessary for proper maturation and secretion of the inflammatory cytokines IL1β and IL18 (Gombault et al., 2012). The production of IL1β is triggered by two processes, the TLR signaling that induce the expression of pro-IL1β and the subsequent increase of extracellular ATP levels. The extracellular ATP signal promotes the activation of purinergic receptors that activate the inflammasome, producing active caspase-1 to process pro-IL1β into its mature form

(Gombault et al., 2012). The most characterized purinergic receptor involved in the inflammasome activation pathway is the P2X7 receptor. Under inflammation, the primary source of extracellular ATP is released by necrotic cells at concentrations high enough to activate P2X7 receptors. Another option is that ATP is released in a controlled manner through the activated Panx1 channels and acts on cells in a paracrine or autocrine fashion. Experiments performed in macrophages show that extracellular ATP released from dying autophagic cells during the phagocytosis process is essential for inflammasome activation in macrophages (Ayna et al., 2012).

Pannexin channels interact with P2X receptors at the cell membrane. Panx1 associates with P2X7 to form a large pore with at the plasma membrane (Poornima et al., 2012). Also, decreased extracellular calcium has been implicated as a trigger for the interaction between P2X7 and Panx1 at the plasma membrane (Poornima et al., 2012), and different splice variants of the P2X7 receptor have different coupling capabilities with Panx1(Xu et al., 2012).

As mentioned above, ATP-mediated inflammasome activation results in the secretion of mature cytokines and occurs in a broad diversity of tissues, mostly in models of acute tissue injury and infection. Bleomycin-induced release of ATP by pulmonary epithelial cells in a murine model of lung inflammation signals through P2X7 receptor resulting in the activation of inflammasome and increased production of IL1β (Riteau et al., 2010;Adamson and Leitinger, 2014). *In vitro*, Panx1 inhibitors block the release of ATP indicating that Panx1 is at least partially responsible for this process (Riteau et al., 2010). In this regard, increased levels of ATP also have been described in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis (Riteau et al., 2010). Moreover, experiments in

Panx1 null mice showed that IL1β upregulation did not occur in this mice (Negoro et al., 2013).

1.8.2 Pannexin 1 and inflammatory cell recruitment

In addition to the inflammasome activation, extracellular ATP triggers the chemotaxis of neutrophils (Chen et al., 2006) and macrophages (Kronlage et al., 2010) by signaling by several purinergic receptors (Corriden and Insel, 2012). Extracellular ATP influences neutrophil migration by increasing chemotactic signals through P2 receptors (Chen et al., 2006;Kawamura et al., 2012). ATP released by dying cells is a chemoattractant of macrophages through the activation of P2Y2 receptors (Elliott et al., 2009). Panx1 mediates ATP release from apoptotic cells as a "find-me" signal for phagocytes (Chekeni et al., 2010). Other studies showed that ATP-induced activation of specific P2Y receptors triggers non-directional movement of immune cells, especially macrophages (Isfort et al., 2011). Furthermore, supporting the role of Panx1-dependent ATP release in leukocyte migration, transwell migration assays showed that Panx1 null thymocytes were unable to recruit peritoneal macrophages (Qu et al., 2011). Also, activation of Panx and the subsequent ATP release from murine atrial myocytes promoted macrophage migration in a transwell assay (Oishi et al., 2012).

It has been described that P2X7 and Panx1 were both needed for the production of multinucleated macrophages stimulated by the inflammatory cytokine GM-CSF (Lemaire et al., 2011). Nevertheless, extracellular ATP release depends on the P2X7 receptor rather than Panx1 expression, and the metabolism of released ATP to adenosine mediated P2X7-dependent macrophage fusion (Lemaire et al., 2011).

Together, several studies showed that controlled release of ATP via Panx1 is an important mechanism for initiating inflammatory signaling and also for the recruitment of leukocytes.

1.8.3 Pannexin 1 and adaptive immunity

Extracellular ATP plays an important role in adaptive immunity, particularly in T cell function. T cell receptor stimulation involved ATP released by Panx1 acting back on P2 receptors to maintain MAPK signaling (Schenk et al., 2008). This TCR signaling feedback loop is mediated by the Panx1-dependent release of ATP which acts specifically on P2X1 and P2X4 receptors on the T cell surface (Woehrle et al., 2010). The elimination of extracellular ATP signaling by ectonucleotidases seems to be an important mechanism through which regulatory T (Treg) cells modulate their immunosuppressive and homeostatic function (Borsellino et al., 2007). In this regard, patients with relapsing-remitting multiple sclerosis, an inflammatory autoimmune disease, have surprisingly reduced numbers of Treg cells that express the ectonucleotidase CD39 (Borsellino et al., 2007). The role of pannexin channels in Treg cell function has yet to be explored.

In addition, extracellular ATP signaling affects dendritic cell function by modulating the production of several cytokines and chemokines. The production of MCP1/CCL2, MiP1α, IL12, IL10, and IL27, IL23 promote a Th2 response or tolerance (Wilkin et al., 2002;Schnurr et al., 2005;Horckmans et al., 2006). Moreover, extracellular ATP recruits and activates lung myeloid dendritic cells that induce Th2 responses in the mediastinal nodes from asthmatic airway mice (Idzko et al., 2007).While, hepatic dendritic cells that lack the ectonucleotidase CD39 have greater proinflammatory and immunostimulatory activity (Yoshida et al., 2013). In cancer models, extracellular ATP increases the capacity of dendritic cells by presenting tumor-associated antigens (Ma et al., 2013). While the role of pannexin

in mediating extracellular ATP signaling to dendritic cells remains to be investigated, Panx1-mediated ATP release may happen in an autocrine manner as has been shown in T cells but may also happen in a paracrine manner between dendritic cells and T cells.

The regulation of immune cells by extracellular ATP has been well characterized (Jacob et al., 2013), the role of Panx1 in controlling immune responses remains to be understood.

1.8.4 Pannexin 1 and resolution of the inflammatory response

As stated above, extracellular ATP is a danger signal that participates in the initiation of the inflammatory response by the activation of the inflammasome and the recruitment of immune cells; however, emergent data suggests that it also plays a role in the resolution of inflammation. The recognition and clearance of dying cells and debris from sites of inflammation are crucial in the resolution of inflammation (Li et al., 2009). Different studies suggest that ATP plays an important in the chemotaxis of immune cells toward damaged cells, which require a subsequent clearance to limit tissue injury (Elliott et al., 2009;Kronlage et al., 2010).

Resolution of inflammation depends upon the decrease of inflammatory cytokine secretion as well as the increase of anti-inflammatory cytokines or injury healing factors. P2X7 receptor signaling contributes to the resolution of inflammation by stimulating the production the proangiogenic factor VEGF from monocytes, which is critical for injury repair (Hill et al., 2010). Pyrophosphates, which are produced by extracellular ATP breakdown, can impair NLR and TLR-mediated inflammatory signaling (Lopez-Castejon et al., 2011). In this context, the genes that control extracellular ATP metabolism are involved with anti-inflammatory M2 macrophage-associated markers (Lopez-Castejon et al., 2011).

1.8.5 Pannexin 1 and infectious diseases

Pannexins channels are expressed at the cell surface of several cell types, similar to other surface receptors (i.e., purinergic receptors), they can also be used by opportunistic pathogens to gain access to the cell. Indirect data, using non- specific blockers of Panx1 channels (carbenoxolone, mefloquine, and probenecid) have been used to decrease the severity of erythrocyte infection and lysis upon exposure to α-toxins from *Escherichia coli* and *Staphylococcus aureus* hemolytic bacteria (Skals et al., 2009;Skals et al., 2011). Considering that P2X receptor antagonists can also block the process of infection, it is probable that the toxins of these bacteria may trigger cell lysis by activation of a purinergic cascade that involves Panx1 and purinergic receptors (Penuela et al., 2014).

Interestingly, new evidence demonstrated that the HIV uses Panx1 channels to enter into the lymphocytes through a mechanism that also involves purinergic receptors (Orellana et al., 2013). HIV-1 can promotes ATP release through Panx1 channels by mechanically stressing the $CD4^+$ host cell membrane upon interaction of the viral envelope protein with host cell receptors (Orellana et al., 2013). The ATP released activates purinergic receptors such as P2Y2 that later activate proline-rich tyrosine kinases. This, in turn, activates membrane depolarization that enables fusion between infected and uninfected target cells, thus permitting the virus to infect neighbor cells (Seror et al., 2011). In this regard, certain evidence showed that HIV isolates prompted the opening of Panx1 channels via binding of the virus to $CD4^+$ and CCR5/CXCR3 (CC chemokine receptor 5/CXC chemokine receptor 3) receptors (Orellana et al., 2013). Also, the blocking of Panx1 by RNAi knockdown and unspecific channel blockers reduced the amount of HIV particles in $CD4^+$infected cells, suggesting that blocking Panx1 channels may be a new target to control and prevent the spread of HIV and AIDS (Penuela et al., 2014). In addition, HIV infection induces a biphasic

activity of Panx1 channels, first during early infection caused by the binding of the virus to its host receptors (5 min – 60 min), and the second late after infection (10 h – 24 h), probably caused by the release of new virions (Velasquez et al., 2016). The role of Panx1 in other viral infections as during human respiratory syncytial virus is poorly understood.

2. HYPOTHESIS

Based on the information summarized above, we propose the following two hypotheses:

1. "HO-1 induction prevents the disease induced by hRSV infection in mice".

2. "Inhibition of Panx1 prevents the disease induced by hRSV infection in mice".

3. GENERAL AND SPECIFIC AIMS

3.1 General Aim

To evaluate whether the endogenous regulators HO-1 and Panx1 confers protection against the disease induced by hRSV infection.

3.2 Specific Aims

3.2.1 Evaluation of the first hypothesis

To evaluate the first hypothesis the following specific aims were carried out.

3.2.1.1 To evaluate *in vitro* whether HO-1 induction prevents hRSV replication and infective viral particle production on airway epithelial cells.

In this aim, A549 cells were treated with CoPP (to induce HO-1 expression), SnPP (a HO-1 inhibitor) or vehicle as a control, to determine whether HO-1 inhibits hRSV replication and infective viral particle production *in vitro*.

3.2.1.2 To evaluate *in vivo* whether HO-1 induction prevents hRSV replication and infective viral particle production on the airways of infected mice.

To test the role of HO-1 induction *in vivo* on the modulation of hRSV replication and infective viral particle production, we developed two experimental approaches. First, we performed a pharmacological induction of HO-1. For this approach, BALB/cJ mice were treated either with CoPP or SnPP 24 h before hRSV infection. Then, animals were intranasally infected with hRSV. At day four postinfection, we performed analysis of viral load and titers in the lung of infected mice. For the second approach, we used a conditional transgenic mouse that expresses HO-1 in MHC-II^{+} cells (tTA-HO-1 mice). These mice were

intranasally infected with hRSV. At day four postinfection, we performed analysis of viral load and titers in the lung of infected mice.

3.2.1.3 To evaluate *in vivo* whether HO-1 induction prevents lung inflammation induced by hRSV in infected mice.

To test whether HO-1 induction modulates lung inflammation *in vivo*, BALB/cJ mice were treated either with CoPP or SnPP 24 h before hRSV infection. Then, animals were intranasally infected with hRSV. At day four postinfection, we performed a measurement of the lung infiltration and histopathological analysis. Also, a similar experiment was performed with the transgenic mice tTA-HO-1.

3.2.1.4 To determine the cytokines and chemokines profile that are involved in the potential protective role of HO-1 against hRSV infection.

To accomplish this aim, we performed a complete secretion analysis of cytokines and chemokines from airways, and we evaluated the T cell response in hRSV infected mice treated with CoPP and transgenic tTA-HO-1 mice.

3.2.2 Evaluation of the second hypothesis

To evaluate the second hypothesis the following specific aims were carried out.

3.2.2.1 To evaluate *in vitro* whether Panx1 inhibition prevents hRSV replication and infective viral particle production on airway epithelial cells.

In this aim, A549 cells were treated with either probenecid or vehicle as a control, to determine whether inhibition of Panx1 activity impairs hRSV replication and infective viral particle production *in vitro*. In addition, inhibition of Panx1 also was addressed using a specific blocking peptide.

3.2.2.2 To evaluate *in vivo* whether Panx1 inhibition prevents viral replication and infective viral particle production of hRSV in the airways.

To test an *in vivo* role of Panx1 activity in the modulation of hRSV replication and infective viral particle production, BALB/cJ mice were treated either with probenecid 24 h before hRSV infection. Then, animals were intranasally infected with hRSV. At day seven postinfection, a lung viral load and titers analysis were performed in infected mice.

3.2.2.3 To evaluate *in vivo* whether Panx1 inhibition prevents lung inflammation induced by hRSV infection.

To test whether the Panx1 activity is required for immune cells recruitment into the lungs, BALB/cJ mice were treated either with probenecid 24 h before hRSV infection. Then, animals were intranasally infected with hRSV. At day seven postinfection, we performed analysis of the lung infiltration.

3.2.1.4 To determine the cytokine profile that are involved in potential the protective role of Panx1 activity against hRSV infection.

To accomplish this aim, we performed an analysis of cytokines involved in the inflammasome activation from airways in hRSV infected mice treated with Probenecid.

4. MATERIALS

4.1 Equipment

- Cell incubator Forma Scientific model 3110
- Biosafety hood Sterile Gard Hood from The Baker Company
- Microcentrifuge Eppendorf 5415R
- Microcentrifuge Refrigerated IEC Micromax RF, Thermo Electron Corporation
- Centrifuge Eppendorf 5702R
- Centrifuge Eppendorf 5804R
- Centrifuge Centra CL3R
- Inverted microscope Olympus, model CKX41
- Freezer -80°C REVCO model Ultima III
- Flow Cytometer BD model FACS Canto II
- Plate heater Lab-Line Instrument
- Thermo regulated water bath LP/BX
- Microm HM 325 Rotary Microtome Thermo Scientific
- Leica ASP300S enclosed, automatic tissue processor
- pH reader Hanna HI 9321PH
- pH reader Hanna HI 2221PH
- Vortex Labnet International, model VX100

- Analytic balance Adam, model AFA-180LC
- 35 Liters Liquid Nitrogen Tank: Taylor Wharton
- Neubauer camera
- Nano Drop 2000 UV-Vis Spectrophotometer, Thermo Scientific
- Maxygene Thermocycler, Axygen.
- Veriti Thermal Cycler, Applied Biosystems
- Real-Time PCR System Applied Biosystem Step OneTM
- Microplate Reader Multiskan EX, Thermo LabSystems
- Microplate Strip Washer ELx50, BioTek
- Tissue Master Homogenizers, Omni International
- Thermo Scientific™ myECL™ Imager.

4.2 Buffers, cell and virus culture reagents

- Heat Inactivated Fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA; catalog # 12484-028)
- Dulbecco's Modified Eagle Medium (DMEM), (Invitrogen Life Technologies, Carlsbad, CA; catalog # 31600034)
- Paraformaldehyde 2% for cell fixation: 2% paraformaldehyde (Merck Millipore Corporation; catalog # 104005) in Phosphate buffered saline (PBS).

- Paraformaldehyde 4% for tissue fixation: 4% paraformaldehyde (Merck Millipore Corporation; catalog # 104005) in PBS.
- PBS: 0.14 M NaCl, 1.47 mM KH_2PO_4, 7.81 mM, Na_2HPO4, 2.68 mM KCl, pH 7.2-7.4, in distilled water.
- Red blood cell lysis buffer: 0.17 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.2
- Staining buffer for flow cytometry: PBS with 10% fetal bovine serum.
- Trypan blue 0.4% (Invitrogen Life Technologies, Carlsbad, CA; catalog # 15250061).
- Washing buffer (ELISA): PBS 0.05% Tween-20.

4.3 Reagents and Chemicals

- Ethanol 100% (Merck)
- TRIZOL (Invitrogen)
- TRUE BLUE Peroxidase (KPL)
- 3,3', 5,5-tetramethylbenzidine (TMB, BD Pharmigen)
- Twenn-20 (Biorad)
- Bovin Serum albumin (BSA, Winkler)
- Lipopolysaccharide (LPS, Sigma)
- Ovalbumin (OVA)
- Protoporphyrin IX cobalt chloride (CoPP, Frontiers Scientific)

- Tin protoporphyrin IX dichloride (SnPP, Frontiers Scientific)
- Ethylenediaminetetraacetic acid (EDTA, US Biological E 2210)
- Na_2HPO_4 (Merck)
- KH_2PO_4 (Winkler)
- NaCl (Merck)
- KCl (Merck)
- H_2SO_4 (Merck)
- NH_4Cl (Winkler)
- $KHCO_3$ (Winkler)
- $NaHCO_3$ (Winkler)
- $CaCl_2$ (Winkler)
- Ketamine 10% (Ketostop)
- Xylaxine 2% (Alfasan)
- Isoflurane (Baxter)
- Kit ImProm Reverse Transcription System (Promega)
- SyBR Green QPCR Master Mix (Life Technology).
- GoTaq G2 Flexi DNA Polymerase (Promega).
- CXCL1/KC ELISA KIT (DuoSet; R&D Systems)

- IL-6 ELISA KIT (OptEIA; BD Pharmingen)
- IL-4 ELISA KIT (OptEIA; BD Pharmingen)
- IL-10 ELISA KIT (OptEIA; BD Pharmingen)
- IFN-γ ELISA KIT (OptEIA; BD Pharmingen)
- CCL3/MIP-1α ELISA KIT (Ready Set Go! Affymetrix)

4.4 Antibodies

For flow cytometry assays:

- Monoclonal anti-mouse CD3 PE (BD Pharmingen, clone 145-2c11; catalog #553064).
- Monoclonal anti-mouse CD11c PECy7 (BD Pharmingen, clone HL3; catalog #558079).
- Monoclonal anti-mouse IA/IE PerCP Cy 5.5 (BD Pharmingen, clone M5; catalog # 562363)
- Monoclonal anti-mouse Ly6G FITC (BD Pharmingen, clone RB6-8C5; catalog # 553126)
- Monoclonal anti-mouse Ly6C PerCP Cy 5.5 (BD Pharmingen, clone AL-21; catalog # 560525)
- Monoclonal anti-mouse heme oxygenase 1 (Abcam, clone HO-1-1; catalog ab13248)
- Monoclonal anti-mouse CD11b Pe-Cy7 (Biolegend, clone M1/70; catalog #101216.).

- Monoclonal anti-mouse CD45 PercP Cy 5.5 (BD Pharmingen, clone 30-F11; catalog #561869).
- Polyclonal anti-respiratory syncytial virus FITC (Abcam; catalog #ab156657)
- Monoclonal antibody anti-hRSV N-AF647 conjugated (clone 1E9/D1)
- Monoclonal anti-mouse CD80 PerCP Cy 5.5 (BD Pharmingen, clone CD28.2; catalog # 560685)
- Monoclonal anti-mouse CD40 PE (BD Pharmingen, clone 2-23; catalog # 561846)
- Monoclonal anti-mouse CD86 APC (BD Pharmingen, clone GL1; catalog # 558703)
- Monoclonal anti-mouse CD4 APC (BD Pharmingen, clone RM4-5; catalog # 553051)
- Monoclonal anti-mouse CD8 FITC (BD Pharmingen, clone 53-6.7; catalog # 553031)
- Monoclonal anti-mouse CD69 PE (BD Pharmingen, clone H1.2F3; catalog # 561932)
- Polyclonal goat anti-mouse IgG-HRP (Invitrogen, Molecular Probes; catalog #:626520)

4.5 Biological Material

- A549 cell line (ATCC), HEp-2 cell line (ATCC), hRSV 13018-8 clinical isolate, BALB/cJ, C57BL/6J mice and tTA-HO-1 mice.

4.6 Plastic material

- 15 mL (Falcon)
- 50 mL centrifuge (Falcon)

- Tube 0.65 mL(Eppendorf)
- Tube 1.5 mL (Eppendorf)
- Tube 2 mL (Eppendorf),
- 5 mL FACS tubes (Falcon)
- 6, 24 and 96 well culture plates (Orange scientific)
- T75 flask (Orange scientific),
- 1000 µl, 200 µl, 20 µl and 2 µl micropipettes (Gilson, Axygen, Eppendorf and Rainin)
- 96-well Flat-bottom ELISA Plates (BD falcon)
- Cell strainer (Fisher scientific)
- Cell scrapers (Thermo scientific)
- 1mL of 25 G 5/8 and 10mL 21 G ½ tuberculin syringe (BD plastipak)
- MicroAmp fast optical 96-well reaction plate 0.1 mL (Life technologies)
- MicroAmp optical adhesive film (Life technologies)
- MicroAmp adhesive film applicator (Life technologies).

5. METHODOLOGY

5.1 General Methodology

5.1.1 Mice

Initial colonies of C57BL/6J and BALB/cJ wild-type mice were obtained from The Jackson Laboratory. pIi-TTA-TetO-HO-1 transgenic mice were generated by crossing the line pIi-TTA with the line TetO-HO-1. pIi-TTA were a kind gift from Christophe Benoist (Harvard Medical School, USA). TetO-HO-1 mice were generated by pronuclear microinjection of CBA/C57BL6 eggs with a DNA fragment containing a Tet-responsive-element downstream a minimal CMV promoter, the human β-globin intron, the human HO-1 cDNA and the bovine growth hormone polyA. All mice were maintained at the animal facility of Pontificia universidad católica de chile and manipulated according to guidelines approved by the institution Bioethical Committee (identification number 151230004) and by the Bioethics Committee of the Fondo Nacional de Desarrollo Científico y Tecnológico de Chile (FONDECYT grant 1150862) and were performed under the supervision of a veterinary.

5.1.2 Virus Preparation

Human laryngeal epidermoid carcinoma #2 (HEp-2) cells (American Type Culture Collection, CCL-23) were used to growth hRSV serogroup A2, strain 13018–8 (clinical isolate obtained from the Instituto de Salud Pública de Chile), as previously described (Espinoza et al., 2013). First, HEp-2 cells monolayers were grown in T75 flasks with DMEM (Life Technologies Invitrogen, Carlsbad, USA) supplemented with 10% FBS. Flasks containing 5 ml of culture medium were inoculated with 2 x 10^5 PFU of hRSV and incubated at 37°C. After viral adsorption (3 h), supernatants were replaced with fresh medium (DMEM 1% FBS) and incubated for 48 h or until visible cytopathic effect was observed. Cells were

harvested, the flask content was pooled and spin twice at 300 x g for 10 min to remove cell debris. In parallel, supernatants of non-infected HEp-2 monolayers were collected as previously described (Espinoza et al., 2013), and used as non-infectious control (mock).

5.1.3 Viral immunoplaque assay

Viral titer of stocks and supernatants in bronchoalveolar lavage (BAL) was determined by immunocytochemistry. Infectious supernatants were serially diluted (10-fold dilutions), added in 96-well plates with HEp-2 monolayers (80% confluence), and incubated for 48 h at 37°C. Later, cells were fixed with 2% paraformaldehyde-PBS and permeabilized 20 min with 0.2% saponin-PBS. Intracellular staining was performed with an anti-N-hRSV (clone 1E9/D1) antibody for 1 h (1 μg/ml 0.2% Saponin-PBS). Cells were washed twice and incubated with anti-mouse IgG-HRP (Invitrogen, Molecular Probes, Eugene, USA) (dilution 1:200) for 45 min. After washing the complex twice, the substrate TRUE-BLUE Peroxidase (KPL, Milford, USA) was added to cells and incubated for 10 min at room temperature.

5.1.4 Quantitative hRSV by ELISA

100 μL of virus-containing supernatants from hRSV-infected A549 cells at a multiplicity of infection (MOI) of 1 and treated with CoPP, SnPP or vehicle (6 to 100 μM) were used to coat ELISA plates for 2 h at 37°C. Then, plates were blocked for 2 h with PBS containing 10% of FBS. After washing three times with PBS tween 20 0.01%, goat anti-hRSV-HRP conjugate (ABCAM, Cambridge, UK) diluted in 1:500 in PBS FBS 10% was added to the wells for 2 h. Finally, after washing five times with PBS-tween 20 0.01%, bound antibody was detected by addition of tetramethylbenzidine peroxidase substrate peroxidase substrate (BD, San Jose, USA), stopped with 1 M H_2SO_4 and analyzed at 450 nm by ELISA plate Reader.

5.1.5 Cell viability assay

A549 cells were seeded in 96-well plates and treated with various concentrations of CoPP, SnPP or vehicle for 24 h at 37°C. Following this incubation, cell viability was assessed by using the AlamarBlue™ Cell Viability Reagent (Biosourse, Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Absorbance was measured at 570 nm by ELISA plate Reader.

5.1.6 Statistical Analyses

All statistical analyses were performed using GraphPad Pris Software version 6.1. Statistical significance was assessed using the Student *t*-test, one-way ANOVA test with a posteriori Bonferroni test and 2way ANOVA or Kruskal-Wallis ANOVA test with a posteriori Mann-Whitney test. Differences were considered significant when $P< 0.05$.

5.2 Methodology for testing hypothesis 1

5.2.1 hRSV infection of A549 cells and pharmacological induction of HO-1

Human alveolar type II-like pulmonary epithelial cells (A549 cells) (kindly provided by Dr. Pedro Piedra, Baylor College of Medicine, USA) were maintained in DMEM medium containing 10% (v/v) FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. A549 cells were treated either with vehicle (NaOH diluted in media), or 50 µM CoPP (HO-1 inducer) (Frontier Scientific, Logan, USA), or 50 µM tin protoporphyrin IX dichloride (SnPP, HO-1 inhibitor) (Frontier Scientific, Logan, USA) and incubated at 37°C in 5% CO_2. After 2 h, supernatants were removed, and cells were inoculated with infectious hRSV at MOI equal to 1 plaque-forming units (PFU)/cell and incubated for 2 h in DMEM 1% FBS medium (pre-

infection). As control, cells were inoculated either with the same media (untreated, UT) or with mock (supernatant of uninfected HEp-2 or with UV-inactivated hRSV). Each treatment was also performed during hRSV infection for 24 h (post-infection). In both cases, after 24 h replication was determined by measuring N transcript copies were quantified by RT-qPCR. In addition, supernatants from infected cells for each treatment were collected at 48 h post-infection to determinate viral titer by an immunoplaque assay.

5.2.2 Quantification of hRSV binding by flow cytometry and western blotting

HEp-2 cells were detached using EDTA 0.48 mM (Sigma Aldrich, Misuri, USA), washed, exposed to 50 μM of CoPP, SnPP, or vehicle, and chilled on ice for 30 min. Cells were washed and exposed to hRSV at a MOI of 1 concomitant with CoPP, SnPP or vehicle for 1 h at 4°C. For flow cytometry analyses, cells were washed, fixed (4% paraformaldehyde) and resuspended in anti-F protein FITC conjugated [0.5 μg/ml, ab20391; abcam, Cambridge, UK] for 1 h at 4°C. For western blotting analyses, hRSV-exposed cells were washed, resuspended in RIPA buffer and incubated at 95°C for 10 min. SDS-PAGE, on 10% BIS/Tris gels and in MES buffer (Invitrogen), was performed and indicated protein was transferred to nitrocellulose membranes. The membranes were then exposed to anti-N-protein ab (1:2000 dilution), anti HO-1 (Abcam ab13248, 1:400 dilution) or anti-β actin (Biolegend, San Diego, USA, 622102, 1:2000) at 0.2 μg/ml, followed by HRP-labeled secondary Ab (goat anti-mouse, Invitrogen, Molecular Probes, Eugene, USA) at 1 μg/ml. Chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare, Little Chalfont, U.K.) was detected using the Thermo Scientific™ myECL™ Imager.

5.2.3 Pharmacological modulation of HO-1 and hRSV challenge *in vivo*.

Six-to-eight-week-old male BALB/cJ WT mice were pretreated intraperitoneally (i.p.) (7.6 μmol/Kg) either with CoPP to induce the HO-1 expression or with SnPP to inhibit

the activity of HO-1 as previous described (Riquelme et al., 2015), 24 h before the viral challenge. Mice, treated with NaOH (diluted in PBS), were included as the vehicle control. Twenty-four hours later mice were anesthetized with ketamine/xylazine (80 mg/kg and 8 mg/kg, respectively) and challenged intranasally with either 1 x 10^6 PFU of hRSV or an equal volume of mock (as non-infectious control). Animal body weight was recorded daily after infection. At day 4 post-infection mice were terminally anesthetized with i.p. injection with a mixture of ketamine and xylazine. BAL and lung tissue samples were collected for further analyses.

5.2.4 Infection of rtTA-HO-1 transgenic mice

Six-to-eight-week-old female or male rtTA-HO-1 mice (**Figure 9**) and littermate mice were treated with 800 µg/ml doxycycline and 36 mg/ml sucrose in the drinking water, protected from the light, to induce the expression of human HO-1 in MHC-II positive cells. 48 h later, mice were anesthetized with ketamine/xylazine (20 mg/kg and 1 mg/kg, respectively) and challenged intranasally with either 1 x 10^6 PFU of hRSV or an equal volume of mock (as non-infectious control). Animal body weight was recorded daily after infection. At day 4 post-infection, mice were terminally anesthetized with i.p. injection with a mixture of ketamine and xylazine. BAL and lung tissue samples were collected for further analyses.

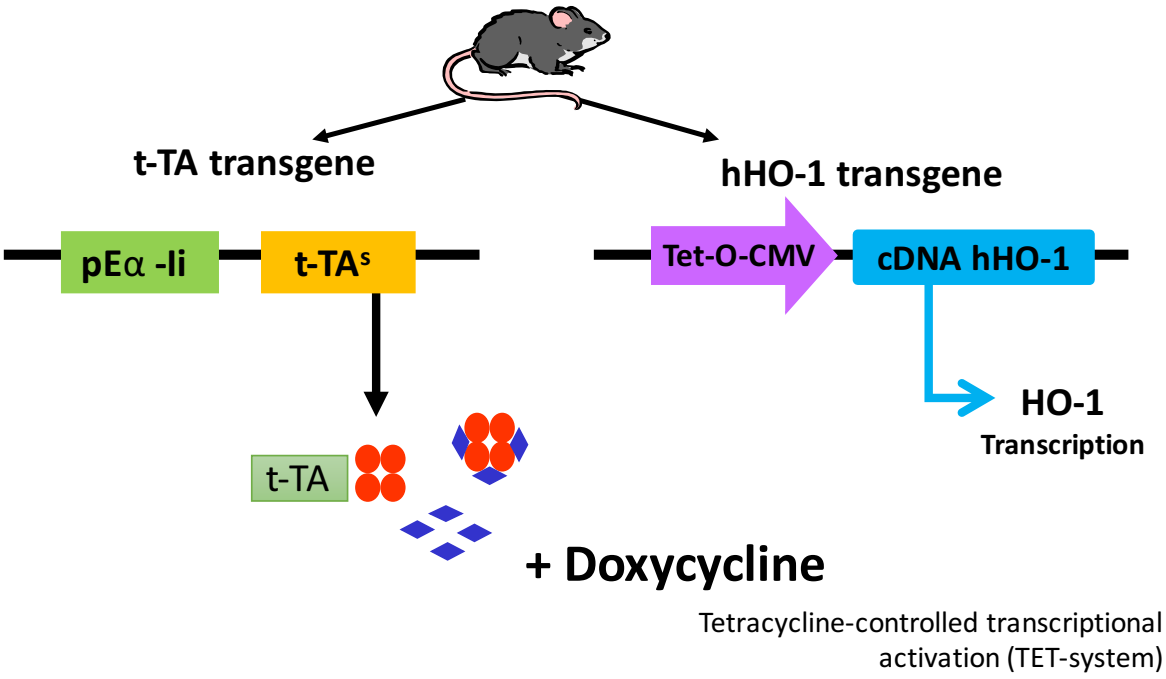

Figure 9. tTA-HO-1 mice model. The figure shows a squematic representation of conditional transgenic mice model. tTA strain expresses the tetracycline-controlled transactivator protein (tTA) under the MHC-II Eα promoter that controls the expression of tTA in MHC-II$^+$ cells. When these transgenic mice are mated to a second transgenic strain carrying a gene of interest (HMOX-1 or human HO-1) coupled to a tetracycline-responsive promoter element (TRE), conditional expression of the target gene in MHC-II$^+$ cells may be controlled by the administration of tetracycline or doxycycline.

5.2.5 Genotyping of tTA-HO-1

Mice were bled from the cheek into 100 µl of heparin (125 U.I./ml). For the purification of the DNA, we used the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and following the manufacture´s instruction. Consequently, we performed a PCR for each transgene inserted in the transgenic mice: tTA and tHO-1 using GoTaq G2 Flexi DNA Polymerase (Promega, Madison, USA). Then, samples were loaded in an agarose electrophoresis. The mice that only had the HO-1 gene but lacked the tTA gene were named littermate (LM), which were used as controls.

5.2.6 Quantitative Real-Time PCR

Total RNA was isolated from tissues or cell cultures by using the Trizol reagent (Life Technologies, Invitrogen, Eugene, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis from total RNAs was performed using the ImProm-II Reverse Transcription kit (Promega, Madison, USA) and random primers. RT-qPCR reactions were carried out using a StepOne plus thermocycler (Applied Biosystems). The abundance of HO-1 and Nrf2 mRNAs were determined by relative expression to the respective housekeeping gene by the 2-ΔΔCt method. For N-gene expression, absolute quantification data was expressed as the number of hRSV N-gene copies for each 5 x 10^3 copies of β-actin transcript, as previously described (Espinoza et al., 2013). The following primers were utilized; hRSV nucleoprotein (N) Fw 5′-GCTAGTGTGCAAGCAGAAATC-3′ and Rv 5′-TGGAGAAGTGAGGAAATTGAGTC-3′, mouse HO-1 5'-CCTCTGACGAAGTGACGCC-3' and Rv 5'-CAGCCCCACCAAGTTCAAA-3', Human HO-1 Fw 5'-AGGCAGAGGGTGATAGAAGAGG-3' and Rv 5'-TGGGAGCGGGTGTTGAGT-3', mouse Nrf-2 Fw 5'-TTC TTT CAG CAG CAT CCT CTC CAG-3' and Rv 5'-ACA GCC TTC AAT AGT CCC GTC CAG-3', Mouse IFN-α Fw 5′-TCC TGA ACC TCT TCA CAT CAA A-3′ and Rv 5′-ACA GGC TTG CAG GTC ATT GAG-3, Mouse IFN-β Fw 5′-AGC TCC AAG AAA GGA CGA ACA-3′ and Rv 5′- GCC CTG TAG GTG AGG TTG AT-3′, Mouse β-actin Fw 5′-ACCTTCTACAATGAGCTGCG-3′ and Rv 5′-CTGGATGGCTACGTACATGG-3.

5.2.7 Flow Cytometry

A549 and HEp-2 cells were inoculated, as mentioned above, and cultured for 24 or 48 h, respectively, in the presence of hRSV, UV-hRSV and mock, then were stained with anti-hRSV N-AF647 conjugated (clone 1E9/D1) in 10% of FBS in PBS as blocking solution.

For HO-1 intracellular staining, fixed cells were incubated with anti-mouse HO-1 mAb (Abcam, Cambridge, UK) in permeabilization buffer (1% Saponin, 10% FBS in PBS) for 45 min at 4°C. Then, cells were washed and stained with goat anti-mouse IgG-AF488 (Invitrogen, Eugene, USA). For cell infiltration analysis, lung samples were homogenized and filtered using a 40-µm cell strainer. BALs were centrifuged at 300 x g for 5 min (Centrifuge Eppendorf), washed and stained with anti-CD11b-APC (clone CBRM1.5, BD Pharmingen, San Jose, USA), anti-CD11c-PE (clone CBRM1.5, BD Pharmingen, San Jose, USA), anti-IA/IE-APC-Cy7 (clone M5; BD PharMingen, San Jose, USA), anti-Ly6C-PercCP 5.5 (clone AL-21, BD Pharmigen, San Jose, USA) and anti-Ly6G-FITC (clone RB6-8C5, BD Pharmingen, San Jose, USA). All antibodies were used at 3µg/ml. In addition, lung samples were stained with HO-1 mAb, as described above. All samples were acquired on a FACS Canto II flow cytometer (BD Biosciences, San Jose, USA) and analyzed using FlowJo 7.6 software.

5.2.8 Lung histopathology analyses

To perform histopathology analyses without losing significant tissue architecture, before BAL collection, the major bronchi of the left lung were clamped using a 10 cm hemostatic forceps. After BAL of the right lung, the left lung was fixed with 4% paraformaldehyde (PFA) and then, paraffin embedded using a Leica ASP300S enclosed, automatic tissue processor (Leica Microsystems, Wetzlar, Germany). Then, 4 µm-thick tissue sections were obtained using a Microm HM 325 Rotary Microtome (Thermo Scientific), and then mounted and stained for histopathology analyses using hematoxylin & eosin (H&E).

5.2.9 Cytokine and chemokine measured by ELISA

Immunoreactive CXCL1/KC were quantified by using a double Ab ELISA kit (DuoSet; R&D Systems, Minneapolis, USA). IL-6, IL-4, IL-10, and IFN-γ were quantified by using Ab ELISA kit (OptEIA; BD Pharmingen, San Jose, USA), and CCL3/MIP-1α detection was performed following the manufacturer's protocol (Ready Set Go! Affymetrix, Göteborg, Sweden).

5.2.10 Measurements of T cell function during hRSV infection

Lymph nodes from infected and mock BALB/cJ mice or tTA-HO-1 conditional transgenic mice of each treatment were removed and mechanically homogenized in 1 X PBS. After erythrocyte lysis with ACK buffer (150 mM NH_4CL, 10 mM $KHCO_3$, 0.15 mM EDTA), cells were resuspended at a final concentration equal to 5 x 10^6 cells per ml in RPMI 1640 medium, supplemented with 10% FBS, 1 mM nonessential amino acids, 2 mM glutamine, 1 mM pyruvate, 10 mg/ml penicillin G, 100 mg/ml streptomycin, 50 mg/ml gentamicin, and 50 mM 2-ME. Then, single-cell suspensions were left untreated or stimulated with ultraviolet hRSV (MOI 1) or anti-CD3ε/CD28 at 10 μg/ml and 2 μg/ml respectively. After 72 h of incubation (37°C, 5% CO_2), culture supernatants were analyzed for IFN-γ production by sandwich ELISA, and the expression of activation marker CD69 was measured on the surface of cells by flow cytometry (FACS Canto II flow cytometer [BD Biosciences, San Jose, CA] and analyzed using FlowJo 7.6 software).

5.2.11 DC maturation and APC assay

Immature DCs from conditional transgenic mice were incubated for 2 h with 50 mM CoPP or 1.5 mg/ml DOX (for all the incubation period). Then, cells were washed once and incubated with 700 ml of fresh RPMI 1640 medium. Maturation of DCs was induced by LPS treatment (1 mg/ml) for 16 h (Escherichia coli 0111; Invitrogen) and the surface expression

of CD40 (CD40-PE; clone 2-23, BD Pharmigen, San Jose, USA), CD80 (CD80-PerCPCy 5.5; clone CD28.2, BD Pharmingen, San Jose, USA) and CD86 (CD86-APC; clone GL APC, BD Pharmingen, San Jose, USA), were measured by flow cytometry. Also, cells were pulsed with 50 mg/ml of OVA protein, non-OVA pulsed cells +/- LPS were used as a control for antigenic presentation. After overnight incubation, cells were washed twice, detached, counted, and plated in round-bottom 96-well plates. Then, 50,000 DCs were incubated with 100,000 purified (over 90%; Miltenyi Biotec, Gladbach, Germany) OVA-specific TCR OT-II T $CD4^+$ cells (relationship 2:1 T cells: DCs). After 48 h of coculture, supernatants were collected for IFN-γ secretion, and T cells were stained for CD69 activation marker.

5.3 Methodology for testing hypothesis 2

5.3.1 Isolation of human PBMCs

Anti-coagulated human blood with 3.8% sodium citrate was obtained from leukopacks from the New Jersey/New York Blood Center. PBMCs were isolated by over-layering with Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) according to the procedure described by the manufacturer.

5.3.2 Dye uptake and time-lapse microscopy to determine pannexin1 channel activity

To characterize the functional state of Panx1 channels, dye-uptake experiments using ethidium (Etd) bromide were performed. Cells were washed twice in HBSS and then exposed to Locke's solution (containing 154 mM NaCl, 5.4 mM KCl, 2.3 mM $CaCl_2$, 5 mM HEPES, pH 7.4) with 5 μM Etd, and time-lapse microscopy was performed. Phase-contrast and fluorescence microscopy with time-lapse imaging were used to record cell appearance and fluorescence-intensity changes in each experimental condition. Fluorescence was recorded every 30 s. The NIH ImageJ program was used for offline image analysis and fluorescence

quantification. For data representation and calculation of Etd uptake slopes, the average of two independent background fluorescence (BF), expressed in arbitrary units (A.U.) was subtracted from mean fluorescent intensity (F1). Results of this calculation (F1−BF), from at least 20 cells, were averaged and plotted against time (min). Slopes were calculated using Microsoft Excel software and expressed as A.U./min. Because we are calculating the slope of the curve between two-time points, dead cells are unable to maintain an increasing Etd uptake resulting in a slope close to 0. Thus, these cells are eliminated from the analysis. Microscope and camera settings remained the same in all experiments. Dead cells or cells with a damaged plasma membrane were clearly identified during the time-lapse microscopy as a result of their nonspecific Etd uptake, determined by lack of time dependency and stability in dye uptake (not inhibited by blockers), and were not quantified.

5.3.3 Peptides and Probenecid.

The Panx1 mimetic blocking peptide Panx1 (WRQAAFVDSY) and the scrambled peptide (FADRYWAQVS) were synthesized by PeproTech (Rocky Hill, NJ). Probenecid water soluble (#P36400) was obtained from Thermo Fisher Scientific.

5.3.4 Phagocytosis assays.

A549 cells were seeded at 2.5×10^5 cells/well on 12-mm-diameter number 1 coverslips in 24-well plates (Falcon, Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada) and grown for 16 h. Then, cells were incubated for 24 h with 500 μM of probenecid and were also incubated with 1-μm-diameter fluorescein-labeled biotinylated polystyrene beads (5×10^6/ml; Molecular Probes, Eugene, USA) diluted in MEM-10% FBS for 1 h at 37°C. As control of the experiment cells were incubated with the mentioned beads for 1 h at 4ºC.

5.3.4 In *vitro* and *in vivo* inhibition assays.

Probenecid (Invitrogen, Eugene, USA), panx1 unspecific pharmacological inhibitor, was resuspended in PBS, and its cellular toxicity on A549 cells was determined by AlamarBlue reagent assay viability (Biosourse, Invitrogen, Carlsbad, USA). For *in vitro* analyses, A549 cells were infected with GFP-hRSV at MOI 1 or increased MOIs of clinical isolated of hRSV. After viral absorption, probenecid was added in the medium in increasing concentrations (250µ to 1mM). At 24 h postinfection, cells were analyzed by flow cytometry, epifluorescence microscopy, and RT-qPCR. For *in vivo* studies to evaluate lung virus burden, probenecid was administered intraperitoneally (i.p.) at 100 mg per kg of weight in BALB/cJ mice 24 h before infection. Further, mice were intranasally inoculated with hRSV. Lungs and BAL was collected 7 days postinfection. For virus titration analyses, BAL was serially diluted, and the titer was determined by a plaque assay on HEp-2 cells for 48 h.

5.3.5 Quantitative Real-Time PCR

Total RNA was isolated from tissues or cell cultures by using the Trizol reagent (Life Technologies, Invitrogen), according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis from total RNAs was performed using the ImProm-II Reverse Transcription kit (Promega, Madison, USA) and random primers. RT-qPCR reactions were carried out using a StepOne plus thermocycler (Applied Biosystems). The abundance of Panx1 mRNAs was determined by relative expression to the respective housekeeping gene by the 2-ΔΔCt method. The following primers were utilized; humanPanx1 Fw 5′-CCGCCCAGCAATATGAATCC-3′ and Rv 5′-CCACGGAGTACGTGTTCTCG-3′, mouse Panx1 5'-GGAGAAGCAGCTTATCTGGGT-3' and Rv 5'-CCACCGAGCCCAAGTTCA-3'

6. RESULTS

6.1 Evaluation of *in vitro* effects of HO-1 induction mediated by CoPP treatment on hRSV infected cells.

6.1.1 HO-1 expression is reduced in hRSV infected cells treated with CoPP.

First, we evaluated whether CoPP, a potent HO-1 inducer, increases HO-1 protein levels in A549 cells. A549 cells were treated with 50 μM CoPP, 50 μM SnPP or vehicle for 2 or 24 h, and then HO-1 protein levels were measured by flow cytometry. Also, we evaluated the effect of hRSV infection on HO-1 protein expression in presence or absence of the treatment mentioned above after 24 h. **Figure 10** shows representative overlaid histograms for flow cytometry analysis for HO-1 protein expression in A549 cell after 24 h of treatment and **Figure 11** shows geometric mean fluorescence intensity for HO-1 expression after 2 h and 24 h of treatment. CoPP administration markedly increased HO-1 protein levels from basal level detected for vehicle alone or untreated cells. Both, 2 h or 24 h of incubation period results in overexpression of HO-1, in infected and uninfected cells. However, the increase of HO-1 protein levels was not homogeneous in the cell population. Interestingly, hRSV infection reduces HO-1 protein levels in CoPP treated cells. Moreover, hRSV infection and SnPP treatment alone not induced changes in HO-1 protein levels from the basal level detected in vehicle treated cells.

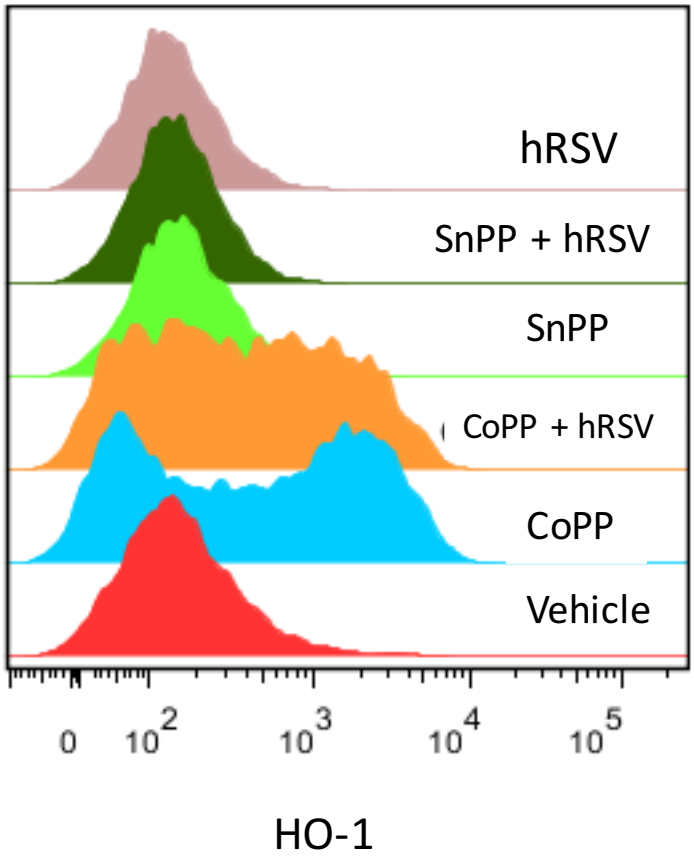

Figure 10. CoPP treatment increases HO-1 expression in A549 cells. A549 cells culture were infected with hRSV and treated with CoPP, SnPP or vehicle for 24 h, then HO-1 expression was analyzed by flow cytometry. The Figure shows representative overlaid histograms for flow cytometry analysis of HO-1 expression for each treatment. Light brown histogram shows hRSV infected cells, dark green histogram shows SnPP treatment in hRSV infected cells, light green histogram shows SnPP treated cells, orange histogram shows CoPP treatment in hRSV infected cells, light blue histogram shows CoPP treated cells and red histogram shows vehicle treated cells. The figure shows representative results of three independent experiments. (N=9)

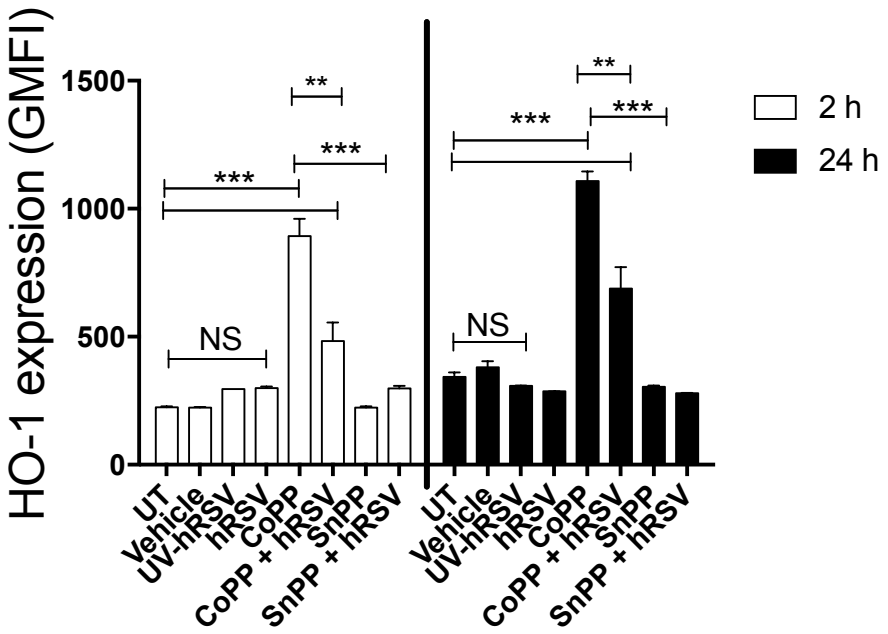

Figure 11. CoPP treatment promotes the overexpression of HO-1 in short and long incubation periods in uninfected and hRSV infected cells. A549 cells were treated with CoPP, SnPP or vehicle solution for 2 or 24 h in uninfected and hRSV infected cells, then HO-1 expression was analyzed by flow cytometry. The graph shows the geometric mean fluorescence intensity of HO-1 after 2 h (white bars) and 24 h (black bars) of each treatment. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by one-way ANOVA and multiple comparisons against the untreated control were performed for statistical analyses (N=9, * $P < 0.05$, ** $P < 0.001$, ***$P < 0{,}0001$, NS means non-significative).

To ensure that CoPP treatment was not cytotoxic to A549 cells, viability assay was performed. A549 cells were treated at different concentrations of CoPP, SnPP or vehicle (6 to 800 μM) for 24 h. Then, cells were washed and incubated with alamarBlue reagent. The active component of this reagent is resazurin, a cell-permeable compound that is converted to resorufin in viable cells, increasing the overall fluorescence and color of the media surrounding cells. The resulting fluorescence is read on a plate reader or fluorescence spectrophotometer. Treatment of A549 cells with concentration below to 100 μM CoPP had no significant effect on cell viability, this is shown in the dose-response curves in **Figure 12.** Conversely, SnPP treatment caused a significant reduction on cell viability in the same range of concentration.

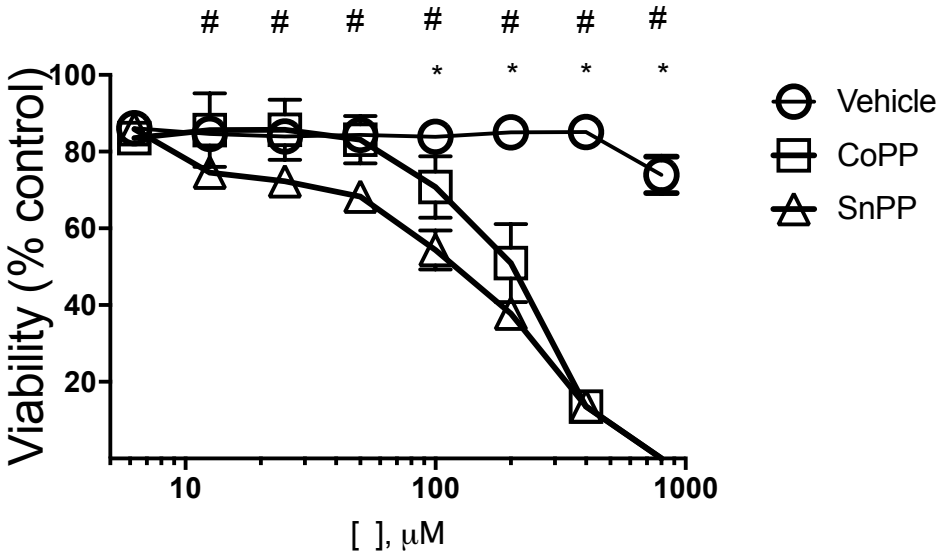

Figure 12. CoPP treatment does not affect cell viability in concentrations below to 100 μM. A459 cells were treated with different concentration of CoPP, SnPP or vehicle solution for 24 h. Then cell viability assay was performed with the alamarBlue reagent. The graph shows the dose-response curves to evaluate viability for A549 cells treated for 24 h with CoPP (white squares), SnPP (white triangles) or vehicle (white circles). Data shown are mean from three independent experiments. Non-significative differences are observed in the range used for further experiments (N=9, 2way ANOVA $P < 0.0001$) * Denotes statiscally signicant difference between vehicle and CoPP groups. # Denotes statiscally significant difference between vehicle and SnPP groups.

6.1.2 CoPP treatment impairs hRSV replication and infective viral particle production in human A549 cells.

Next, we evaluated whether CoPP treatment impairs hRSV replication, total viral particle production and infective viral particle production. A549 cells were pretreated with different concentrations of CoPP, SnPP or vehicle for 2 h. Then, the medium was removed, and the cells were washed with fresh medium and infected with hRSV at a MOI of 1 for 2 h. After viral adsorption, cells were washed with medium to remove unbound virus. After 48 h, total virus particle production was measured by detection of the hRSV F protein by ELISA in the supernatants of infected cells. We observed that treatment with increasing CoPP doses causes a significant reduction in the amount of total virus particles in the supernatants of infected A549 cells compared with vehicle treated cells as shown the dose-response curves in **Figure 13** (white squares). Conversely, treatment with increasing doses of SnPP causes a significant increase in the amount of total virus particles in supernatants from A549 cells infected cells compared to vehicle-treated cells (**Figure 13**, white triangles).

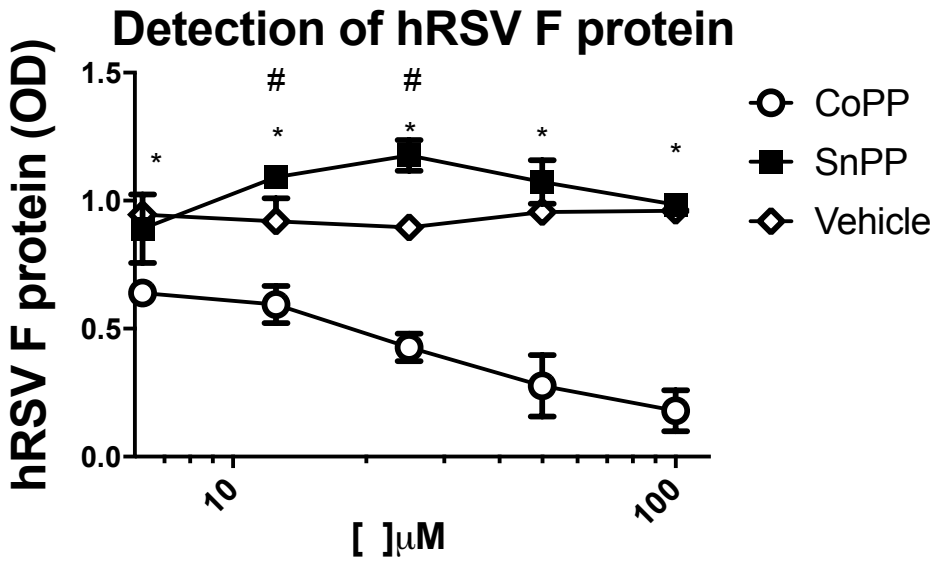

Figure 13. CoPP treatment decreased the levels of total viral particles in the supernatants of A549 infected cells compared with vehicle treated cells. A549 cells were treated with different doses of CoPP, SnPP or vehicle solution and were infected with hRSV. Then, after 48 h the supernatants of cell cultures was collected and ELISA for hRSV F protein detection was performed. The graph shows the dose-response curves for each treatment; CoPP (white squares, 450 nm), SnPP (white triangles), or vehicle (white circles). Data shown are mean from three independent experiments. (N=9, Student t-test). * Denotes statiscally signicant difference between vehicle and CoPP groups. # Denotes statiscally significant difference between vehicle and SnPP groups.

Considering that treatment with 50 μM of CoPP reduces in more than a 60% of hRSV production compared with vehicle treatment without affecting cell viability, we confirm this concentration for further experiments. Furthermore, to evaluate whether HO-1 overexpression affects the generation of infective hRSV viral particles, supernatants from infected A549 cells treated with CoPP, vehicle, or SnPP were used to quantify virus titers by immune-plaque assays. We found that treatment with CoPP reduces the viral titers by 3 log units as compared with vehicle-treated cells **(Figure 14).**

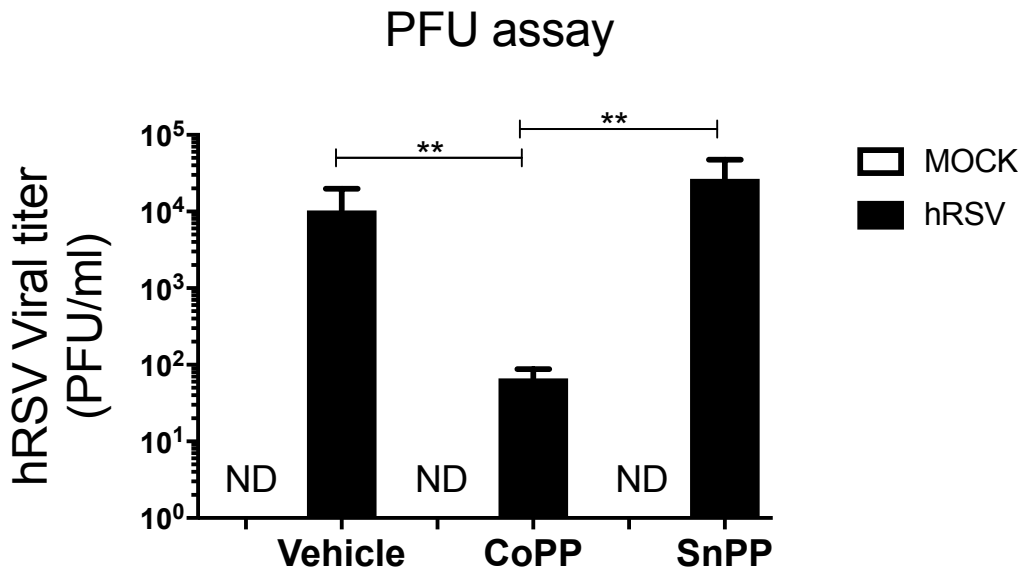

Figure 14. CoPP treatment decreased the levels of infective viral particles in supernatants of hRSV infected A549 cells in compared with vehicle treated cells. A549 cells were treated with CoPP, SnPP or vehicle solution and were infected with hRSV. 48 h postinfection, the supernatants were collected to measure viral titers. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by oneway ANOVA and Bonferroni posttest (N=9, *P < 0.05, **P < 0.001, ***P < 0.0001).

Also, because N transcription can be considered a measurement of hRSV viral replication within infected cells, we evaluated the transcription of N by RT-qPCR in cells treated with CoPP, SnPP or vehicle during 2 h before infection (pre-infection treatment) or for 24 h after infection (post-infection treatment). We observed a significant reduction in viral N mRNA amounts in cells treated with CoPP, both added before infection and after infection compared with vehicle treatment **(Figure 15).** Conversely, treatment with SnPP increased the amounts of hRSV N-transcripts in infected cells compared with vehicle treatment, suggesting that HO-1 activity is necessary to control viral replication.

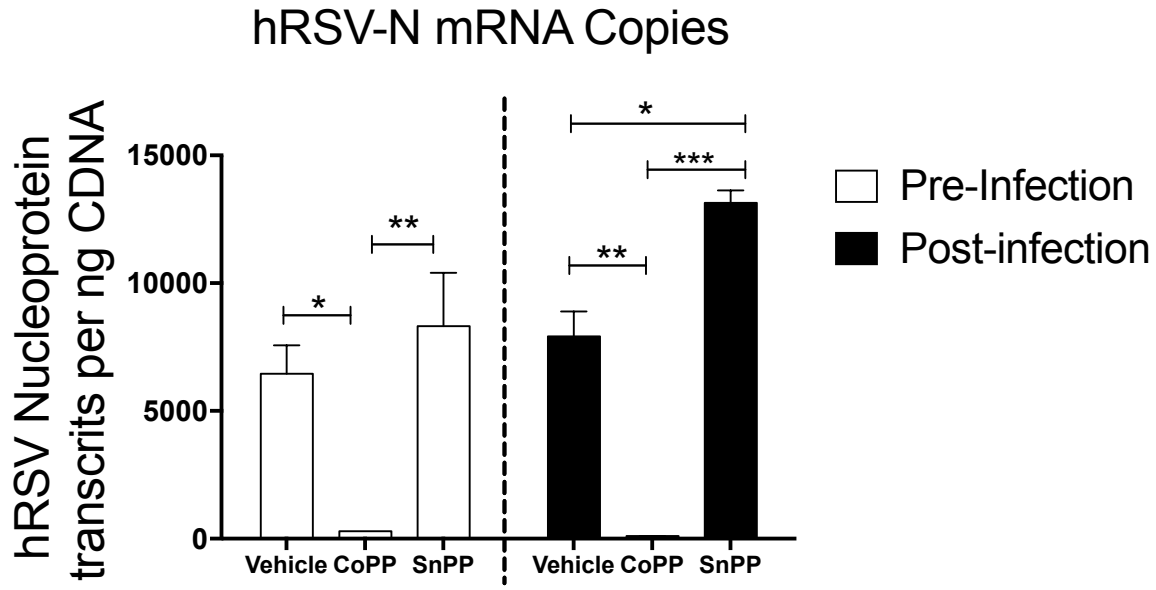

Figure 15. CoPP treatment impairs hRSV replication in infected A549 cells *in vitro*. A549 cells were treated with CoPP, SnPP or vehicle solution after or before infection with hRSV. 24 h postinfection, the viral load was measured by RT-qPCR. The graph shows the copy number for hRSV-N RNA in infected A549 cells per ng of cDNA. Preinfection treatment with drugs (white bars), or treatment with drugs postinfection (black bars). Data shown are mean +/- SEM from three independent experiments. Data were analyzed by one-way ANOVA and Bonferroni posttest (N=9; *P <, 0.05, **P < 0.001, ***P < 0.0001).

6.1.3 CoPP treatment does not impair the entry and binding processes of hRSV.

To determine the stage of the hRSV infection cycle that was affected by HO-1 overexpression, we evaluated whether HO-1 induction, produced by CoPP, impairs hRSV binding and entry process. Previous reports have described that the hRSV fusion is an active process that is inhibited at 4°C in HEp-2 cell line (Srinivasakumar et al., 1991), whereas viral binding to cell membrane still occurs. Viral binding was evaluated by western blotting, detecting the hRSV-N protein on total cell lysates (**Figure 16A, Table 1**), as well as by flow cytometry analyses of surface expression of the hRSV F-protein (**Figure 16B and 16C**). No significant differences were observed for hRSV-N expression in cell lysates obtained from CoPP treated cells (**Figure 16A**) nor the surface expression of the hRSV-F protein (**Figure 16B and 16C**), suggesting that HO-1 induction does not affect hRSV binding. However, further experiments are required to demonstrated that HO-1 does not impair binding process.

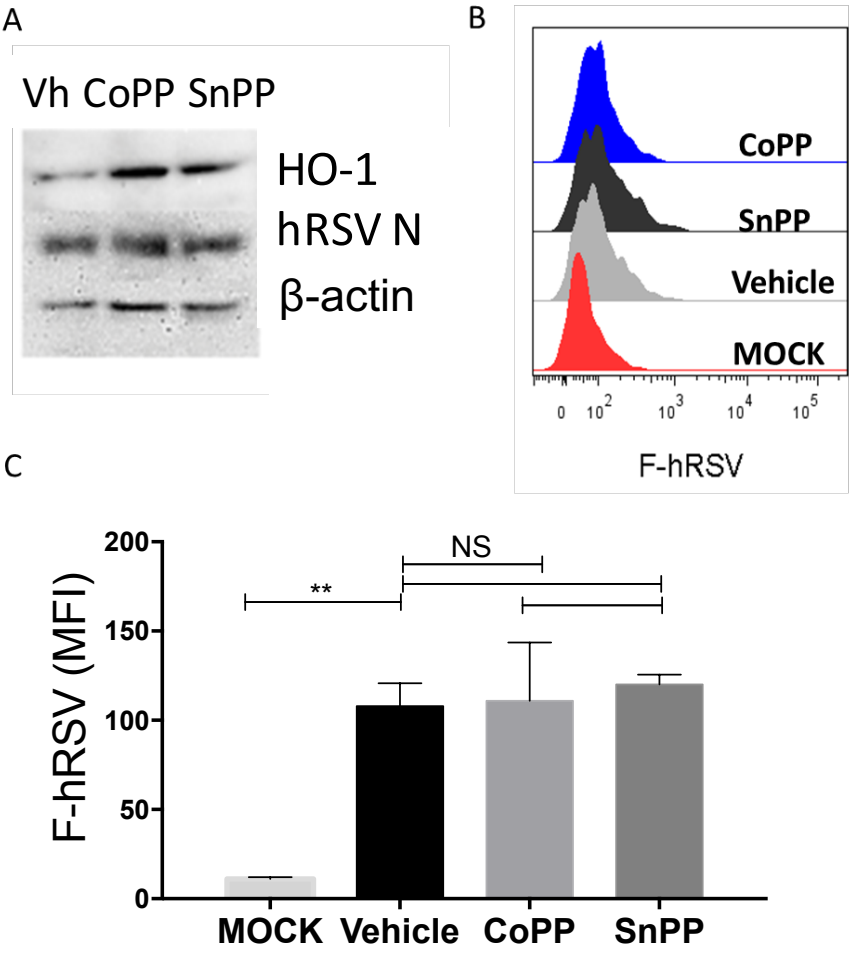

Figure 16. CoPP treatment does not impair hRSV binding process. HEp-2 cells were infected at MOI 5 with hRSV, an incubated for absorption for 2 h at 4°C in the presence of CoPP, SnPP or vehicle. Then, detection of F hRSV protein was performed by western blotting and flow cytometry to asses viral binding. (**A**) The figure shows representative western blotting from cell lysates for HO-1 (upper panel), hRSV-N (middle panel), and β-actin (lower panel) for each treatment. (**B**) The figure shows representative overlaid histograms of flow cytometry analyses for surface hRSV F protein expression in HEp-2 cells for each treatment. The blue histogram shows CoPP treated cells, dark gray shows the SnPP treatment, the light gray histogram shows vehicle treatment and red histogram shows MOCK treated cells. (**C**) The graph shows the geometric mean fluorescence intensity of surface hRSV F protein–expressing cells for each treatment. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by one-way ANOVA and Bonferroni posttest (N=3; *P <, 0.05, **P < 0.001, ***P < 0.0001, NS means non-significant).

Vehicle	CoPP	SnPP	Target
16649 ± 1250	30838 ± 845	22770 ± 2075	HO-1
2249 ± 71.42	2446 ± 1.414	2122 ± 14.14	HRSV N

Table 1. Protein levels of HO-1 and hRSV-N in hRSV infected A549 cells treated with vehicle, CoPP and SnPP. Values are densitometric data expressed as ratios of HO-1 or hRSV-N to β-actin (mean ± SD)

Next, to evaluate viral entry, cells were infected at MOI 5 and maintained at 4°C for 1 h to allow viral binding. Then, cells were washed and treated with CoPP, vehicle, or SnPP and incubated at 37°C for 5 h. Viral entry was determined by detecting intracellular expression of hRSV-F protein by flow cytometry. As shown in **Figure 17A and 17B**, no effect was observed in the intracellular expression of the hRSV-F protein in CoPP-treated cells compared with vehicle-treated or SnPP treated cells. However, further experiments are required to demonstrated that HO-1 does not impair entry process.

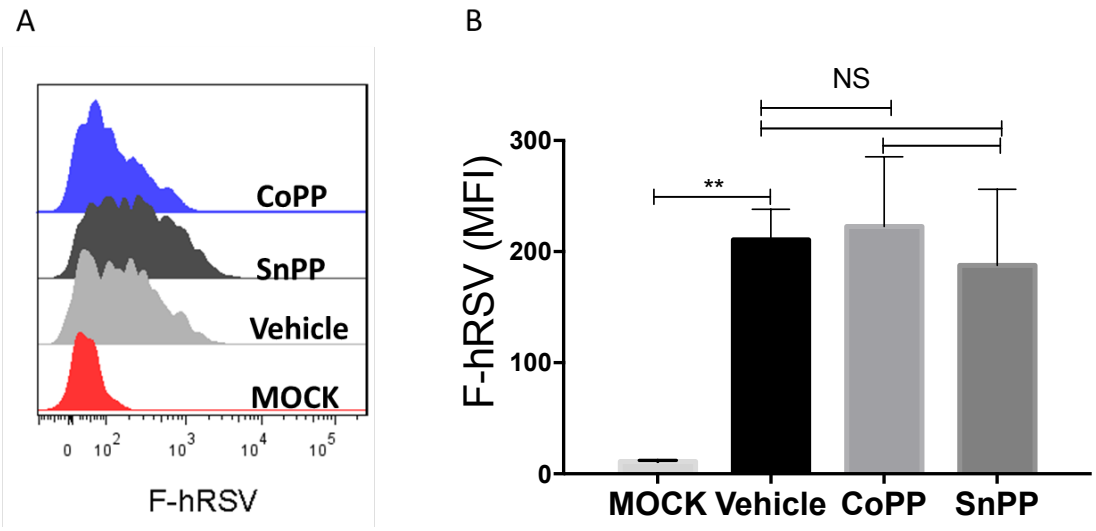

Figure 17. CoPP treatment does not impair hRSV entry process. HEp-2 cells were infected at MOI 5 with hRSV, an incubated for 2 h to allow viral absorption at 4°C. after viral absorption, cells were incubated for 5 h at 37°C in the presence of CoPP, SnPP or vehicle to assess viral entry. (**A**) The figure shows representative overlaid histograms of flow cytometry analyses for intracellular hRSV F protein expression in HEp-2 cells for each treatment. Blue histogram shows CoPP treated cells, the dark gray histogram shows SnPP treated cells, the light gray histogram shows vehicle-treated cells and the red histogram shows MOCK treated cells (**B**) Data graph shows geometric mean fluorescence intensity of surface hRSV F protein–expressing cells for each treatment. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by one-way ANOVA and Bonferroni posttest (N=3; *P < 0.05, **P < 0.001, ***P < 0.0001, NS means non-significant).

Importantly, hRSV infection in HEp-2 cells was affected similarly as A549 cells with the CoPP treatment (**Figure 18**). Thus, these results support the notion that although HO-1 induction negatively modulates hRSV replication, the inhibition of HO-1 activity promotes replication of this virus in these cells. However, HO-1 induction does not affect hRSV binding or fusion in infected cells.

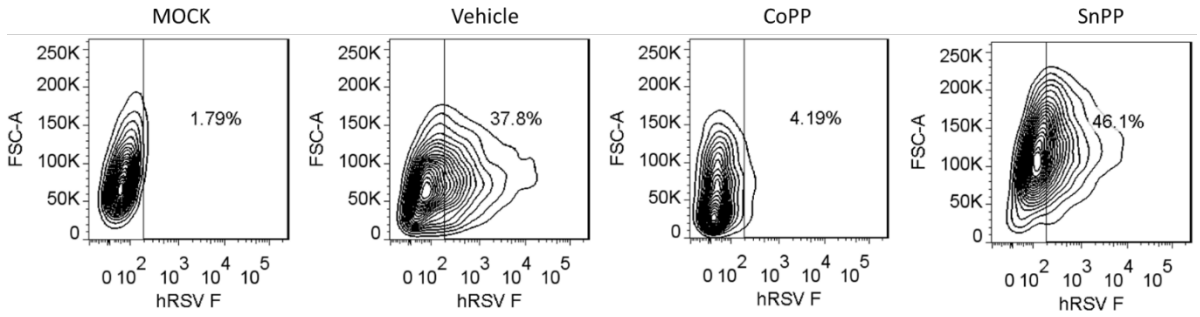

Figure 18. HRSV propagation is impaired in HEp-2 cell line similarly treated with CoPP that A549 cell line. HEp-2 cells were treated with 50 µM of CoPP, SnPP or Vehicle and were infected with hRSV at MOI of 1 for 24 h. Then infection was evaluated by flow cytometry. The figure shows representative dot plots for flow cytometry analyses for HEP-2 cell infected for each treatment from three independent experiments. (N=3)

6.2 Evaluation of the role of HO-1 induction in hRSV infection *in vivo*.

6.2.1 Pharmacological induction of HO-1 prevents viral replication and protects mice from hRSV-induced disease.

To evaluate whether the pharmacological induction of HO-1 could modulate hRSV disease parameters *in vivo.* BALB/cJ mice were treated either with CoPP, SnPP or vehicle for 24 h. After treatment administration, mice were intranasally infected with hRSV ($1x10^6$ PFUs) or equivalent volumes amount of MOCK solution (as a non-infectious control). Mice body weight was monitored for 7 days postinfection. As shown in **Figure 19**, a marked weight loss was observed after hRSV infection in vehicle-treated mice. On the contrary, CoPP-treated mice displayed accelerated kinetics of body weight recovery after hRSV infection. Significant differences between CoPP-treated and vehicle-treated mice were observed at days 2, 3, and 4 postinfection with hRSV.

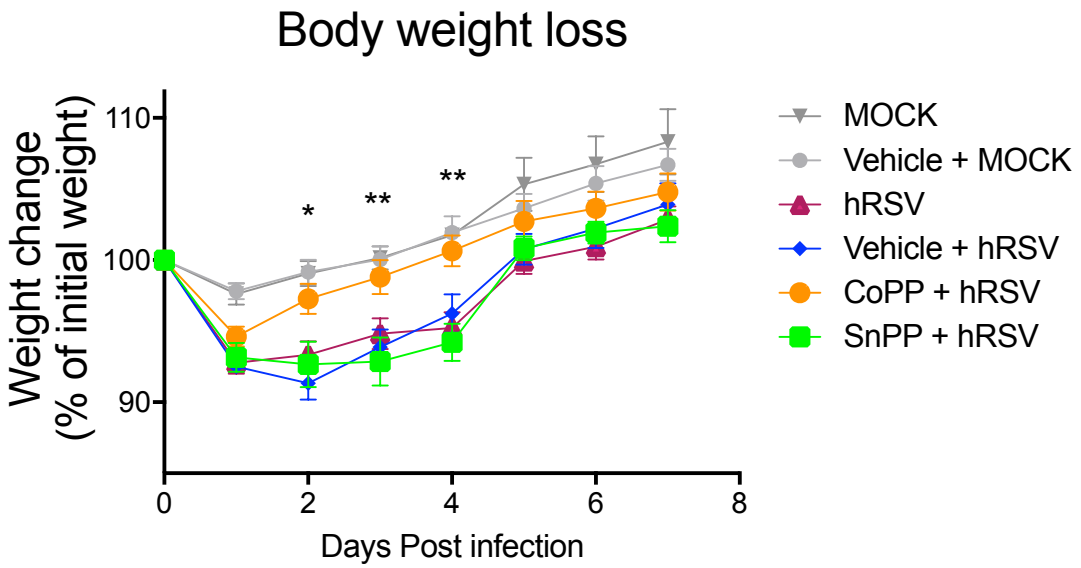

Figure 19. CoPP treatment prevents body weight loss after hRSV infection in mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle, 24 h after the treatment, mice were intranasally infected with hRSV, and the disease progression was monitored by determining values for animal body weight loss over 7 days. The orange circle shows CoPP -hRSV infected mice, the green square shows SnPP- hRSV infected mice, the blue diamond shows Vehicle-hRSV infected mice, the purple triangle shows hRSV infected mice. The graph shows curves for animal weight loss over 7 d. [N= 9; *P < 0.05, Student t-test was applied between CoPP + hRSV (orange circle) and hRSV (purple triangle)].

Further, we evaluated whether HO-1 induction mediated by CoPP treatment could prevent hRSV replication in the airways of infected mice. Mice were intranasally infected with $1x10^6$ PFUs of hRSV, then lungs and BAL were harvested at day 4 postinfection. Viral loads were assessed by detection of viral RNA by RT-qPCR. CoPP treatment results in a significant reduction of hRSV-N-RNA levels in the lungs compared with mice treated with vehicle solution (**Figure 20**). On the contrary, SnPP-treated mice showed high amounts of viral RNA in the lungs compared with vehicle-treated mice (**Figure 20**).

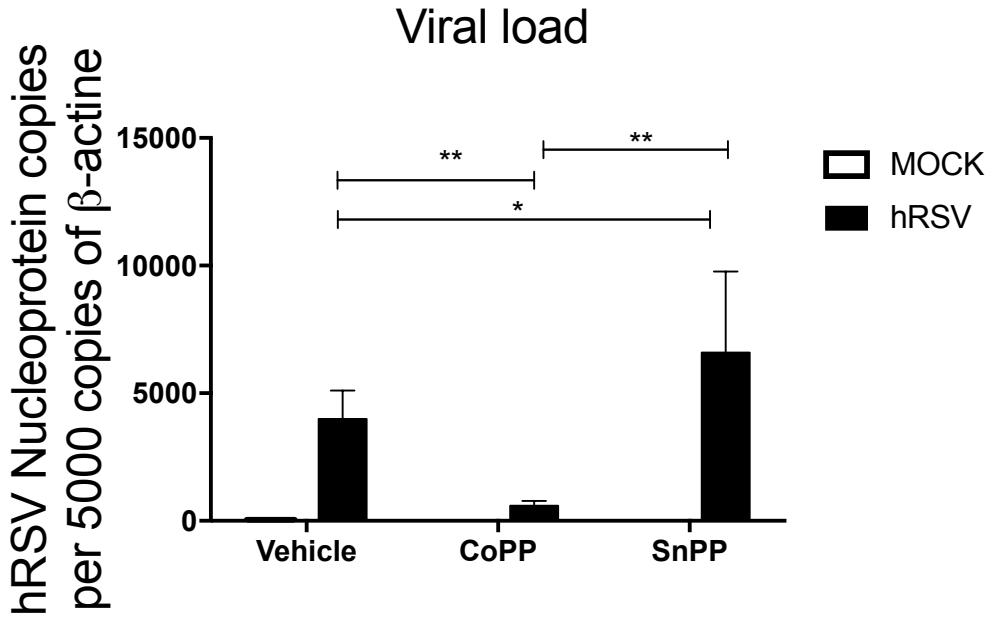

Figure 20. CoPP treatment prevents replication in the lung of hRSV infected mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle, 24 h after were intranasally infected with hRSV. Then, lung homogenates of each experimental group of infected mice were collected at day 4 postinfection and quantified for viral copy number assessing hRSV-N RNA per 5000 copies of β-actin of by RT-qPCR. White bars represent the N RNA copy numbers for MOCK controls, and black bars represent hRSV infected mice. Data shown are mean +/- SEM from three independent experiments, each with three mice per group (N = 9). Data were analyzed by 2way ANOVA and Tukey´s posttest, and multiple comparisons against the vehicle control were performed for statistical analyses (*$P < 0.05$, **$P < 0.001$).

Furthermore, the production of infective viral particles was analyzed by immune-plaque assay in BAL obtained from hRSV infected mice at 4 d postinfection. CoPP-treated mice showed a 2-log reduction in viral titers, as compared with vehicle-treated mice. SnPP treatment does not increase the production of infective viral particles compared with vehicle-treated mice. (**Figure 21**).

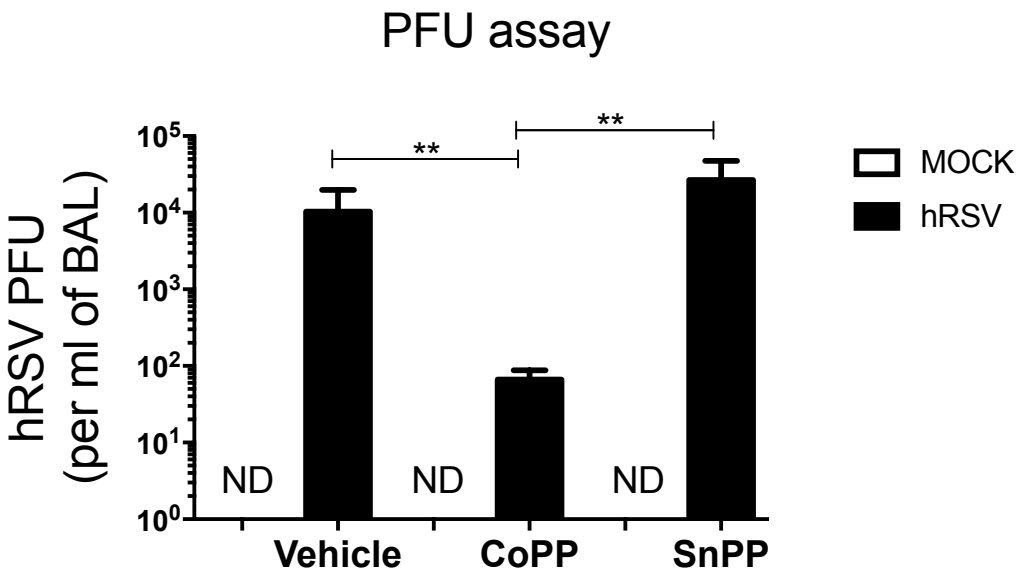

Figure 21. CoPP treatment prevents hRSV infective viral particle production in airways of infected mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle, 24 h after were intranasally infected with hRSV. Then, the BAL from vehicle, CoPP-, and SnPP-treated mice were titrated on HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed as PFUs per milliliter). Data shown are mean +/- SEM from three independent experiments, each with three mice per group (N = 9). Data were analyzed by 2way ANOVA and Tukey´s posttest, and multiple comparisons against the vehicle control were performed for statistical analyses (**P < 0.001).

In addition, we evaluated whether CoPP treatment increases HO-1 in lung tissue. Lung homogenates were analyzed by RT-qPCR analyses, our results showed a significant increase in HO-1 mRNA levels in the lungs of CoPP-treated, hRSV-infected, and CoPP-uninfected mice as compared with control mice at day 4 post-infection (**Figure 22**). These data suggest that the administration of CoPP was effective at inducing HO-1 expression in the lungs of treated mice.

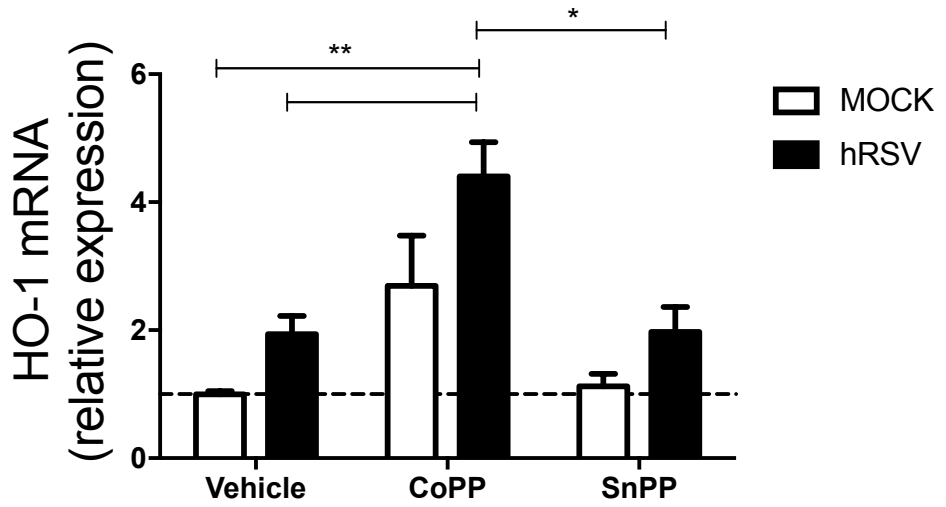

Figure 22. HO-1 mRNA is increased at day 4 postinfection in the lungs of CoPP treated mice compared with vehicle control mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle, 24 h after were intranasally infected with hRSV. Then, lung homogenates of each experimental group of mice were collected at day 4 post-infection, and HO-1 relative mRNA expression levels were evaluated. Relative levels of mRNA HO-1 were normalized to β-actin levels by using the 2-ΔΔCt method (vehicle-treated mice were used as a reference control). Data shown are mean +/- SEM from three independent experiments, each with three mice per group (N = 9). (*$P < 0.05$, **$P < 0.001$. 2way ANOVA and Tukey´s posttest).

Furthermore, to determine the specific cell type that overexpresses HO-1 in response to CoPP treatment in the lung tissues flow cytometry analyses were performed. We analyzed HO-1 expression in EPCAM positive cells that are considered epithelial cells and in CD45 positive cells that are classified as immune cells. Our data indicated that CoPP treatment markedly increased the HO-1expression levels in epithelial Epcam-positive cells (**Figure 23**).

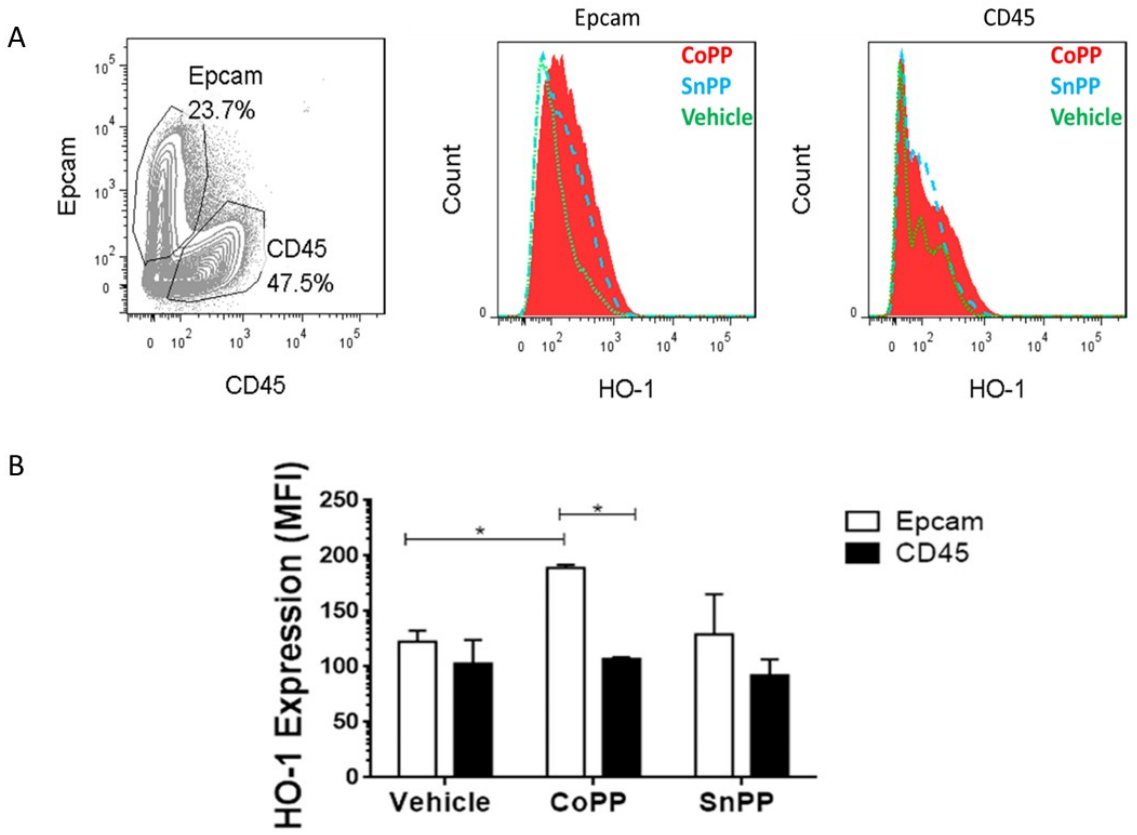

Figure 23. HO-1 protein levels are increased in at day 4 postinfection in EPCAM positive cells from lungs in CoPP treated mice compared with vehicle control mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle, 24 h after were intranasally infected with hRSV. Whole lung cell suspension was analyzed by flow cytometry for HO-1 expression at day 4 post-infection. (**A**) Representative dot plots of flow cytometry analyses, showing gating strategy to separate Epcam epithelial cells and CD45 immune cells. Then, representative histograms show expression of HO-1 enzyme in each population. (**B**) MFI of HO-1 in whole lung cell suspensions of Epcam and CD45 cell populations. Values were analyzed by 2way ANOVA and the Tukey´s post-test (*, $P < 0.05$). Data shown are means +/- SEM from three independent experiments (N=9).

6.2.2 Transgenic expression of human HO-1 in MHC-II+ cells impairs viral replication and confers protection against hRSV infection in mice.

Fist, to address whether the treatment with DOX induces the expression of exogenous human HO-1in MHC-II+ cells in the conditional transgenic mice tTA-tHO-1 (**Figure 24A**), RT-qPCR analyses of the human HO-1 transcripts were performed from lung tissue obtained from mice of each experimental group (**Figure 24A**). The detection of human HO-1 in sorted pulmonary MHC-II+ cells further validated this model (**Figure 24B**).

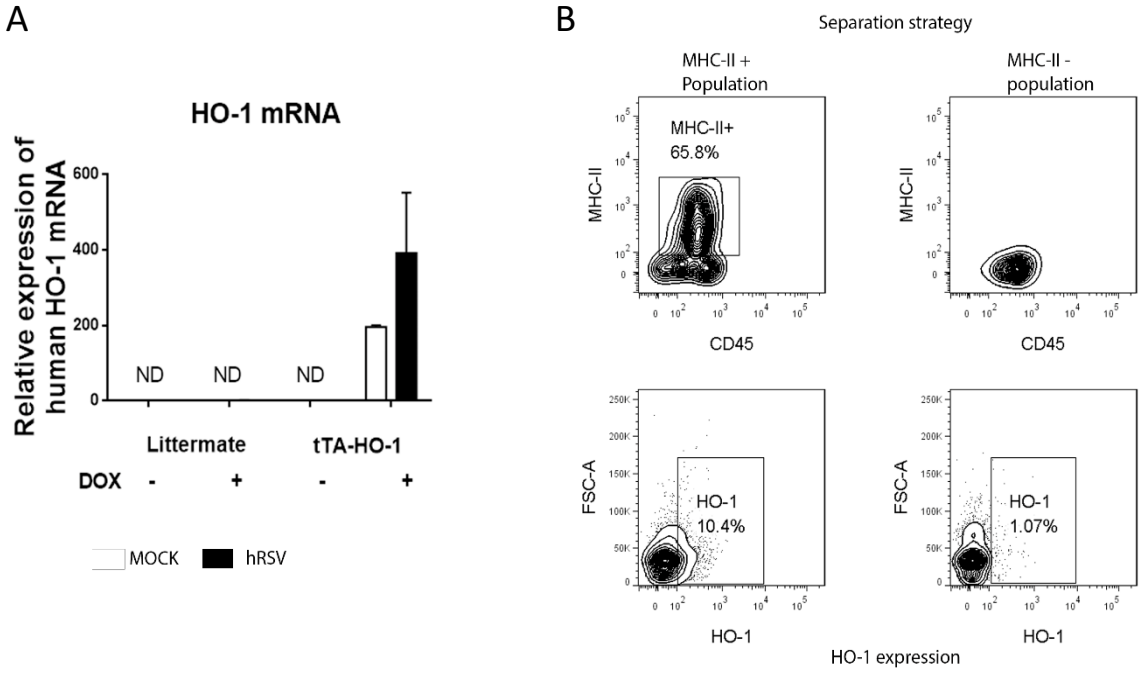

Figure 24. Human doxycycline-induced HO-1 expression is limited to MHC-II+ cells in conditional transgenic mice tTA-HO-1.

(A) Lung homogenates of conditional transgenic tTA-HO-1 and littermate mice with or without administration of DOX were obtained to obtain mRNA. Data in the graph shows the relative expression of human HO-1 in each experimental group. **(B)** Single-cell suspensions were obtained from the spleen of conditional transgenic mice that receive DOX in drinking water for 24 h. Then, cells suspensions were separated by MACs in a negative MHC-II population (right upper panel) and MHC-II-enriched (left upper panel). HO-1 expression was measured by flow cytometry. The percentage of HO-1 expression was measured in each population (bottom panel). Data shown are means +/- SEM from two independent experiments with three animals for each group (N=6).

To validate the protective role of HO-1 during hRSV infection through pharmacological induction, conditional transgenic mice overexpressing the HMOX-1 gene in MHC-II+ cells (tTA-tHO-1) or littermate mice were instilled intranasally either with hRSV (1 x10^6 PFU) or mock. The conditional expression of hHO-1 is regulated by the administration of doxycycline in the drinking water. Mice body weight was monitored as described above, in this transgenic mouse model expressing human HO-1. Consistent with the effects observed in CoPP treatment, the expression of human HO-1 induced by DOX resulted in an accelerated kinetic of body weight recovery at days 3 and 4 postinfection, as compared with littermate controls and untreated transgenic mice (**Figure 25**). No significant unspecific effects were observed for DOX treatment in uninfected mice (**Figure 25**).

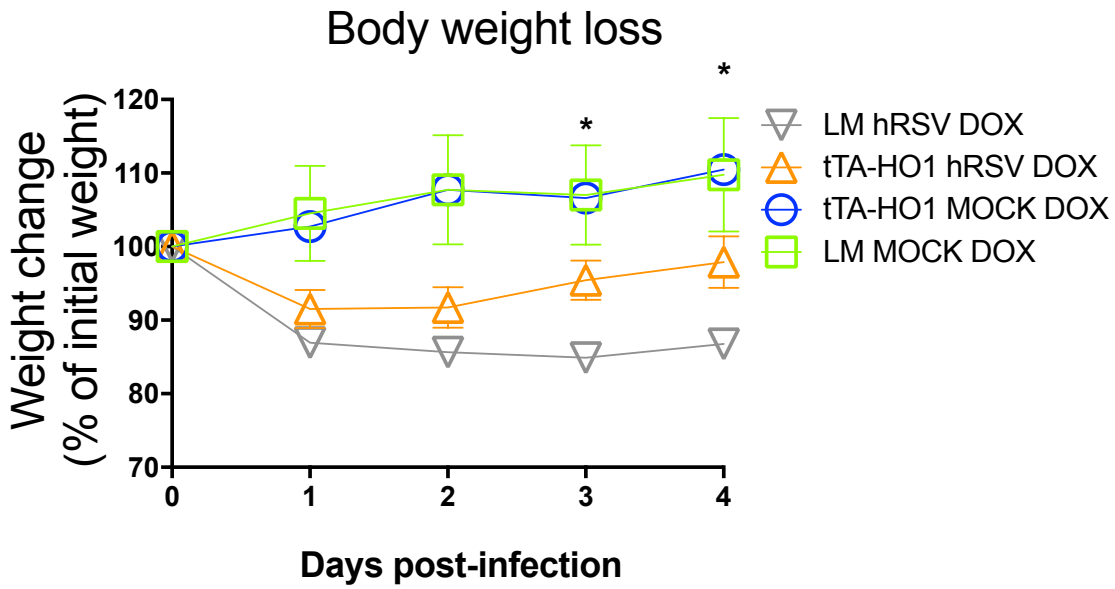

Figure 25. HO-1 overexpression in the MHC-II+ cell subset prevents disease induced by hRSV in tTA-HO-1 infected mice. tTA-HO-1 and littermate mice were intranasally infected with hRSV and disease progression was monitored by analyzing animal weight loss during 4 d postinfection. DOX and sucrose were added to the drinking water of mice to induce HO-1 expression. The graph shows the curve of body weight change for each treatment. Gray triangle shows Littermate + DOX mice, Orange triangle shows tTA-HO-1 + DOX mice, Blue circles shows tTA-HO-1 MOCK + DOX mice and green squares shows Littermate mock mice treated with DOX. (N=6; *p < 0.05, Student t test between LM DOX hRSV + tTA-HO-1 DOX hRSV values).

Moreover, we evaluated whether the transgenic expression of hHO-1 could prevent hRSV replication in the airways of infected mice. Transgenic and littermate mice were intranasally infected with $1x10^6$ PFUs of hRSV, and the lungs and BAL were harvested at day 4 postinfection. Viral loads were assessed by detection of viral RNA by RT-qPCR. Lower levels of hRSV-N RNA were observed at day 4 postinfection in the lungs of transgenic mice infected with hRSV induced with DOX compared with littermate mice (**Figure 26**).

Furthermore, the production of infective viral particles was analyzed by immune-plaque assay in BAL obtained from hRSV infected mice at 4 d postinfection. Transgenic mice treated with DOX showed a significant reduction in viral titers, as compared with littermate mice (**Figure 27**).

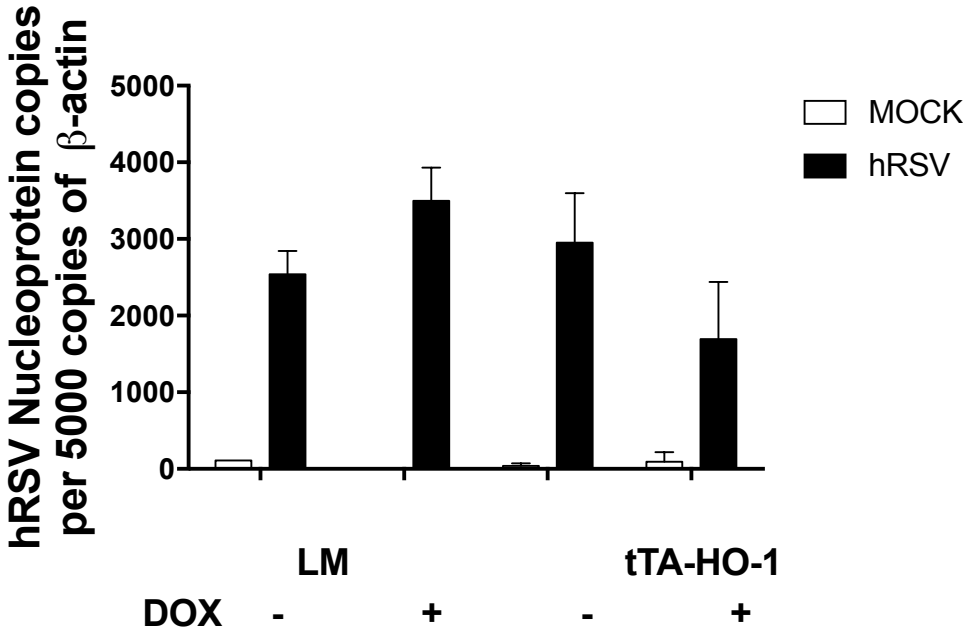

Figure 26. HO-1 overexpression in the MHC-II+ cell subset slightly prevents viral replication in lungs of infected mice. tTA-HO-1 and littermate mice were intranasally infected with hRSV. DOX and sucrose were added to the drinking water of littermate or tTA-HO-1. Lung homogenates of each experimental group of mice were collected at day 4 postinfection, and quantified for viral RNA by RT-qPCR, using primers targeting the hRSV-N gene. Data in the graph show N-RNA copy numbers per 5000 copies of β-actin. Data shown are from two independent experiments, each with three mice per group (N = 6).

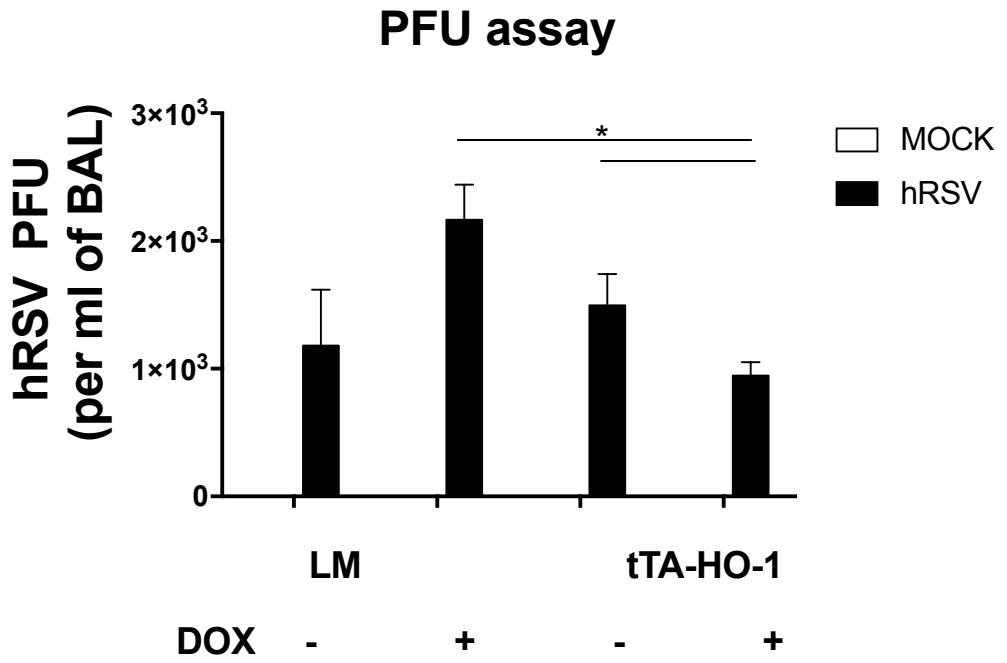

Figure 27. HO-1 overexpression in the MHC-II+ cell subset impairs infective viral particles production in airways of infected mice. tTA-HO-1 and littermate mice were intranasally infected with hRSV. DOX and sucrose were added to the drinking water of littermate or conditional transgenic mice (tTA-HO-1). Then, the BAL from each experimental group were titrated on HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed as PFUs per milliliter). The graph shows the PFU/ml in BAL for each experimental group. Data shown are from two independent experiments, each with three mice per group (N = 6). Mann–Whitney U test (*P < 0.05, **P < 0.01).

6.2.3 Pharmacological induction of HO-1 prevents neutrophil infiltration and lung inflammation in hRSV infected mice.

To evaluate whether HO-1 induction prevents hRSV-mediated airway inflammation and lung damage, lung tissue from mice of each experimental group were analyzed by hematoxylin and eosin (H&E) and PAS staining at day 4 postinfection. hRSV-induced histopathologic abnormalities gradually decreased during the first week after inoculation (Jafri et al., 2004). The histologic changes induced by hRSV infection as we observed in **Figure 28,** upper panel**,** included perivascular edema and the margination of neutrophils and mononuclear cells, which progressed to a sparse perivascular infiltrate and, later, to dense perivascular and peribronchial/peribronchiolar inflammatory infiltrates that were composed of mononuclear cells and scattered neutrophils. Then, these inflammatory infiltrates extended into surrounding alveolar septa in a stellate manner, with patchy involvement of the parenchyma and abundant macrophages and occasional lymphocytes and neutrophils in alveolar spaces (Jafri et al., 2004). CoPP-treated mice displayed reduced inflammatory infiltration in both bronchoalveolar airspaces and the lung interstitium and less perivascular edema compared with vehicle-treated mice, suggesting that HO-1 induction protects mice against bronchopneumonia and interstitial pneumonia. Conversely, SnPP treatment caused a slight increase in the signs of lung damage compared with vehicle-treated mice. No significant mucus production was detected by PAS staining for all the treatments. (**Figure 28,** bottom panel**)**.

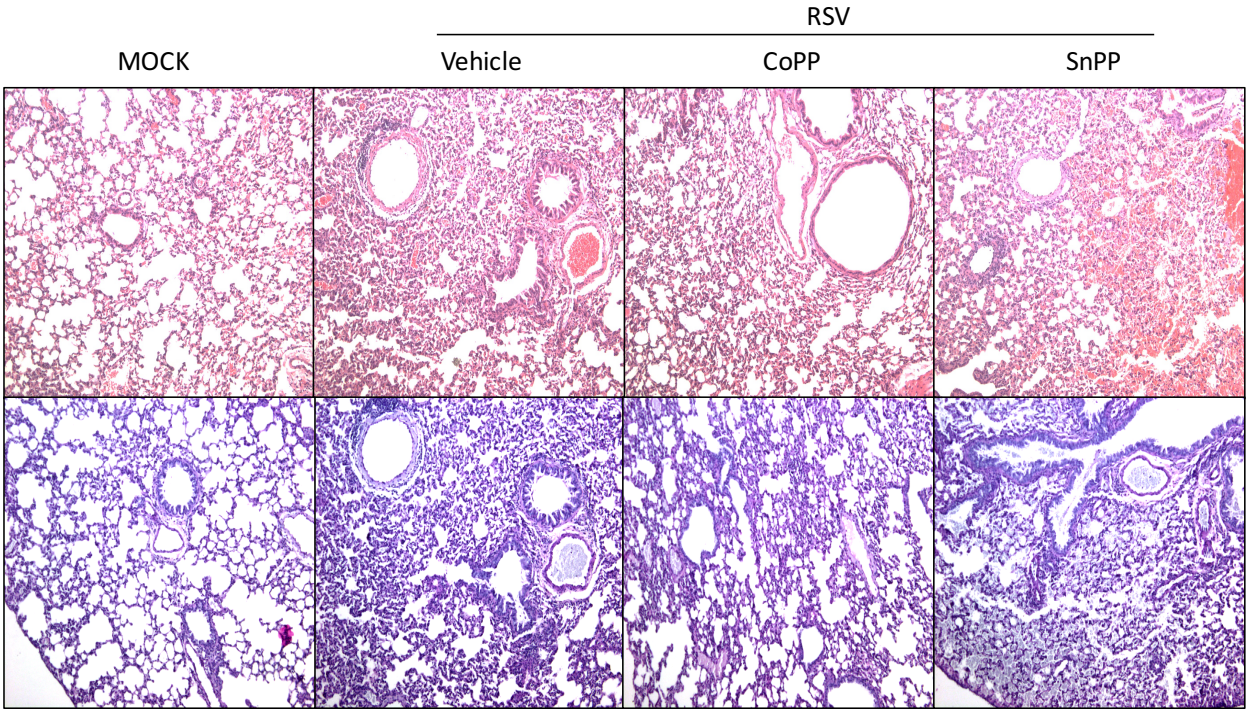

Figure 28. CoPP prevents lung inflammation in hRSV infected mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, the mice were infected with hRSV. Then, histopathology analyses of lung sections from each experimental group was performed at day 4 postinfection. MOCK, vehicle-treated, CoPP- treated and SnPP-treated and hRSV-infected mice for (upper panel) H&E and (bottom panel) PAS staining, original magnification 10X.

Next, we evaluate whether HO-1 induction mediated by CoPP treatment could prevent leukocyte recruitment in lungs after hRSV infection. BAL was collected at day 4 postinfection and evaluated by flow cytometry for leukocyte infiltration. We observed that hRSV infection caused significant infiltration of inflammatory cells into the airways of vehicle-treated mice (**Figure 29**). Further, we analyzed the frequency of total leukocytes ($CD45^+$) (**Figure 29**) and neutrophils (MHC-II negative [$MHC\text{-}II^-$] $CD11c^+$ $CD11b^+$ Ly-$6G^+$/Ly-$6C^+$) in the BAL of hRSV-infected mice (**Figure 30**). A significant reduction in the total cell counts of total leukocytes and neutrophils in BAL was observed for CoPP-pretreated mice upon hRSV challenge, at day 4 postinfection compared with vehicle-treated mice (**Figure 30**). However, SnPP-treated mice showed a higher number of neutrophils in BAL at day 4 post hRSV infection compared with vehicle-treated mice (**Figure 29**).

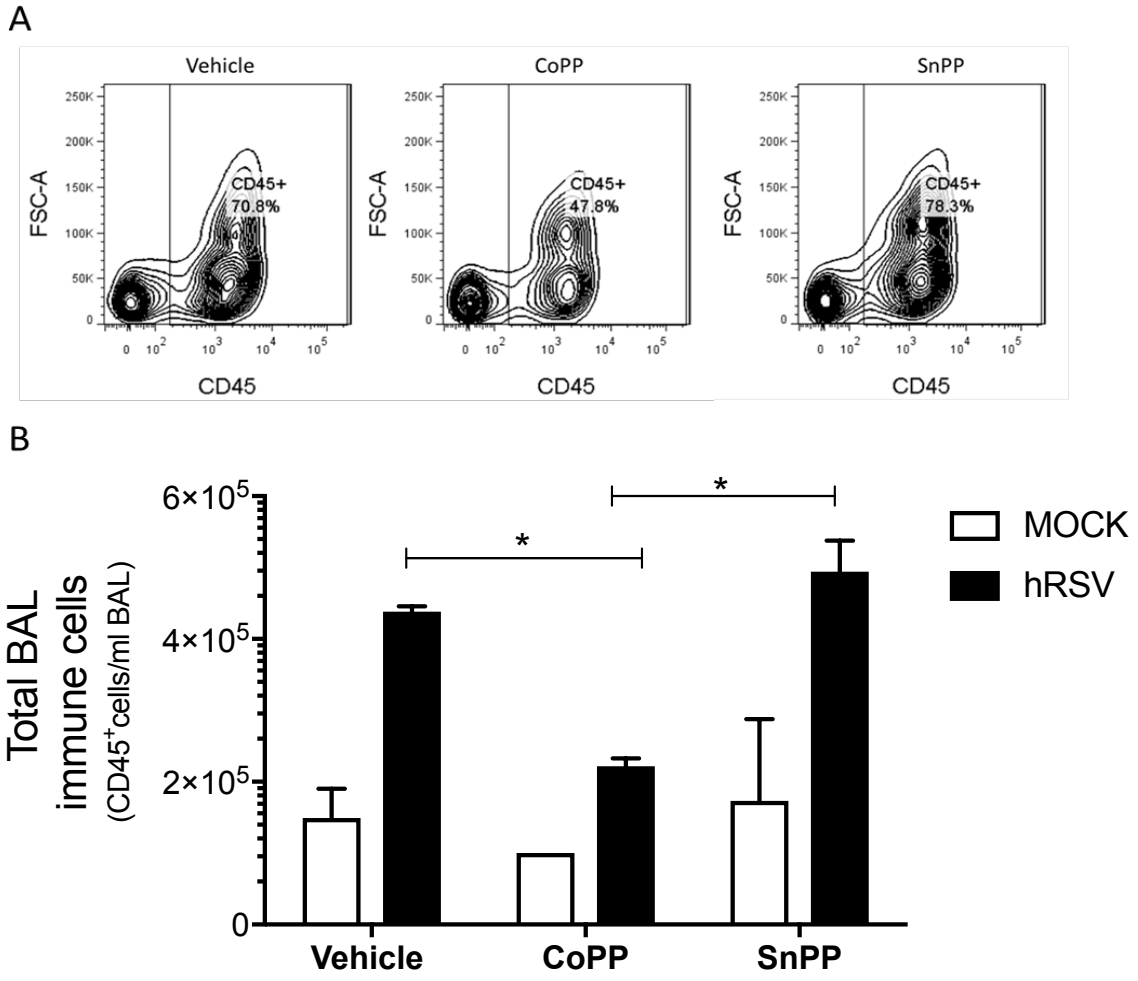

Figure 29. CoPP treatment prevents total leukocyte infiltration on airways of hRSV infected mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL was collected and analyzed by flow cytometry. (**A**) Representative dot plots of flow cytometry analysis for $CD45^+$ for vehicle, CoPP-, and SnPP-treated mice (upper panel). (**B**) Absolute number cell count (bottom panel) of total $CD45^+$ infiltration at day 4 postinfection in BAL for the mentioned conditions. Data shown are mean +/- SEM from three independent experiments, each with three mice per group (N= 9). Values were analyzed by 2way ANOVA and Tukey´s posttest (*$P < 0.05$).

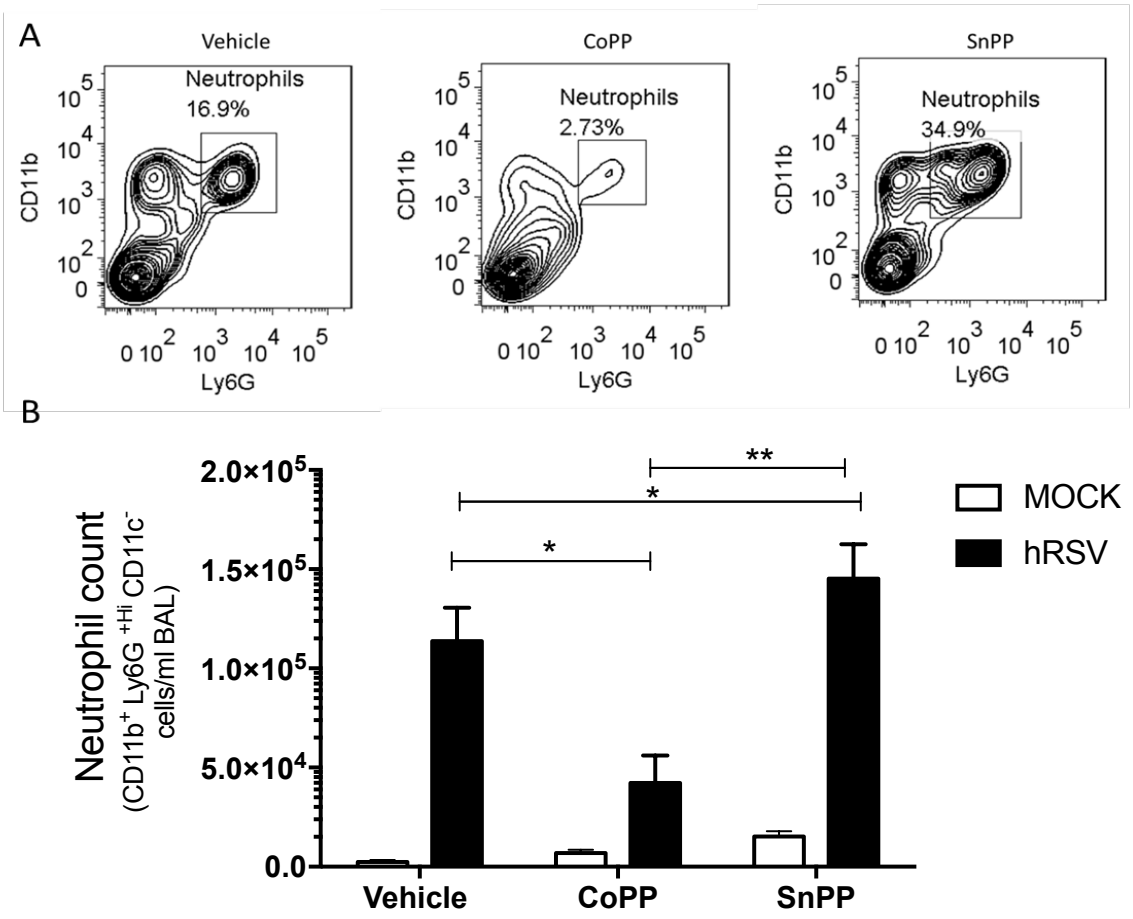

Figure 30. CoPP treatment prevents neutrophil infiltration on airways of hRSV infected mice. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL was collected and analyzed by flow cytometry. (**A**) Representative dot plots of flow cytometry analysis for neutrophils cell infiltration for vehicle, CoPP-, and SnPP-treated mice (upper panel). (**B**) Absolute number cell count (bottom panel) for neutrophil cell infiltration at day 4 postinfection in BAL (MHC-II$^-$ CD11c$^+$ CD11b$^+$ Ly-6G/Ly-6C$^+$) for the mentioned conditions. Data shown are mean +/- SEM from three independent experiments, each with three mice per group (N=9). Values were analyzed by 2way ANOVA and Tukey´s posttest (*P< 0.05, **P < 0.01, ***P < 0.0001).

6.2.4 Transgenic expression of human HO-1 in MHC-II+ cells impairs neutrophil infiltration and lung inflammation in hRSV infected mice.

To confirm the anti-inflammatory effect of HO-1 induction observed in hRSV infected mice treated with CoPP, transgenic and littermate mice were intranasally infected with $1x10^6$ PFUs of hRSV, then lungs and BAL were harvested at day 4 postinfection to evaluate lung inflammation and inflammatory cells infiltration respectively. Lung histological analyses were performed to evaluate whether the transgenic expression of human HO-1 could prevents inflammatory lung damage. As shown in **Figure 31**, transgenic mice treated with DOX displayed significantly less inflammation than hRSV-infected littermate controls and untreated transgenic mice (**Figure 31**). According to these data, we found a decreased neutrophil infiltration in the airways of tTA-HO-1 mice treated with DOX, as compared with littermates treated with DOX and infected with hRSV (**Figure 32**). Therefore, these data suggest that exogenous expression of human HO-1 in MHC-II+ cells prevents neutrophil infiltration and lung inflammation in the conditional tTA-HO-1 transgenic mice.

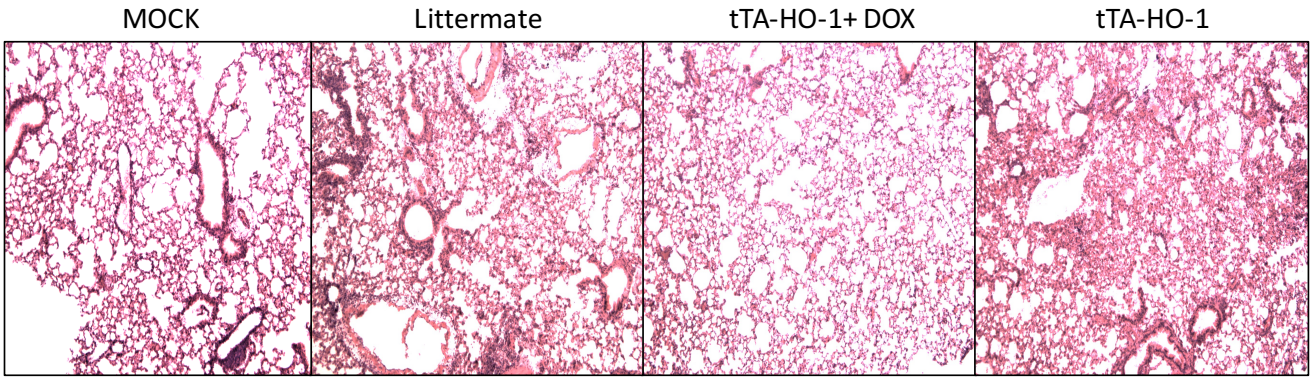

Figure 31. HO-1 overexpression in the MHC-II+ cell subset prevents inflammatory damage in lungs of hRSV infected mice. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 postinfection, histopathology analyses of lung sections for each experimental group were performed (H&E, original magnification X10).

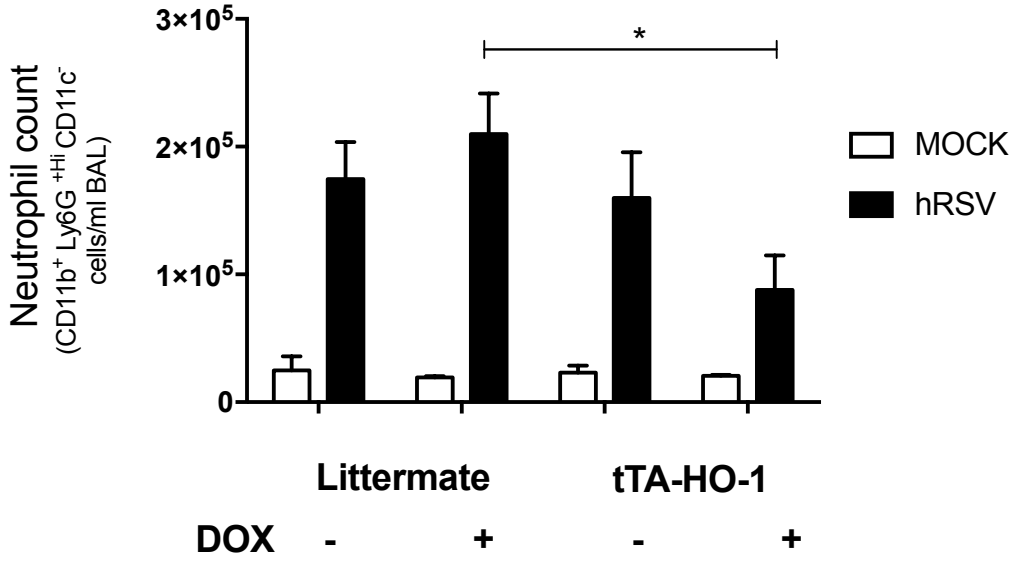

Figure 32. HO-1 overexpression in the MHC-II+ cell subset prevents neutrophil infiltration on airways of hRSV infected mice. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. Neutrophil infiltration was measured in BAL at day 4 postinfection. The figure shows the neutrophil absolute cell count infiltration (MHC-II$^+$ CD11c$^+$ CD11b$^+$ Ly-6G/Ly-6C$^+$) at day 4 in BAL for mock and hRSV-infected mice for each experimental group. Data shown are from two independent experiments, each with three mice per group (N = 6). Mann–Whitney U test (*P < 0.05, **P < 0.01).

6.3 Evaluation of the mechanism behind HO-1 protective role against hRSV infection.

6.3.1 CoPP impairs hRSV –inducible cytokines and chemokines secretion and promotes IL-10 secretion in airways from hRSV infected mice.

To address whether the described anti-inflammatory effects of HO-1 in the course of hRSV infection were associated with the modulation of cytokines or chemokines secretion in the airways, protein levels of IL-6 (**Figure 33**), IL-4 (**Figure 34**), IFN-γ (**Figure 35**), IL-10 (**Figure 36**), CCL3/MIP-1a (**Figure 37**), and CXCL1/KC (an IL-8 homolog) (**Figure 38**) were measured by ELISA in BAL of infected and MOCK-treated mice for each experimental group. Overall, the protein concentrations of all hRSV-inducible cytokines (IL-6, IL-4, and IFN-γ; **Figure 33-35**) and chemokines (CCL3/MIP-1α and CXCL1/KC; **Figure 37 and 38**) decreases in mice treated with CoPP, as compared with vehicle-treated mice. Conversely, CoPP-treated mice displayed increased secretion of the immunomodulatory cytokine IL-10, even in mock controls (**Figure 36**). Interestingly, SnPP-treated mice showed increased levels of CCL3/ MIP-1α (**Figure 37**), suggesting that the increase in neutrophil recruitment can be mediated by a higher concentration of this chemokine.

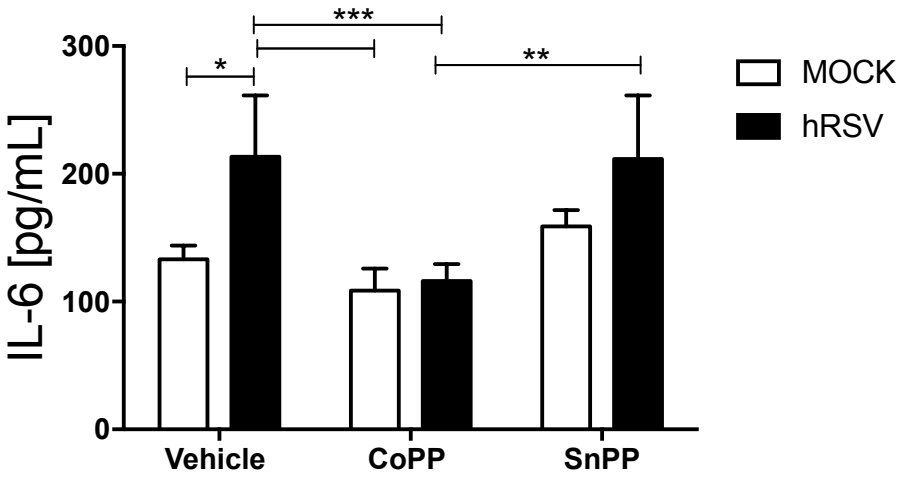

Figure 33. CoPP treatment inhibits hRSV-induced IL-6 secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of IL-6. Data shown are mean +/- SEM of three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N= 9, * P < 0.05, **P < 0.001, ***P < 0.0001).

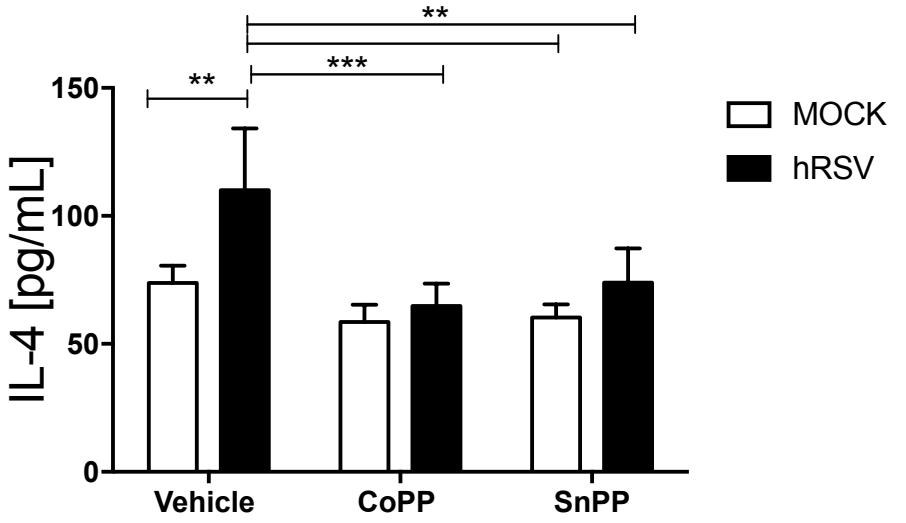

Figure 34. CoPP treatment inhibits hRSV-induced IL-4 secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of IL-4. Data shown are mean +/- SEM of three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9, *P < 0.05, **P < 0.001, ***P < 0.0001).

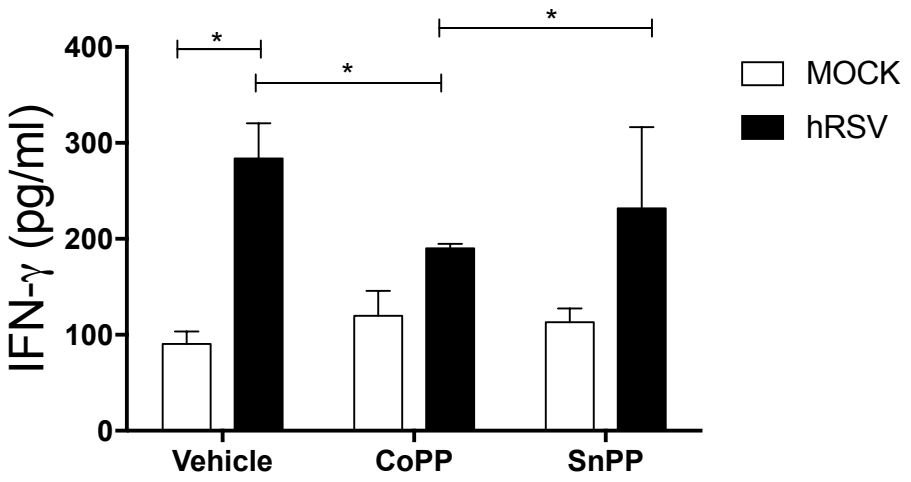

Figure 35. CoPP treatment impairs IFN-γ secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of IFN-γ. Data shown are mean+/- SEM of three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest t (N=9; *P < 0.05, **P < 0.001, ***P < 0.0001).

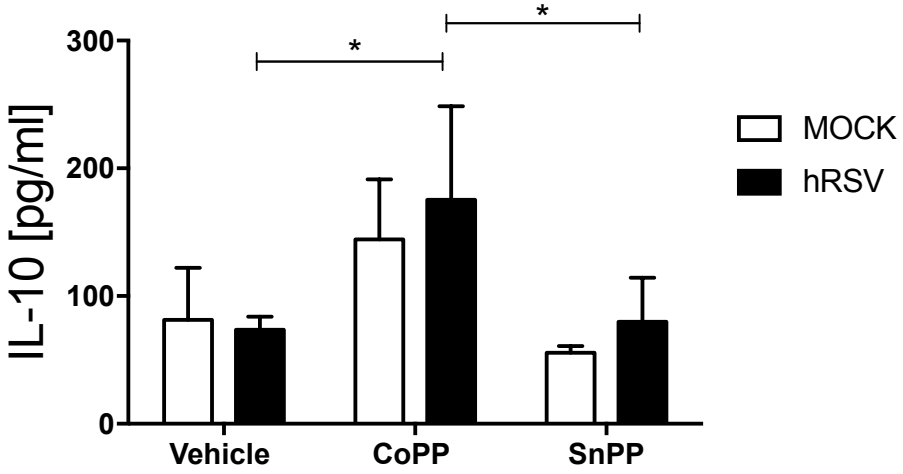

Figure 36. CoPP treatment promotes IL-10 secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of IL-10. Data shown are mean+/- SEM of three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05, **P < 0.001, ***P < 0.0001).

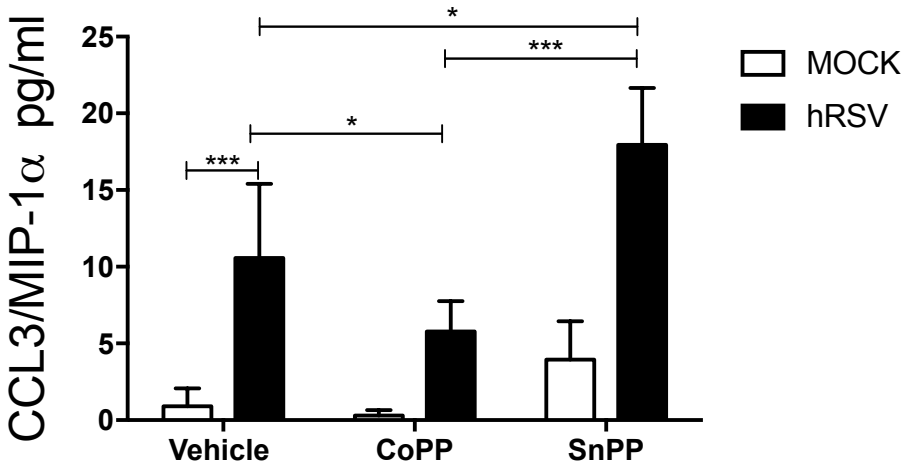

Figure 37. CoPP treatment inhibits hRSV-induced CCL3/MIP-1α secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of CCL3/MIP-1α. Data shown are mean +/- SEM of three independent experiments.Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9, *P < 0.05, **P < 0.001, ***P < 0.0001).

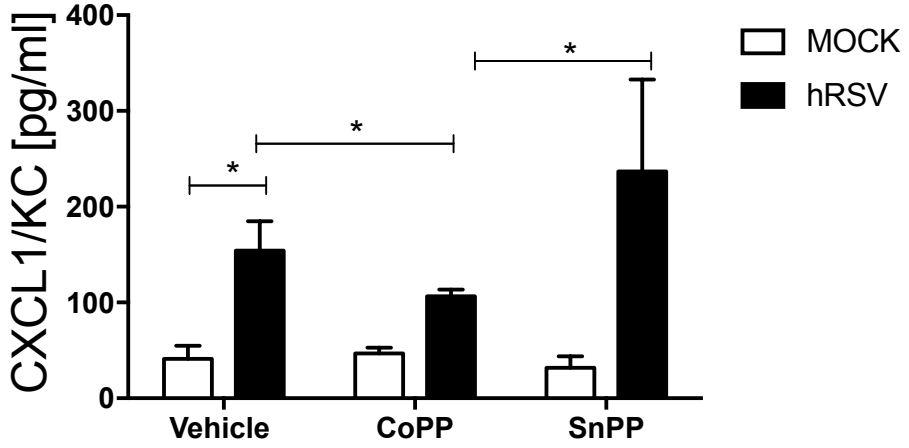

Figure 38. CoPP treatment inhibits hRSV-induced CXCL1/KC secretion in the airways of mice experimentally infected with hRSV. BALB/cJ mice were treated with CoPP, SnPP or vehicle. After 24 h, mice were infected with hRSV. At day 4 postinfection, BAL samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of CXCL1/KC. Data shown are mean +/- SEM of three independent experiments.Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05, **P < 0.001, ***P < 0.0001).

6.3.2 Transgenic expression of human HO-1 in MHC-II+ cells reduces hRSV – inducible cytokines and chemokines secretion and promotes IL-10 secretion in airways from hRSV infected mice.

To confirm that HO-1 induction impairs the secretion of proinflammatory cytokines and chemokines and promotes IL-10 secretion in airways, as we observed in CoPP treated mice. Transgenic and littermate mice were intranasally infected with $1x10^6$ PFUs of hRSV, then BAL were collected at day 4 postinfection to measure cytokine and chemokine protein levels by ELISA in each experimental group. In a similar manner that CoPP treated mice, transgenic mice showed a decrease in IL-6 (**Figure 39**), IL-4 (**Figure 40**) and the chemokine CCL3/MIP-1α (**Figure 41**). Furthermore, tTA-HO-1 mice showed an increase in IL-10 secretion in a similar manner that CoPP-treated mouse (**Figure 42)**. All these data, suggest that HO-1 expression induces an anti-inflammatory effect associated with IL-10 production and the concomitant reduction in proinflammatory cytokines and chemokines.

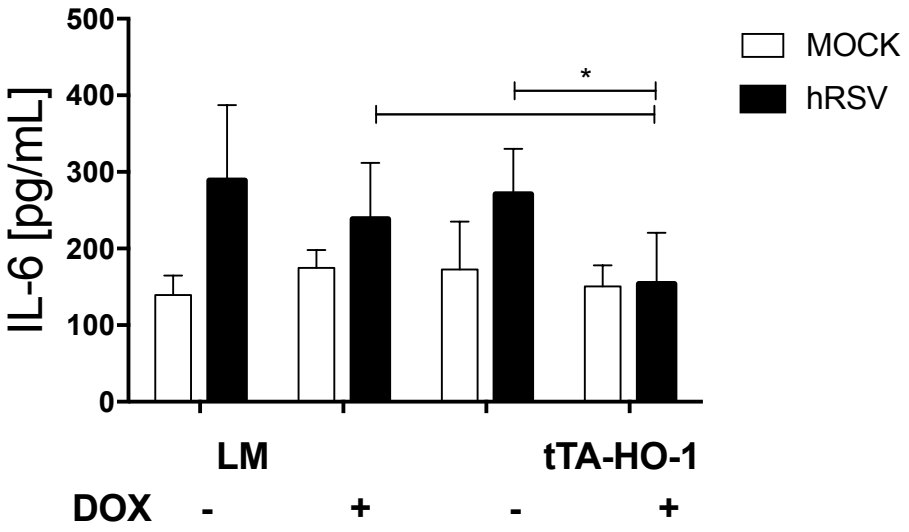

Figure 39. HO-1 overexpression in the MHC-II+ cell subset inhibits IL-6 secretion in the airways of mice experimentally infected with hRSV.tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, BAL samples were obtained from mice from each experimental group to measure concentrations of IL-6. Data shown are mean+/- SEM of two independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05).

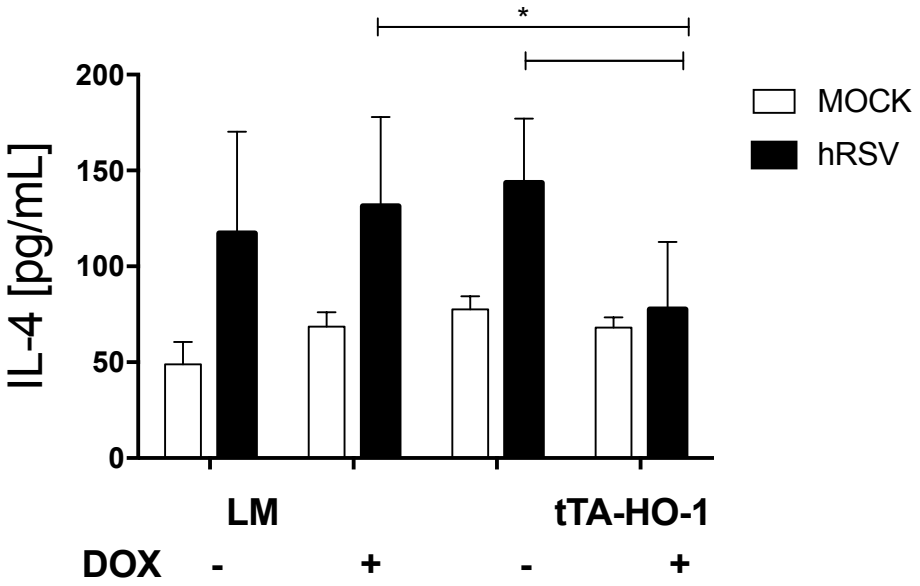

Figure 40. HO-1 overexpression in the MHC-II+ cell subset inhibits IL-4 secretion in the airways of mice experimentally infected with hRSV. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, BAL samples were obtained from mice from each experimental group to measure concentrations of IL-4. Data shown are mean+/- SEM of two independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05).

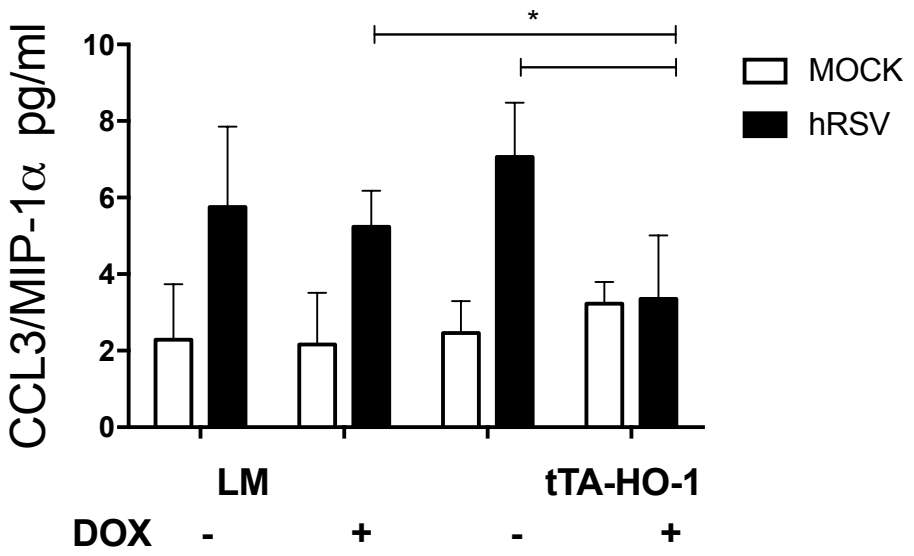

Figure 41. HO-1 overexpression in the MHC-II+ cell subset inhibits CCL3/MIP-1α secretion in the airways of mice experimentally infected with hRSV. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, BAL samples were obtained from mice from each experimental group to measure concentrations of CCL3/MIP-1α. Data shown are mean+/- SEM of two independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05).

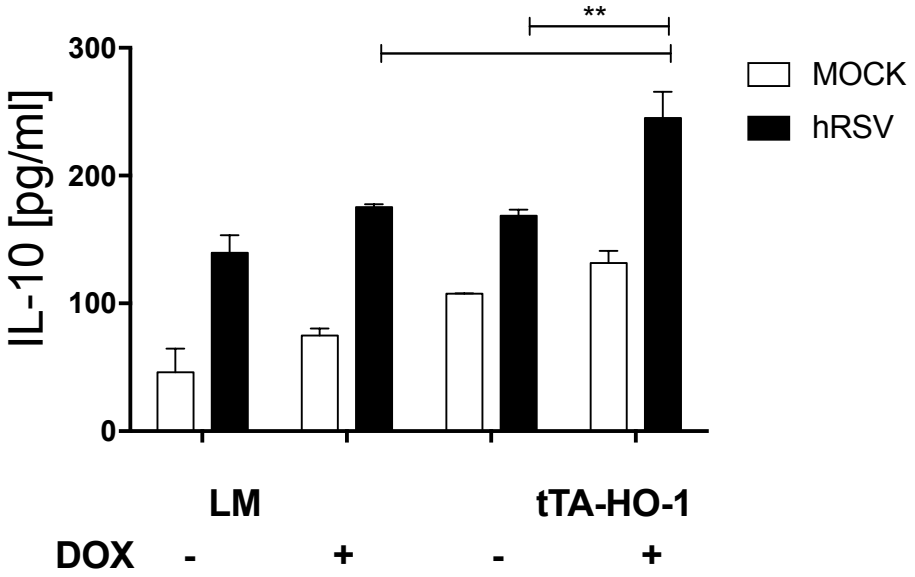

Figure 42. HO-1 overexpression in the MHC-II+ cell subset promotes IL-10 secretion in the airways of mice experimentally infected with hRSV. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, BAL samples were obtained from mice from each experimental group to measure concentrations of IL-10. Data shown are mean+/- SEM of two independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05, **P < 0.001).

6.3.3 Pharmacological induction of HO-1 increases antiviral type I IFN response during hRSV infection *in vivo*.

HO-1 induction has been reported to induce IFN responses against virus replication (Lehmann et al., 2010). To investigate whether anti-hRSV effect of HO-1 is mediated by antiviral IFN responses, we evaluated whether CoPP-mediated HO-1 induction could promote an antiviral type I IFN response *in vivo* during hRSV infection. Expression of IFN-α and β was measured in lungs from both hRSV-infected and control mice by RT-qPCR. The results of the RT-qPCR analysis indicated that CoPP treatment increased mRNA levels of IFN-α (**Figure 43**) and IFN-β (**Figure 44**) in the lungs of hRSV infected mice at day 4 postinfection. Also, CoPP treatment also increased IFN-α/β mRNA expression in the lungs of mock (uninfected) control animals. Interestingly, SnPP treatment failed to modulate this type I IFN response. These data suggest that CoPP by itself induces an antiviral state in airway cells and that the upregulation of IFN-α/β requires HO-1activity.

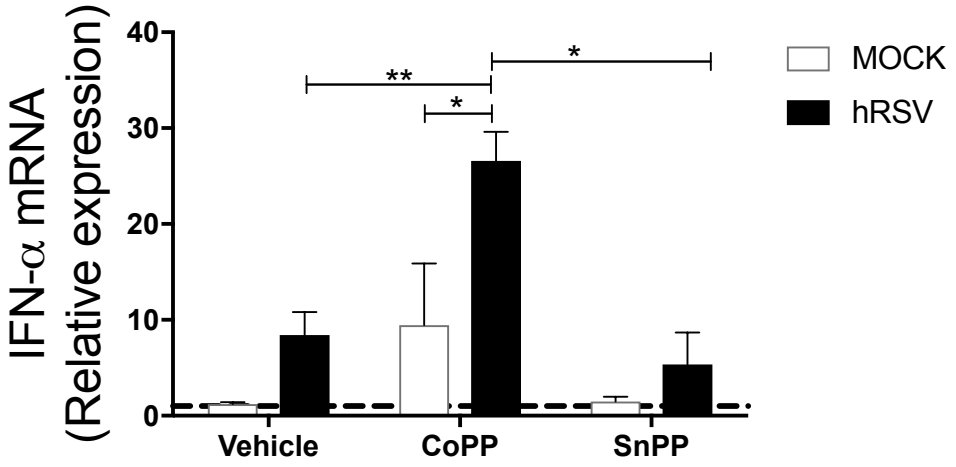

Figure 43. CoPP treatment promotes the upregulation of IFN α in lungs. BALB/cJ mice were treated for 24 h with CoPP, SnPP, or vehicle then inoculated intranasally, either with mock or hRSV (1x 10^6 PFU). RNA from lungs of each experimental group was collected at day 4 and analyzed by RT-qPCR for IFN-α mRNA levels. IFN-α relative mRNA expression levels for vehicle and CoPP or SnPP pharmacological-treated experimental groups. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05, **P < 0.001).

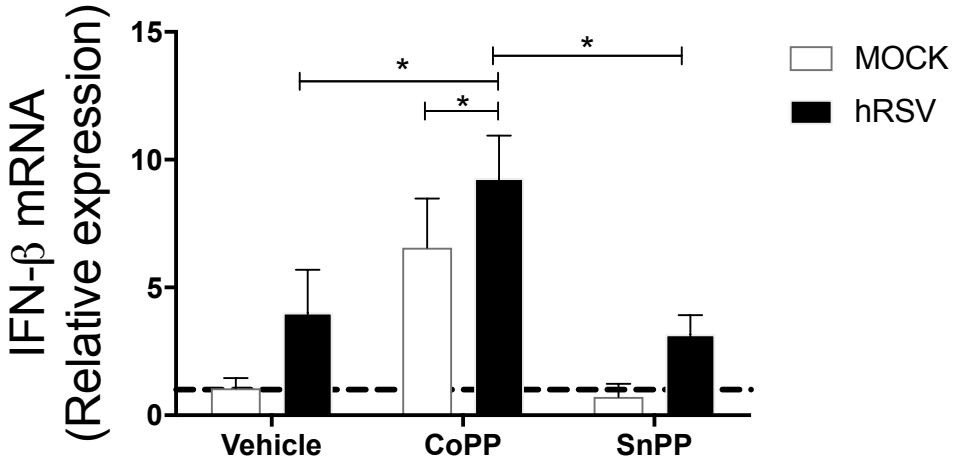

Figure 44. CoPP treatment promotes the upregulation of IFN- β in lungs. BALB/cJ mice were treated for 24 h with CoPP, SnPP, or vehicle then inoculated intranasally, either with mock or hRSV (1x 10^6 PFU). RNA from lungs of each experimental group was collected at day 4 and analyzed by RT-qPCR for IFN-β mRNA levels. IFN-β relative mRNA expression levels for vehicle and CoPP or SnPP pharmacological-treated experimental groups. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05, **P < 0.001).

6.3.4 Transgenic expression of human HO-1 in MHC-II+ cells increases antiviral type I IFN response during hRSV infection *in vivo*.

To evaluate whether HO-1 expression increases the antiviral IFN response independent of the CoPP treatment, we evaluated whether the exogenous expression of HO-1 in the transgenic mice promote an antiviral type I IFN response *in vivo* during hRSV infection. Expression of IFN-α and β was measured in lungs from both hRSV-infected and control mice by RT-qPCR. The data of the RT-qPCR analysis indicated that mRNA levels of IFN-α (**Figure 45**) and IFN-β (**Figure 46**) are increased in the lungs of hRSV infected tTA-HO-1 mice treated with DOX at day 4 postinfection. In addition, transgenic mice showed an increase in IFN-α/β mRNA expression in the lungs of mock (uninfected) control animals. These data suggest that HO-1 induces an antiviral state in airway cells and that the upregulation of IFN-α/β is independent of CoPP pharmacological effect.

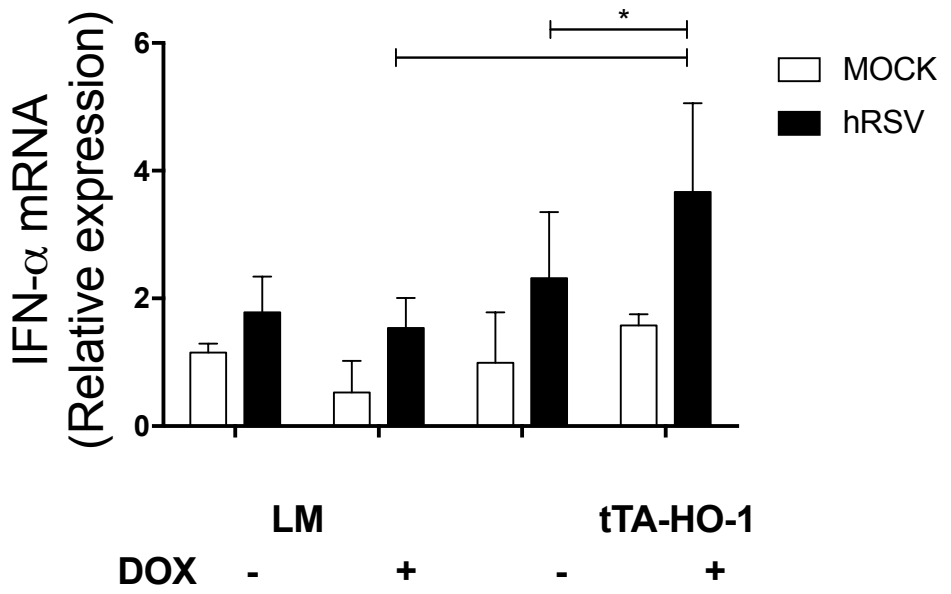

Figure 45. HO-1 overexpression in the MHC-II+ cell subset promotes the upregulation of IFN-α in lungs. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, RNA from lungs of each experimental group was collected at day 4 and analyzed by RT-qPCR for IFN-α mRNA levels. Data in graph shows the IFN-α relative mRNA expression levels for each experimental group. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05).

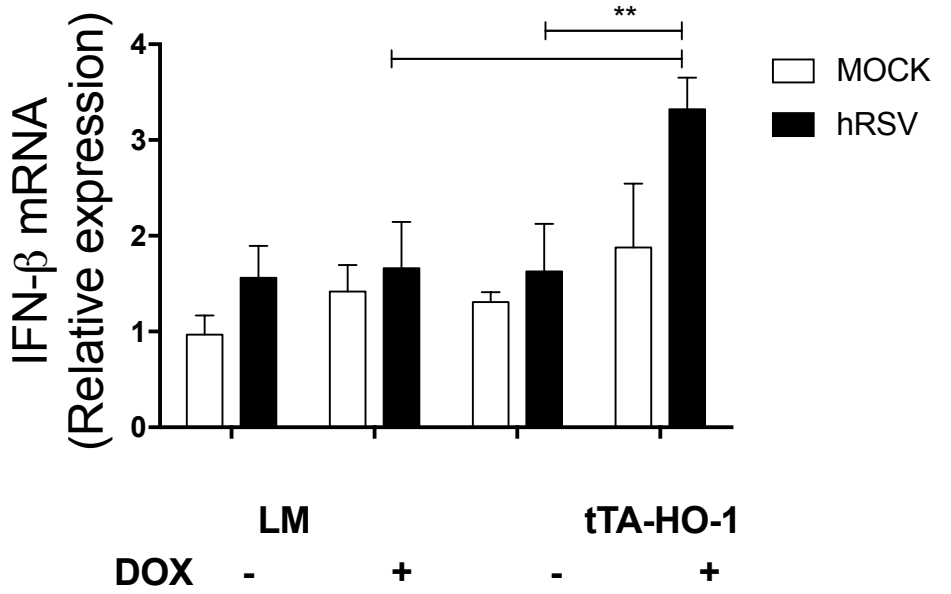

Figure 46. HO-1 overexpression in the MHC-II+ cell subset promotes the upregulation of IFN-β in lungs. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water. At day 4 post-infection, RNA from lungs of each experimental group was collected at day 4 and analyzed by RT-qPCR for IFN-β mRNA levels. Data in the graph shows the IFN-β relative mRNA expression levels for each

experimental group. Data were analyzed 2way ANOVA and Tukey´s posttest (N=9; **P < 0.001).

6.3.5 Pharmacological induction of HO-1 slightly impairs T cell activation and function during hRSV infection

HO-1 exerts several immunomodulatory effects on T cell–mediated adaptive response, by impairing T cell activation, proliferation, and their effector functions. To evaluate whether CoPP-mediated HO-1 induction modulates T cell responses during hRSV infection, single-cell suspensions were obtained from mediastinal lymph nodes of each experimental group and were stimulated for 72 h with ultraviolet-hRSV or with anti-CD3ε/CD28 (1 mg/ml) or left untreated. T cell activation in single-cell suspensions was determined by CD69 expression in $CD4^+$ and $CD8^+$ T cells as a parameter for early activation. A significant increase in CD69 expression was observed in both $CD4^+$ (**Figure 47**) and $CD8^+$ (**Figure 48**) T cells stimulated with a polyclonal stimulus (anti-CD3ε/CD28) for all treatments. However, CoPP treatment led to a reduction of CD69 expression on anti-CD3ε/CD28-stimulated cells (**Figure 47 and 48**). No significant changes were observed in ultraviolet-hRSV stimulation, as evidenced by a non-significant increase in CD69 expression, which had been previously described by our group. In agreement with these data, a significant amount of IFN-γ was secreted in response to anti-CD3ε/CD28 stimulation with a mild decrease in the CoPP treatment (**Figure 49**).

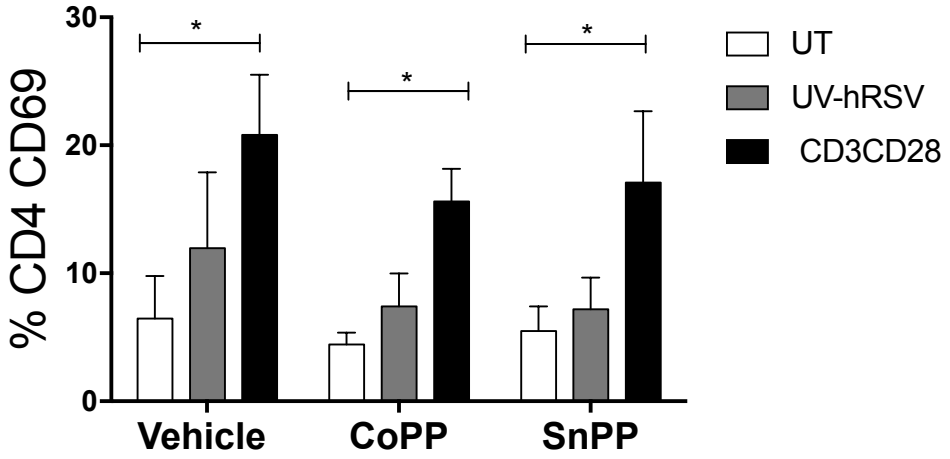

Figure 47. CoPP treatment slightly impairs CD4+ T cell activation during hRSV infection. BALB/cJ mice were treated with CoPP, SnPP or vehicle for 24 h, then were intranasally infected with hRSV. At day 4 postinfection, single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell activation in infected mice treated with vehicle, CoPP, or SnPP. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28, or left unstimulated (UT) for 72 h. Then, cells were analyzed by flow cytometry to measure surface expression of $CD69^+$ in $CD4^+$ cells. Data in graph percentage of CD69 expression by $CD4^+$T cells derived from each experimental group. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=6; *P < 0.05).

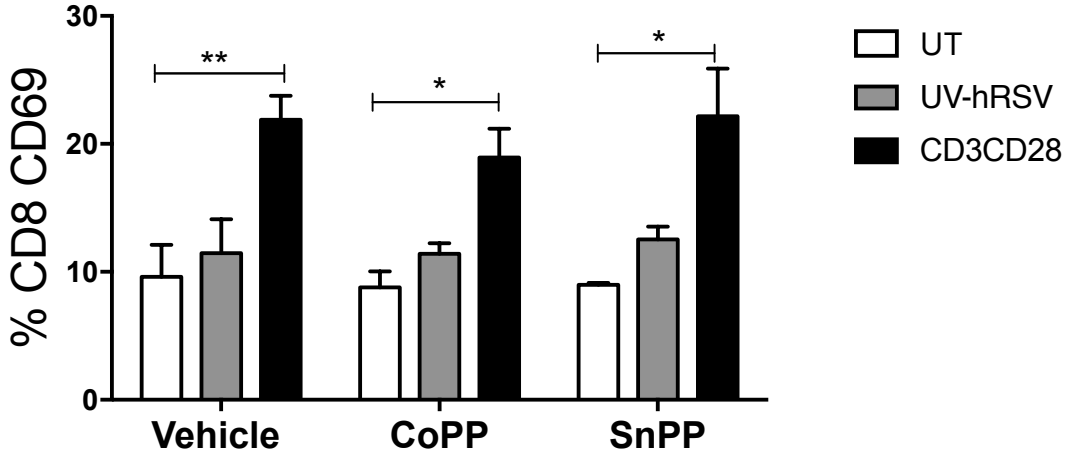

Figure 48. CoPP treatment slightly impairs CD8+ T cell activation during hRSV infection. BALB/cJ mice were treated with CoPP, SnPP or vehicle for 24 h, and then were intranasally infected with hRSV. At day 4 postinfection, single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell activation in infected mice treated with vehicle, CoPP, or SnPP. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28, or left UT for 72 h. Then, cells were analyzed by flow cytometry to measure the surface expression of $CD69^+$ in $CD8^+$ cells. Data in graph percentage of $CD69^+$ expression by $CD8^+$T cells derived from each experimental group. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05).

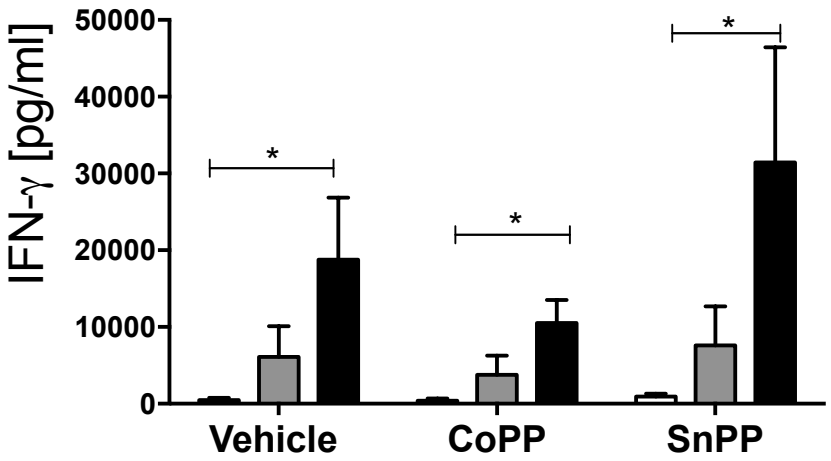

Figure 49. CoPP treatment slightly impairs IFN-γ secretion during hRSV infection. BALB/cJ mice were treated with CoPP, SnPP or vehicle for 24 h, then were intranasally infected with hRSV. At day 4 postinfection, single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell function in infected mice treated with vehicle, CoPP, or SnPP. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28, or left UT for 72 h. Then, the collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28 or left unstimulated for 72 h. IFN-γ secretion was measured by ELISA in the supernatant of lymph nodes at 72 h poststimulation with hRSV or anti-CD3ε/CD28. Data shown are mean +/- SEM from three independent experiments. Data were analyzed by 2way ANOVA and Tukey´s posttest (N=9; *P < 0.05).

6.3.6 Transgenic expression of human HO-1 in MHC-II+ cells modulates T cell function during hRSV infection

Further, we evaluate whether the exogenous expression of HO-1 in $MHCII^+$ cells in the conditional transgenic mice model could impair the function of APCs and their ability to process and present Ags to T cells. First, we evaluated the maturing capacity of DCs from tTA-HO-1. tTAHO-1 DCs were treated with vehicle, CoPP, or DOX for 2 h. Cells were washed, and LPS (1 mg/ml) was added to the medium to induce maturation. After 16 h, DC maturation was measured by surface expression of $CD80^+$, $CD40^+$, and $CD86^+$ molecules by flow cytometry. Overall, all assessed surface markers were upregulated in LPS-pulsed DCs, suggesting that HO-1 did not alter the maturation process of DCs in conditional transgenic mice after HO-1 induction by DOX (**Figure 50**).

To evaluate whether the ability of DCs to process and present Ags to T cells could be altered by the transgenic expression of HO-1, OVA-pulsed mature DCs from tTA-HO-1 mice were cocultured with OT-II $CD4^+$ T cells, which recognize the OVA-derived peptide OVA323–339/I-Ab complex as a cognate ligand. Thus, LPS-DCs treated with CoPP showed impairment in T cell activation after 48 h of coculture, suggesting that CoPP pretreated DCs were unable to prime OT-II T cells (**Figure 51**). On the contrary, mature DCs from the conditional transgenic mice that were treated with DOX were capable of processing and presenting OVA-derived peptides, resulting in OT-II T cell priming. However, the activation of OT-II T cells in the coculture with DCs from conditional transgenic mice treated with DOX was lower compared with cocultures between vehicle-treated DCs and OT-II cells (**Figure 51**), suggesting that the expression of HO-1 in DCs from conditional transgenic mice slightly affects the T cell priming.

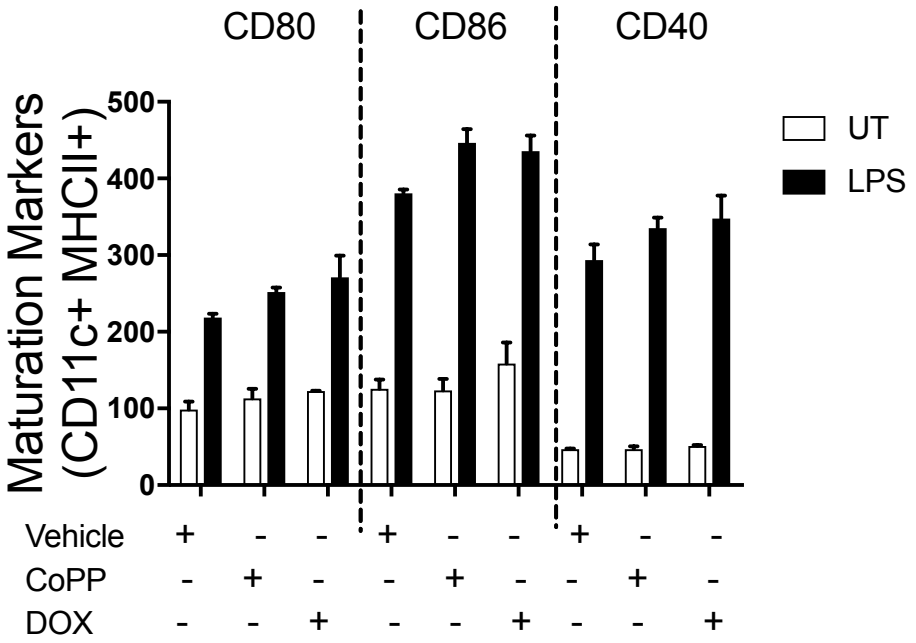

Figure 50. DCs from tTA-HO-1 conditional transgenic mice have a normal maturation process. DCs derived from bone marrow progenitors from tTA-HO-1 mice were stimulated with LPS for 16 h in the presence or absence of CoPP, DOX or vehicle to the evaluated maturation process. Flow cytometry analyses of surface expression of the maturation markers $CD40^+$, $CD80^+$, and $CD86^+$ in LPS-stimulated or unstimulated DCs. Data were expressed as MFI. Data shown are means +/- SEM of two independent experiments. (N=6).

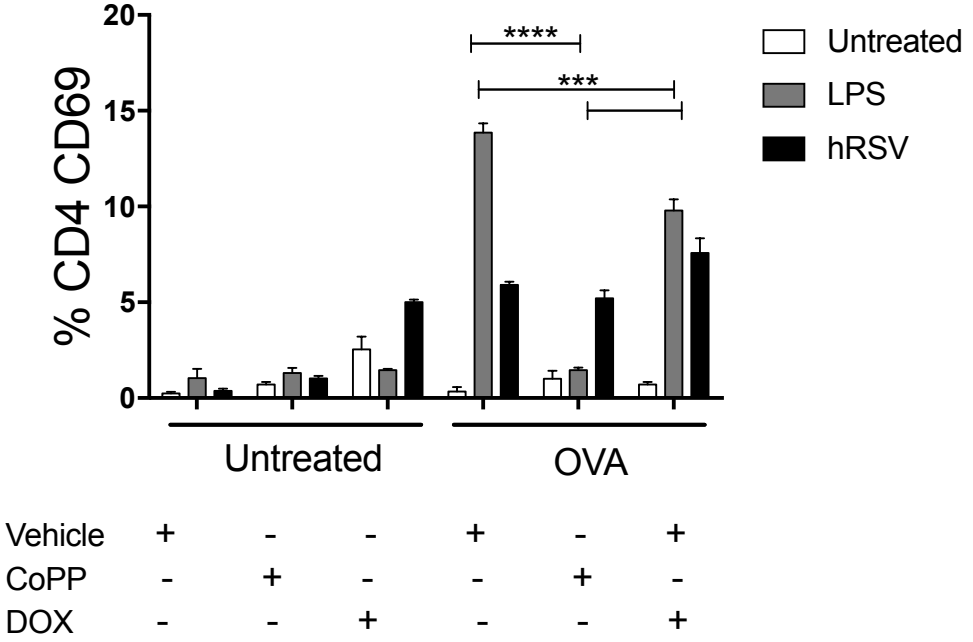

Figure 51. DCs from tTA-HO-1 conditional transgenic mice are impaired for antigen processing/presentation. tTA-HO-1 DCs were stimulated with OVA in the presence or absence of CoPP, DOX or vehicle 24 h. Next, OVA-DCs were co-cultured with OT-II T cells for 48 h, and surface expression of CD69 was evaluated by flow cytometry. Results in the graph are the percentages of $CD4^+/CD69^+$ cells. Data shown are means +/- SEM of two independent experiments. Values were analyzed by 2way ANOVA and Tukey posttest (N=6; ***$P < 0.0001$).

Moreover, to determine whether expression of HO-1 in MHC-II+ cells modulates T cells priming *in vivo* during hRSV infection, littermate, untreated or DOX-treated tTA-tHO-1 mice were infected with hRSV and 4 d postinfection. Then, single-cell suspensions were obtained from mediastinal lymph nodes and stimulated for 72 h with ultraviolet-hRSV or with anti-CD3ε/CD28(1 mg/ml) or left untreated. To address T cell activation in the cells suspensions, CD69 expression was measured in CD4+T cells. No significant increases in CD4 or CD69 expression were observed for cell suspensions from anti-CD3ε/CD28-stimulated DOX-induced transgenic mice as compared with unstimulated DOX-induced

transgenic mice, suggesting that T cell activation is affected by HO-1 overexpression in MHC-II+ cells (**Figure 52**). In agreement with these data, no significant IFN-γ secretion could be measured in supernatants of cell suspensions derived from DOX-induced transgenic mice (**Figure 53**), suggesting that T cell function can also be modulated by HO-1 overexpression in MHC-II+ cells.

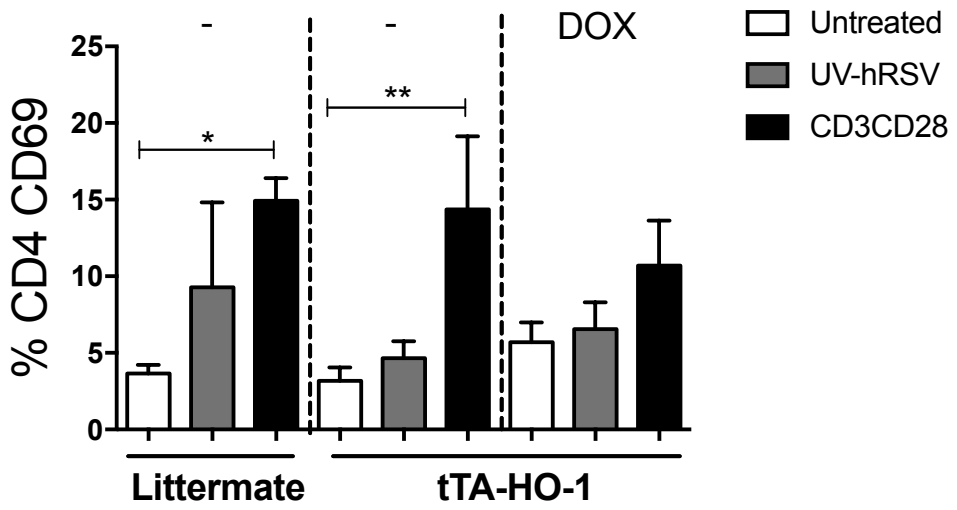

Figure 52. Exogenous HO-1 expression in MHC-II+ cells of conditional transgenic mice impairs CD4+ T-cell activation during hRSV infection. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water of hRSV infected transgenic mice (tTA-HO-1). Single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell activation, in the conditional transgenic mice during hRSV infection. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28 or left unstimulated for 72 h. Data in graph shows flow cytometry detection of $CD69^+$ expression on $CD4^+$ T cells derived from mice with each treatment. Data shown are mean +/- SEM from two independent experiments. Values were analyzed by 2way ANOVA and Tukey posttest (N=6, *P < 0,5 **P < 0.01).

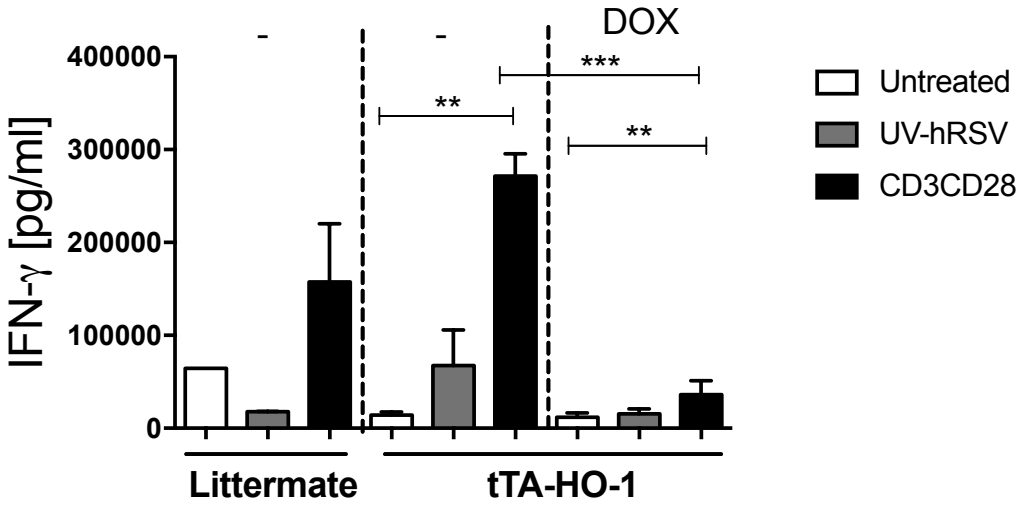

Figure 53. Exogenous HO-1 expression in MHC-II+ cells of conditional transgenic mice impairs IFN-γ secretion during hRSV infection. tTA-HO-1 and littermate mice were infected with hRSV. DOX and sucrose were added to the drinking water of hRSV infected transgenic mice (tTA-HO-1). Single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell response, in the conditional transgenic mice during hRSV infection. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28 or left unstimulated for 72 h. IFN-γ secretion was measured by ELISA in the supernatant of lymph nodes at 72 h poststimulation with each treatment. Data shown are mean +/- SEM from two independent experiments. Values were analyzed by 2way ANOVA and Tukey posttest (N=6, **P < 0.01, ***P < 0.0001).

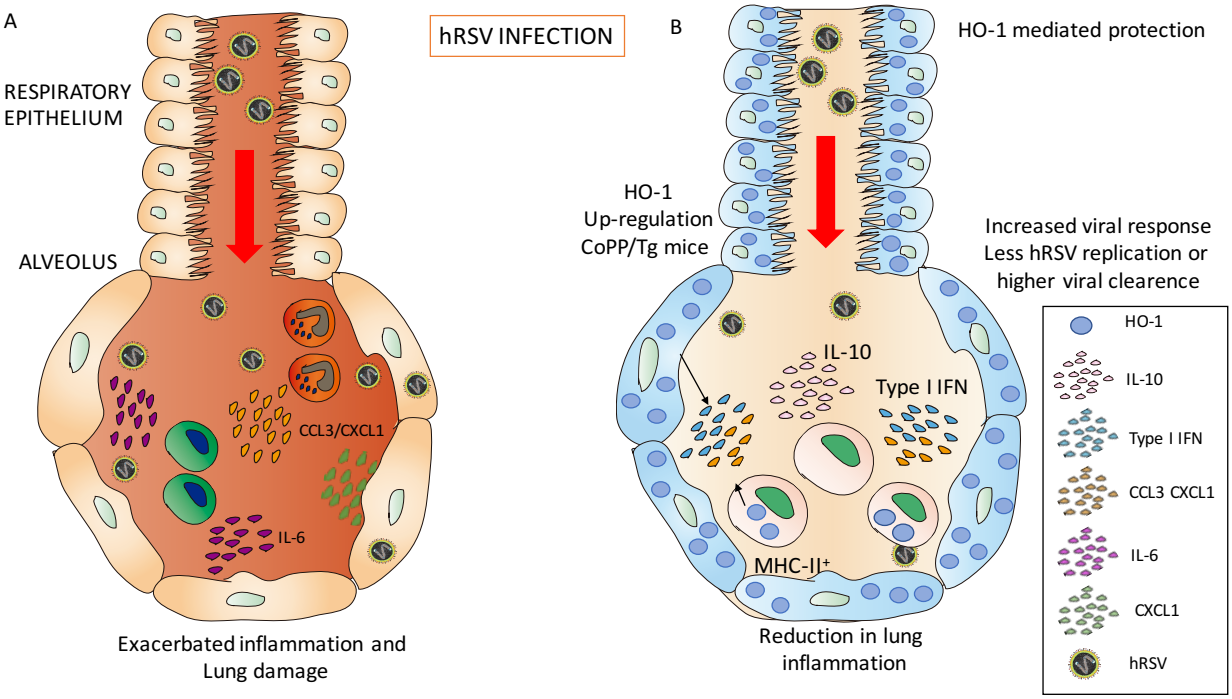

Figure 54. Summary of main results for hypothesis 1. Figure (**A**) shows the exacerbated inflammation in lung alveoli after hRSV infection according to the main results obtained in this thesis. Figure (**B**) shows the protective effects of HO-1 upregulation on lung inflammation and viral replication of hRSV mediated by IFN type 1 response and IL-10 secretion (original figure).

6.4 Evaluation of *in vitro* effects of Panx1 activity on hRSV infection.

6.4.1Panx1 expression is increased during hRSV infection *in vitro*.

Panx1 expression was examined by RT-qPCR in A549 cells (**Figure 55**) and DCs (**Figure 56**) treated with MOCK, hRSV or UV-hRSV for 48 h. We found high levels of Panx1 in both cultures and a significantly increased mRNA expression after hRSV infection. Conversely, UV inactivated hRSV does not increase Panx1 levels. In addition, the effect of hRSV infection on Panx1 expression was evaluated in A549 cells infected at three different MOIs for 48 h (**Figure 57**). Our data showed a significant increase of Panx1 mRNA levels for infected cells with a MOI between 1 and 5. However, at MOI 25 we observed a slight reduction in the Panx1 expression compared with MOI 5 (**Figure 57**). These results suggest that epithelial lung cells and DC can be affected by hRSV infection by increasing the expression of Panx1 mRNA.

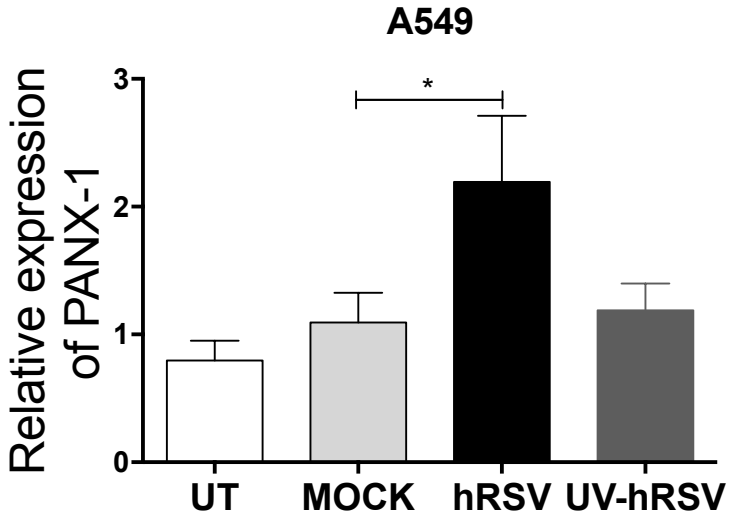

Figure 55. Panx1 mRNA is increased in hRSV infected A549 cells. A549 cells were infected with hRSV at MOI 1 (black bar) or treated with MOCK (light gray) or UV-hRSV (dark gray) for 48 h. After the incubation period, RNA from A549 cells of each experimental group was collected and analyzed by RT-qPCR for Panx1 mRNA levels. Data in the graph shows the Panx1 relative mRNA expression levels for each experimental group, with β-actin as housekeeping (UT: Untreated). Data shown are mean +/- SEM from one experiment. (one-way ANOVA and Tukey´s multiple comparation test (N=3; *P < 0.05))

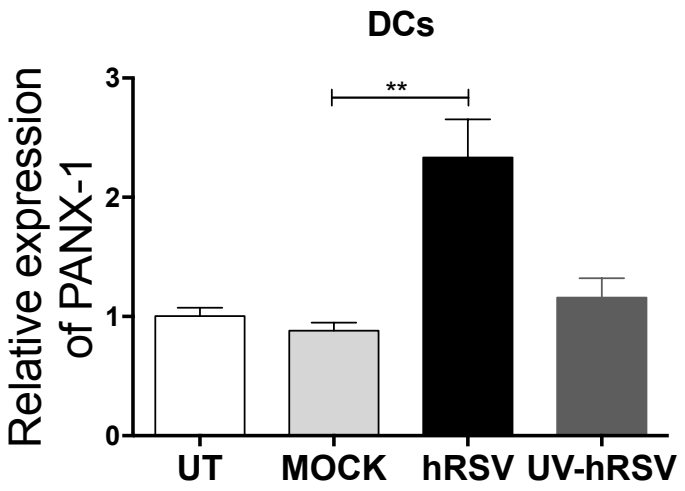

Figure 56. Panx1 mRNA is increased in hRSV infected DCs. DCs were infected with hRSV at MOI 1 (black bar) or treated with MOCK (light gray bar) or UV-hRSV (dark gray bar) for 48 h. After the incubation period, RNA from DCs of each experimental group was collected and analyzed by RT-qPCR for Panx1 mRNA levels. Data in the graph shows the Panx1 relative mRNA expression levels for each experimental group, with β-actin as housekeeping (UT: Untreated). Data shown are mean +/- SEM from one experiment. (one-way ANOVA and Tukey´s multiple comparation test (N=3; *P < 0.05))

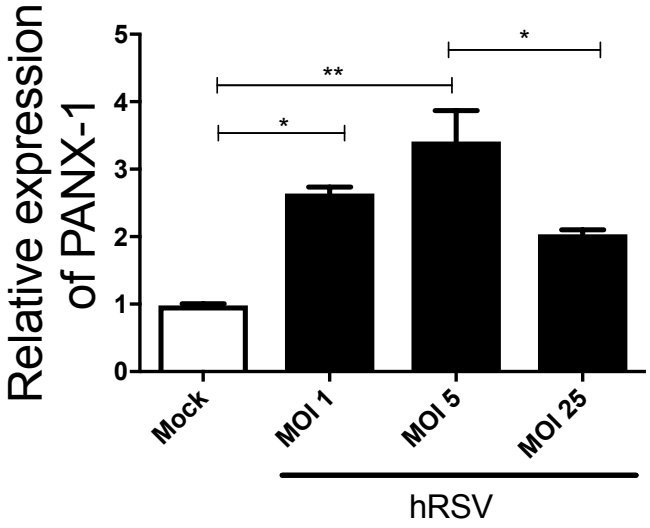

Figure 57. hRSV infection induces Panx1 mRNA increases in a MOI dependent manner. A549 cells were infected with hRSV at different MOIs for 48 h. After the incubation period, RNA form A549 cells were collected and analyzed by RT-qPCR for Panx1 mRNA levels. Data in the graph shows the Panx1 relative mRNA expression for each experimental group. Data shown are mean +/- SEM from one experiment. (one-way ANOVA and Tukey´s multiple comparation test (N=3; *P < 0.05))

6.4.2 hRSV infection induces Panx1 channel opening.

To determine whether hRSV modifies Panx1 channel activity, PBMCs isolated from healthy donors were infected with hRSV at MOI 1 or treated with MOCK and ethidium bromide uptake assays were performed. Ethidium bromide only crosses the plasma membrane in healthy cells by passing through relatively non-specific large channels, such as connexin and pannexin hemichannels, and becomes fluorescent upon binding to intracellular nucleotides. In this experiment PBMCs were used as model for ethidium bromide uptake assay (Orellana et al., 2013). Time-lapse fluorescence imaging revealed that PBMCs cultured under control conditions (MOCK) exhibited a basal ethidium bromide uptake (**Figure 58A**). After hRSV stimulation, the uptake of ethidium bromide exhibited increased ethidium bromide (**Figure 58B**), and this response persists at 60 minutes post exposure to the virus. These data suggest that hRSV exposure induces the ethidium bromide entry into PBMCs that could be related with Panx1.

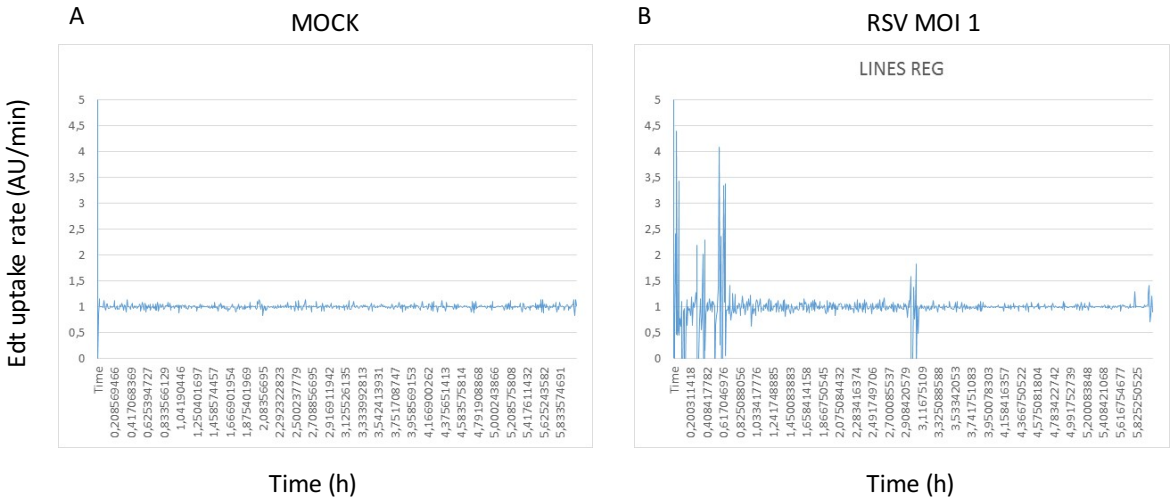

Figure 58. hRSV induces the uptake of ethidium bromide in infected PBMCs. Ethidium bromide uptake measurements were performed in human PBMCs under MOCK control conditions (**A**) or after hRSV exposure (**B**). (AU: Arbitrary units) Data shown are from one experiment.

6.4.3 Probenecid prevents hRSV propagation and replication *in vitro*.

The effect of probenecid treatment on hRSV virus propagation was evaluated in A549 cells treated with 500 µM probenecid and infected with hRSV that contains the reporter GFP (GFP-hRSV) at MOI 1 for 24 h. Treatment of A549 cells with probenecid significantly reduced the number of GFP positive cells compared with untreated infected cells (**Figure 59**). A slight reduction of GFP infected cells was also observed in cells treated with a specific blocking peptide against Panx1 (200µM Panx-1 peptide.). Treatment with the scrambled peptide did not affect the number of GFP hRSV positive cells. These data suggest that Panx 1 activity contributes to the viral propagation of hRSV.

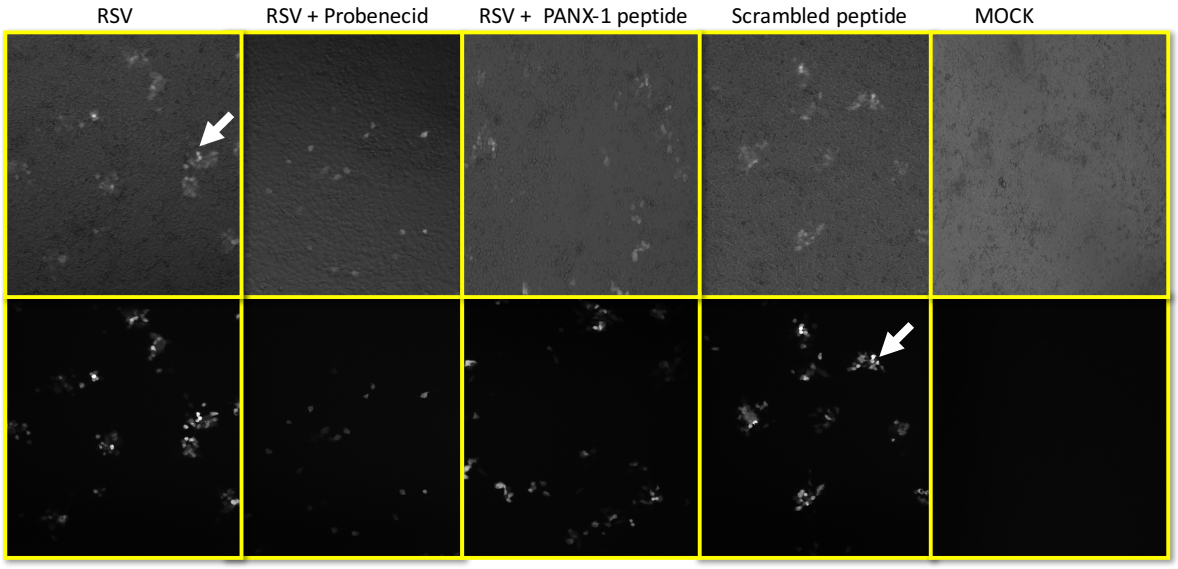

Figure 59. Blocking of Panx1 hemichannel inhibits GFP-hRSV propagation in A549 cells. A549 cells were infected with GFP-hRSV and treated with probenecid, a blocking peptide specific for Panx1 or a scrambled peptide for 24 h. Then, infection was evaluated by epifluorescence detection of GFP-hRSV positive cells. White arrows show representative GFP-hRSV infected cells. The figure shows representative epifluorescence microscopy images for each treatment. Data shown are from one experiment (N=3, original magnification 10X).

Similar results were observed infected A549 cells with the clinical isolate from hRSV. After 24 h, infection was detected by flow cytometry to measure F hRSV surface expression. Probenecid treatment causes a substantial reduction of F hRSV protein expression (**Figure 60**). Further, virus copy number was measured by RT-qPCR in infected A549 cell treated with probenecid. The treatment significantly reduced virus copies number of infected cells (**Figure 61**)

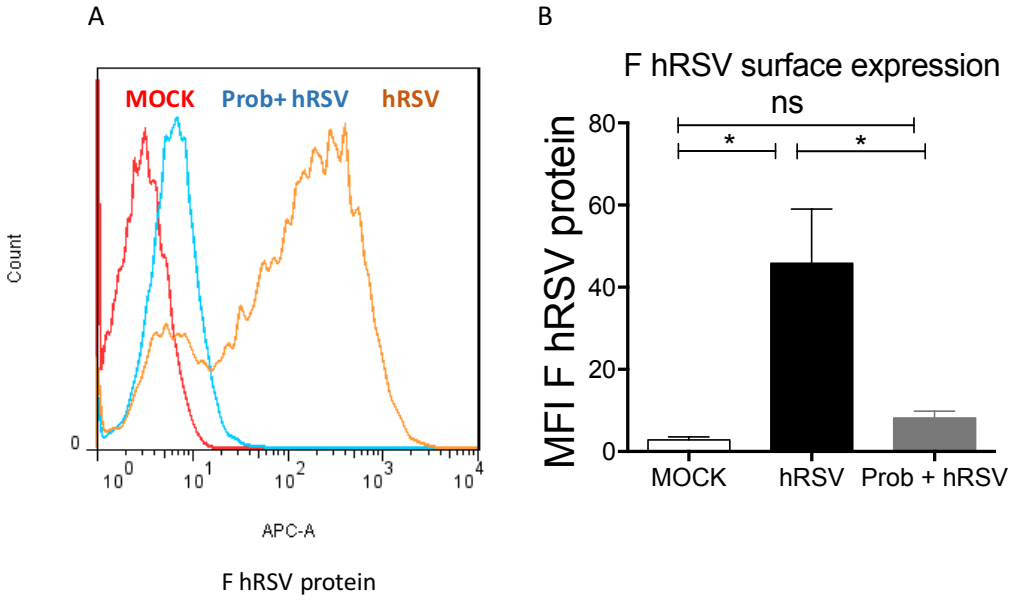

Figure 60. Treatment with probenecid decreased the levels F hRSV surface expression in A549 cells infected cells compared with vehicle control. A549 cells were infected with hRSV at MOI 1 and treated with Probenecid for 24 h. Then, infection was measured by flow cytometry to detect F hRSV surface expression. (**A**) The figure shows a representative histogram of A549 cells for each treatment. (**B**) The graph shows the MFI for F hRSV surface expression for each treatment. Graphs represent results from three independent experiments. Statistical analyses were performed using one-way ANOVA and Tukey´s multiple comparation test. (N=9, ***, P< 0.0001).

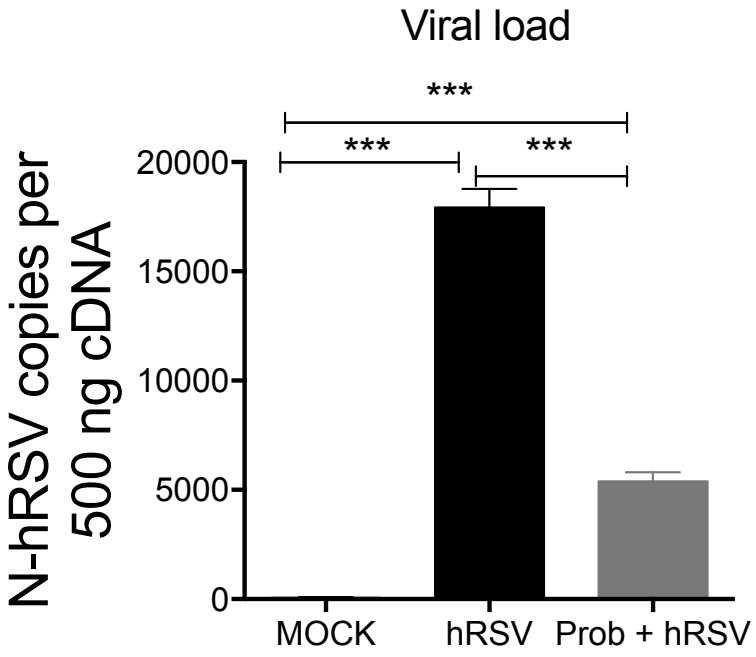

Figure 61. Treatment with probenecid prevents hRSV replication *in vitro*. A549 cells were treated with probenecid and infected with hRSV at MOI 1. At 24 h following infection, total RNA was collected and hRSV virus copy numbers were assessed using RT-qPCR. Graphs represent results from three independent experiments. Statistical analyses were performed using one-way ANOVA and Tukey´s multiple comparation test. (N=9, ***, P< 0.0001).

Furthermore, we added Probenecid treatment at different time points after hRSV exposure to evaluate which step of the viral cycle is affected by this drug. For this purpose, infection was measured by flow cytometry to detect F hRSV surface expression at 24 h after viral absorption. The probenecid treatment was added immediately after 30 minutes of viral exposure (viral fusion and entry), 2 h (viral adsorption and initiation of viral replication) or 6 h post viral exposure (transcription and translation). Our data showed that the percentage of hRSV infected cells without treatment reach to 80% (**Figure 62**). Probenecid reduces the percentage of infection by a 50% when the treatment was added immediately after viral absorption. The percentage of reduction reached to 60% when the treatment with probenecid was between 30 minutes and 2 h after viral absorption. However, the percentage of reduction reached to 30% when the probenecid treatment was added after 6 h of viral absorption. These

data suggest that probenecid act during the first hours after viral infection or impairs the propagation to neighbouring cells.

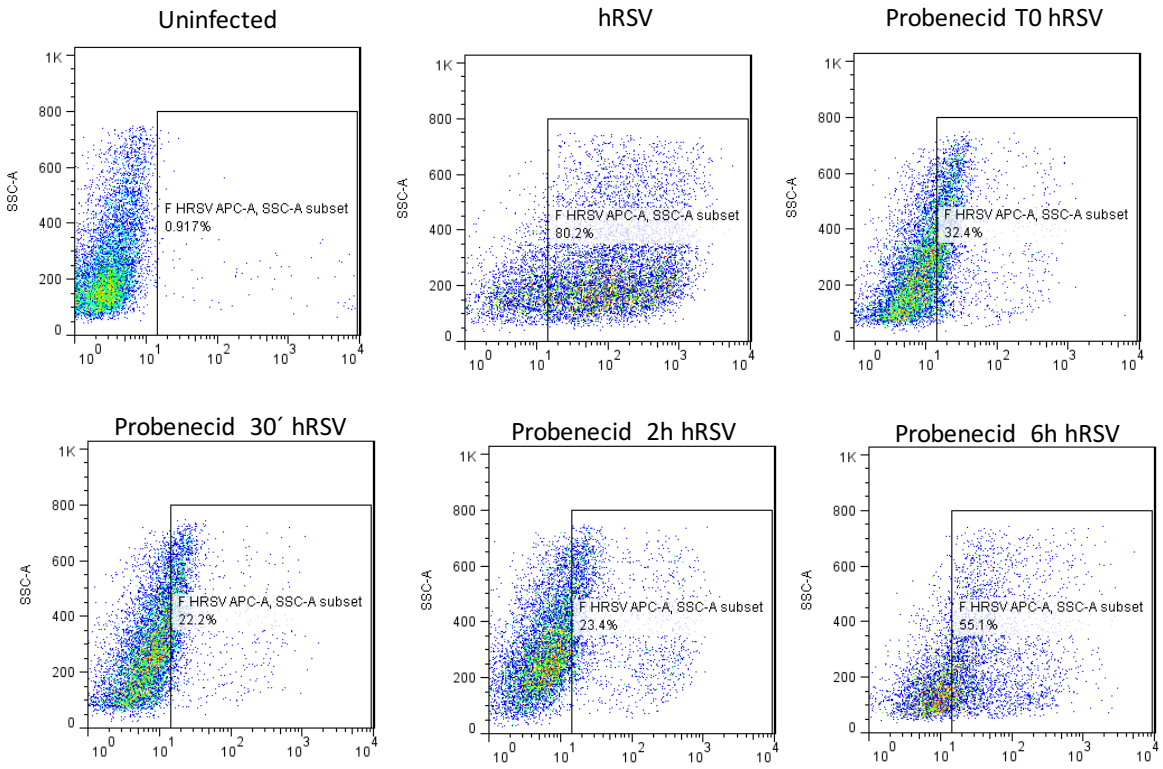

Figure 62. Probenecid treatment acts at an early stage during hRSV infection. A549 cells were infected with hRSV at MOI 1 a time of addition experiment was performed. Probenecid was added at different time points after viral exposure. At 24 h following infection, F hRSV surface expression was measured by Flow cytometry. Data shown are from one experiment (N=2).

6.4.4. The viability and functionality of A549 is normal in the presence of probenecid.

Cytotoxicity assays were performed using A549 cell culture treated with increasing doses of probenecid for 24 h. Probenecid had a low level of cytotoxicity even used at higher concentrations (1 mM, **Figure 63**). Further, functionality assays were performed using fluorescent latex beads. Previous studies have demonstrated type II alveolar cell line A549 can internalize foreign particles by phagocytosis (Wasylnka and Moore, 2002;Luther et al.,

2008). After 1 h of incubation at 37º C, beads uptake was measured by flow cytometry (**Figure 64**). As a negative control of the experiment, the uptake assay was performed at 4ºC.

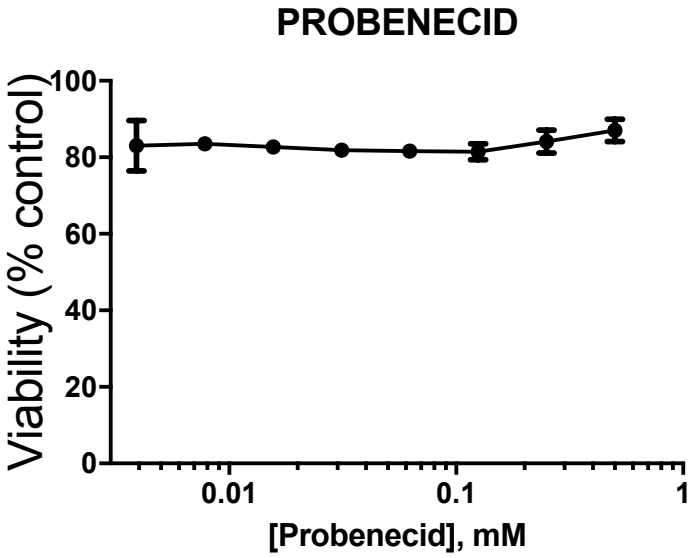

Figure 63. Probenecid demonstrated low cellular cytotoxicity as evaluated by Alamarblue assay. A549 cell cultures were treated with Probenecid at increasing concentration for 24h. Then, cell viability assay was performed with the alamarBlue reagent. The graph shows the dose-response curves to evaluate viability for A549 cells treated with Probenecid. Data shown are mean +/- SEM from one experiment (N=3).

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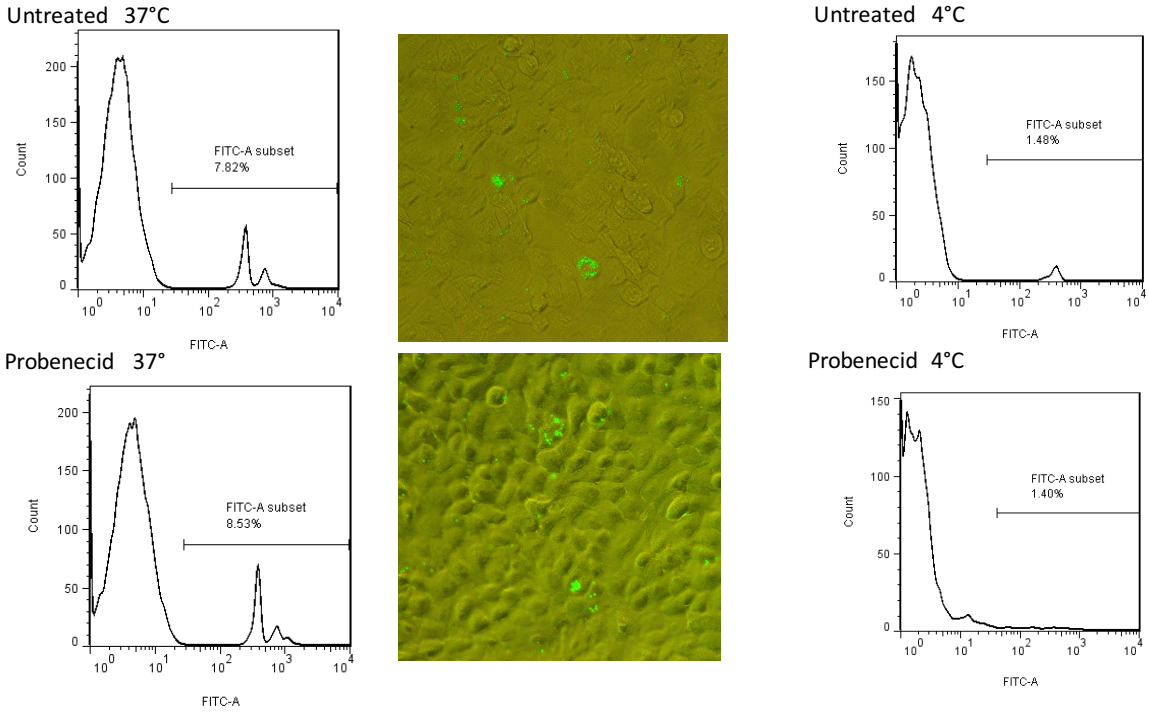


Figure 64. Functionality of A549 cells is normal in presence of Probenecid. A549 cells were treated for 24 h with 500 µM of probenecid. Then, FITC fluorescent latex beads were added to the cell culture. Beads uptake was measured by flow cytometry and epifluorescence microscopy. Data shown are mean +/- SEM from one experiment (N=3).

6.5 Evaluation of the role of Panx1 modulation in hRSV infection *in vivo*.

6.5.1Treatment with probenecid confers protection against hRSV-induced disease and prevents infective viral particle production.

To examine whether treatment with probenecid improves the outcome of hRSV pneumonia, BALB/cJ mice were pretreated probenecid (100 mg per kg body weight) and infected intranasally with hRSV (3×10^6 PFUs). Then, mice body weight was monitored for 7 days postinfection. As shown in **Figure 65**, a marked weight loss was observed after hRSV infection (red curves). Conversely, probenecid treated mice displayed accelerated kinetics of body weight recovery after hRSV infection (blue curves). Remarkably, treatment with probenecid resulted in an improved clinical outcome.

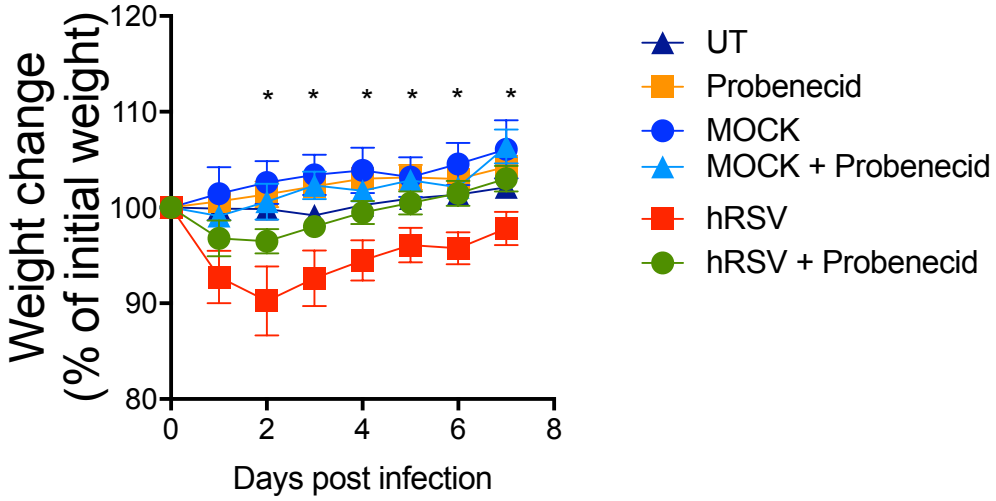


Figure 65. Probenecid treatment prevents body weight loss after hRSV infection in mice. BALB/cJ mice were treated with probenecid and infected with hRSV. Body weight was monitored for 7 days post infection. Data shown are mean+/- SEM of three independent experiments. Data shown are from two independent experiments, each with three mice per group. One-way ANOVA and Bonferroni posttest (N=6, *P < 0.001). * Denotes statiscally significant difference between hRSV and hRSV + probenecid.

As probenecid was effective in limiting replication of hRSV virus *in vitro*, its effects were evaluated in BAL from BALB/cJ mice infected with hRSV with or without probenecid was analyzed to measure viral titers for plaque assay. Our data shows that probenecid causes a significant reduction in viral titers compared with vehicle treated mice (**Figure 66**).

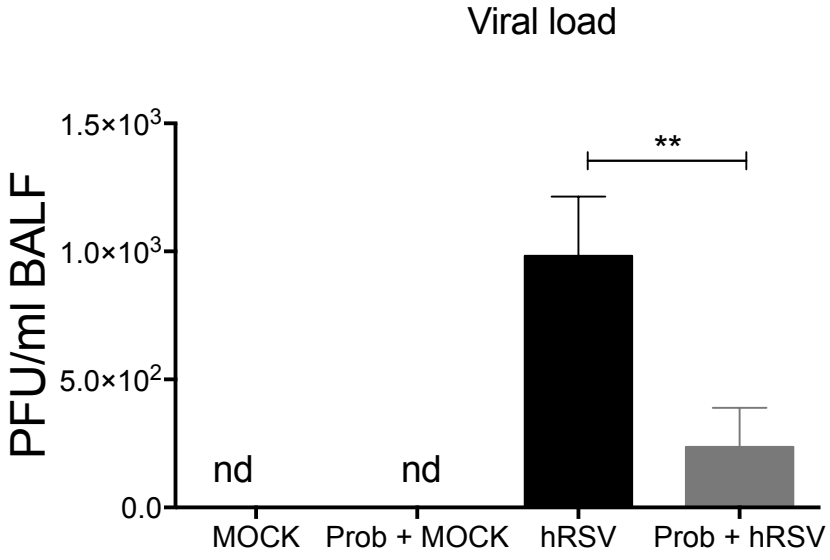
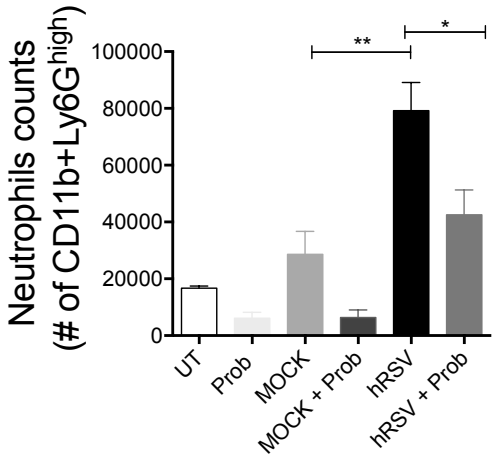


Figure 66. Probenecid treatment prevents infective viral particle production in airways of hRSV infected mice. BALB/cJ mice treated with Probenecid and intranasally infected with hRSV. Then, at day 7 postinfection, the BAL from each experimental group were titrated on HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed as PFUs per milliliter). The graph shows the PFU/ml in BAL for each experimental group. Data shown are from two independent experiments, each with three mice per group. One-way ANOVA, Bonferroni posttest (N=6, **P < 0.001).

6.5.2 Probenecid-treatment prevents lung inflammation induced by hRSV infection in mice.

As mentioned before, acute hRSV infection resulted in neutrophil dominated inflammation. Here, treatment with probenecid decreases the influx of several immune cells into the airways upon viral infection. BAL analyses by flow cytometry show a low number of neutrophils and macrophages in lungs of probenecid-treated mice (**Figure 67A** and **67B**, respectively).

A



B

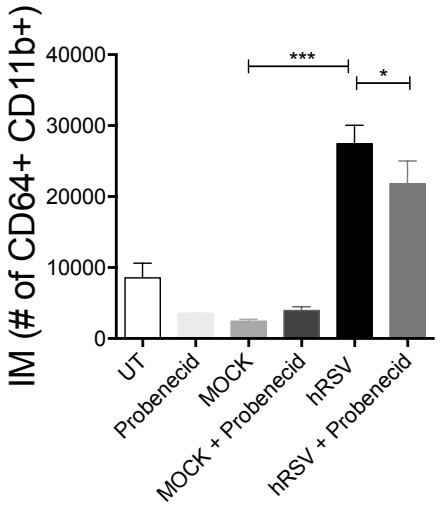


Figure 67. Treatment with probenecid prevents the influx of immune cells into the lungs. Numbers of neutrophils (**A**) and macrophages (**B**) were determined in BAL fluids 7 days post-infection with hRSV. (Prob: Probenecid) Data shown are from one experiment with three mice per group. One-way ANOVA, Bonferroni posttest test (N=3, *P < 0.05, **P < 0.005, ***P < 0.0006).

6.6 Evaluation of the mechanism behind Panx1 inhibition in the protection against the disease induced by hRSV.

6.6.1 Treatment with probenecid resulted in lower pulmonary levels of IL-1β compared with vehicle control.

As stated above, Panx1 plays an important role in the activation of the inflammasome and the subsequent maturation of proinflammatory cytokines, mainly the production of mature IL-1β. This cytokine contributes to the immunopathology caused by hRSV infection. Then, to evaluate a possible mechanism involved in the reduction of the inflammation caused by probenecid treatment, concentration of IL-1β was measured in lungs of BALB/J mice treated or not with probenecid. The levels of IL-1β were significantly reduced in lungs of probenecid-treated mice compared to untreated mice (**Figure 68**). These data suggest that inhibition of panx1 activity mediated by probenecid decreases secretion of IL-1β in infected mice.

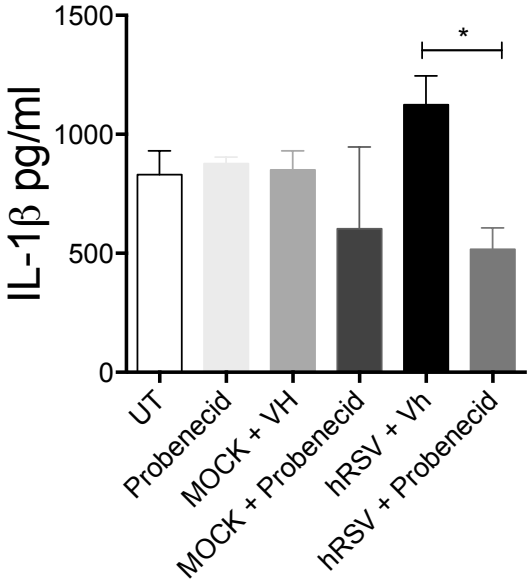


Figure 68. Concentrations of the inflammatory cytokine IL-1β are reduced in lungs of mice treated with probenecid compared with vehicle control. Concentrations of IL-1β were measured by ELISA in lungs homogenates from BALB/cJ mice after 7 days postinfection. Data shown are from two independent experiments with three mice per group. One-way ANOVA, Bonferroni posttest (N=6, *P < 0.05).

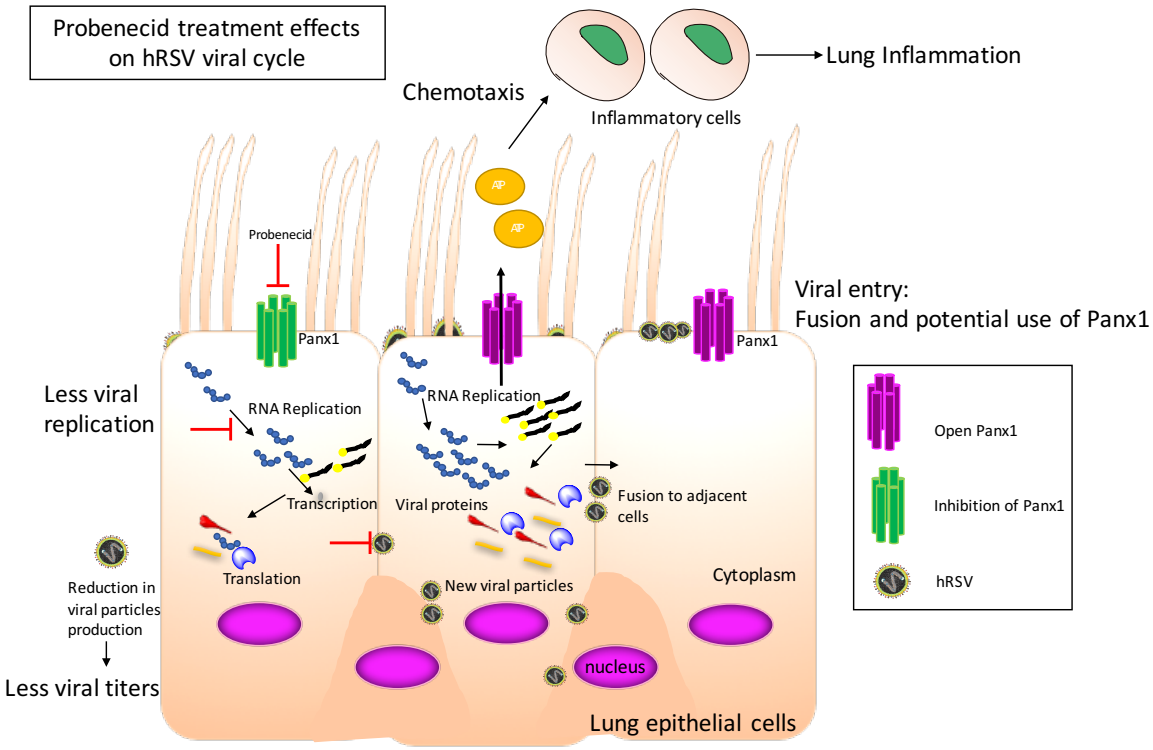


Figure 69. Summary of main results for hypothesis 2. The figure shows the activation of Panx1 in response to hRSV exposure, that results in the release of ATP as a danger signal that recruits' neutrophils and macrophages. In addition, the Panx1 activity promotes IL-1β production by the inflammasome pathway, increasing inflammation in infected cells. On the other hand, the virus could use Panx1 opening to promote the viral fusion to the host membrane. The inhibition of Panx1 decreases replication and infective viral particle production. (original figure)

7. DISCUSSION

Immune system is a complex network of interactions that occur between subsets of innate and adaptive immune cells that are necessary to initiate a functional and specific immune response. A network can be represented as a map of interactions using a terminology of nodes and links (Quintana and Cohen, 2011). The nodes are the units that interact, and the links are the interactions between the units (Quintana and Cohen, 2011).

Recently, several pieces of evidence have been demonstrated that besides the immune cells, cytokines, and chemokines, the immune system network also has other endogenous molecules that can act as natural immune regulators (Hasko and Cronstein, 2004;Thakar et al., 2009). These molecules are nodes in the intracellular molecular networks and linking molecules in the intercellular immune networks. These endogenous immune regulators can accomplish a variety of cell functions in different tissues and also modulates the immune response. These molecules have an important therapeutic potential, particularly to arrest pathogenic inflammation or promote a specific type of immune response. In this thesis, we studied the role of two endogenous regulators; heme oxygenase 1 and pannexin 1, both in the context of found new targets for prevent and control the disease caused by hRSV infection.

7.1.1 Evaluation of *in vitro* effects of HO-1 induction mediated by CoPP treatment on hRSV replication and infective viral particle production.

Several studies described that increased levels of HO-1 display antiviral activity (Espinoza et al.). In this study, we demonstrate that induction of HO-1 enzyme suppresses hRSV replication in A549 cells culture assays. The efficacy of HO-1 against hRSV *in vitro* and the fact that HO-1 expression can be induced by several drugs already approved for human use (Hill-Batorski et al., 2013), for example, the FDA-approved drug Panhematin®

that contain hemin as the active component (Huang et al., 2016), makes to HO-1 an interesting target to the formulation of antiviral drugs against hRSV. To evaluate our hypothesis that overexpression of HO-1 impairs hRSV growth, we used the potent HO-1 inducer CoPP to induce high levels of HO-1 in A549 cell line (an adenocarcinomic human alveolar basal epithelial cell line). CoPP treatment was administrated before or after infection (pre-infection and postinfection treatment), in both cases hRSV growth was significantly suppressed, indicating that hRSV is sensitive to the antiviral effects of HO-1. However, the postinfection scheme was more effective to suppress hRSV replication and viral particle production. Additionally, hRSV attenuate CoPP-induced HO-1 levels similarly that HCV and HIV (Abdalla et al., 2004;Protzer et al., 2007). These data suggest that the virus directly target HO-1 for downregulation to optimize its viral replication.

The mechanism involved in the antiviral properties of HO-1 induction against hRSV in the *in vitro* assays is still unknown. Other studies for the antiviral effects of HO-1 sustain that the specific mechanism is the disruption of the viral replication cycle. For instance, HO-1 directly target the transcription/replication process (Devadas and Dhawan, 2006;Shan et al., 2007;Zhu et al., 2008). Our data are consistent with this mechanism considering the reduction in the amount of N transcripts. However, the experiments performed to evaluate the effect of CoPP treatment on the binding and entry process of hRSV are insufficient to sustain that mechanism of inhibition is not related with both processes. Additional experiments are required to clarify whether CoPP impairs binding and entry. Furthermore, recent studies support the antecedent that HO-1 enzymatic products may directly interfere with viral proteins involved in viral replication (Lehmann et al., 2010;Zhu et al., 2010) or may lead to the upregulation of IFN-stimulated genes and triggers an antiviral state in cells

(Ma et al., 2016). In the case of hRSV, further experiments are needed to propose a direct effect of HO-1 on hRSV viral proteins, and the hypothesis of the antiviral state seems more likely with our results. Another interesting possibility is that the antioxidant effects of HO-1 can contribute to the inhibition of the viral infection. Indeed, recent reports have shown that antioxidants are efficient to ameliorate hRSV infectivity (Castro et al., 2006;Shin et al., 2013). On the other hand, our data showed that SnPP-inhibition of the enzymatic activity promotes viral replication in A540 infected cells, suggesting that the enzymatic activity of HO-1 is essential to control the infection in the epithelial cells. To confirm this hypothesis, a measure of the enzymatic activity of HO-1 could be included in further experiments.

7.1.2 Induction of HO-1 inhibits viral replication and confers protection against hRSV infection in mice.

Previous evidence described that increased levels of HO-1 could attenuate disease severity of influenza infection in mice (Hashiba et al., 2001). In addition, HO-1–deficient ($HO^{-/-}$) mice exerted decreased survival rates after influenza infection, as compared with wild-type mice (Cummins et al., 2012). In the present study, we demonstrated that the HO-1 induction decreases disease severity and virus load in the lungs of hRSV infected mice. Our data indicated that treatment with CoPP promotes a significant faster body weight recovery after hRSV infection, as compared with control mice. Similar results were observed in conditional transgenic tTA-HO-1 mice that overexpress human HO-1 in MHC-II^{+} cells. On the contrary, inhibition of HO-1 activity mediated by SnPP administration did not increase body weight loss as a parameter of disease, suggesting that HO-1 enzymatic activity is not necessarily the unique component involved in the protective outcome observed in the body weight recovery induced by CoPP. Further, our data showed that CoPP administration decreases viral mRNA amounts and the titers of infective viral particles in the lungs of hRSV-

infected mice, an outcome that coincided with an increase in HO-1 expression in lungs of mice at day 4 postinfection. Conversely, we found that SnPP administration caused a slight increase in viral loads in the lungs during the same period, however, viral progeny (PFU) was not raised. All these data suggest that the HO-1 enzymatic activity or its products are partially necessary in the modulation of viral replication and clearance in the mice.

The increased levels in HO-1 expression were mainly observed in Epcam-positive cells from the lungs of CoPP pretreated mice, this data is consistent with the notion that a reduction in viral loads promoted by HO-1 could be related to the promotion of an antiviral response in airway epithelial cells.

7.1.3 Induction of HO-1 prevents neutrophil infiltration and lung inflammation in hRSV infected mice.

HO-1 is the rate-limiting enzyme in heme degradation, but recent reports have emphasized the importance of HO-1 in modulating the magnitude of inflammation (Hashiba et al., 2001). In this regard, HO-1 expression or CO administration display strong anti-inflammatory effects in monocytes and macrophages, probably restraining these cells from inducing tissue damage and modulating their role in the initiation of immune responses (Tzima et al., 2009). Several reports have described the protective properties of the HO-1/CO system; CO inhibits the proinflammatory and stimulates the anti-inflammatory response of macrophages, a cell type that controls the balance of inflammation in many conditions (Bermingham and Collins, 1999;Tzima et al., 2009). The present study indicated that HO-1 upregulation results in the attenuation of lung inflammation, associated with a decrease in the inflammatory cell infiltration and an inhibition of proinflammatory cytokine or chemokine secretion during hRSV infection. Cytokine expression likely affects the balance

between viral clearance and progression of the disease. Various studies have reported that hRSV infection actively induces activation of NF-kB in the lung tissue, leading to the secretion of proinflammatory mediators (Choudhary et al., 2005;Xie et al., 2012).

Considering that HO-1 can inhibit NF-κB activity (Brunt et al., 2009), we hypothesized that HO-1 induction or its by-products prevents lung inflammation through the inhibition of NF-kB, which results in lower levels of proinflammatory cytokines and chemokines. Specifically, our results showed a decrease in the cytokines IL-6, IL-4, IFN-γ and the chemokines CCL3/MIP-1α and CXCL1/KC. IL-6, among other cytokines, such as IL-1β and IL-8, play an important role in neutrophil and macrophage chemotaxis. The cellular inflammatory response during severe hRSV infection is characterized by the massive recruitment of neutrophils and macrophages, suggesting that the expression of IL-6 in the airways is a crucial feature in disease progression (Levitz et al., 2012). In agreement with this statement, the observed reduction in the levels of IL-6 in BAL from infected mice promoted by HO-1 induction contributes to the decrease of hRSV disease severity.

Furthermore, IL-4 has an important contribution in the immunopathology induced by hRSV infection by increasing Th2-type inflammation (Moore et al., 2009). Thus, the decreases in IL-4 levels in BAL mediated by HO-1 limits the immunopathology reducing lung damage as evidence the lung histopathology. Also, we observed a reduction in the secretion of IFN-γ, this cytokine is mainly produced by NK cells and activated $CD4^+$ and $CD8^+$ T cells, which promotes cell-mediated immune responses against viral infections (Lee et al., 2008). This result can be explained by the observation that the administration of a HO-1 inducer, as CoPP, to wild type mice results in suppressions of T cell-mediated cytotoxicity and Th1-mediated cytokine production (Woo et al., 1998). To complete the analysis of

cytokine and chemokine profile, we include the measure of CCL3/MIP1α and CXCL1/KC. The chemokine CCL3/MIP1α is secreted following hRSV infection and is mostly chemotactic for both T cells NK cells (Tregoning et al., 2010).Then, a reduction in the levels of this chemokine could be related to a diminished T cell response in the lung of hRSV infected mice. Finally, the chemokine CXCL1/KC (an IL-8 homolog) and its receptor CXCR2 mediates the recruitment and activation of neutrophils to the lungs (Sawant et al., 2015). Here, we observed an important reduction of CXCL1/KC after induction of HO-1 and a tendency to the increases of this chemokine when HO-1 is inhibited by SnPP treatment. This result suggests that enzymatic activity of HO-1, maybe by CO production, controlling chemokines production in airways. This idea is supported by the histopathologic analysis of lung sections, when the inhibition of HO-1 enzymatic activity seems increases lung RSV microscopic inflammatory damage caused by hRSV and increases the neutrophil recruitment observed by flow cytometry analysis in BAL.

On the other hand, HO-1 induction increases IL-10 secretion in BAL of hRSV infected mice, supporting an anti-inflammatory role for HO-1 during hRSV infection. In agreement with these results, Lee and Chau identified a potential interaction between IL-10 and HO-1 in the inhibition of LPS-induced inflammatory responses and provided evidence that HO-1 mediates the anti-inflammatory function of IL-10 both *in vivo* and in *vitro*, and that IL-10 and HO-1 activate a positive feedback pathway to augment the anti-inflammatory response (Lee and Chau, 2002;Tzima et al., 2009). In this regard, HO-1 overexpression or CO administration promotes the secretion of high levels of IL-10 in LPS-treated macrophages (Otterbein et al., 2000). In addition, the increase in IL-10 levels in the macrophages is known to be necessary for the resolution of neutrophilic migration in the

lung, overexpression of exogenous HO-1 is thought to provide its cytoprotection at least via the mediation of IL-10 production (Jin and Choi, 2005). In agreement with our results, these reports support the hypothesis that HO-1 antiinflammatory effects described in this work could in part be mediated by IL-10 production.

Similar results to the obtained in this work have been described for influenza virus H1N1, where as a result of the adenoviral-mediated HO-1 gene transfer infected mice display a decrease in neutrophil infiltration and lung injury (Hashiba et al., 2001). These findings demonstrate that HO-1 activity may be important for modulating lung inflammation during viral pulmonary pathologies (Cummins et al., 2012). This notion was further supported by the observation that mice deficient for Nrf2 ($Nrf2^{-/-}$), a transcription factor controlling HO-1 gene expression, showed a significantly reduced hRSV clearance, a higher bronchopulmonary inflammation, and a reduced body weight gain as compared with control mice (Cho et al., 2009).

Moreover, the protection against hRSV infection observed for the pharmacological induction of HO-1 mediated by CoPP treatment was validated in a second mice model. This model consists in a conditional transgenic mouse named tTA-HO-1. The mice express HMOX under a Tetracycline-Controlled Transcriptional Activation system in $MHC\text{-}II^{+}$ cells. Our results showed that HO-1 overexpression in $MHC\text{-}II^{+}$ subsets in vivo displayed a decrease in lung inflammation in hRSV-infected tTA-HO-1 transgenic mice. These data suggest that the HO-1 enzyme is responsible for the protective effect observed in CoPP treated-mice. Importantly, although the MHC-II molecule is constitutively expressed by APCs, its expression is not exclusive of immune cells. In fact, intestinal and pulmonary epithelial cells can also express this molecule under inflammatory conditions (Kambayashi

and Laufer, 2014). Then, in the conditional transgenic mice used in this work, the upregulation of HO-1 can occur in epithelial cells and immune cells expressing MHC-II and contributes to suppressing the inflammation triggered by the hRSV infection.

8.1.4 Induction of HO-1 increases antiviral type I IFN response during hRSV infection *in vivo*.

Furthermore, we observed that CoPP treatment induced an upregulation of IFN-α/β, in the lungs of hRSV-infected mice, indicating that HO-1 plays an important role during the development of the antiviral type I IFN response in the airways. The type I IFN system presents important innate immunity to effectively block virus replication, and then all these data suggest that HO-1 induction could be promoting an antiviral state that prevents or limits viral replication and propagation of new virus particles. A recent study indicated a novel function for HO-1 in myeloid cells as an upstream mediator of the IRF3/IFN-β response. These data showed that HO-1 forms a complex with IRF3 and is essential for IRF3 activation and subsequent gene expression in response to TLR3/TLR4 stimulation (Tzima et al., 2009).

Furthermore, IRF3 activation and subsequent IFN-β production were severely impaired in HO-1 knockout macrophages (Tzima et al., 2009). Herein, HO-1 can be detected in the nucleus of cultured cells after exposure to hypoxia and modified the binding of transcription factors involved in oxidative stress (Lin et al., 2007). Enhance of IFN type I response mediated by HO-1 have been validated in another study (Ma et al., 2016). This work also described that HO-1 is necessary for the initiation of IFN immune responses against virus infection in macrophages via interaction with IRF3 (Ma et al., 2016). Also, this work described that the increases of IFN-α/β is mediated by the HO-1 expression is efficient to restrict influenza virus replication *in vitro* and *in vivo* thought the induction of ISGs such as

IFITM, IFIT, OAS1, and PKR (Ma et al., 2016). Also, the activation of IFN type response mediated by the interaction of IRF3 and HO-1 is a potent mechanism to explain the suppression of the hRSV replication observed in A549 cells cultures and mice infected lungs described in this work.

8.1.5 Induction of HO-1 modulates T cell function during hRSV infection

HO-1 displays immunomodulatory effects on adaptive immunity, by impairing T-cell activation, T-cell proliferation, and effector functions (Soares et al., 2009). Several reports have characterized the effects of HO-1 induction on T cell response, showing that the activation $CD4^+$ T cells are impaired by HO-1/CO axis (Pae et al., 2004). Similar results are observed for $CD8^+$ T cells (Pae et al., 2004). On the other hand, pharmacological induction of HO-1 expression and activity impairs mouse, rat, and human DC activation and their immunogenicity (Riquelme et al., 2016). Then, the T cell function can be modulated directly by HO-1 or by the alteration in DC activation.

Here, we observed that both; the HO-1 upregulation mediated by CoPP treatment and the exogenous expression of human HO-1 in $MHC\text{-}II^+$ cells in the conditional transgenic mice slightly affects T cell activation and function. Thus, it indicates that Ag processing and presentation by DCs was impaired by HO-1, in agreement with the previous data supporting the role of HO-1 on adaptive immunity (Soares et al., 2009). These results suggest that the lower viral titers measured in lungs of infected mice treated with CoPP and in the conditional transgenic mice, are not associated with an increase in the cytotoxic activity of $CD8^+$ T cells. On the contrary, the T cells response is reduced in both models of HO-1 induction. This effect could contribute to the reduction in the lung inflammation observed after the overexpression of HO-1 and may generate a better outcome in the respiratory disease of infected mice.

There are several potential approaches to the therapeutic use of HO-1. It is an inducible gene, and several synthetic molecules, such as heme arginate (Sasaki et al., 2006) or synthetic protoporphyrins (Emtestam et al., 1989), could be used to regulate its expression or activity. In addition, there is an emergent number of synthetic molecules that are thought to exert their therapeutic effects through a mechanism that is functionally linked to their ability to induce the expression of HO-1, a phenomenon coined as the 'therapeutic funnel'(Bach, 2005). Treatment based on HO-1 upregulation is currently used as a therapeutic approach. Hemin is the most powerful inducer of HO-1. Although intravenous hemin (1–4 mg/kg/day for 3–14 days) has been approved by the US Food and Drug Administration for the treatment of acute intermittent porphyria, it has also been used to treat thalassemia intermedia, myelodysplastic syndrome, and liver allograft failure in erythropoietic protoporphyria. However, this strategy has not been approved for the treatment of infectious diseases.

In summary, the current study demonstrates that hRSV infection can be modulated by the expression of HO-1 both *in vitro* and in *vivo*. Thus, HO-1 expression and activity may play a critical role during hRSV infection. HO-1 induction could protect the host from the pulmonary pathology developed upon hRSV infection, by reducing viral replication and lung inflammation, thus favoring disease resolution. Therefore, our results shed light on the potential role of the therapeutic induction of HO-1 in this viral pneumonia and suggest new avenues for the immunomodulatory treatment of hRSV-infected patients. Furthermore, genetics variations on HMOX1 gene such as the polymorphism at position −413 and shorter lengths of $(GT)_n$ repeats [$n \leq 27$] closer to the transcription starting point, have been reported to promotes HMOX1 gene expression (Bonkovsky et al, 2010). Specifically, these variants

correlate with less severe chronic inflammatory diseases, including chronic obstructive pulmonary disease, coronary artery disease, diabetes mellitus, and arthritis (Bonkovsky et al, 2010). However, the effects of genetic variants on viral infection progression and the outcomes of viral infection diseases are still unknown. According with our results, we hypothesized that patients with genetic variants that increases HMOX1 gene expression would present a better outcome during hRSV infection disease. On the contrary, longer lengths of $(GT)_n$ repeats polymorphism in the promoter of the gene HMOX1 have been associates with worse outcomes in several inflammatory diseases (Gibbons et al, 2017). Sequences containing longer polyGT repeats had lower transcriptional activity than sequences with fewer repeats meaning that longer polyGT repeat alleles result in lower HO-1 protein. According with this background, patients with genetic variants that induces a deficient expression of HO-1 would had a worse outcome during hRSV infection disease.

7.2.1 Evaluation of *in vitro* effects of Panx 1 inhibition mediated by probenecid treatment on hRSV replication and propagation.

Several studies demonstrated that ATP release by Panx1 channels facilitates HIV-1 infection of $CD4^+$ cells (Seror et al., 2011;Orellana et al., 2013;Velasquez et al., 2016). HIV-1 envelope proteins interact with specific $CD4^+$ cell receptors and elicit the release of ATP via Panx1 channels into the extracellular milieu where ATP then acts on P2Y2 purinergic receptors and activates Pyk2 kinase (Penuela et al., 2013). This interaction produces membrane depolarization and facilitates membrane-to-membrane fusion to allow viral entry into the cell as well as cell-to-cell transmission (Seror et al., 2011). Interestingly, Panx1, P2Y2, and Pyk2 are all physically recruited to the contact site between Env-containing and

CD4/CDXCR4-containing membranes suggesting that they form part of the infection synapse that is orchestrated by the virus to facilitate infection (Seror et al., 2011). Inhibition of any of the factors of this interaction could decrease HIV-1 infection and impair viral replication demonstrating their potential use as a novel antiretroviral therapy(Penuela et al., 2013). Here, we demonstrate that hRSV infection can increases the expression of Panx1 in epithelial cells and DCs *in vitro.* Suggesting that Panx1 hemichannel activity is beneficial for the viral propagation. Supporting this idea, exposure to hRSV promotes the opening of Panx1 and possibly other related hemichannels during the first 60 minutes after the contact with the virus. All these data suggest that Panx1 favors infection, may be contributing to viral entry or the infection of neighbor cells. In this regard, the inhibition of Panx1 by probenecid and a specific peptide that block Panx1 activity impairs replication and propagation in A549 cells. We observed a decrease in surface expression of F hRSV protein, less amount of viral RNA and low GFP positive cells after infection with GFP-hRSV virus. Together, these results suggested that viral propagation and replication is limited by probenecid treatment. Then, probenecid or other blocking molecules against Panx1 are attractive strategies to control hRSV infection.

7.2.2 Inhibition of Panx1 impairs viral replication and confers protection against hRSV infection in mice.

Previous evidence described that probenecid treatment could attenuate disease severity in influenza A infected mice (Perwitasari et al., 2013). Similar results have been described for *Pseudomonas aeruginosa* pneumonia (Wonnenberg et al., 2014). In this thesis, we demonstrated that probenecid treatment has beneficial effects on the outcome of the disease caused by hRSV, reducing body weight loss and decreasing virus titers in lungs of

infected hRSV mice. Our results suggest that the block of Panx1 and potentially others related hemichannels with probenecid is a potential therapeutic strategy against hRSV infection.

7.2.3 Inhibition of Panx1 prevents neutrophil infiltration and lung inflammation in hRSV infected mice.

HRSV infection causes an exacerbated inflammation of the respiratory tract that leads to lung injury, the formation of edema, and loss of lung function. The excessive inflammation trigger by hRSV not induces and effective viral clearance and increased lung pathology. Here, we observed that the blocking of Panx1 mediated by probenecid decrease neutrophils and macrophages infiltration in the airways of infected mice. This effect can contribute to decreasing the excessive lung inflammation observed in the hRSV disease. The reduction of inflammatory cells infiltration mediated by probenecid can be explained by the loss in the extracellular ATP that acts as a strong chemoattractant for neutrophils and other inflammatory cells (Lohman et al, 2015). According with our results, experiments performed in sepsis murine models showed that the block of Panx1 decrease the tissue damage by the reduction of PMN activation, however this treatment also decrease the immune response against bacterial infection (Lohman et al, 2015).

7.2.4 Inhibition of Panx1 inhibits production of IL-1β in lungs of infected mice.

It has been reported that IL-1β produced in the respiratory tract may act as one of the main inflammatory cytokines during the acute phase of hRSV infection(Hornsleth et al., 2001). IL-1β may also promote histamine-induced bronchoconstriction in infants during hRSV infection because it is known that injection of IL-1β into mice induces the formation of histidine decarboxylase, the enzyme that forms histamine, in various tissues, including

lung tissue (Endo, 1989). Furthermore, increased levels of IL-1β have been associated with hRSV disease severity in studies performed in nasopharyngeal wash samples from infected children (Tabarani et al., 2013). In the same way, the reduction in the secretion of IL-1β observed in mice treated with probenecid, maybe caused by the blocking of inflammasome activation, helps to decrease the disease severity in infected mice by the prevention of the inflammatory lung damage. Other studies suggest that excessive inflammation during bacterial infection contributes to decreased bacterial clearance and increased pathology whereas blocking of inflammasome activation results in improved bacterial clearance and diminished pathology (Wonnenberga et al, 2014). In this regard, the absence or reduction in endogenous IL-1β activity improves host defense against bacteria by suppressing the inflammatory response (Wonnenberga et al, 2014). Furthermore, a strong host response is required to control infections and to eliminate pathogens. However, exacerbated inflammation leads to tissue damage and pathology. The presented findings support the hypothesis that exacerbated inflammation reduces the efficiency of the host to eliminate pulmonary pathogens as hRSV.

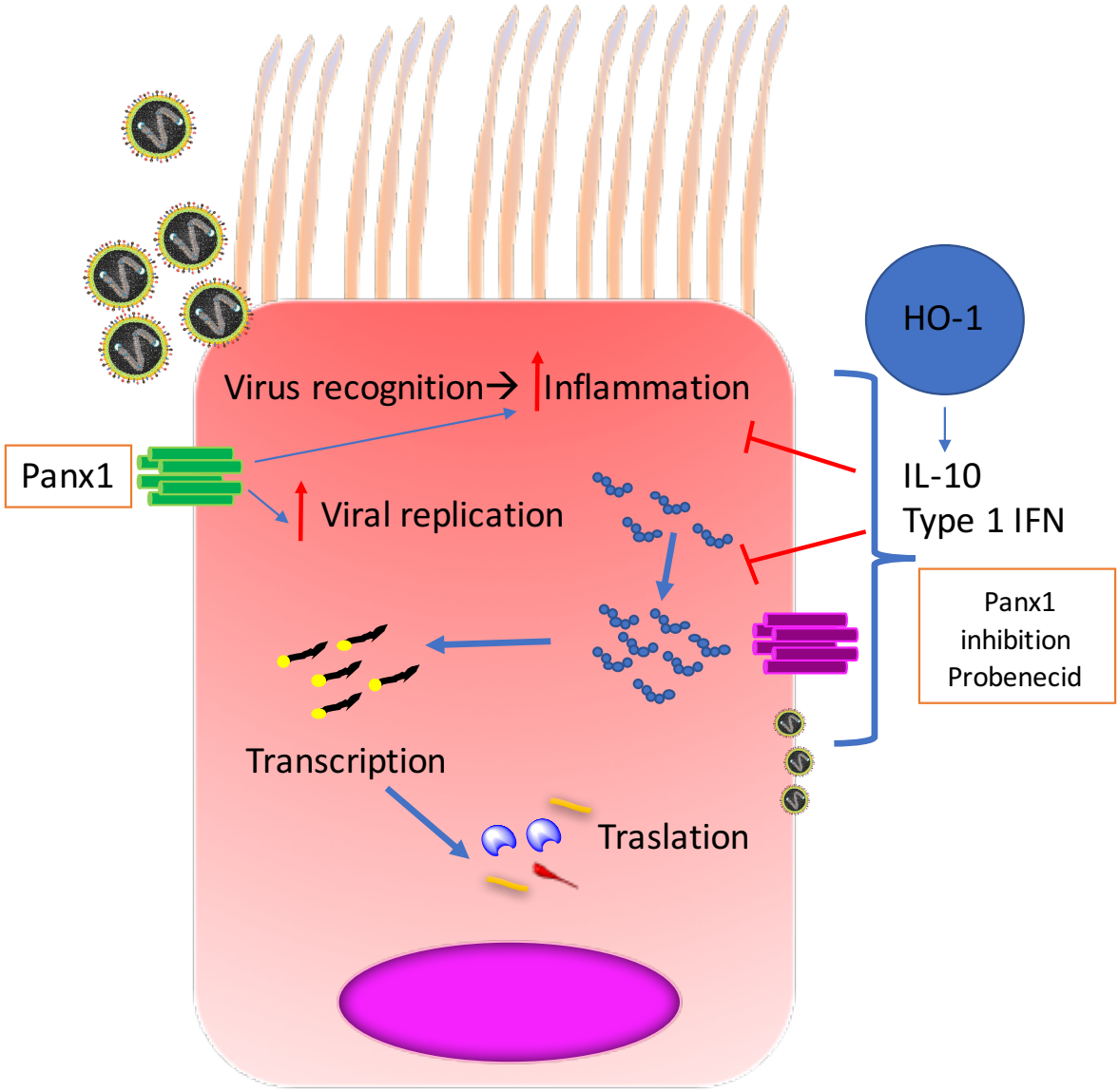


Figure 70. Proposal model for antiviral and anti-inflammatory effects of HO-1 induction and probenecid treatment against hRSV infection. Summary and potential mechanism behind HO-1 and Panx1 modulation of disease induced by hRSV infection.

8. CONCLUSIONS

The major goal of this thesis was to investigate the modulatory effects of two endogenous regulatory molecules: HO-1 and Panx1, during hRSV infection.

For the HO-1 study, we found that the HO-1 inductor CoPP, pre- and postinfection significantly suppressed hRSV replication in a manner dependent upon HO-1 upregulation and activity. Also, our results suggest that the enzymatic activity of HO-1 is crucial to control hRSV replication, considering that the treatment with the competitive inhibitor SnPP promotes viral load in A549 cells assays. In addition, we demonstrated that viral binding and entry process are not affected by HO-1 induction. To evaluate whether CoPP had a direct virucidal effect on hRSV or its components further experiments are required.

Further, we showed that the induction of HO-1 *in vivo* display a protective effect in hRSV infected mice. This idea was evidenced by the reduction in the loss of body weight duo hRSV-induced disease, a decrease in the viral load and lungs and a reduction in inflammatory lung damage in mice treated with the CoPP and in transgenic mice that express HO-1 in MHC-II^{+} cells. The expression of HO-1 could protect the host from the pulmonary pathology developed upon hRSV infection, by reducing viral replication reducing inflammation and thus, favoring disease resolution.

Also, we showed that HO-1 induction increases IL-10 secretion and decreases pro-inflammatory cytokines and chemokines secretion in airways. Concomitantly, HO-1 induction promotes IFN type I response in lungs. Both effects generated an anti-inflammatory and antiviral environment in the lung that results in a better outcome in hRSV disease mediated by HO-1 in infected mice.

For the Panx1 study, we found that Panx1 is overexpressed after hRSV infection in A549 cells and DCs. Similar results are observed *in vivo* in lungs from infected mice. Furthermore, Panx1 opening is triggered by hRSV exposure, and the blocking of the activity of Panx1 by probenecid impairs hRSV infection *in vitro*.

Next, we showed that the inhibition of Panx1 *in vivo* display a beneficial effect in the outcome in the disease of hRSV infected mice, demonstrated by the reduction in the loss of body weight, a decrease in the viral titers and a reduction neutrophils infiltration in airways. Then, Panx1 is necessary and favors hRSV infection and blocking its activity could protect the host from the pulmonary pathology allowing disease resolution.

Furthermore, we showed that probenecid treatment decreases IL-1β secretion and this effect could be related to the inhibition of inflammasome. Then, lower levels of IL-1β favors a reduction in lung inflammation.

Finally, this work is the first evidence that HO-1 and Panx1, both endogenous regulatory elements can be modulated to efficiently control hRSV infection *in vivo* and in *vitro* and reduce the pulmonary disease induced by this pathogen. Therefore, induction of HO-1 and inhibition of Panx1 are potent therapeutic strategies to the development of new antiviral drugs against hRSV infection.

9. LIST OF PUBLICATION GENERATED IN THIS THESIS

1. **Modulation of host adaptive immunity by hRSV proteins. Espinoza JA,** Bohmwald K, Céspedes PF, Riedel CA, Bueno SM, Kalergis AM. Virulence. 2014 Oct 1;5(7):740-51. doi: 10.4161/viru.32225.
2. **Induction of protective effector immunity to prevent pathogenesis caused by the respiratory syncytial virus. Implications on therapy and vaccine design. Espinoza JA,** Bueno SM, Riedel CA, Kalergis AM. Immunology. 2014 Sep;143(1):1-12. doi: 10.1111/imm.12313.
3. **Central nervous system alterations caused by infection with the human respiratory syncytial virus**. Bohmwald K1, **Espinoza JA**, González PA, Bueno SM, Riedel CA, Kalergis AM. Rev Med Virol. 2014 Nov;24(6):407-19. doi: 10.1002/rmv.1813. Epub 2014 Oct 14. Expert Opin Investig Drugs. 2015;24(12):1613-30. doi: 10.1517/13543784.2015.1099626.
4. **Inflammatory damage on respiratory and nervous systems due to hRSV infection.** Bohmwald K, **Espinoza JA**, Becerra D, Rivera K, Lay MK, Bueno SM, Riedel CA, Kalergis Current Opinion in Immunology 2015, 36:14–21 doi: 10.1016/j.coi.2015.05.003
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11. ANNEX: ARTICLES PUBLISHED DURING THIS THESIS

frontiers in Immunology

REVIEW
published: 27 November 2017
doi: 10.3389/fimmu.2017.01643

Functional Impairment of Mononuclear Phagocyte System by the Human Respiratory Syncytial Virus

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The mononuclear phagocyte system (MPS) comprises of monocytes, macrophages (MΦ), and dendritic cells (DCs). MPS is part of the first line of immune defense against a wide range of pathogens, including viruses, such as the human respiratory syncytial virus (hRSV). The hRSV is an enveloped virus that belongs to the *Pneumoviridae* family, *Orthopneumovirus* genus. This virus is the main etiological agent causing severe acute lower respiratory tract infection, especially in infants, children and the elderly. Human RSV can cause bronchiolitis and pneumonia and it has also been implicated in the development of recurrent wheezing and asthma. Monocytes, MΦ, and DCs significantly contribute to acute inflammation during hRSV-induced bronchiolitis and asthma exacerbation. Furthermore, these cells seem to be an important component for the association between hRSV and reactive airway disease. After hRSV infection, the first cells encountered by the virus are respiratory epithelial cells, alveolar macrophages (AMs), DCs, and monocytes in the airways. Because AMs constitute the predominant cell population at the alveolar space in healthy subjects, these cells work as major innate sentinels for the recognition of pathogens. Although adaptive immunity is crucial for viral clearance, AMs are required for the early immune response against hRSV, promoting viral clearance and controlling immunopathology. Furthermore, exposure to hRSV may affect the phagocytic and microbicidal capacity of monocytes and MΦs against other infectious agents. Finally, different studies have addressed the roles of different DC subsets during infection by hRSV. In this review article, we discuss the role of the lung MPS during hRSV infection and their involvement in the development of bronchiolitis.

Keywords: human respiratory syncytial virus, dendritic cells, macrophages, infection, immunity

OPEN ACCESS

Edited by:
Luciana Balboa, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Robert Braidwood Sim, University of Leicester, United Kingdom
Silvia Beatriz Boscardin, University of São Paulo, Brazil

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *14 August 2017*
Accepted: *10 November 2017*
Published: *27 November 2017*

Citation:
Bohmwald K, Espinoza JA, Pulgar RA, Jara EL and Kalergis AM (2017) Functional Impairment of Mononuclear Phagocyte System by the Human Respiratory Syncytial Virus. Front. Immunol. 8:1643. doi: 10.3389/fimmu.2017.01643

INTRODUCTION

Mononuclear Phagocyte System (MPS): Background

Since the 1960s, the term MPS was defined as a family of cells differentiated from a common committed progenitor derived from the bone marrow (1–3). The MPS is composed of three major cell types, including monocytes, macrophages (MΦs), and dendritic cells (DCs) (2, 4). These cells share common morphologic and functional features, such as stellated form and the endocytic capacity (2).

In addition, MPS cells express a heterogeneity of cell surface markers based on the tissue where they are located (3, 5, 6).

In the lungs, the cells of the MPS play a key role during host defense and homeostasis (7, 8). MΦs are found mostly in the alveolus adjacent to the epithelium and less frequently in the terminal airways and interstitial space, while most DCs are located in the pulmonary interstitium (7). Finally, this cellular system play critical roles in pulmonary host defense against viral pathogens, such as human respiratory syncytial virus (hRSV), which will be discussed in detail below.

Monocytes

Monocytes originate in the bone marrow from a common myeloid progenitor that is shared with granulocytes and macrophages (9). Furthermore, recruitment of monocytes is critical for an effective control and clearance of viral infections (10). It has been described that, in the bone marrow, the earliest monocytic precursor needs between two or three generations before becoming a mature monocyte that can be released into the peripheral blood. Once in the blood, these cells circulate for several days before entering the tissues and replenishing tissue macrophage populations (10). In the absence of an inflammatory process, it is thought that migration of monocytes into tissues is a random phenomenon (10). Once there, monocytes are able to differentiate into tissue-specific-resident phagocytes (8, 10). Monocytes can differentiate *in vivo* and *in vitro* into other myeloid cells, such as MΦs or DCs in response to cytokines including granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor (11–13).

In humans, monocytes can be classified into three groups according to molecular markers and their function: (1) classical ($CD14^{++}CD16^{-}$); (2) intermediate ($CD14^{++}CD16^{+}$); and (3) non-classical ($CD14^{+}CD16^{+}$) (12, 14). While classical monocytes exert a high myeloperoxidase and intermediate phagocytic activity, non-classical monocytes are important during inflammatory and antiviral responses (12, 15). On the other hand, murine monocytes are classified into two groups: (1) $LyC6^{low}$ and (2) $LyC6^{High}$ (12). While the $LyC6^{High}$ subpopulation is responsible for the inflammatory and antimicrobial response (11, 12), $LyC6^{low}$ monocytes contribute mainly to immune surveillance and to tissue repair (12).

Macrophages (MΦs)

Macrophages are characterized by their phagocytic capacity, which is required for the removal of cellular debris during tissue repair processes (16). MΦs are present in different tissues, such as the brain, bone marrow, lung, and liver, among others. During an inflammatory response, MΦs can migrate into various tissues from the peripheral blood (16).

Macrophages can be activated both by a microbial infection or by endogenous stimuli, which include inflammatory cytokines, such as IFN-γ, IL-4, and IL-13 (16). These cells can display two different activation profiles known as M1 (classic) and M2 (alternative) (17). The M1 MΦ subset displays higher antimicrobial, inflammatory and antigen-presenting capacity (17). Meanwhile, the M2 MΦ subset mainly displays anti-inflammatory activity (16–18). In addition, M1 MΦs are stimulated by IFN-γ, while the activation of M2 MΦs requires IL-4 and IL-13 (17, 19). Importantly, M1 and M2 polarization can be modulated by viral infections (20). The role of these cells during hRSV infection will be discussed below.

Dendritic Cells

Dendritic cells are specialized cells whose main function is to modulate the communication between the innate and acquired immune responses (21). These cells are considered as professional antigen-presenting cells (APCs) with a low phagocytic capacity, as compared to other cells of the MPS (7). In mice, two major subsets of DCs have been identified: (1) conventional DCs (cDCs) or "myeloid" DCs and (2) plasmacytoid DCs (pDCs) (**Table 1**). While cDCs locate mainly in lymphoid and non-lymphoid tissues, pDCs can be found in blood, lymph nodes (LN), and lymphoid tissues (22). Additionally, murine cDCs can be divided in two subtypes: $CD103^{+}$ cDCs and $CD11b^{+}$ cDCs (**Table 1**) (22, 23). Further,

TABLE 1 | Dendritic cell (DC) subsets, location, and their surface markers.

DC subset	Location	Surface markers	Reference
Mouse conventional DC (cDC) $CD103^{+}$	Lymphoid organs and non-lymphoid organs	$CD11c^{+}$ MHC-II^{+} $F4/80^{-}$ $CD11b^{-}$ $CD103^{+}$	(22)
Mouse cDC $CD11b^{+}$	Lymphoid organs	$CD11c^{+}$ MHC-II^{+-} $CD103^{-}$ $F4/80^{-}$ integrin $\alpha E\beta 7^{+}$ $CD11b^{+}$	(22)
Mouse cDC $CD8\alpha^{+}$	Lymphoid organs	$CD11c^{+}$ $CD205^{+}$ MHC-II^{+} $CD11b^{-}$ $CD8\alpha^{+}$	(22, 24)
Human myeloid DC $CD1c^{+}$	Lymphoid organs	$CD1c^{+}$ $CD11b^{+}$ $CD11c^{high}$ BDCA-1^{+} $CD206^{+}$ $CD207^{+}$	(14, 25, 26)
Human myeloid DC $CD141^{+}$	Lymphoid organs	$CD141^{+}$ $CD11c^{+}$ $CD11b^{-}$ BDCA-3^{+} CLEC9A^{+} XCR1^{+} $CD123^{+}$	(14, 25, 26)
Plasmacytoid DC (pDC)	Blood, lymph nodes, and lymphoid tissues	$CD11c^{low}$ mPDCA-1^{+}	(22)
Human pDC	Blood and bone marrow	Lin^{-} MHC-II^{+} $CD123^{+}$ $CD4^{+}$ $CD303^{+}$ $CD304^{+}$	(14, 25, 26)

cDCs can be separated in lymphoid tissues in two subsets: $CD8^+$ and $CD11b^+$ cDCs. $CD8^+$ cDCs express the CD8α transcript and protein, but not CD8αβ heterodimer, which is most commonly expressed by $CD8^+$ T cells (**Table 1**) (22).

As for the case of human DCs, these cells can be divided into pDCs and myeloid DCs. These latter cells are additionally classified into two subsets: $CD1c^+$ and $CD141^+$ (**Table 1**) (14, 26). Here, $CD1c^+$ and $CD141^+$ are analogous to the mouse tissue-resident $CD11b^+$ and $CD103^+$ DCs, respectively (27).

With respect to the function of the various DCs subsets, cDCs display an increased ability of sensing tissue damage independent of their role in the capture, processing, and presentation of antigens (22). Equivalent to other MPS members, DCs are found in the lungs as is the case for $CD103^+$ cDCs, $CD11b^+$ cDCs, and pDCs subsets, which are distributed in the lamina propria (28). Furthermore, both subsets of cDCs ($CD103^+$ and $CD11b^+$) are found in the alveoli, allowing their migration to the mediastinal LNs (28). Consistent with this notion, it is known that lung DCs play an active role in the pulmonary pathogenesis caused by viral infection and asthma (28).

On the other hand, pDCs are able to secrete large amounts of type I IFN during viral infections and contribute to the maintenance of immune tolerance (22). The latter activity of pDCs is achieved through the expression of molecules, such as the inducible tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO), the inducible costimulator ligand, and/or the programmed death 1 ligand. These molecules promote the expansion of regulatory T cell (Treg) and the suppression of self-specific and alloreactive lymphocytes (28–31).

EPIDEMIOLOGY FEATURES OF hRSV INFECTION

Viral infections are the most important cause of acute lower respiratory tract infection (ALTRI), affecting mainly young children and the elderly (32, 33). Up to date, the main agent causing this pathology is the hRSV (33–37). Human RSV produces a broad spectrum of clinical manifestations, ranging from mild, such as rhinitis, to more serious symptoms that include bronchiolitis and pneumonia (38, 39). Clinical symptoms not only are due hRSV but also involve host risk factors, such as preterm birth, immunosuppression, congenital heart disease, and chronic lung disease (40–43). Importantly, it is known that almost 100% of children have been infected with hRSV before 2 years old, due to the fact that this virus is highly contagious and efficient at disseminating from one individual to the next (44, 45). Up to date, infections due to hRSV remain still as one of the most important global public health burdens affecting humans in all countries (45). Annually, approximately 33 millions new cases worldwide are associated to ALTRI caused by hRSV infection alone, affecting mainly children under 5 years old (33, 46). Moreover, hospitalization events due to a severe bronchiolitis or pneumonia caused by hRSV infection have increased and reached about a 10% of the total number of cases (46). Importantly, the annual cost of hospitalizations due to hRSV outbreaks is about 394 million USD, a situation that repeats every year (33, 47).

In young children, the immune system fails to establish a protective response against hRSV, which leads to frequent reinfections (33, 47–49). Lack of protective immunity is explained by an impaired induction of cellular and humoral immune memory after the primary exposure to hRSV (34, 39, 50). Furthermore, hRSV is capable of modulating phagocytic cell function, leading to the respiratory immunopathology that is a characteristic of the infection by this virus (44).

The most severe clinical manifestation caused by hRSV is bronchiolitis (51), which is mainly characterized by a distal bronchiole inflammation and obstruction, which reduces the airflow into small airways and impairs the exhalation capacity (52). All these alterations promote an abnormal lung function that is manifested as airway hyperexpansion, increased mucus production, atelectasis, and wheezing (52, 53). The bronchiolitis caused by hRSV infection also can produce long-term pathologies and sequelae, such as asthma and respiratory hyperreactivity (54).

After that hRSV encounters the airway epithelial cells (AECs), this virus gets in contact with innate immune cells, such as monocytes, MΦ, and DCs located at the lung tissue (10). These immune cells produce significant amounts of pro- inflammatory cytokines after a viral infection that is involved in controlling adaptive immunity by their interaction with helper T cells (10, 55). In addition to contributing to the clearance of microbial pathogens, monocytes and MΦs also play an important role as APCs to prime T lymphocytes (56). Consistently with this notion, monocytes, MΦs and DCs not only are involved during the acute inflammatory phase of hRSV-induced bronchiolitis but also contribute to the promotion of reactive airway disease caused by this virus (57–59).

MONOCYTES ARE REQUIRED TO INITIATE THE IMMUNE RESPONSE AGAINST hRSV

Monocytes are part of the first line of the host immune defense against viral pathogens (60). In response to infection with hRSV, human AEC-derived cell lines secrete cytokines and chemokines *in vitro*, including IL-6, IL-8, CCL2, CCL3, and CCL5 that promote the recruitment of monocytes and eosinophils to the site of infection (**Figure 1**) (60, 61). In addition, infection of BEAS2B cells (human lung epithelial cell line) with hRSV kept them from inhibiting the secretion of pro-inflammatory cytokines by monocytes, such as TNFα (62). During homeostasis, AECs are able to inhibit the function of inflammatory monocytes, a feature that is impaired in hRSV-infected AECs (62). Interestingly, monocytes can be directly infected by hRSV, reducing the expression of the intercellular adhesion molecule 1 and its ligand, the lymphocyte function-associated antigen 1, which alters the collaboration between monocytes and other immune cells (10, 63). These observations suggest that monocytes infected with hRSV can display a reduced capacity to induce a protective immune response against hRSV (10).

Patients infected with hRSV show a frequency increase for $CD14^+CD16^+$ monocytes in the blood (37). Furthermore, an

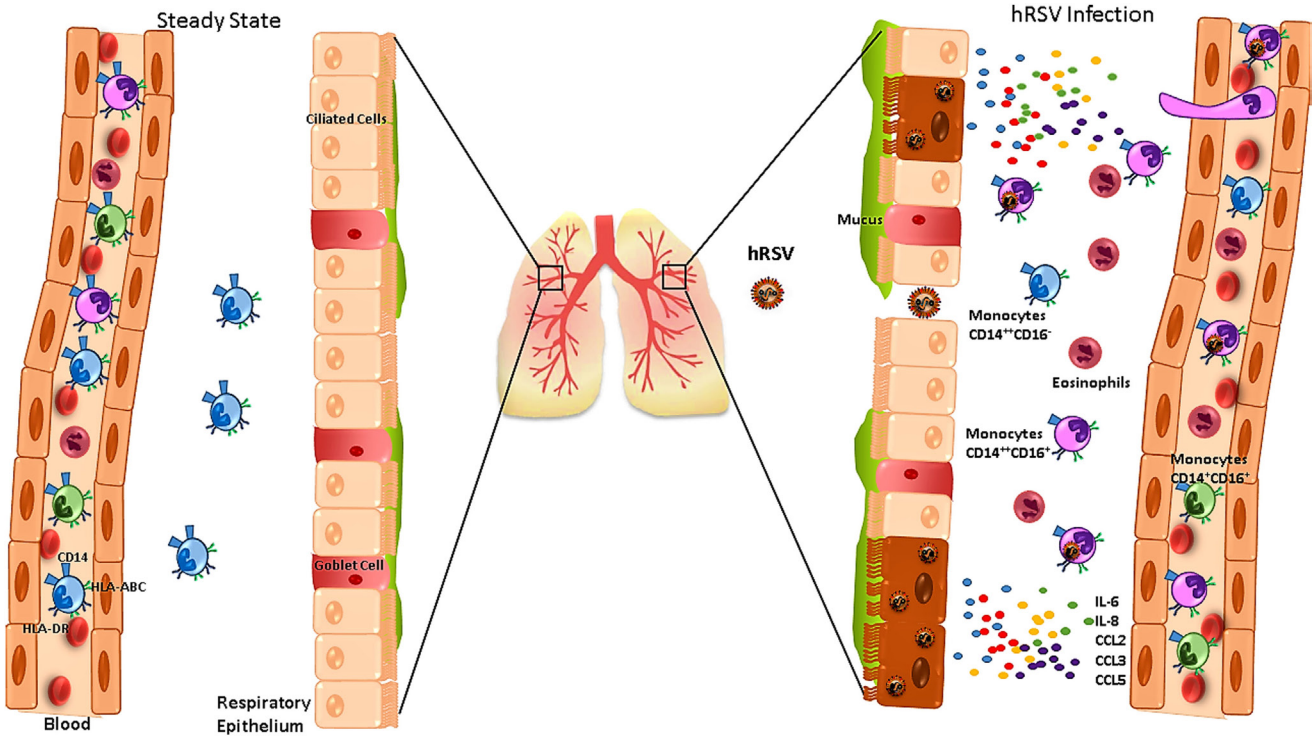

FIGURE 1 | Human respiratory syncytial virus (hRSV) infection alters the function of monocytes and promotes their infiltration into the lungs. During the steady state (left), the main monocytes subset found in lungs are $CD14^{+}CD16^{-}$. During hRSV infection (right), infected airway epithelial cells secrete IL-6, IL-8, CCL2, CCL3, and CCL5, which promote monocytes and eosinophils infiltration into the airways. In addition, blood monocytes express increased levels of CD14 and HLA-ABC and reduced levels of HLA-DR, which is associated with severe bronchiolitis.

increase of CD14 expression has been observed for all the monocyte subsets (**Figure 1**) (37, 64), suggesting that these cells can display an enhanced capacity to secrete cytokines and to migrate into the airways, probably to replace alveolar MΦ during hRSV infection (37, 64). Moreover, monocytes from hRSV-infected patients show a diminished expression of HLA-DR (**Figure 1**), correlating with disease severity (37, 65).

As mentioned above, hRSV infection causes bronchiolitis in children under 2 years old (66). Moreover, the pathology is worsened by some cytokines produced by monocytes during the early state of hRSV infection (67). Monocytes from of hRSV-infected patients presenting bronchiolitis in the convalescent stage of the infection secreted large amounts of IL-10 in response to stimulation with LPS and IFN-γ *in vitro* (67). Furthermore, authors showed a significant correlation between the monocyte-produced IL-10 and the number of wheezing episodes (10, 67).

Similar to MΦ and DCs, monocytes express TLR8 that promotes endosomal activation and IL-12p70 release upon binding to viral RNA (68). Monocytes derived from hRSV-infected infants displayed reduced expression of TLR8 during the acute phase of infection (68). Additionally, this study showed that monocytes from hRSV-infected infants produced reduced levels of TNF-α as compared to monocytes from healthy controls (68). Taken together, these results suggest that hRSV infection interferes with the normal expression of TLR8 and perhaps with the cytokines production that are important to initiate the immune response against hRSV (68).

The data relative to the role of monocytes during the immune response induced by the hRSV infection suggest that these cells are important to initiate the immunity against this pathogen. Further, monocytes are also involved in the development of bronchiolitis and the recurrent wheezing. Thus, it is likely that these cells could contribute to chronic respiratory sequelae caused by hRSV, such as asthma and airway hyperreactivity.

ALVEOLAR MACROPHAGES (AMs) ARE CRUCIAL TO CONTROL hRSV-CAUSED DISEASE

Lung-resident macrophages consist of two distinct populations namely (1) AMs and (2) interstitial macrophages (IMs) (69). AMs locate in the luminal surface, while IMs reside in the interstitial space of the lung parenchyma (70). AMs are the most abundant phagocytic resident cells in the lungs, which uptake foreign particles, remove cellular debris, initiate immune responses against pathogens and contribute to restoring homeostasis in the lung epithelium (70). During the steady state (**Figure 2**), AMs can display an immunosuppressive effect by directly inhibiting

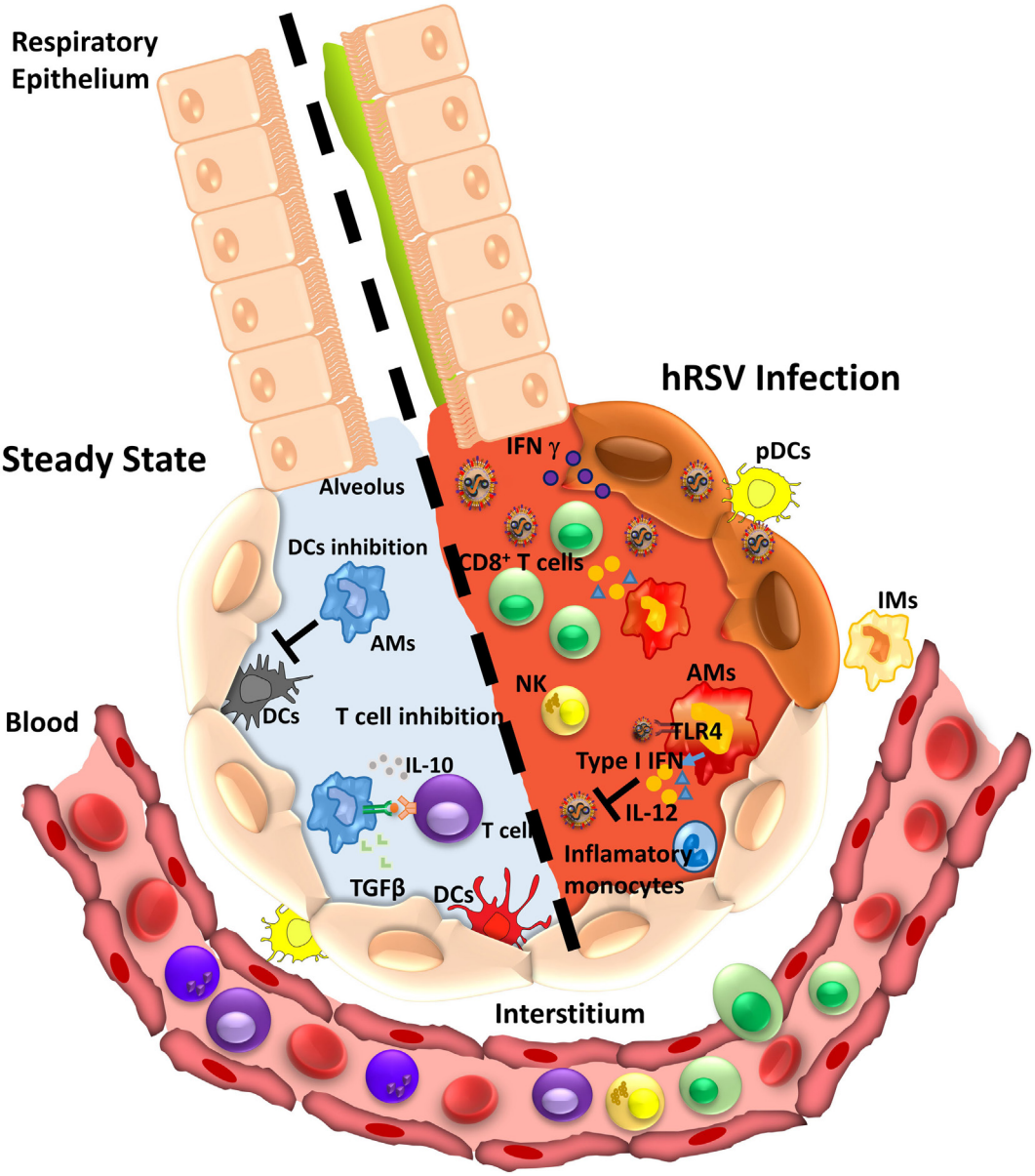

FIGURE 2 | Alveolar macrophage (AM) function during homeostasis or infection by human respiratory syncytial virus (hRSV). During the steady state (left), AMs perform an anti-inflammatory function leading to immune tolerance. AM suppresses the response of T cell in an antigen-dependent manner and secretes cytokines as IL-10 and transforming growth factor-β (TGFβ). Also, AMs impair dendritic cells (DCs) activity inhibiting T cell presentation. Under hRSV infection (right), AMs can recognize the virus by TLR4 and respond by secreting type I IFNs that induce an antiviral state in epithelial cells. In addition, the secretion of pro-inflammatory cytokines promotes recruitment of inflammatory monocytes, $CD8^+$ T cells, and NK cells that contribute with viral clearance.

the antigen-presenting function of lung DCs (71) or by inducing $CD4^+$ T cell unresponsiveness in an antigen-specific manner (72). Furthermore, AMs can secrete several immunomodulatory molecules, such as IL-10, nitric oxide, prostaglandins, and transforming growth factor-β (**Figure 2**), which reduce inflammation in the lungs (73).

The role of AMs during hRSV infection has been characterized in murine models by depleting these cells through the administration of liposomes containing clodronate, a molecule that promotes the apoptosis of AMs (70, 74, 75). It was shown that AMs are crucial for the clearance of hRSV and for the control of lung inflammation (70). The depletion of AMs during hRSV infection leads to an increased viral replication and an exacerbated lung immunopathology (70). These results were consistent with a dramatic increase of neutrophils and inflammatory DCs recruitment to the lungs (70, 74). Then, in mice, AMs display a protective function during hRSV infection and contribute to attenuating lung inflammation and bronchiolitis

triggered by this pathogen (76). Similar results were obtained in New Zealand Black (NZB) mice, which lack normal macrophage function and show an enhanced lung immunopathology upon hRSV exposure (76). Although the mechanisms responsible for the beneficial effect of AMs to control hRSV infection remain unknown, the available data suggest that the phagocytic and microbicidal capacity of AMs together with the secretion of type I IFN are the main elements contributing to the protection against this virus (76).

Alveolar macrophages are the main producers of type I IFNs in the airways during hRSV infection, even more than other cells, such as epithelial cells and pDCs (75). Type I IFN production by AMs is triggered by hRSV recognition and mediated by cytosolic mitochondrial antiviral signaling protein-coupled retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) (77). The type I IFN production promotes the monocytes-derived inflammatory cells recruitment (**Figure 2**), which further contributes to controlling hRSV infection and reducing lung pathology (77).

On the other hand, *ex vivo* experiments showed that hRSV infects both murine and human AMs (78). However, in both cases, the infection failed to lead to an increase of viral particle production (70, 79). These results suggest that the infection of AMs by hRSV might be abortive, allowing that AMs maintain a sentinel activity. Further, it is thought that abortive replication may allow AMs to resist the effects of hRSV NS protein, which inhibits the activity of RIG-I-like receptors (RLR) (77). Along these lines, AMs can restrict hRSV replication even in the absence of type I IFNs (75). However, the exposure of AMs to hRSV can result in a reduced phagocytic capacity during subsequent infections (79).

Moreover, AMs are essential for the activation of the early immune response against hRSV (70). Infection of human AMs by hRSV leads to the secretion of several pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β, and IL-8 (78, 80, 81). Conversely, similar experiments have described the secretion of IL-10 by these cells (82). The AMs response to hRSV is mediated mainly by the activation of NF-κB through recognition of non-replicative viral particles and surface viral proteins by TLR4 at early times postinfection (**Figure 2**) (83). Based on the available data about the role of TLR4 in the infection with hRSV, it has been hypothesized that in the beginning, surfactant protein A-opsonized hRSV can bind to TLR4 expressed on the surface of alveolar epithelial cells and AMs (84). Next, additional TLR4 and CX3CR1 molecules are recruited to the virus attachment site (84). Then, the hRSV F and G proteins interact with TLR4 and CX3CR1, respectively. Furthermore, both proteins interact with heparan sulfate structures (84), an interaction that is followed by the recruitment of caveolin-1 and the formation of caveolae with the subsequently recruitment of RhoA to the binding site (84). Moreover, it has been suggested that the binding of the hRSV F protein to TLR4 may activate the signaling pathway for this receptor and NF-κB translocation (84, 85). However, the role of TLR4 during the hRSV entry into target cells remains controversial, as well as the interaction with viral proteins that trigger the activation of the NF-κB pathway (86). A study performed in TLR4-positive cells (HEK 293 reporter cell lines) showed that infection with hRSV does not activate the NF-κB signaling pathway through the TLR4/MD-2/CD14 complex (87). However, in the context of the AM-directed immune response, studies have shown that in the absence of TLR4, the NF-κB signaling pathway is not activated (83) and that the latter is required for the polarization of MΦ toward the M2 phenotype (88). On the other hand, the establishment of this initial condition allows an effective lymphocyte recruitment and proper antiviral activity. Along these lines, human neonatal AMs infected with hRSV showed an impairment in the IFN-γ and IL-12 production (81). An inefficient secretion of IFN-γ has been associated with an increase of severe illness in infants (89). Considering that IFN-γ is necessary for the activation of AMs, a reduced production of IFN-γ in neonates has been observed to impair AMs activation, affecting the phagocytic capacity of these cells and exacerbating the hRSV-mediated bronchiolitis (90, 91). Also, an impaired AMs function reduces the T and NKT cells recruitment to the lungs, contributing to higher viral loads (91).

According with the data described above, AMs are important for the elicitation of an early immune response against hRSV, contributing to the viral clearance mainly mediated by the type I IFN secretion and the coordination of the adaptive response against this pathogen. Thus, an impaired function or absence of AMs can increase hRSV-induced bronchiolitis, both in mouse models and infants.

In contrast to the significant research efforts to understand the role of AMs during the infection with hRSV, the participation of IMs has only been poorly studied (92). Qi et al. evaluated the role of AMs and IMs in the production of IL-33 during hRSV infection (93). This study showed that the absolute number of IMs in lungs of hRSV-infected mice remained constant during the hRSV infection, in contrast to the increase observed for the absolute number of AMs (93). Furthermore, IMs from lungs of hRSV-infected mice showed an increase in the expression of both TLR3 and TLR7 mRNA (93). Considering that the number of IL-33-producing IMs in the lungs of mice was affected by hRSV infection, authors concluded that the IMs may not be the source of IL-33 during hRSV infection (93).

Therefore, additional studies are required to better understand the contribution of IMs to hRSV infection and pathogenesis.

DCs AS COMMANDERS OF THE IMMUNE RESPONSE DURING hRSV INFECTION

The infection with hRSV can induce different immune responses depending on the type of DC subset infected (94). According to this notion, it has been described that hRSV infection promotes CDs maturation by increasing the expression of CD80, CD86, CD40, and MHC-II in the lungs, which leads to a decrease of phagocyte function (95). Moreover, some studies in mice have reported that during the acute phase of hRSV infection, the frequency of mature DCs in the lungs is increased (95). On the other hand, it has been shown that hRSV has the capacity to infect and replicate inside DCs but in a non-productive manner (96–98). Importantly, it has been described that toll-like receptors expressed by DCs can interact with hRSV proteins. TLR-2 interacts with the viral fusion glycoprotein (F) and TLR-4 with

both the F and the attachment G protein (99, 100). TLR4 activation promotes the secretion of IL-6 and TNF-α, as well as antigen cross-presentation *in vivo* and *in vitro* (101). Additionally, hRSV G glycoprotein interacts with DC- and L-SIGN, inducing both DC/L-SIGN-dependent and -independent phosphorylation of ERK1 and ERK2. As a result, DCs activation is impaired (102). This mechanism can be considered as a possible explanation for the reduced immunity induced by hRSV reinfections.

To understand as to how DCs become infected with hRSV, *in vitro* and *in vivo* experiments were performed in mouse models, which showed the contribution of Fcγ receptors (FcγRs), mainly FcγRIII, to infection by this virus (103). Human RSV-infected FcγRIII KO mice showed reduced airway inflammation as compared to infected wild-type mice, suggesting that FcγRIII plays a pro-inflammatory role during hRSV infection (103). On the other hand, it is known that hRSV infection induces only weak immune memory in the host (50). To understand this phenomenon, a possible impairment of the immunological synapsis between hRSV-infected DCs and T cells was evaluated *in vitro*. It was observed that hRSV infection of DCs not only impaired the assembly of the immunological synapsis with T cells but also the activation of naïve antigen-specific T cells (50, 104). The hRSV virulence factor that seems responsible for the inhibition of immunological synapse assembly is the nucleoprotein (N) (104). The N protein was found on the DCs membrane and by itself could interfere with the assembly of the immunological synapsis (104). Further, the N protein was located nearby to the TCR–pMHC complexes at the DC-T cell synapse interface (50, 104).

On the other hand, it has been reported that the mTOR protein on bone marrow-derived dendritic cells (BMDCs) plays an important role during hRSV infection (105). According to this notion, mTOR inhibition by rapamycin in hRSV-infected BMDCs decreased the number of $CD8^+CD44^{high}$ T cells, suggesting that mTOR is necessary for the proliferation of the T cell memory subset (105). Moreover, the treatment of the hRSV-infected BMDCs with rapamycin did not affect maturation and increased the survival when DCs were cocultured with T cells, suggesting that this phenomenon requires the contact of both cell types (105).

$CD103^+$ cDCs are the most prevalent population of DCs in the lungs, which locate directly underneath the airway epithelium (14). $CD103^+$ cDCs express the integrin $\alpha_E\beta_7$ and are found mainly at the lamina basal of the bronchial epithelia and arterioles (106). This DCs subset efficiently loads virus-derived peptides onto MHC-I molecules, inducing a potent proliferation of naïve $CD8^+$ T cells (**Figure 3**) (107). Therefore, $CD103^+$ cDCs work as key mediators of immunity to intracellular pathogens infecting the lungs (108, 109). In several studies with hRSV, neonatal mice have been used to better compare the human clinical features with mouse models of the disease (110). $CD103^+$ DCs from neonatal mice infected with hRSV showed lower expression of co-stimulatory molecules, CD80 and CD86, as compared to the

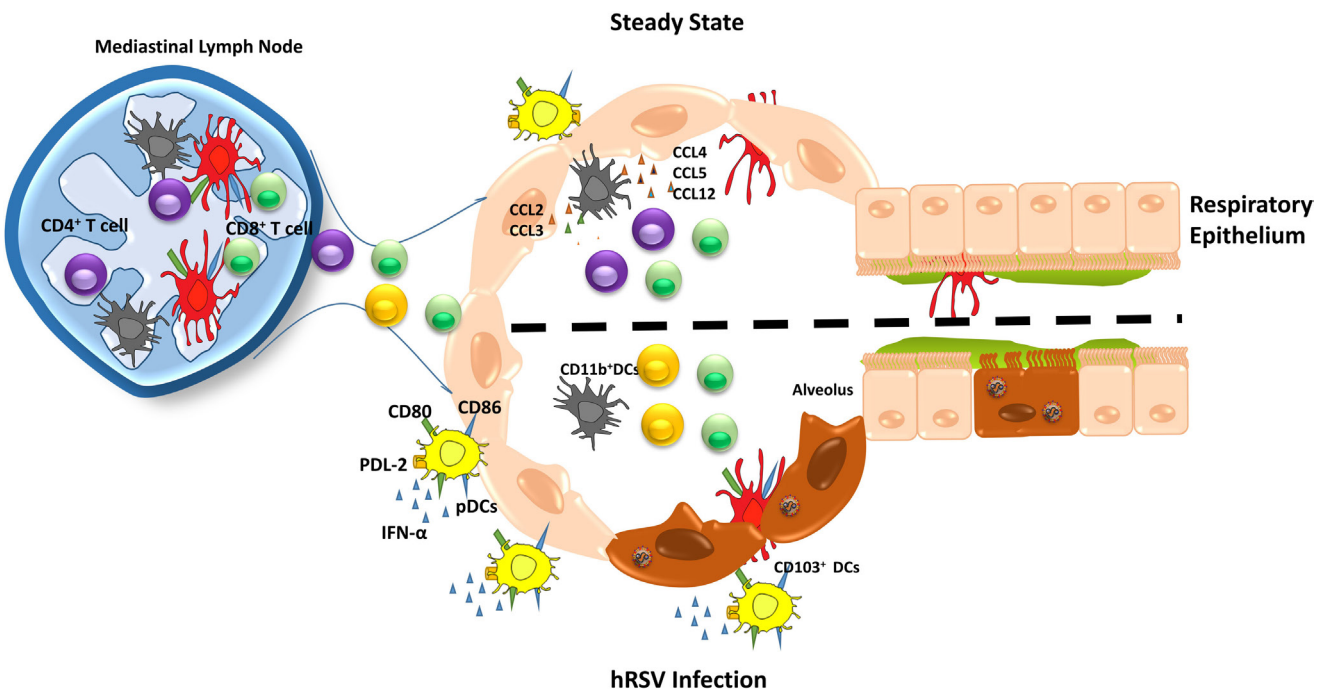

FIGURE 3 | Different roles of dendritic cells (DCs) subsets during human respiratory syncytial virus (hRSV) infection. During the steady state (upper), DCs subset maintain the lung homeostasis and when an infection occurs, they are capable to migrate to mediastinal lymph node to present antigens to T cells, promoting an immune response. Upon hRSV infection, plasmacytoid DCs (pDCs) increase in numbers, as well as the expression of co-stimulatory molecules, such as CD80, CD86. In addition, pDCs secretes high levels of IFN-α and PDL-2 expression is reduced. Moreover, in response to hRSV infection, $CD11b^+$ DCs fail to activate $CD4^+$ T cells leading a poor response against the virus. On the other hand, $CD103^+$ DCs show a lower expression of CD80 and CD86, which are required for an appropriate antiviral immune response.

adult counterparts, affecting the T cell synapsis quality (110). For this reason, neonatal mice infected with hRSV generated a distinct $CD8^+$ T cell response as compared to adult mice, suggesting a key role of $CD103^+$ DCs (110). Furthermore, the immunization with F virus-like particles (VLP) of hRSV-infected mice showed high levels of $CD103^+$ DCs in bronchoalveolar lavage fluids (BALFs) and lungs (23). Moreover, the mediastinal LN from F VLP-immunized mice showed higher levels of $CD103^+$ DCs and resident $CD8\alpha^+$ DCs (23). It is known that after hRSV infection is resolved, is possible to develop subsequent asthma during childhood (111, 112). Here, $CD103^+$ DCs play a protective role during asthma/allergic-related symptoms by producing IL-12 (113).

As mentioned above, another DCs cell subset, $CD11b^+$ cDCs locate in the lung parenchyma (114, 115). In addition to the uptake of extracellular pathogens, the main function of these cells is to present antigens to $CD4^+$ T cells (116). Studies were carried out in mice to understand the role of $CD11b^+$ DCs during hRSV infection. In lungs from mice infected with hRSV, an increase was observed for the frequency of $CD11b^+$ DCs (114). Moreover, it was determined that the ability of $CD11b^+$ DCs to migrate to LN remained intact (114). One of the mechanisms proposed for the accumulation of $CD11b^+$ DCs in the airways is that during hRSV infection, the high levels detected for CCL20 in the lungs can attract $CD11b^+$ $CCR6^+$ DCs (117, 118). To evaluate the role of $CD11b^+$ $CCR6^+$ DCs during hRSV infection, studies were performed in CCR6-deficient mice, showing an increase of viral clearance, lower levels of Th2 pro-inflammatory cytokines, as well as reduced mucus production (117, 118). Overall, these findings suggest that the $CD11b^+$ $CCR6^+$ DCs promote a Th2 immune response upon hRSV infection (117, 118). Additional studies performed in the neonatal murine model described that lung $CD11b^+$ DCs express higher levels of the IL-4 α receptor (IL-4Rα) as compared to adult mice (119). *In vitro* experiments using neonatal murine $CD11b^+$ DCs showed that IL-4Rα promotes the differentiation of T cells into a Th2 phenotype (119). Furthermore, when the IL-4Rα was deleted, maturation of these cells increased, suggesting that neonatal $CD11b^+$ DCs are less prone to maturation. This feature of neonatal DCs could impair their capacity to induce a protective hRSV-specific immune response (119). Similar results were observed *in vivo* experiments; however, the deletion of IL-4Rα could be observed in several cells types including AMs, $CD11b^+$ DCs, and $CD103^+$ DCs (119).

Plasmacytoid DCs are the other prominent subset present in the lungs, which are an important source of IFN-α/β, fundamental antiviral cytokines during an infection, both in humans and mice (120, 121). The role of this DCs subset was elucidated by performing experiments with hRSV-infected bone marrow-derived pDCs, which expressed high levels of IFN-α, CD80, and CD86 and lower PD-L2 levels (**Figure 3**) (122). Moreover, in the same study, authors showed that the number of pDCs was increased early after infection and then decreased with the resolution of the disease, suggesting that pDCs are required during early stages of infection by hRSV (122). Also, it has been demonstrated that when pDCs are depleted using a 120G8 antibody, the lungs of hRSV-infected mice displayed an enhanced inflammation consisting mainly of mononuclear cells and lymphocytes, together an increase of viral loads (123). Consistently with these findings, it was observed that BALFs from hRSV-infected preterm born children showed reduced recruitment of pDCs into the lungs. These data are in agreement with the notion that low numbers of pDCs could work as a risk factor for severe bronchiolitis (124).

Additionally, three pDCs subsets have been characterized that include $CD8\alpha^-\beta^-$, $CD8\alpha^+\beta^-$, and $CD8\alpha^+\beta^+$ (125). The frequency of these subsets is 61, 22, and 6% in the lungs of healthy mice, respectively (125). However, beside all the current knowledge about the role of the different subset of DCs during hRSV infection, there are no data about the contribution of these cells to the hRSV-induced pathology.

In summary, all DCs subsets seem to be important for an antiviral immune response. However, hRSV is able to modulate the function of these cells by promoting an imbalance between these subsets, which could be critical for the resolution of the disease caused by this virus.

CONCLUDING REMARKS

The MPS consists of a family of cells that include monocytes, MΦ, and DCs, among others (1–3). These cells are characterized by their high mobility, phagocytic capacity, and ability to secrete a broad spectrum of immunomodulatory molecules (2). MPS exerts several functions in health and disease in several tissues (2). In the lungs, MPS plays an important role in the maintenance of homeostasis during steady state. During an infection, the MPS works as the first line of immune response against pathogens (2). Among the respiratory pathogens, hRSV is considered the most important cause of respiratory illness in infants and young children (33). The main severe clinical manifestation due to the hRSV infection is bronchiolitis (33). In this context, the MPS can contribute to the development of the immunopathology induced by hRSV (10). This viral pathogen is able to infect the MPS cells, altering the proper immune response required for viral clearance and the later acquisition of an antiviral immune memory (10). During the acute phase of the hRSV infection, monocytes are important to initiate the innate immune response, secreting cytokines and chemokines that recruit other immune cells, such as MΦ, eosinophils, and neutrophils (126). The normal function of monocytes during hRSV infection can be impaired not only by the pathogen itself but also by the contact with virus-infected AECs, contributing to the development of severe bronchiolitis (62).

During hRSV infection, MΦs play a protective role against infection and, finally, these cells are required for proper virus clearance (77). This notion is supported by experimental data obtained from various experimental models, such as neonatal mice and MΦ-depleted animals.

One of the effector mechanisms of MΦs is the secretion of IFN-γ and IL-12, which are reduced in human neonates infected by hRSV, contributing to development of bronchiolitis (81). Also, the inefficient recruitment of T and NKT cells into the airways (91) contributes to the development of bronchiolitis. As a consequence, there is a significant decrease of the protective capacity of the immune response triggered by hRSV and promoting virus spreading (91).

The ability of hRSV to infect DCs seems to be a major virulence mechanism used by this pathogen. The hRSV-infection of DCs impairs the correct immunological synapsis, which is required for T cell activation (104). These findings may contribute to explaining the lack of an effective immunological memory against hRSV, which allow subsequent reinfections throughout life. Moreover, pDCs are the major source of type I IFNs, cytokines necessary to induce an appropriate antiviral immune response (122). Consistently with a lack of protective immunity to hRSV, DCs infected by this virus show an impaired capacity to produce type I IFNs (122). Based on the current knowledge relative to the role of DCs in the hRSV-infection, these cells could be important for the promotion of an exacerbation of the inflammation during bronchiolitis. Besides the actual knowledge about the MPS in the hRSV immunopathology, is still necessary to further understand the mechanisms involved in the impairment of MPS function by hRSV virulence factors.

AUTHOR CONTRIBUTIONS

AK wrote, revised, and edited the article and figures. KB, JE, RP, and EJ wrote the article and drew the figures.

FUNDING

The grants are Millennium Institute in Immunology and Immunotherapy and Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) CONICYT/FONDECYT POSTDOCTORADO No. 3150559.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 October 2017
doi: 10.3389/fmicb.2017.02108

Pharmacological Induction of Heme Oxygenase-1 Impairs Nuclear Accumulation of Herpes Simplex Virus Capsids upon Infection

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 13 June 2017
Accepted: 16 October 2017
Published: 31 October 2017

Citation:
Ibáñez FJ, Farías MA, Retamal-Díaz A, Espinoza JA, Kalergis AM and González PA (2017) Pharmacological Induction of Heme Oxygenase-1 Impairs Nuclear Accumulation of Herpes Simplex Virus Capsids upon Infection. Front. Microbiol. 8:2108. doi: 10.3389/fmicb.2017.02108

Heme oxygenase-1 (HO-1) is an inducible enzyme that is expressed in response to physical and chemical stresses, such as ultraviolet radiation, hyperthermia, hypoxia, reactive oxygen species (ROS), as well as cytokines, among others. Its activity can be positively modulated by cobalt protoporphyrin (CoPP) and negatively by tin protoporphirin (SnPP). Once induced, HO-1 degrades iron-containing heme into ferrous iron (Fe^{2+}), carbon monoxide (CO) and biliverdin. Importantly, numerous products of HO-1 are cytoprotective with anti-apoptotic, anti-oxidant, anti-inflammatory, and anti-cancer effects. The products of HO-1 also display antiviral properties against several viruses, such as the human immunodeficiency virus (HIV), influenza, hepatitis B, hepatitis C, and Ebola virus. Here, we sought to assess the effect of modulating HO-1 activity over herpes simplex virus type 2 (HSV-2) infection in epithelial cells and neurons. There are no vaccines against HSV-2 and treatment options are scarce in the immunosuppressed, in which drug-resistant variants emerge. By using HSV strains that encode structural and non-structural forms of the green fluorescent protein (GFP), we found that pharmacological induction of HO-1 activity with CoPP significantly decreases virus plaque formation and the expression of virus-encoded genes in epithelial cells as determined by flow cytometry and western blot assays. CoPP treatment did not affect virus binding to the cell surface or entry into the cytoplasm, but rather downstream events in the virus infection cycle. Furthermore, we observed that treating cells with a CO-releasing molecule (CORM-2) recapitulated some of the anti-HSV effects elicited by CoPP. Taken together, these findings indicate that HO-1 activity interferes with the replication cycle of HSV and that its antiviral effects can be recapitulated by CO.

Keywords: heme oxygenase-1, carbon monoxide, pharmacological induction, antiviral drug, herpes simplex virus, capsid

INTRODUCTION

Heme oxygenase-1 (HO-1), also known as heat shock protein 32 (Hsp32) in mammals, is an inducible enzyme expressed in numerous cell types in response to increasing amounts of its substrate heme, as well as various stress stimuli, such as reactive oxygen species (ROS; Keyse and Tyrrell, 1989), ultraviolet radiation (Keyse and Tyrrell, 1987), hyperthermia (Shibahara et al., 1987), hypoxia (Murphy et al., 1991), as well as cytokines (Terry et al., 1998), and lipopolysaccharide (LPS; Camhi et al., 1995), among others (Ryter et al., 2006). Additionally, the activity of HO-1 can be modulated by numerous chemical compounds, such as protoporphirins either to increase or inhibit its activity. While cobalt protoporphyrin (CoPP) is known to promote the expression and activity of HO-1 (Bonkovsky et al., 1990; Shan et al., 2000), tin protoporphirin (SnPP) blocks its activity, although it may also elicit increased HO-1 expression (Bonkovsky et al., 1990; Ewing et al., 2005). Once induced, HO-1 has been described to localize at multiple sites within the cell, such as the endoplasmatic reticulum (Yoshida et al., 1988), plasma membrane (Kim et al., 2004), mitochondria (Slebos et al., 2007; Riquelme et al., 2015b), and nucleus (Lin et al., 2007), with its distribution likely playing different and specific roles at distinct organelles (Kim et al., 2004). HO-1 degrades iron-containing pro-oxidant heme (preferably heme *b* and *c* and hematoheme) into equimolar quantities of ferrous iron (Fe^{2+}), carbon monoxide (CO) and biliverdin, with the latter rapidly being converted into bilirubin by NADPH-dependent biliverdin reductase (Tenhunen et al., 1970; Maines and Kappas, 1977). Importantly, numerous products of HO-1 catalysis are cytoprotective displaying anti-apoptotic, anti-oxidant, anti-inflammatory, and anti-cancer effects, among others (Ryter et al., 2006). Ferrous iron ions derived from HO-1 can participate in important cell processes that depend on this metal and high concentrations can modulate the stability of particular mRNAs, either dampening or promoting their translation (Eisenstein et al., 1991). Increased intracellular levels of iron also promote the expression of ferritin, which has been described to exert, *per-se* numerous cytoprotective effects against cell-damaging agents (Vile et al., 1994). Eventually, elevated cellular concentrations of iron derived from HO-1 activity may also activate cytoprotective NF-κB pathways, that support resistance to Fas-mediated apoptosis (Choi et al., 2004). While biliverdin and bilirubin display cytoprotective effects and act as strong anti-oxidants, at high concentrations they can alter mitochondrial function and be toxic for organs such as the brain (Menken et al., 1966; Clark et al., 2000; Kapitulnik, 2004). Lastly, carbon monoxide (CO) has acquired considerable attention as a molecule that modulates inflammatory processes, as well as cellular apoptosis (Motterlini and Otterbein, 2010). Among others, CO has been shown to dampen the expression of pro-inflammatory molecules on the cell surface (Riquelme et al., 2015a), alter endosome-lysosome fusion (Tardif et al., 2013), modulate mitochondrial function (Riquelme et al., 2015b), inhibit T cell activation (Mackern-Oberti et al., 2015) and alter cellular ion channel function (Peers et al., 2015).

Because cell infection with viruses often involves the modulation of stress-related processes that can favor or dampen virus replication, assessing the potential role of HO-1 over virus infection has acquired significant interest in the recent years. Indeed, new studies have shown that HO-1 displays important antiviral properties against human viruses, such as the human immunodeficiency virus (HIV; Devadas and Dhawan, 2006), influenza (Hashiba et al., 2001), human respiratory syncytial virus (RSV, Espinoza et al., 2017), hepatitis B (HBV; Protzer et al., 2007), hepatitis C virus (HCV; Lehmann et al., 2010), enterovirus 71 (EV71; Tung et al., 2011), dengue virus (DENV; Olagnier et al., 2014; Tseng et al., 2016), and Ebola virus (EBOV; Hill-Batorski et al., 2013). An antiviral role for HO-1 has also been reported for bovine viral diarrhoa virus (BVD; Zhang et al., 2015) and porcine reproductive and respiratory syndrome virus (PRRSV; Xiao et al., 2014) *in vitro*. While the mechanisms underlying the antiviral effects of HO-1 have remained elusive for some viruses, in other cases its antiviral effects have been identified (Schmidt et al., 2012). For instance, bilirubin derived from HO-1 has been reported to inhibit the protease activity of HIV (Liu et al., 2016), the activity of the non-structural 3/4A protease of HCV (Zhu et al., 2010) and non-competitively inhibit the protease of DENV (Olagnier et al., 2014). Interestingly, biliverdin derived from HO-1 has also been shown to elicit an increased interferon response against HCV (Lehmann et al., 2010). On the other hand, iron derived from HO-1 has been suggested to inhibit the non-structural 5B (NS5B) RNA-dependent RNA polymerase of HCV, through the inhibition of divalent cation binding (Fillebeen et al., 2005). Finally, CO has been shown to dampen ROS generation in EV71-infected cells and consequently diminish virus replication in these cells (Tung et al., 2011).

Given that the products of HO-1 can exert significant anti-viral effects, beyond their anti-oxidant and cytoprotective properties, studying the role of this enzyme over the replication of viruses may help identify novel therapeutic approaches that hamper viral infection. Herpes simplex viruses (HSV-1 and HSV-2) are highly prevalent in the human population with scarce treatment options for drug-resistant variants that may arise in immunosuppressed individuals and at present, there are no available vaccines to prevent infection (Suazo et al., 2015). While HSV-1 is the primary cause of infectious blindness in developed countries, HSV-2 is a major catalyst of HIV infection and spread (Suazo et al., 2015). Building on a previous study that reported that bilirubin, a product of heme metabolism by HO-1 can dampen HSV replication *in vitro* (Santangelo et al., 2012), we sought to assess the role of HO-1 activity on the infection of epithelial and neuronal cells, to identify other potential antiviral products of this enzyme and assess possible mechanisms of action. By using HSV viruses that encode structural and non-structural green fluorescent protein (GFP) genes, here we assessed the effects of the pharmacological induction of HO-1 activity over cell infection with HSV-2 and found that treatment with CoPP impaired virus propagation. Furthermore, CoPP treatment protected epithelial cells from suffering morphological cytopathology after infection. Interestingly, many effects elicited by CoPP were recapitulated by CO. Taken together, these results propose an important role for HO-1 and its product CO in

blocking HSV infection thus, opening potential new treatment opportunities against this virus. Noteworthy, numerous CO-releasing molecules (CORMs) intended for clinical use are currently being developed (Zobi, 2013; Abeyrathna et al., 2017).

MATERIALS AND METHODS

Cells and Viruses

Vero (ATCC #CCL-81) cells were used to propagate HSV-2 (strain G; Dolan et al., 1998). Briefly, T75 flasks with Vero cell monolayers were grown in RPMI (Thermo Fisher Scientific), 5% FBS (Fetal Bovine Serum Gibco®, Thermo Fisher Scientific) supplemented with 1 mM piruvate (Thermo Fisher Scientific), 2 mM Glutamine (Thermo Fisher Scientific) and 100 IU/mL Penicilin/Streptomycin (Thermo Fisher Scientific) to 80% confluence, inoculated with an MOI of 0.01 of virus in 10 ml Opti-MEM media (Thermo Fisher Scientific) and incubated at 37°C for 1 h. Then, supernatants were replaced with fresh Opti-MEM medium for 24–36 h until abundant visible cytopathic effect was observed. The contents of the flasks were pooled, and cells removed twice by centrifugation at 400 g for 10 min. Pellets were sonicated for 5 min in a sonicator waterbath (Branson 1210, Branson Ultrasonics), aliquoted in cryotubes and stored at −80°C until use. Virus dilutions were titered over Vero cells cultured in flat-bottom 96 well plates and screened for plaque formation after cell fixation with 1% paraformaldehyde (PFA, Winkler) in PBS and a 0.04% crystal violet (Sigma-Aldrich) staining solution.

Modulation of HO-1 Activity and CORM-2

Vero and HeLa cells (ATCC, #CCL-2) were grown in RPMI supplemented as indicated above, to 80% confluence before application of the treatments indicated below. Vero cells were treated with 60 μM CoPP (Frontier Scientific, Inc.), 60 μM SnPP (Frontier Scientific, Inc.) or an equivalent volume (2.24 μL per mL) of NaOH 0.1 M (vehicle for CoPP and SnPP) in Opti-MEM (Thermo Fisher Scientific) for 6 h, then the cells were washed with PBS. HeLa cells were treated with 10 μM CoPP, 10 μM SnPP, or an equivalent volume of NaOH 0.1 M (vehicle for CoPP and SnPP) in Opti-MEM for 14 h, then washed with PBS. Next, Vero or HeLa cells were inoculated with HSV-2 at the indicated MOI for 1 h in Opti-MEM media at 37°C, then washed with PBS and finally incubated in Opti-MEM for additional periods, as indicated for each experiment, and prepared for western blot analysis, flow cytometry, plaque formation, or laser confocal microscopy. Tricarbonyldichlororuthenium(II) dimer, also known as CORM-2 (Sigma Aldrich, catalog number #288144), was used as a carbon monoxide donor. CORM-2 was applied to cells 6 h after infection with HSV at a final concentration of 100 μM in Opti-MEM medium, as previously reported by others (Zhang et al., 2017). Lyophilized CORM-2 was pre-dissolved DMSO at a final concentration of 100 mM and then diluted as needed in Optimem before application onto the cells. DMSO was used as a vehicle when assessing the effect of CORM-2. CORM-2 was inactivated (iCORM-2) with 0.1 M HCl for 2 h and then the neutralized with 0.1 M NaOH. As a control, CORM-2 was diluted with equivalent amounts of NaCl at neutral pH.

Western Blot Analyses

Western blot analyses were performed to assess the expression of HO-1 and viral proteins. Briefly, protein preparations from 0.4×10^6 cells were extracted using RIPA protein extraction RIPA buffer (2006). Proteins in the soluble fraction were then quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Twenty-five micrograms of protein was loaded onto SDS-PAGE 12% polyacrylamide gels (miniprotean II, Bio-Rad Laboratories) and transferred onto nitrocellulose membranes (Promega). After transfer, membranes were blocked with 5% Bovine Serum Albumin (BSA, Winkler, Chile) and incubated, either with an anti-HO-1 monoclonal antibody (Abcam, clone ab13248) at a dilution of 1:400 at 4°C, an anti-gD monoclonal antibody (Virusys, clone HA025) at a dilution of 1:50,000 at 4°C, an anti-VP16 monoclonal antibody (Santa Cruz Biotechnology, clone 1-21) at a dilution of 1:1000 at 4°C overnight or an anti-β-actin (Biolegend, clone 2F1-1) at a dilution of 1:1,500 for 2 h at room temperature. After incubation, membranes were washed thrice with TBS-Tween 0.01% (Calbiochem, Inc. La Jolla) and incubated with an anti-mouse-IgG HRP-conjugated polyclonal antibody (Biolegend, Poly4053) for 1 h at room temperature at a dilution of 1:2,500. After incubation with the secondary antibody, membranes were washed, membranes were washed thrice with TBS-0.01% Tween and incubated with a luminol:coumaric acid solution to detect membrane-bound antibodies. Quimioluminiscence derived from this reaction was visualized using a MyECL™ Imager (Thermo Fisher scientific) digital documentation system. Band intensity was calculated using UN-SCAN-IT gel 6.1 software (Silk Scientific Corporation).

Quantitative PCR (qPCR)

Vero and HeLa cells were left untreated or treated with HO-1-modulating drugs at 60 and 10 μM, respectively and then infected with HSV-2 G strain at an MOI 10. Samples were collected at the indicated time-points after infection and processed for DNA extraction. Briefly, cells and cell supernatants were ultracentrifugated at 21,000 × g to pellet both, cells and virus particles in the supernatants. The pellet was then processed according to the method described for Extraction and Precipitation of DNA in Appendix 3C of the Current Protocols Humman Genetics 2001. The DNA was then used for qPCR analysis using 100 ng of DNA per reaction with the following primers and probe for the U_L30 gene: Fwd GGCCAGGCG CTTGTTGGTGTA, Rev-ATCACCGACCCGGAGAGGGA and Probe CCGCCGAACTGAGCAGACACCCGC and an Applied Biosystems StepOnePlus thermocycler, as previously described (Petro et al., 2016).

Flow Cytometry

To evaluate HO-1 expression by flow cytometry, $0.5–1.0 \times 10^6$ cells were detached using 0.25% W/V Trypsin (Thermo Fisher Scientific) for 10 min at 37°C, centrifugated at 400 g, washed with PBS and fixed for 15 min at room temperature with 4%

paraformaldehyde (PFA). Then cells were permeabilized with 0.05% saponin in PBS (Sigma-Aldrich, St Louis USA) for 45 min at 4°C, washed with PBS and incubated with an anti-HO-1 antibody (Abcam, clone ab13248) at a dilution of 1:400 in PBS-saponin 0.1% for 1 h. Cells were then washed twice with PBS and subsequently incubated with an anti-mouse-IgG-APC antibody (Biolegend, clone Poly4053) for 45 min at 4°C. Finally, cells were washed twice with PBS and resuspended in PBS for flow cytometry analysis. To evaluate GFP-derived fluorescence from HSV GFP-capsids (structural reporter) or the non-structural GFP reporter encoded within the virus genome, 0.5×10^6 cells Vero or HeLa were infected with the indicated amounts of virus for 1 h in Opti-MEM, washed with PBS and then incubated with Opti-MEM for an additional 16 h prior to trypsinization and fixation as indicated above, but for 20 min. Cell viability was assessed by flow cytometry using the Zombie-NIR Fixable Viability Kit (BioLegend). All flow cytometry analyses were performed on a FACSCANTO II flow cytometer (BD Beckton Dickinson).

Multi-Mode Plate Reader

GFP-derived fluorescence from HSV GFP-capsids or GFP encoded within the virus genome was assessed within SH-SY5Y cells treated with CORM-2 or inactivated CORM2 (iCORM2) on a Synergy Neo HTS Multi-Mode Reader (Biotek Instruments, Inc.).

Laser Confocal Microscopy

1×10^4 Vero or HeLa cells were seeded onto Slide 8-well FLux Hybridwell™ microchambers (SPL Life Sciences Co., Korea) for 16 h at 37°C in RPMI, then cooled to 4°C for 5 min and incubated with HSV VP26-GFP at an MOI of 400 for 1 h to 4°C. Later, cells were transferred to a culture chamber at 37°C to synchronize virus internalization. Two hours later, cells were washed twice with PBS and fixed with 2% PFA at 4°C for 20 min. Then the cells were washed twice and stained with Hoechst (Thermofisher Scientific, #H21492) in PBS at a final concentration of 2 μg/ml for 15 min. Then, the cells were washed twice and stained 10 min with Alexa Fluor 594 wheat germ agglutinin, (WGA, Thermofisher Scientific, #W11262) in PBS at final concentration of 5 μg/ml. Finally, the cells were washed twice and mounted with Prolong Diamond Antifade Mountant (Thermofisher Scientific, #P36970). Microscopic analyses were performed at 63x in a Ti Eclipse, Nikon laser confocal microscope. An average of 15 fields and 150–200 cells were analyzed per experiment. The distribution of capsid-associated fluorescence between treatments was assessed in a blinded manner. To determine capsid-derived fluorescence in the cytoplasm of infected cells, the images obtained by confocal microscopy were analyzed with Image J and the FIJI plugin, according to a report by McCloy et al. (2014). Briefly, both regions of interest consisting on cells, based on WGA-staining (cell membrane label), as well as regions free of cells, to determine background fluorescence were selected. The selected regions were then analyzed in the green channel (GFP) and converted to an 8 bit format. Then, the area and the integrated fluorescence density of each region of interest (cell) were calculated in z-stack images. Finally, the value of the TCCF in the complete z-axis of the cell was calculated. TCCF is the integral density of fluorescence of the region of interest minus the equivalent of the cell area*mean fluorescence intensity of the background.

Statistical Analysis

Statistical significance between experimental groups was assessed by unpaired Student's *t*-test (bar graphs), one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test (three or more groups) or two-way analysis of variance with Tukey's multiple comparison test (two independent variables) using GraphPad Prism (GraphPad Software, La Jolla California USA). Values were considered statistically significant for $p \leq 0.05$.

RESULTS

HO-1 Expression in CoPP-Treated and HSV-Infected Epithelial Cells

To determine whether HSV modulates the expression of HO-1 in cells that are permissive for infection with this virus, we assessed the expression of HO-1 by western blot and flow cytometry in Vero and HeLa cells treated with CoPP or SnPP. Vero cells are monkey kidney epithelial cells (*Cercopithecus aethiops;* green monkey), frequently used for expanding viruses, while HeLa is a human cervix epithelial cell line that is susceptible to herpes simplex virus infection. As shown in **Figures 1A,B**, Vero cells and HeLa cells, respectively up-regulated the expression of HO-1 in response to CoPP. The concentration of CoPP that induced optimal HO-1 expression, altogether without compromising cell viability was both, based on previous reports and determined experimentally by performing dose-response curves (**Supplementary Figure 1**; Zhang et al., 2015; Espinoza et al., 2017). Overall, the expression of HO-1 did not significantly vary upon HSV-2 infection at an MOI 1 in the absence of treatment in these cells as compared to non-infected controls. Other MOIs tested and incubation periods with the virus did not significantly alter the expression of HO-1 in these cells (data not shown). Nevertheless, Vero cells treated with CoPP evidenced a significant increase in the expression of HO-1, which decreased after HSV-2 infection when assessed by western blot; however, this was not the case for HeLa cells. Treatment with SnPP, a drug that inhibits the activity of HO-1 (Sardana and Kappas, 1987), yet may up-regulate its expression in some cells (Ewing et al., 2005), increased the expression of this enzyme in Vero cells without infection (western blot). HeLa cells treated with SnPP did not express HO-1 with or without infection, suggesting that the modulation of HO-1 expression by SnPP is likely restricted to certain cells types and/or culture conditions (**Figure 1A**). Flow cytometry analyses of HO-1 expression in these cells overall revealed similar results to those observed by western blot, that is no variation in HO-1 expression after HSV-2 infection and significant HO-1 expression after CoPP treatment (**Figures 1C,D**). Similar results were also observed in SH-SY5Y cells, which are human neuronal cells derived from the SK-N-SH neuroblastoma cell line (**Supplementary Figures 2A,B**).

To evaluate whether increased expression of HO-1, induced by CoPP modulates the replication cycle of HSV-2 within

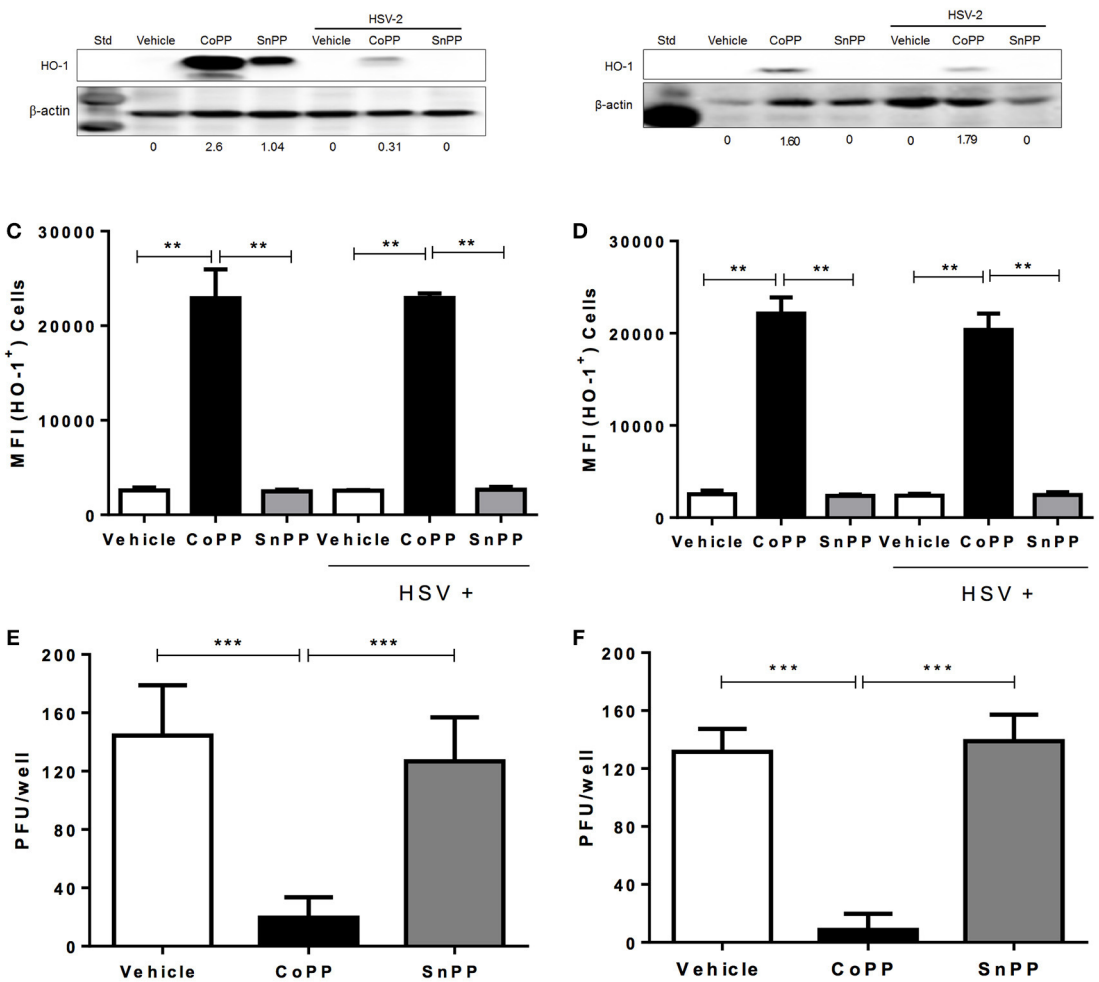

FIGURE 1 | HO-1 expression in epithelial cells treated with CoPP or SnPP and infected with HSV. **(A,B)** Western blot analyses of HO-1 expression in Vero (left) and HeLa cells (right) after treatment with HO-1-modulating drugs and/or infection with HSV-2 at an MOI 1 for 16 h. **(C,D)** Flow cytometry analyses of HO-1 expression in Vero (left) and HeLa cells (right) after treatment with HO-1-modulating drugs and/or infection with HSV-2 at an MOI 1 (MFI, mean fluorescence intensity). **(E,F)** Virus plaque formation (PFU, plaque forming units) in Vero (left) and HeLa cells (right) treated with HO-1-modulating drugs and then infected with 150 PFU of HSV-2. Plaque forming units were determined at 18 and 36 h post-infection for Vero and HeLa cells, respectively. Data are means ± SEM of three independent experiments. Representative images are shown for western blots. Two-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (**$p < 0.01$, ***$p < 0.001$).

permissive cells, we infected Vero and HeLa cells with a fixed amount of virus (150 PFU) and measured virus plaque formation. As shown in **Figures 1E,F**, Vero and HeLa cells treated with CoPP and infected with HSV-2 displayed significantly less plaque forming units (PFU) at 18 and 36 h after infection, as compared to untreated cells or cells treated with SnPP. Similar results were observed with the SH-SY5Y neuronal cell line, which released significantly less infectious HSV particles at 24 h post-infection when treated with CoPP (**Supplementary Figure 2C**).

Taken together, these results suggest that HSV does not modulate HO-1 expression *per-se* in infected cells, nor significantly interferes with its expression after pharmacological induction with CoPP. Furthermore, we observe that CoPP treatment reduces virus plaque formation in Vero and HeLa cells, while SnPP does not, suggesting that the effect of HO-1 over HSV replication depends on its catalytic activity.

CoPP Restricts HSV-Encoded Gene Expression in Infected Cells

To determine whether reduced virus plaque formation in cells treated with CoPP was due to reduced viral protein expression within inoculated cells, we assessed the expression of both, a reporter gene encoded within the virus genome, as well as viral genes in CoPP-, SnPP-treated and untreated cells. The reporter gene used in this assay consists of a non-structural GFP controlled by a constitutive, HSV-independent strong promoter

(human cytomegalovirus, CMV; Wang et al., 2012). Noteworthy, Vero cells (**Figure 2A**) and HeLa cells (**Figure 2B**) treated with increasing concentrations of CoPP and then inoculated with HSV at an MOI 1, expressed significantly less GFP than cells treated with vehicle alone or SnPP. A similar result was observed for SH-SY5Y cells treated with these drugs and inoculated with the virus (**Supplementary Figures 3A,B**). To exclude the possibility that these results may be a consequence of increased cell death after treatment with CoPP or SnPP, we assessed cell viability for all treatments. Importantly, neither CoPP nor SnPP treatment significantly altered the viability of Vero, HeLa, or SH-SY5Y cells at the drug concentration used (**Figures 2C,D** and **Supplementary Figure 3C**, respectively).

To determine whether reduced expression of the virus-encoded GFP protein also applied to virus-encoded viral proteins, we performed western blot assays for HSV glycoprotein D (gD) and VP16 in cells treated or not with vehicle, CoPP and SnPP. As shown in **Figure 2E** the expression of gD and VP16 was significantly reduced in Vero cells treated with CoPP, as compared to untreated cells and cells treated with SnPP. However, the latter also displayed a slight reduction in viral protein expression upon treatment. Similar results were observed for HeLa cells, although treatment with SnPP increased viral protein expression (**Figure 2F**). Equivalent findings to HeLa cells were observed for SH-SY5Y cells (**Supplementary Figure 3D**), suggesting that pharmacological induction of HO-1 with CoPP in epithelial and neuronal cells interferes with the expression of HSV-encoded proteins. To assess whether CoPP treatment also affected the quantity of viral genomes present in Vero and HeLa cells, we performed qPCR for the U_L30 viral gene on total DNA obtained from each treatment. As shown in **Figures 2G,H**, CoPP-treatment reduced the amount of HSV genome copies recovered at 16 h after infection of both, Vero and HeLa cells with an MOI 10, although the differences were less marked in HeLa cells and in both cases did not reach statistical significance.

CoPP Treatment Does Not Interfere with Virus Binding or Entry

To gain insights on the mechanism of action of HO-1 activity over impaired HSV replication and viral protein expression in CoPP-treated cells, first we assessed whether equivalent amounts of virus bound to the surface of drug-treated and untreated epithelial cells, in such a way to determine whether reduced virus binding to the cell surface may account for lesser infection and hence, diminished virus gene expression. To evaluate virus binding to the cell surface, we performed a previously described virus-binding assay that assesses attachment of different amounts of virus to the cell surface at 4°C (Cheshenko et al., 2013). As shown in **Figures 3A,B**, western blot analyses of gD evidenced equivalent amounts of virus bound to the surface of untreated, CoPP- and SnPP-treated Vero and HeLa, respectively suggesting that pharmacological treatment with these drugs does not interfere with the expression of virus-binding proteins on the surface of epithelial cells, nor blocks virus binding to these cells.

To further dissect how the pharmacological induction of HO-1 with CoPP interferes with HSV replication, we used an HSV virus that encodes a GFP-VP26 fusion protein (structural reporter) and measured capsid-derived GFP fluorescence early after infection to evaluate capsid entry into the cytoplasm of treated and untreated cells. To minimize the detection of GFP fluorescence that may derive from virus particles bound to the cell surface, that have not been internalized into the cytoplasm, cells were washed and treated with trypsin before flow cytometry analyses. As shown in **Figure 3**, equivalent amounts of capsid-derived GFP fluorescence was detectable intracellularly for all treatments in Vero (**Figure 3C**) and HeLa cells (**Figure 3D**), indicating that similar quantities of virus internalize in treated and untreated cells. These results suggest that CoPP-treatment does not interfere with the capacity of the virus to enter target cells and thus, that interference with gene expression likely occurs further downstream of this process.

Viral Capsid Distribution in CoPP-Treated Infected Cells

The following process associated to the virus infectious cycle that we assessed was capsid accumulation at the outer nuclear membrane in virus-inoculated cells. Upon infection, HSV capsids entering the cytoplasm migrate from the inner side of the plasma membrane to the nucleus to deliver the viral genome into this compartment. To evaluate whether CoPP-treatment interferes with the accumulation of viral capsids around the nucleus, we performed confocal microscopy analyses on cells inoculated with a virus containing GFP-fluorescent capsids 2 h after viral entry was allowed. Importantly, we observed that CoPP-treated Vero cells displayed less capsid-derived GFP fluorescence distributed adjacent to the nucleus, as compared to non-treated cells and that the viral capsids in CoPP-treated cells were rather homogenously dispersed in the cytoplasm (**Figures 4A,C**). A similar result was observed for CoPP-treated HeLa cells (**Figures 4B,D**). Because the intensity of the virus-derived fluorescence seemed somewhat lower in cells treated with CoPP, as compared to vehicle-treated cells, we quantified the total intensity of this fluorescence in individual cells using z-stack confocal imaging. As shown in **Figures 4E,F**, the integral density of fluorescence of individual Vero and HeLa cells that were infected with HSV was calculated in three dimensions and found not to be significantly different between treatments. This result indicates that similar amounts of virus entered the cells, which is consistent with the flow cytometry data in **Figures 3C,D**. However, it is noteworthy to mention that vehicle-treated epithelial cells displayed a sphere-shaped phenotype after HSV infection which was not observed in CoPP-treated cells. Indeed, cells treated with CoPP retained their epithelial-like phenotype after drug treatment and HSV infection which may impact the distribution and concentration of viral capsids within these cells and negatively affect the virus replication cycle (**Figures 4E,F**).

Carbon Monoxide Recapitulates the Effects of CoPP in Epithelial Cells

Because HO-1 yields three enzymatic products as a consequence of its activity, we sought to assess whether one of its products, namely carbon monoxide (CO), which is known to modulate the

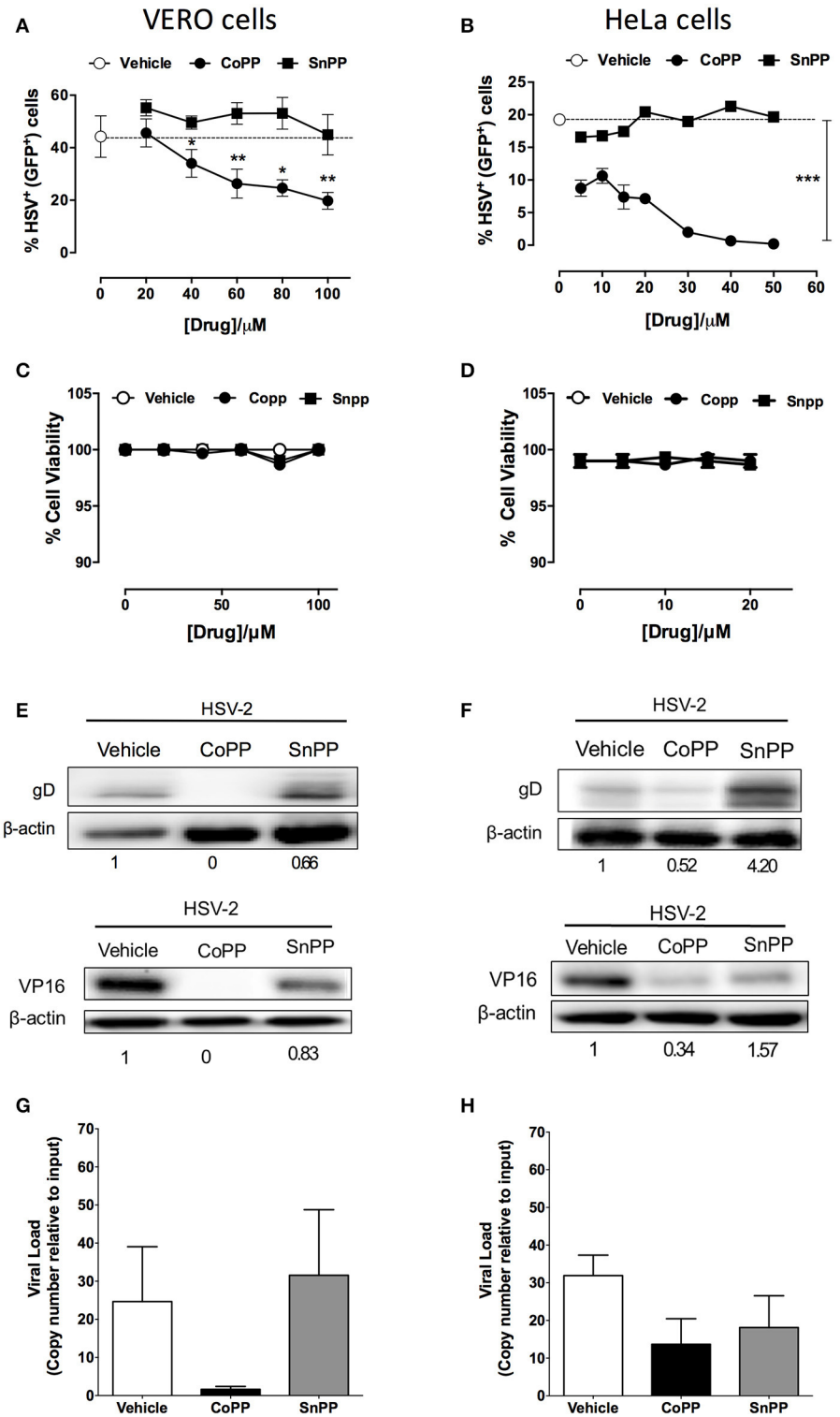

FIGURE 2 | Pharmacological induction of HO-1 restricts the expression of HSV-encoded genes in epithelial cells. **(A,B)** Flow cytometry analyses of GFP-derived fluorescence in Vero (left) and HeLa cells (right), respectively infected at MOI 1 with HSV-2 that encodes a non-structural GFP reporter gene under the control of a constitutive promotor, in response to varying concentrations of HO-1-modulating drugs. **(C,D)** Cell viability was assessed for Vero (left) and HeLa cells (right) for treatment with varying concentrations of HO-1-modulating drugs. **(E,F)** Western blot analyses for viral proteins gD and VP16 in Vero (left) and HeLa (right) cells at 16 h post-infection with HSV-2 at an MOI 1 after treatment with 60 and 10 μM, respectively of HO-1-modulating drugs. **(G,H)** Quantification of viral genome copies in Vero (left) and HeLa cells (right) by qPCR at 18 h post-infection at MOI 10. Data are means ± SEM of three independent experiments for experiments **(A–F)** and two independent experiments for **(G,H)**. Representative images are shown for Western blots. Two-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses **(A)**: statistics is shown for the CoPP treated group vs. SnPP-treated and untreated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

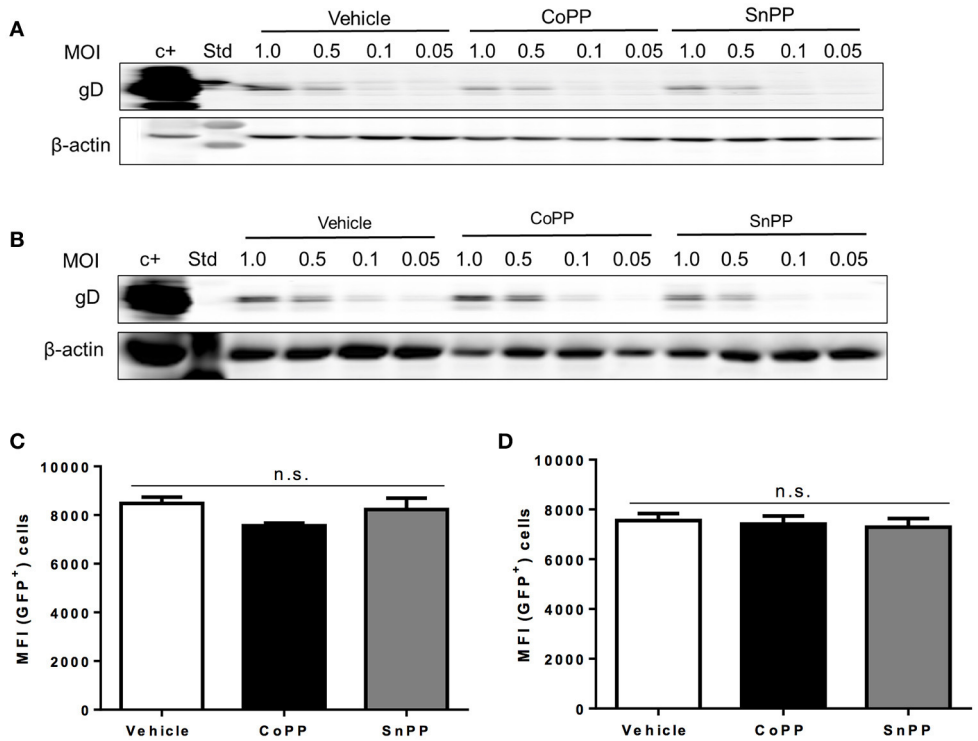

FIGURE 3 | Pharmacological induction of HO-1 activity does not interfere with virus binding to the cell surface nor capsid entry into the cytoplasm. Western blot analyses for determining virus binding to the surface of **(A)**. Vero cells and **(B)**. HeLa cells. Cells were incubated with varying doses of virus for 5 h at 4°C, washed with saline buffer and then immediately prepared for protein extraction. HSV gD protein expression was assessed to determine the amount of virus bound to the surface of cells. **(C,D)** Flow cytometry analyses measuring virus-derived GFP fluorescence of capsids internalized into the cytoplasm 2 h post-infection with a MOI of 100 after treatment with CoPP, SnPP, or vehicle in Vero cells (left) and HeLa cells (right). Cells were thoroughly washed with saline buffer and treated with trypsin to remove any surface-bound virus before analysis. Data are means ± SEM of three independent experiments. Representative images are shown for Western blots. Two-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (n.s., non-significant differences).

expression of pro-inflammatory molecules by the cell (Riquelme et al., 2015a), alter endosome-lysosome fusion (Tardif et al., 2013) and modulate mitochondrial function (Riquelme et al., 2015b), among others plays a role in the results observed above. As shown in **Figures 5A,B**, HSV titered out at significantly higher virus dilutions in CORM-2-treated Vero and HeLa cells, respectively than untreated cells. The effect conferred by CORM-2 treatment was similar to that observed for CoPP. Importantly, inactivation of CORM-2 (iCORM-2) reestablished the titers obtained with vehicle alone. Furthermore, Vero and HeLa cells treated with CORM-2 displayed significantly less virus-related GFP fluorescence (non-structural reporter) than cells treated with vehicle alone (**Figures 5C,D**, respectively). Again, inactivation of CORM-2 (iCORM-2) yielded GFP-fluorescence levels similar to those obtained with the vehicle. A similar result was observed in SH-SY5Y cells, which displayed less virus-derived fluorescence after treatment with CORM-2 **Supplementary Figure 4**). Importantly, inactivation of CORM-2 (iCORM-2) restored the GFP fluorescence to similar levels as those observed in vehicle-treated cells. Taken together, these results suggest that the HO-1 product CO mediates numerous of the effects elicited by CoPP treatment.

DISCUSSION

Recent studies report significant antiviral properties for HO-1 and its enzymatic products. Here, we observed that pharmacological induction of HO-1 activity hampers HSV propagation in epithelial cells and neuronal cells. Furthermore, we found that carbon monoxide, a product of HO-1 activity could reproduce numerous of the effects of this enzyme.

A previous study that assessed the effect of bilirubin over HSV infection suggested that its inhibition over HSV replication may be mediated through the production of NO, which displays virucidal effects (Croen, 1993; Akaike and Maeda, 2000; Santangelo et al., 2012). Interestingly, CO can bind to target proteins that harbor heme groups, such as caveolar NO synthase (NOS) which promotes NO production, and modulate their functions by increasing for example their activity (Boczkowski et al., 2006). Interestingly, NO produced by NOS has been described to promote the activity of HO-1 leading to an activating positive loop between both gases (Zuckerbraun et al., 2003; Wegiel et al., 2010). Thereby, it is possible that the effect of CO observed herein might relate directly to the production of NO and relate to the previous

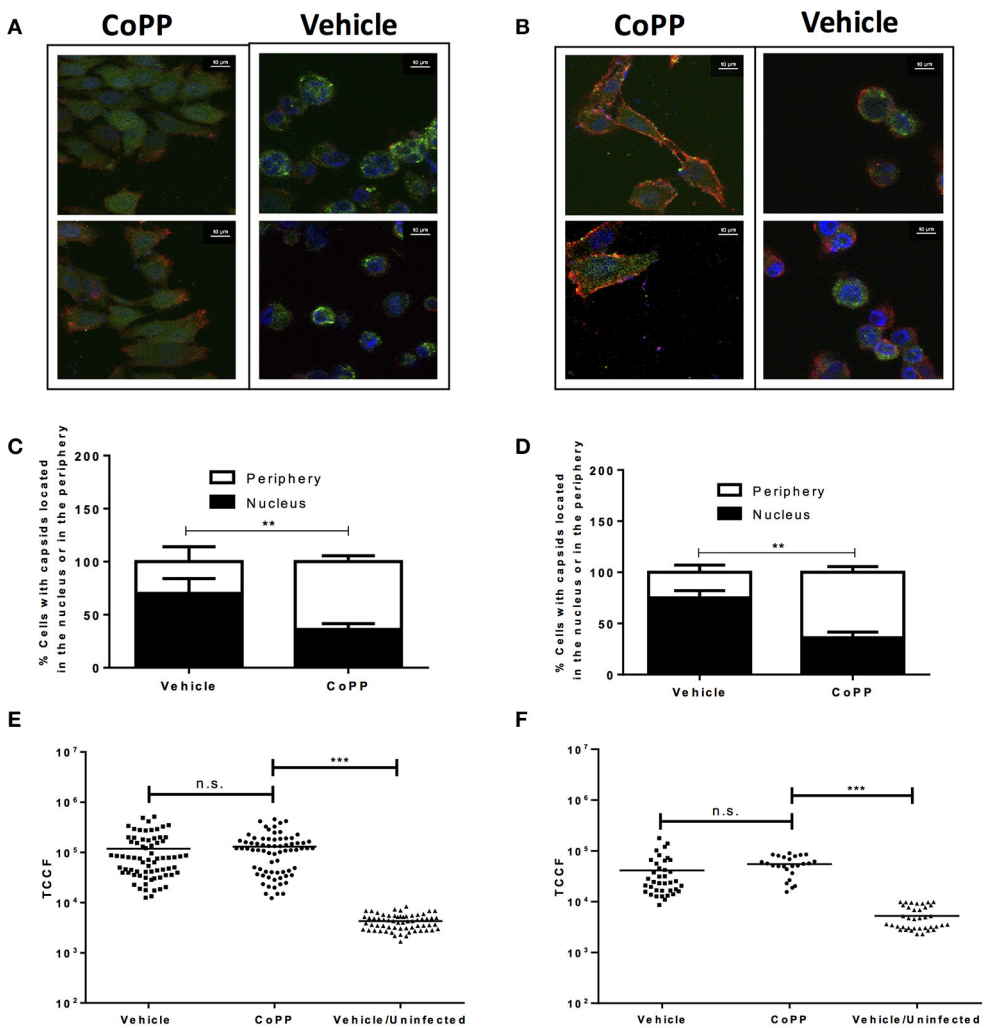

FIGURE 4 | Pharmacological induction of HO-1 with CoPP modulates capsid distribution in infected cells and elicits differential cell morphology after treatment after HSV-infection. Representative confocal microscopy images of **(A)**. Vero and **(B)**. HeLa cells treated or not with CoPP and infected with an HSV virus encoding a structural GFP fluorescent capsid (green: GFP-capsid fusion protein). Cell nuclei were stained with Hoescht (blue), and membrane were stained with WGA (red) **(C,D)**. Quantification of the distribution of capsid GFP-fluorescence in treated and infected Vero (left) and HeLa cells (right), respectively relative to the position of the nucleus. An average of fifteen fields and 150–200 cells were analyzed per experiment in a blind manner **(E,F)**. Quantification of total green fluorescence (virus encoded structural GFP fluorescent capsid) in confocal microscopy images of HSV-infected Vero (left) and HeLa cells (right), respectively treated or not with CoPP. TCCF is the integral density of fluorescence of the region of interest minus the equivalent of the cell area*mean fluorescence intensity of the background in the complete z-axis of the analyzed cells. Representative confocal microscopy images at 63X magnification are shown. Data are means ± SEM of two independent experiments. One way ANOVA, Two-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (**$p < 0.01$, ***$p < 0.001$).

results reported with bilirubin and HSV (Santangelo et al., 2012).

Additionally, CO displays numerous other molecular targets within cells with varying consequences, such as guanylyl cyclases (sGC), heme-containing channels, surface NADPH oxidase, and heme-containing transcription factors, such as NPAS2 (Dioum et al., 2002; Boczkowski et al., 2006). CO can also modulate the activity of mitochondrial proteins, such as mitochondrial cytochrome c oxidases (Desmard et al., 2007; Zuckerbraun et al., 2007) and promote mitochondria-derived ROS, although at very low levels, which have been described to act as signaling molecules (Almeida et al., 2015). Thus, it is likely that depending on the concentration of CO generated by HO-1 within the cell, that multiple functional outcomes by this molecule may arise.

Previous studies have also reported anti-microbial effects for CO, namely against several viruses. CO-mediated inhibition of EV71 virus replication in neuronal cells was suggested to occur through the inhibition of ROS levels that are deliberately induced by this virus in these cells (Tung et al., 2011). Distinct from the EV71 virus, HSV viruses are likely susceptible to pro-oxidizing environments, as they carry catalases in the virion that are intended to neutralize ROS in infected cells (Newcomb and Brown, 2012). Furthermore, inducing ROS in infected cells

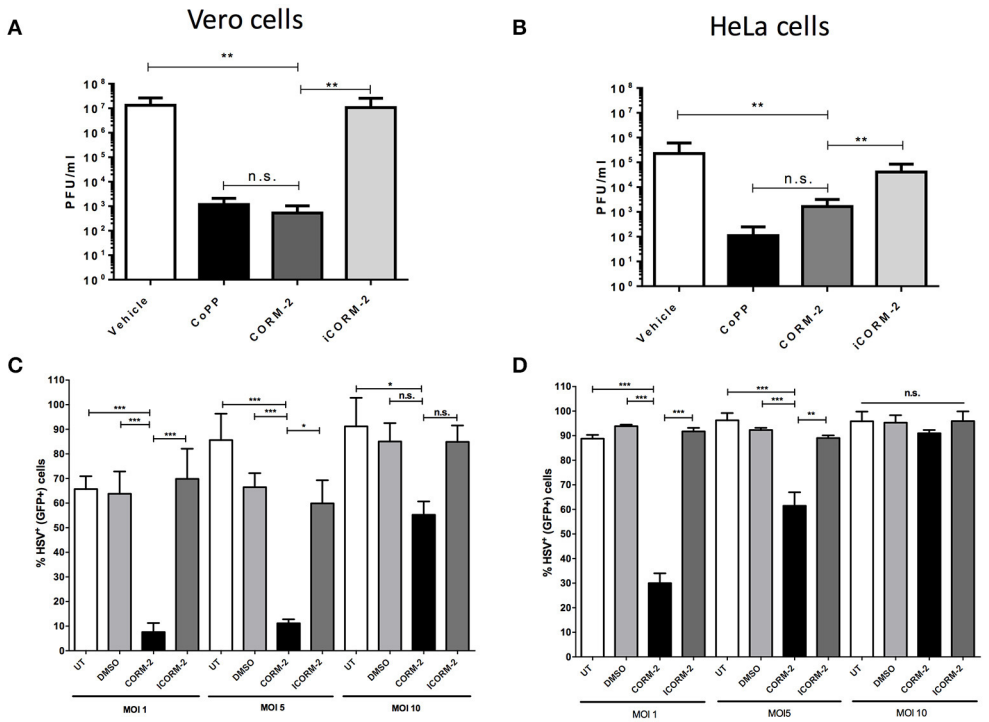

FIGURE 5 | Treatment with a carbon monoxide-releasing molecule recapitulates the antiviral effects of CoPP after HSV infection. Quantification of plaque forming units (PFU) after HSV-2 titration over **(A)**. Vero and **(B)**. HeLa cells treated with CORM-2 and inactivated CORM-2 (iCORM2). Virus-encoded GFP fluorescence (non-structural GFP reporter) determined by flow cytometry in **(C)**. Vero and **(D)**. HeLa cells infected with HSV at different MOIs at 20 h post-infection. Data are means ± SEM of three independent experiments. One-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

with trimeric and tetrameric derivatives of stilbenoids have been shown to inhibit HSV replication and thus, inhibition of ROS in target cells could rather favor these viruses (Chen et al., 2012).

While elevated levels of CO may dampen ATP production by the mitochondria and glycolysis separately, low levels of CO may also promote adequate levels of ATP production because of adaptive feedback loops triggered by mechanisms that sense hypoxic states in the cell (Lavitrano et al., 2004; Tsui et al., 2007). Importantly, reduced levels of ATP could impact cellular processes that require increased levels of this molecule, such as cargo transporters between cellular compartments (Dodding and Way, 2011; Kaczara et al., 2015; Riquelme et al., 2015b). Because HSV binds to ATP-consuming molecular motors, such as dynein within the cell, reduced levels of ATP could interfere at some level with the activity of this molecule, although the role of microtubules in capsid transport in epithelial cells is still a matter of discussion (Lee et al., 2006; Wolfstein et al., 2006; Abaitua et al., 2012; Matthews et al., 2013). Another process that requires elevated levels of ATP and is required for optimal HSV genome delivery into the nucleus is proteasome activity. Indeed, blocking energy-consumption by this protein complex has been described to inhibit optimal capsid migration within infected cells (Delboy et al., 2008). Hence, it is possible that altered availability of ATP within infected cells treated with CoPP or CORM-2, may compromise optimal HSV capsid accumulation around the nucleus, which is required early after infection for the initiation of an infectious replication cycle.

On the other hand, it is important to note that HO-1 and its product CO have been reported to directly interfere with the activity of host heat shock protein 90 (HSP90), which plays a crucial role in the localization of HSV capsids and their association with the nucleus (Lee et al., 2014; Zhong et al., 2014). Thus, it is also possible that CO could inhibit HSP90 activity, in such a way to interfere with the capacity of this protein to interact with HSV capsids and alter their localization within infected cells.

An interesting finding in this study was that while cells treated with CoPP retained their epithelial-like phenotypes after infection with HSV, vehicle-treated cells infected with this virus acquired a rounded-like phenotype (cytopathic-like phenotype). Importantly, the transition of an epithelial-like phenotype into a rounded cell morphology may better support viral capsid encounter with nuclear components that are needed for viral genome delivery into this compartment, as a role for microtubules in viral capsid transport to the nucleus remains controversial (Lee et al., 2006; Wolfstein et al., 2006; Abaitua et al., 2012; Matthews et al., 2013). Noteworthy, a recent study reported that the induction

of HO-1 significantly increased the expression of adhesion molecules in cancer cells, namely E-cadherin and β-catenin, which can modulate the morphology of cells (Gueron et al., 2014).

Taken together, the results obtained in this study suggest an antiviral effect for HO-1 over HSV, which is at least partially mediated by its product CO. Although, our results point at the unusual distribution of viral capsids within infected cells as a possible mechanism by which HO-1 activity may interfere with the replication cycle of HSV, further experiments will be needed to determine whether this phenomenon overall accounts for reduced virus yield. Additionally, it will be of interest to determine if the effects elicited by HO-1 activity, and more specifically CO over HSV infection apply in the context of *in vivo* infections. Importantly, at present there are several CO-releasing molecules (CORMs) that are being tested in pre-clinical settings that replicate the effects of HO-1, when CO has been identified as a relevant effector of this enzyme (Motterlini and Otterbein, 2010; Schatzschneider, 2015).

AUTHOR CONTRIBUTIONS

FI, MF, and PG designed experiments. FI, MF, and ARD conducted experiments. FI, MF, ARD, JE, AK, and PG analyzed the data. FI, AK, and PG wrote the manuscript. All authors reviewed the manuscript.

FUNDING

Authors are supported by Grants CRP-ICGEB 2762-011 CRP/CHI14-01, FONDECYT no. 1140011, FONDEQUIP EQM130158, FONDEQUIP EQM-130092 from CONICYT Chile, as well as CRP-ICGEB CRP/CHI14-01 and the Millennium Institute on Immunology and Immunotherapy (no. P09/016-F). AK is Chaire De La Region Pays De La Loire, Chercheur Etranger d'Excellence and a CDD-DR INSERM. ARD and JE are CONICYT Fellows.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02108/full#supplementary-material

Suppulementary Figure 1 | HO-1 expression in Vero and HeLa cells treated with low doses of CoPP. **(A,B)** Quantification of HO-1 expression in Vero (left) and HeLa cells (right), respectively by flow cytometry after treatment with varying drug concentrations. Optimal HO-1 expression in Vero cells was achieved with 60 μM CoPP at 6 h as previously described, while maximum expression of HO-1 in HeLa cells was achieved with 15 μM CoPP at 14 h after treatment. Data are means ± SEM of three independent experiments. One-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (***$p < 0.001$).

Suppulementary Figure 2 | HO-1 expression in SH-SY5Y cells treated with CoPP or SnPP and infected with HSV and quantification of virus released into the media. **(A)** Western blot analysis of HO-1 expression in SH-SY5Y cells after treatment with CoPP, SnPP, or vehicle and/or infection with HSV-2 at an MOI 1 for 24 h. **(B)** Flow cytometry analysis of HO-1 expression in SH-SY5Y cells 14 h after treatment with CoPP, SnPP, or vehicle (10 μM) and/or infection with HSV-2 at an MOI 1 for 24 h. C. Quantification of virus plaque forming units (PFU) in the supernatants of SH-SY5Y cells at 6, 12, and 24 h post-infection. PFU determination was done over Vero cells. Data is means ± SEM of three independent experiments. A representative plug is shown for the western blot. One-way ANOVA, and Tukeys's multiple comparison test were used for statistical analyses (**$p < 0.01$, ***$p < 0.001$).

Suppulementary Figure 3 | Pharmacological induction of HO-1 activity dampens HSV-encoded gene expression in SH-SY5Y cells. **(A)** Fluorescence microscopy of SH-SY5Y cells treated with HO-1 modulators and infected with a GFP-encoding HSV virus at an MOI 1 (Representatives images are show; 5X magnification). **(B)** Quantification of virus-derived fluorescence in HSV-infected SH-SY5Y cells treated with HO-1 modulating drugs by flow cytometry. **(C)** Viability of SH-SY5Y cells treated with HO-1 modulating drugs before and after infection with HSV at an MOI 1. **(D)** Western blot analyses for HSV proteins gD and VP16 in SH-SY5Y cells at 24 h after infection with HSV-2 at an MOI 1. Representative images are shown for Western blots. One-way ANOVA, and Tukeys's multiple comparison test were used for statistical analyses (*$p < 0.05$, **$p < 0.01$).

Suppulementary Figure 4 | Treatment with a carbon monoxide-releasing molecule reduces HSV-encoded gene expression in SH-SY5Y cells. GFP-derived fluorescence from the HSV-encoded reporter was measured in SH-SY5Y cells treated with CORM-2, 1 h before infection, or inactivated CORM-2 (iCORM-2) at an MOI 1. Data are means ± SEM of three independent experiments. Two-way ANOVA, and Tukey's multiple comparison test were used for statistical analyses (*$p < 0.05$, ***$p < 0.001$).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Journal of Immunology

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J Immunol published online 31 May 2017
http://www.jimmunol.org/content/early/2017/05/31/jimmunol.1601414

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published May 31, 2017, doi:10.4049/jimmunol.1601414

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Human respiratory syncytial virus (hRSV) is the leading cause of severe lower respiratory tract infections in children. The development of novel prophylactic and therapeutic antiviral drugs against hRSV is imperative to control the burden of disease in the susceptible population. In this study, we examined the effects of inducing the activity of the host enzyme heme oxygenase-1 (HO-1) on hRSV replication and pathogenesis on lung inflammation induced by this virus. Our results show that after hRSV infection, HO-1 induction with metalloporphyrin cobalt protoporphyrin IX significantly reduces the loss of body weight due to hRSV-induced disease. Further, HO-1 induction also decreased viral replication and lung inflammation, as evidenced by a reduced neutrophil infiltration into the airways, with diminished cytokine and chemokine production and reduced T cell function. Concomitantly, upon cobalt protoporphyrin IX treatment, there is a significant upregulation in the production of IFN-α/β mRNAs in the lungs. Furthermore, similar antiviral and protective effects occur by inducing the expression of human HO-1 in MHC class II$^+$ cells in transgenic mice. Finally, in vitro data suggest that HO-1 induction can modulate the susceptibility of cells, especially the airway epithelial cells, to hRSV infection. ***The Journal of Immunology*****, 2017, 199: 000–000.**

The human respiratory syncytial virus (hRSV) is the leading cause of lower respiratory tract illness (LRTI) in infants and children worldwide. hRSV produces reinfections throughout life, generating frequent milder respiratory infections in adults as well as severe LRTIs in pediatric, elderly, and immunocompromised patients (1). LRTI can manifest as bronchiolitis or pneumonia, with the risk of death due to respiratory failure (2). Epidemiological studies suggest that hRSV contributes to nearly 33.8 million new episodes of LRTI in children <5 y of age each year, with 3.4 million annual hospital admissions worldwide (3). Furthermore, hRSV infection is linked to neurologic symptoms in patients, as well as learning impairment in animal models (4, 5)

Currently, there are still no licensed vaccines nor specific antiviral drugs for the prophylaxis of hRSV disease in children and other susceptible populations (6). The only approved therapeutic approaches are palivizumab (7), a humanized mAb that protects against hRSV infection in high-risk infants, and ribavirin, an antiviral nucleoside analogue that is rarely used due to toxicity concerns and questionable benefits (7). For these reasons, intense research has focused on finding novel vaccines or antiviral agents to prevent hRSV infection.

During hRSV disease, epithelial cells in the distal airway respond to viral infection by secreting proinflammatory cytokines and chemokines that promote an exacerbated recruitment of infiltrating cells, mainly neutrophils, leading to inflammation and tissue damage (8). Therefore, the design of new strategies to prevent hRSV diseases must consider parameters such as the inhibition of viral replication and reduction of lung inflammation.

Heme oxygenase-1 (HO-1) is a metabolic enzyme that catalyzes the degradation of heme into carbon monoxide, biliverdin, and free iron (9). This enzyme has anti-inflammatory and antioxidant properties, which modulate host innate and adaptive immune responses (10). The immunomodulatory capacity of HO-1 has been demonstrated in several models, such as the LPS-induced acute lung inflammation in which HO-1 activation decreased the migration of polymorphonuclear leukocytes to the lung, reducing oxidative tissue damage (11). Furthermore, the pharmacological induction of HO-1 inhibits dendritic cell (DC) activation and immunogenicity (12), suppressing cytokine secretion and the capacity to prime T cells (13). Also, recent studies have shown that HO-1 can display important antiviral properties. Specifically, upregulation of HO-1 was shown to diminish infection by several viruses, including Ebola, influenza, enterovirus, hepatitis C virus

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Received for publication August 15, 2016. Accepted for publication April 24, 2017.

This work was supported by grants from Comisión Nacional de Investigación Científica y Tecnológica/Fondo Nacional de Desarrollo Científico y Tecnológico (Postdoctorado 3140455, 1140011 and 1150862), Instituto Milenio en Inmunología e Inmunoterapia (P09-016-F). J.A.E. and R.S.G. are Comisión Nacional de Investigación Científica y Tecnológica de Chile Fellows.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; CoPP, cobalt protoporphyrin IX; ΔΔ Ct, ΔΔ threshold cycle; DC, dendritic cell; DOX, doxycycline; HCV, hepatitis C virus; HEp-2, human laryngeal epidermoid carcinoma number 2; HO-1, heme oxygenase-1; hRSV, human respiratory syncytial virus; LM, littermate; LRTI, lower respiratory tract illness; MHC-II, MHC class II; MHC-II$^-$, MHC-II negative; MHC-II$^+$, MHC class II positive; MOI, multiplicity of infection; N, nucleoprotein; qPCR, quantitative PCR; SnPP, tin protoporphyrin IX dichloride; UT, untreated.

(HCV), hepatitis B virus, and HIV. During infection by these viruses, HO-1 induction protects infected tissues, such as the liver and lungs, from virus-induced oxidative injury (10). Although the mechanisms of action for HO-1 during viral infection have not been elucidated, available data suggest a direct effect on virus proteins or on the activation of cellular processes that interfere with virus replication, such as the type I IFN response (14).

The HO-1 expression can be highly induced by analogs of heme, such as hemin and other metalloporphyrins. The best-characterized inductor of HO-1 is cobalt protoporphyrin (CoPP), which promotes the upregulation of HO-1 gene expression (15). Thus, the primary mechanism involved in the upregulation of the HO-1 enzyme seems to be the enhancement of gene transcription (15, 16). Allosteric HO-1 inhibitors can also induce upregulation of HO-1 expression, as occurs with tin protoporphyrin IX dichloride (SnPP) (17). However, despite inducing HO-1 expression, SnPP irreversibly inhibits the activity of this enzyme (18).

Taking into consideration the multifunctional properties of HO-1, in this study we examined the effects of HO-1 induction on the pathogenesis caused by hRSV in vitro and a mouse infection model. Specifically, we assessed the modulatory effects of HO-1 on airway epithelial cells infected with hRSV. Additionally, we evaluated whether either CoPP or transgenic induction of HO-1 displayed antiviral and anti-inflammatory effects in the airways of hRSV-infected mice.

Materials and Methods

Mice and hRSV production

C57BL/6J and BALB/cJ wild-type mice were obtained from The Jackson Laboratory for the generation of pIi-TTA-TetO-HO-1 transgenic mice. PIi-TTA mice were a kind gift from Christophe Benoist (19). TetO-HO-1 mice, located upstream of the cDNA sequence and located downstream of the cDNA, was cloned at the Not-I/Xho-I sites into the pBluKSM-tet-*O*-CMV vector containing the followed by the human β-globin intron and the bovine growth hormone polyA.

TetO-HO-1 transgenic mice were generated by pronuclear microinjection of CBA/C57BL6 eggs with a DNA fragment containing a Tet-responsive element downstream a minimal CMV promoter, the human β-globin intron, the human HO-1 cDNA, and the bovine growth hormone polyA. All mice were maintained at the pathogen-free facility of the Pontificia Universidad católica de Chile and manipulated according to guidelines approved by the institution's Bioethical Committee. hRSV serogroup A2, strain 13018–8, is a clinical isolate obtained from the Instituto de Salud Pública de Chile. In most experiments, a mock control was included consisting of supernatants collected from uninfected human laryngeal epidermoid carcinoma number 2 (HEp-2) cells kept in culture for the same period of time as infected cells.

Virus preparation

HEp-2 cells (CCL-23; American Type Culture Collection) were used to propagate hRSV serogroup A2, strain 13018–8 (clinical isolate obtained from the Instituto de Salud Pública de Chile), as previously described (4). Briefly, HEp-2 cell monolayers were grown in T75 flasks with DMEM (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Flasks containing 5 ml of culture medium were inoculated with 2×10^5 PFU of hRSV and incubated at 37˚C. After viral adsorption (3 h), supernatants were replaced with fresh medium (DMEM 1% FBS) and incubated for 48 h or until the visible cytopathic effect was observed. Cells were harvested, the flask content was pooled, then spun twice at $300 \times g$ for 10 min to remove cell debris. In parallel, supernatants of noninfected HEp-2 monolayers were collected as previously described, and used as noninfectious control (mock). Viral titer of supernatants was determined by immunohistochemistry. Ultraviolet-inactivated hRSV (ultraviolet-hRSV) was obtained exposing ice-packed virus preparation vials for 45–60 min at 302 nm using a 15 W lamp *trans*-illuminator.

hRSV infection of A549 cells and pharmacological modulation of HO-1 expression

Human alveolar type II–like pulmonary epithelial cells (A549 cells) (kindly provided by Dr. P. Piedra, Baylor College of Medicine) were maintained in DMEM medium containing 10% (v/v) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. A549 cells were treated with vehicle (NaOH diluted in media), or 50 μM CoPP (HO-1 inducer) (Frontier Scientific), or 50 μM SnPP (HO-1 inhibitor) (Frontier Scientific), and incubated at 37˚C in 5% CO_2 (19). After 2 h, supernatants were removed, and cells were inoculated with infectious hRSV at a multiplicity of infection (MOI) equal to 1 PFU per cell and incubated for 2 h in OptiMem I Reduced Serum Medium (preinfection). As a control, cells were inoculated either with the same media (untreated [UT]) or with mock (supernatant of uninfected HEp-2 or with ultraviolet-inactivated hRSV). Each treatment was also performed during the hRSV infection for 24 h (postinfection). In both cases, after 24 h replication was determined by measuring the nucleoprotein (N) RNA, and copies were quantified by real-time quantitative PCR (qPCR). In addition, supernatants from infected cells for each treatment were collected at 48 h postinfection to determine viral titer by immuno-plaque assay.

Viral immuno-plaque assay

The viral titer in supernatents of bronchoalveolar lavage fluid (BALF) was determined by immunocytochemistry. Briefly, infectious supernatants were serially diluted (10-fold dilutions), added to 96-well plates with HEp-2 monolayers (80% confluence), and incubated for 48 h at 37˚C. Later, cells were fixed with 2% paraformaldehyde-PBS and permeabilized 20 min with 0.2% saponin-PBS. Intracellular staining was performed with an anti-N-hRSV (clone 1E9/D1) Ab for 1 h (dilution 1:500, 0.2% saponin-PBS). Cells were washed twice and incubated with anti-mouse IgG-HRP (dilution 1:200; Invitrogen, Molecular Probes) for 45 min. After washing the complex twice, the substrate TRUE-BLUE peroxidase (KPL) was added to cells and incubated for 10 min at room temperature.

Quantitative hRSV by ELISA

Briefly, 100 μl of virus-containing supernatants from hRSV-infected A549 cells at an MOI of 1, and treated with CoPP, SnPP, or vehicle (6–100 μM) were used to coat ELISA plates for 2 h at 37˚C. Then, plates were blocked for 2 h with PBS containing 10% of FBS. After washing three times with PBS-tween 20 0.01%, goat anti-hRSV-HRP conjugated (Abcam) diluted in 1:500 in PBS FBS 10% was added to the wells for 2 h. Finally, after washing five times with PBS-tween 20 0.01%, bound Ab was detected by addition of tetramethylbenzidine peroxidase substrate (BD), stopped with 1 M H_2SO_4, and analyzed at 450 nm by an ELISA Plate Reader.

Quantification of hRSV binding by flow cytometry and Western blotting

HEp-2 cells were detached using versene solution (Sigma-Aldrich), washed, exposed to 50 μM of CoPP, SnPP, or vehicle, and chilled on ice for 30 min. Cells were washed and exposed to hRSV (MOI = 1) concomitantly with CoPP, SnPP, or vehicle for 1 h at 4˚C. For flow cytometry analyses, cells were washed, fixed (4% paraformaldehyde), and anti–hRSV F-protein FITC–conjugated Ab (1:200, ab20391; Abcam) was added and incubated for 1 h at 4˚C. For Western blotting analyses, hRSV- exposed cells were washed, resuspended in RIPA buffer, and incubated at 95˚C for 10 min. SDS-PAGE, on 10% Bis/Tris gels and in MES buffer (Invitrogen), was performed and the indicated protein was transferred to nitrocellulose membranes. The membranes were then exposed to anti-N protein Ab, anti HO-1 (ab13248; Abcam), or anti–β actin (6221102; BioLegend), followed by HRP-labeled secondary Ab (goat anti-mouse; Invitrogen, Molecular Probes). Chemiluminescence (Amersham ECL Prime Western blotting Detection Reagent; GE Healthcare, Little Chalfont, U.K.) was detected using a thermal imaging system.

DC infection and pharmacological modulation of HO-1

Bone marrow–differentiated DCs from C57BL/6 mice were prepared as previously described (20, 21). Analyses for expression of surface markers by flow cytometry revealed a typical phenotypic profile for immature DCs (>75% of $CD11c^+$ cells). On day 5 of culture, DCs were inoculated with hRSV or ultraviolet-hRSV for 2 h at an MOI equal to 1 PFU per cell. As controls, DCs were left UT or inoculated with similar volumes of supernatants from uninfected HEp-2 cultures (mock). Then 48 h postinfection, the viability of DCs was determined by trypan blue exclusion. In contrast, immature DCs (1×10^6 cells) were pulsed for 2 h with 50 μM CoPP, SnPP, or vehicle control. Cells were then washed twice and cultured 4 h before hRSV inoculation. Then, HO-1 mRNA and protein levels were analyzed by qPCR and flow cytometry or immunofluorescence, respectively.

Pharmacological modulation of HO-1 expression and hRSV challenge in vivo

Male 6–8 wk old BALB/cJ wild-type mice were pretreated i.p. (7.6 μmol/kg) either with CoPP to induce the HO-1 expression, or with SnPP to inhibit the activity of HO-1 as previously described (22), 24 h before viral challenge.

Mice, treated with NaOH (diluted in PBS), were included as the vehicle control. After 24 hr mice were anesthetized with ketamine or xylazine (80 and 8 mg per kg, respectively) and challenged intranasally with either 1×10^6 PFU of hRSV or an equal volume of mock (as non-infectious control). Animal body weight was recorded daily postinfection. At day 4 postinfection, mice were terminally anesthetized by i.p. injection with a mixture of ketamine and xylazine. BALF and lung tissue samples were collected for further analyses.

Infection of rtTA-HO-1 transgenic mice

Female or male 6–8 wk old rtTA-HO-1 mice and littermate mice were treated with 800 μg/ml doxycycline (DOX) and 36 mg/ml sucrose in drinking water, protected from the light, to induce the expression of human HO-1 in MHC class II positive (MHC-II$^+$) cells. Then 48 h later, mice were anesthetized with ketamine/xylazine (20 and 1 mg/kg, respectively) and challenged intranasally with either 1×10^6 PFU of hRSV or an equal volume of mock (as non-infectious control). Animal body weight was recorded daily postinfection. At day 4 postinfection, mice were terminally anesthetized by i.p. injection with a mixture of ketamine and xylazine. BALF and lung tissue samples were collected for further analyses.

Genotyping of rTA-HO-1

Mice were bled from the cheek into 100 μl of heparin (125 UI/ml). For DNA purification, we used the DNeasy Blood and Tissue Kit following the manufacturer's instructions. Consequently, we performed a PCR for each transgene inserted in the transgenic mice: rtTA and tHO-1 using GoTaq G2 Flexi DNA Polymerase (Promega). Then, samples were loaded in an agarose electrophoresis. The mice that only had the tHO-1 gene, but lacked the tTA gene were named littermate (LM), which were used as controls.

Quantitative real time RT-PCR

Total RNA was isolated from tissues or cell cultures by using the Trizol reagent (Life Technologies, Invitrogen), according to the manufacturer's instructions. cDNA synthesis from total RNAs was performed using the ImProm-II Reverse Transcription kit (Promega) and random primers. qRT-PCR reactions were carried out using a StepOne plus thermocycler (Applied Biosystems). The abundance of HO-1 and Nrf2 mRNAs was determined by relative expression to the respective housekeeping gene by the 2-ΔΔ threshold cycle (ΔΔCt) method. For N-gene expression, absolute quantification data were expressed as the number of hRSV N-gene copies for each 5×10^3 copies of β-actin transcript, as previously described (4). The following primers were used: hRSV N Forward 5′-GCTAGTGTGCAAGCAGAAATC-3′ and Reverse 5′-TGGAGAAGTGAGGAAATTGAGTC-3′, mouse HO-1 5′-CCTCTGACGAAGTGACGCC-3′ and Reverse 5′-CAGCCCCACCA-AGTTCAAA-3′, human HO-1 Forward 5′-AGGCAGAGGGTGATA-GAAGAGG-3′ and Reverse 5′-TGGGAGCGGGTGTTGAGT-3′, mouse Nrf-2 Forward 5′-TTC TTT CAG CAG CAT CCT CTC CAG-3′ and Reverse 5′-ACA GCC TTC AAT AGT CCC GTC CAG-3′, mouse IFN-α Forward 5′-TCC TGA ACC TCT TCA CAT CAA A-3′ and Reverse 5′-ACA GGC TTG CAG GTC ATT GAG-3′, mouse IFN-β Forward 5′-AGC TCC AAG AAA GGA CGA ACA-3′ and Reverse 5′-GCC CTG TAG GTG AGG TTG AT-3′, mouse β-actin Forward 5′-ACCTTCTACAATGAGCTGCG-3′ and Reverse 5′-CTGGATGGCTACGTACATGG-3′.

Laser confocal microscopy

DCs (1×10^6 cells) were produced as described above. Briefly, DCs were grown over 12 mm microscope cover glasses (Marienfeld-Superior, DE). Then, DCs were inoculated, as mentioned above, with hRSV, ultraviolet-hRSV or mock, and cultured for 48 h at 37°C. Inoculated DCs were prepared for confocal microscopy analysis, as previously described (23). Briefly, DCs were washed and fixed with 2% p-formaldehyde for 15 min at 4°C. Then, cells were permeabilized with 0.05% saponin-PBS for 15 min at 4°C. Next, cover glasses were passed to a cold chamber and DCs were double-stained with 1/200 anti-mouse HO-1 mAb (Abcam), already dissolved in 0.05% saponin-PBS. These preparations were incubated overnight at 4°C in darkness. The next day, cells were washed with PBS and stained with 1/200 goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen) and 1/200 goat-anti rabbit IgG-AF555 (Invitrogen) secondary Abs, and incubated for 3 h at 4°C in darkness. After washing the complex, cells were dried and mounted with DABCO mounting medium for confocal microscopy. Fluorescence measurements were performed on a FluoView FV1000 confocal microscope (Olympus). After image recording (at 40× magnification), each channel (HO-1, nuclei, and transmission) was analyzed separately using Olympus Fluoview version 3.0 software.

Flow cytometry

DCs, A549, and HEp-2 cells were inoculated, as mentioned above, and cultured for 24 or 48 h, respectively, in the presence of hRSV, ultraviolet light-inactivated hRSV, and mock. DCs were then stained with anti-CD11c-PE-Cy7 (clone HL3; BD Pharmingen), anti-IA/IE-PerCP-Cy7 (clone M5; BD Pharmingen), and anti-hRSV N-AF647 conjugated (clone 1E9/D1) in 10% of FBS in PBS as a blocking solution. For HO-1 intracellular staining, fixed cells were incubated with anti-mouse HO-1 mAb (Abcam) in permeabilization buffer (1% saponin, 10% FBS in PBS) for 45 min at 4°C. Then, cells were washed and stained with goat anti-mouse IgG-AF488 (Invitrogen). For cell infiltration analysis, lung samples were homogenized and filtered using a 40 μm cell strainer. BALF was centrifuged at $300 \times g$ for 5 min, washed, and stained with anti-CD11b-APC (clone CBRM1.5; BD Pharmingen), anti-CD11c-PE (clone CBRM1.5; BD Pharmingen), anti-IA/IE-APC-Cy7 (clone M5; BD Pharmingen), anti-Ly6C-PercCP 5.5 (clone AL-21; BD Pharmingen), and anti-Ly6G-FITC (clone RB6-8C5; BD Pharmingen). In addition, lung samples were stained for HO-1 mAb, as described above. For the A549 cell line, cells were stained with anti hRSV F-Alexa Fluor 647, conjugated, and washed for further analysis. All samples were acquired on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo 7.6 software.

Lung histopathology analyses

To perform histopathology analyses without losing significant tissue architecture, before BALF collection, the major bronchus of the left lung was clamped using 10 cm Kelly hemostatic forceps. After BALF of the right lung, the left lung was fixed with 4% paraformaldehyde, then paraffin embedded using a Leica ASP300S enclosed, automatic tissue processor (Leica Microsystems, Wetzlar, Germany). Then, 4 μm-thick tissue sections were obtained using a Microm HM 325 Rotary Microtome (Thermo Scientific), before being mounted and stained for histopathology analyses using H&E.

Cytokines and chemokines measured by ELISA

Immunoreactive CXCL1/KC were quantified by using a double Ab ELISA kit (DuoSet; R&D Systems, Minneapolis, MN). IL-6, IL-4, IL-10, and IFN-γ were quantified by using Ab ELISA kit (OptEIA; BD Pharmingen), and CCL3/MIP-1α detection was performed following the manufacturer's protocol (Ready Set Go!; Affymetrix).

Measurements of T cell function during hRSV infection

Lymph nodes from infected and mock BALB/cJ mice or tTA-HO-1 conditional transgenic mice of each treatment were removed and mechanically homogenized in 1 × PBS. After erythrocyte lysis with ACK buffer (150 mM NH_4CL, 10 mM $KHCO_3$, 0.15 mM EDTA), cells were resuspended at a final concentration equal to 5×10^6 cells per ml in RPMI 1640 medium, supplemented with 10% FBS, 1 mM nonessential amino acids, 2 mM glutamine, 1 mM pyruvate, 10 μg/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 50 μM 2-ME. Then, single-cell suspensions were left untreated or stimulated with ultraviolet-hRSV or anti-CD3ε/CD28. After 72 h of incubation (37°C, 5% CO_2), culture supernatants were analyzed for IFN-γ production by sandwich ELISA, and the expression of activation marker CD69 was measured on the surface of cells by flow cytometry (FACS Canto II flow cytometer [BD Biosciences, San Jose, CA] and analyzed using FlowJo 7.6 software).

DC maturation and APC assay

Immature DCs from conditional transgenic mice were incubated for 2 h with 50 μM CoPP or 1.5 μg/ml DOX (for all the incubation period). Then, cells were washed once and incubated with 700 μl of fresh RPMI 1640 medium. Maturation of DCs was induced by LPS treatment (1 μg/ml) for 16 h (*Escherichia coli* 0111; Invitrogen) and the surface expression of CD40, CD86, and CD80 molecules was measured by flow cytometry. Also, cells were pulsed with 50 μg/ml of OVA protein, non-OVA pulsed cells ± LPS were used as a control for antigenic presentation. After overnight incubation, cells were washed twice, detached, counted, and plated in round-bottom 96-well plates. Then, 50,000 DCs were incubated with 100,000 purified (over 90%; Miltenyi Biotec) OVA-specific TCR OT-II T CD4$^+$ cells (relationship 2:1 T cells:DCs). After 48 h of coculture, supernatants were collected for IFN-γ secretion, and T cells were stained for CD69 activation marker.

Statistical analyses

All statistical analyses were performed using GraphPad Prism Software version 6.1. Statistical significance was assessed using the Student *t* test and one-way ANOVA test with a posteriori Bonferroni test or Kruskal–Wallis ANOVA test with a posteriori Mann–Whitney *U* test. Differences were considered significant when $p < 0.05$.

Results

HO-1 induction inhibits hRSV replication and virus particle production in vitro

Upregulation of HO-1 suppresses infection of several viruses, including HIV, HCV, hepatitis B virus, Ebola virus, and influenza virus (16). To evaluate whether HO-1 induction reduces hRSV infection in vitro, we pretreated the human airway epithelial cell line A549 with increasing concentrations of CoPP (an HO-1 inducer), vehicle, or SnPP (an allosteric HO-1 inhibitor) for 2 h (Fig. 1A, 24). Supernatants of cells were then removed and cells were washed with fresh medium and infected with hRSV at an MOI equal to 1 for 2 h. After viral adsorption, cells were washed with medium to remove unbound virus. For postinfection treatment, cells were infected as described above, and CoPP was added to the cells and incubated for 24 or 48 h. Induction of HO-1 by CoPP was assessed by flow cytometry and Western blotting (Fig. 1D, Supplemental Fig. 1), respectively. Supernatants were harvested 48 h postinfection, and infective virus particle production was measured by detection of the hRSV F protein by ELISA. We observed that production of virus particles in supernatants was inversely correlated with increasing CoPP doses postinfection in A549 cells (Fig. 1A, white circles). Conversely, treatment with increasing doses of SnPP was directly associated with hRSV production in the supernatant of the treated A549 cells (Fig. 1A, black squares). Because treatment with 50 μM of CoPP resulted in more than a 60% reduction in hRSV production, as compared with cells treated with vehicle, we selected this concentration for further experiments. Moreover, to address whether HO-1 upregulation affects the generation of the viable progeny of hRSV viral particles, supernatants from infected A549 cells treated with CoPP, vehicle, or SnPP were harvested to quantify virus titers by immune-plaque assays. We found that hRSV-infected A549 cells treated with 50 μM of CoPP postinfection showed reduced virus titers by 3 log units as compared with cells treated with vehicle

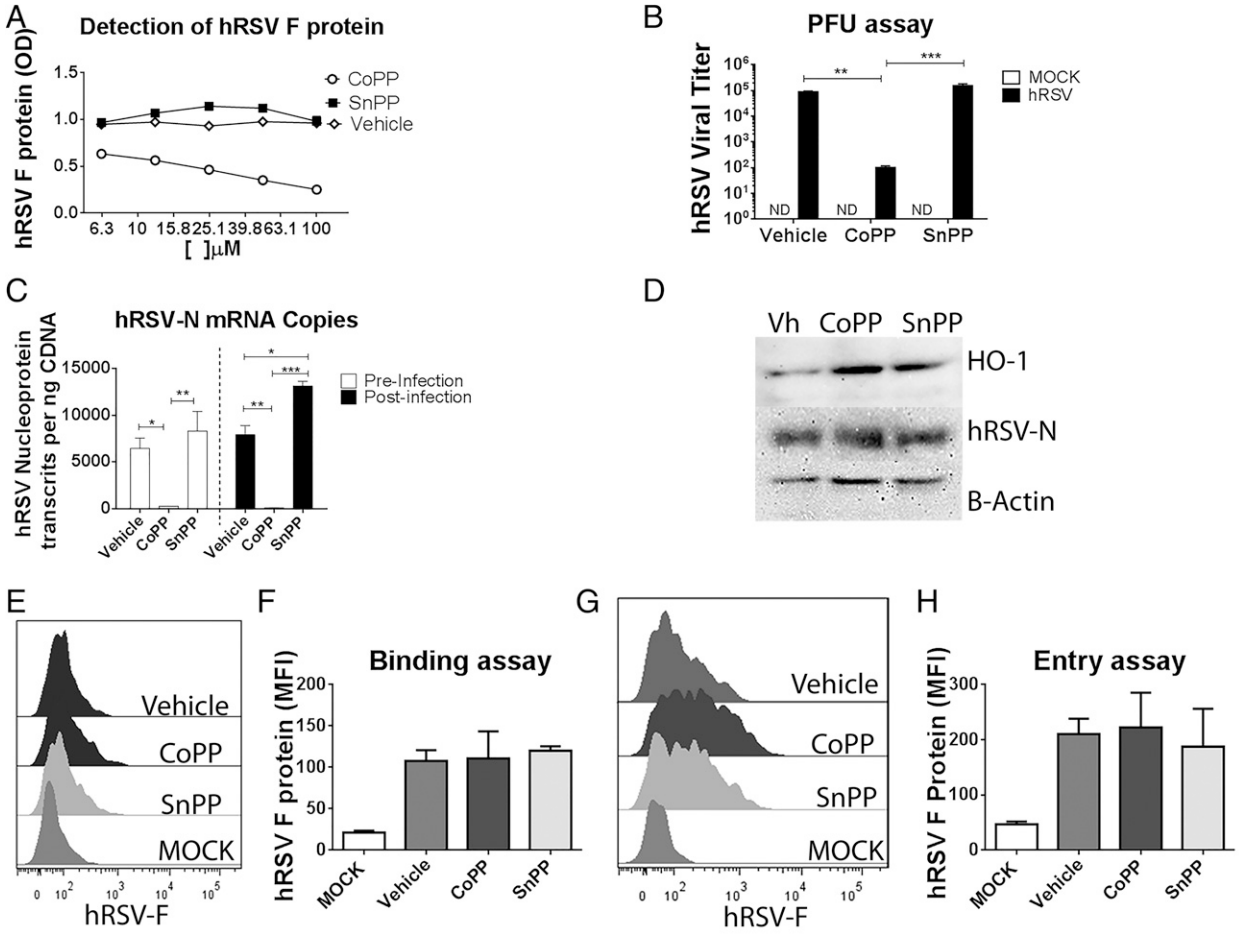

FIGURE 1. HO-1 induction reduces hRSV replication in human A549 cells. A549 cells were infected with hRSV at MOI 1 in the presence or absence of CoPP (HO-1 inducer), SnPP (HO-1 inhibitor) or vehicle control. (**A**) Dose-response curves measured by ELISA to detect hRSV F protein in supernatants from infected A549 cells treated with CoPP (white circles, 450 nm), SnPP (black squares), or vehicle (white diamonds). (**B**) Viral titers of supernatants from infected cells treated with vehicle, CoPP, or SnPP 48 h postinfection with hRSV. (**C**) Copy number for hRSV-N RNA in infected A549 cells per ng of cDNA at either 24 h postinfection for both cells, preinfection treatment with drugs (white bars), or treatment with drugs postinfection (black bars). (**D**) Representative Western blotting from total protein homogenates for HO-1 (upper panel), hRSV-N (middle panel), and β-actin (lower panel), in HEp-2 cells after 2 h of viral absorption at 4˚C to assess viral binding, in the presence of vehicle, CoPP, or SnPP. (**E**) Overlaid histograms of flow cytometry analyses for surface hRSV F protein expression in HEp-2 cells after 2 h of viral absorption at 4˚C to assess viral binding, in the presence of vehicle, CoPP, or SnPP. (**F**) Mean fluorescence intensity of surface hRSV F protein–expressing cells after 2 h of viral absorption at 4˚C, to assess viral binding in the presence of vehicle, CoPP or SnPP. (**G**) Overlaid histograms of flow cytometry analyses for surface hRSV F protein expression in HEp-2 cells after 2 h of viral absorption at 4˚C and 5 h of incubation at 37˚C, to assess viral entry in the presence of vehicle, CoPP, or SnPP. (**H**) Mean fluorescence intensity of surface hRSV F protein–expressing cells after 2 h of viral absorption at 4˚C and 5 h of incubation, to assess viral entry, in the presence of vehicle, CoPP, or SnPP. Data shown are mean ± SEM from three independent experiments. Data were analyzed by one-way ANOVA and Bonferroni posttest (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

(Fig. 1B). Furthermore, because N transcription can be considered a measurement of hRSV viral replication within infected cells (25), we evaluated this transcript in cells treated as indicated above. We observed a significant reduction in viral N mRNA amounts in cells treated with CoPP, both added preinfection and postinfection (Fig. 1C). Conversely, treatment with SnPP increased the amounts of hRSV N-transcripts in infected cells. Equivalent to A549, virus particle production and viral replication were similarly influenced by the HO-1 expression in HEp-2 cells (data not shown). In addition, to address the stage of the hRSV replication cycle that was affected by HO-1, we evaluated the effects of HO-1 induction, mediated by CoPP, on hRSV binding and entry. Previous reports have described that the active fusion process of hRSV is inhibited in HEp-2 epithelial cells at 4˚C, whereas viral binding still occurs (26, 27). Viral binding was assessed by Western blotting, measuring the hRSV-N protein over total protein collected from these cells (Fig. 1D), as well as by flow cytometry analyses of surface expression of the hRSV F-protein (Fig. 1E, 1F). Interestingly, no significant changes were observed for the expression of hRSV-N (Fig. 1D, middle panel) nor the surface expression of the hRSV-F protein (Fig. 1E, 1F), suggesting that HO-1 induction has no effect on hRSV binding. To evaluate viral entry, cells were infected at 4˚C for 1 h, as described above, washed and treated with CoPP, vehicle, or SnPP. Cells were then incubated at 37˚C for 5 h. Viral entry was measured by detecting intracellular expression of hRSV-F protein by flow cytometry. When CoPP-treated cells were compared with vehicle-treated or SnPP-treated cells, no significant differences were observed (Fig. 1G, 1H). Virus particle production and viral replication in HEp-2 cells were affected similarly as for A549 cells (data not shown).

Thus, these results suggest that although HO-1 induction negatively modulates hRSV replication in human alveolar epithelial cells, the inhibition of HO-1 activity promotes replication of this virus in these cells. However, HO-1 induction does not affect hRSV binding nor fusion in infected cells.

hRSV infection increases HO-1 expression in DCs

Because HO-1 can modulate the function of APCs (12, 28) and previous reports have shown that hRSV infects DCs, impairing their capacity to prime T cells (14, 29), we evaluated whether HO-1 was expressed in DCs upon hRSV infection. Bone marrow–derived DCs were exposed to hRSV, ultraviolet-hRSV, or mock. HO-1 expression was determined by qPCR, flow cytometry, and immunofluorescence. As shown in Fig. 2A, a significant increase in HO-1 mRNA expression levels was observed in hRSV-infected DCs, as compared with mock or untreated cells at 48 h postinfection. On the contrary, ultraviolet-hRSV–inoculated DCs did not show an upregulation of HO-1 mRNA levels, suggesting that HO-1 induction depends on hRSV gene transcription. Consistent with this observation, flow cytometry analyses exhibited a significant increase in the expression of HO-1 in hRSV-inoculated DCs (Fig. 2B). Confocal microscopy experiments further confirmed these findings, showing significantly higher HO-1 levels in hRSV-infected DCs, as compared with control cells (Fig. 2C). The HO-1 expression levels seen in hRSV-infected DCs were equivalent to the HO-1 levels induced by CoPP (Fig. 2C), suggesting that hRSV is a potent stimulus for HO-1 expression in DCs. Of note, the subcellular location of HO-1 was mainly situated near the plasma membrane in CoPP-treated DCs, but displayed a homogeneous distribution throughout the cytoplasm and plasma membrane in hRSV-infected DCs, suggesting that the differential distribution of HO-1 could be associated with different functions.

HO-1 induction limits viral replication in vivo and provides protection against hRSV infection in mice

Considering hRSV infection was significantly reduced in vitro as a result of HO-1 induction in airway epithelial cells and that this enzyme can display a potent anti-inflammatory activity (30–32), we evaluated whether the pharmacological induction of HO-1 in vivo can modulate hRSV infection in mice. Thus, BALB/cJ mice were treated either with CoPP or SnPP 24 h before hRSV infection. Then, animals were challenged intranasally with hRSV (1×10^6 PFU) or with equivalent volumes of a mock solution (as a non-infectious control). Changes in body weight were monitored as a parameter of disease progression for 7 d postinfection (4). As shown in Fig. 3A, a noticeable weight loss was observed after hRSV infection in vehicle-treated mice. Remarkably, CoPP-treated mice displayed accelerated kinetics of body weight recovery after hRSV infection, as compared with vehicle-treated mice (Fig. 3A). Consistently, significant differences between

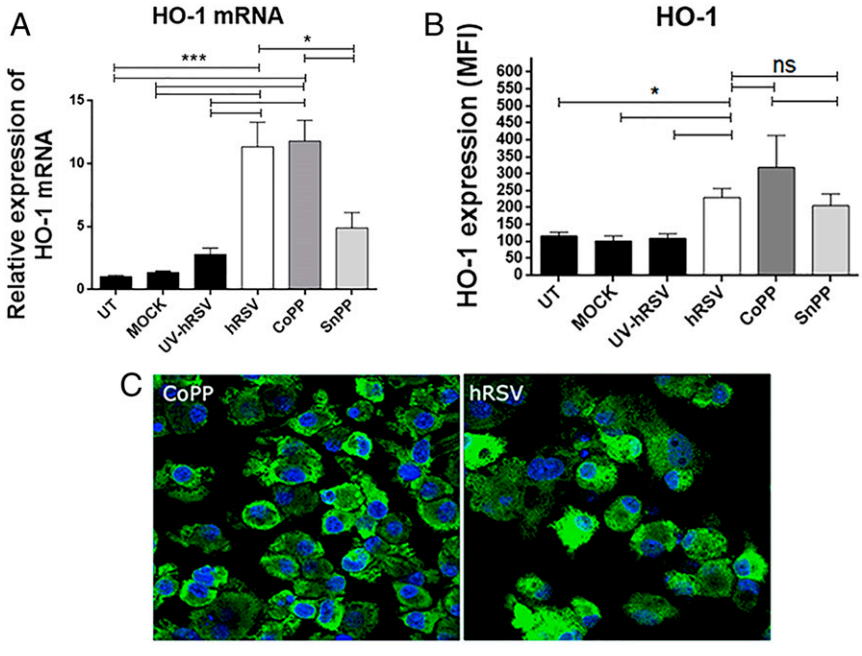

FIGURE 2. hRSV infection induces HO-1 expression in DCs. DCs were incubated with mock, ultraviolet-hRSV or hRSV at MOI 1, CoPP was used as a control of HO-1 induction and SnPP as an inhibitor of HO-1 activity, then cells were analyzed after 48 h. (**A**) HO-1 mRNA levels quantified by qPCR. Expression of relative mRNAs for target genes was normalized to β-actin levels using the 2-ΔΔCt method (UT cells were used as a reference control) (***$p < 0.0001$, *$p < 0.0154$; one-way ANOVA, Bonferroni posttest). (**B**) Mean fluorescence intensity (MFI) for HO-1 in MHC-II$^+$ cells for each treatment (*$p < 0.05$, ns, $p < 0.0575$; one-way ANOVA, Bonferroni posttest). (**C**) Confocal microscopy images show merged channels for HO-1 expression (green fluorescence) in DC cytoplasm of hRSV-infected and CoPP-treated DCs. In addition, the nuclei are shown in blue. Data shown are mean ± SEM from three independent experiments. **$p < 0.005$; one-way ANOVA, Bonferroni posttest.

CoPP-treated and vehicle-treated mice were observed at days 2, 3, and 4 postinfection with hRSV. In agreement with the body weight recovery data, a significant reduction of hRSV-N RNA levels was observed at day 4 postinfection in the lungs of hRSV-infected mice that were pretreated with CoPP (Fig. 3B). In contrast, mice receiving SnPP as pretreatment showed high viral loads in the lungs (Fig. 3B). Furthermore, viral titers in BALF from mice at 4 d postinfection were analyzed by immuno-plaque assays. CoPP-treated mice showed a 2-log reduction in viral titers, as compared with vehicle-treated mice (Fig. 3C). Further, qPCR analyses confirmed a significant increase in HO-1 mRNA levels in the lungs of CoPP-treated, hRSV-infected, and CoPP-uninfected mice as compared with control mice at day 4 postinfection (Supplemental Fig. 2A). These results suggest that the administration of CoPP was effective at inducing HO-1 expression in the lungs of mice. Furthermore, mRNA expression data were supported by flow cytometry analyses, which showed that CoPP treatment increased the amount of HO-1 protein in epithelial Epcam-positive cells (Supplemental Fig. 2B). Therefore, our data suggest that pharmacological induction of HO-1 expression, before virus challenge, reduces viral replication and virus particle production.

Induction of HO-1 expression reduces neutrophil infiltration and lung inflammation during hRSV infection

To assess whether the pharmacological induction of HO-1 modulates hRSV-mediated airway inflammation, histopathological analyses were performed in mice for each treatment at day 4 postinfection. Consistent with body weight loss and viral titration data, CoPP-treated mice displayed reduced inflammation after hRSV infection, as compared to vehicle-treated control animals (Fig. 4A, middle panel). Furthermore, CoPP-treated mice exhibited reduced inflammatory infiltration in both bronchoalveolar airspaces and the lung interstitium, suggesting that HO-1 induction protects mice against bronchopneumonia and interstitial pneumonia. Conversely, SnPP treatment caused a slight increase in lung inflammation (Fig. 4A, bottom panel). BALF collected at day 4 postinfection and evaluated by flow cytometry for leukocyte infiltration were consistent with previous data (21), showing that hRSV challenge caused significant infiltration of inflammatory cells into the airways of vehicle-treated mice (Fig. 4B, 4C). Further, we analyzed the frequency of total leukocytes ($CD45^+$) and neutrophils (MHC-II negative [$MHC\text{-}II^-$] $CD11c^-$ $CD11b^+$ $Ly\text{-}6G^+/Ly\text{-}6C^+$) in the BALF of hRSV-infected mice (Fig. 4B, 4C). A significant decrease in the total cell counts of total leukocytes and neutrophils in BALF was observed for CoPP-pretreated mice upon hRSV challenge, at day 4 postinfection (Fig. 4B, 4C). Contrarily, SnPP-treated mice showed a higher amount of neutrophils in BALF at day 4 post hRSV infection (Fig. 4C). Additionally, hRSV infection increased the production of several cytokines and chemokines in BALF, which are associated with lung inflammation (23). Thus, to evaluate whether the observed anti-inflammatory effects of HO-1 in the course of hRSV infection were due to the modulation of cytokines or chemokines in BALF, protein levels of IL-6, IL-4, IFN-γ, IL-10, CCL3/MIP-1α, and CXCL1/KC (an IL-8 homolog) were measured by ELISA. Overall, the protein concentrations of all hRSV-inducible cytokines (IL-6, IL-4, and IFN-γ; Fig. 5A–C) and chemokines (CCL3/MIP-1α and CXCL1/KC; Fig. 5D, 5E) were lower in mice that were treated with CoPP, as compared with vehicle-treated mice. Conversely, CoPP-treated mice displayed increased secretion of the immunomodulatory cytokine IL-10, even in mock controls (Fig. 5F), as previously described (33). Interestingly, SnPP-treated mice showed increased levels of CCL3/MIP-1α (Fig. 5D), suggesting that the increase in neutrophil recruitment can be mediated by a higher concentration of this chemokine.

Taken together, these data suggest that the pharmacological induction of HO-1 expression before virus challenge prevents the development of pulmonary inflammation by modulating the airway immunological milieu during hRSV infection, as evidenced by a reduction in lung inflammation and neutrophil infiltration in BALF.

HO-1 induction increases antiviral type I IFN response during hRSV infection in vivo

Previous reports indicate that HO-1 can regulate early innate immunity by modulating type I IFN production (14), as evidenced

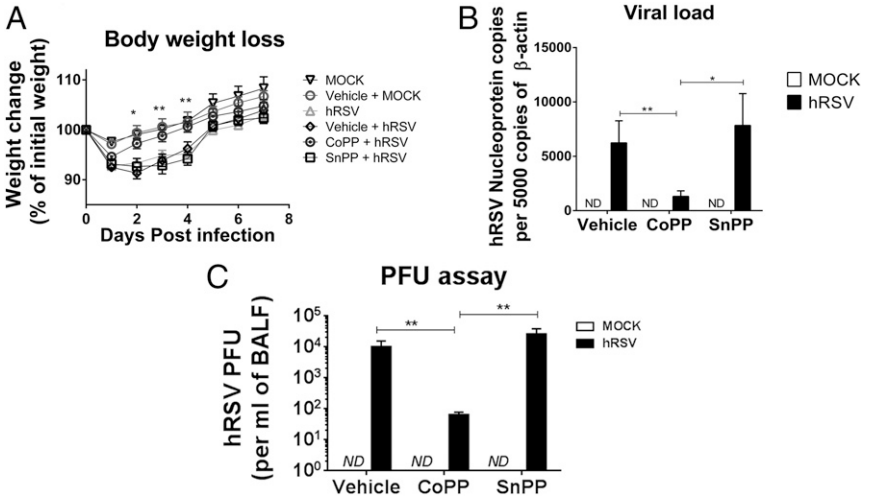

FIGURE 3. HO-1 promotes disease resolution and viral clearance in mice experimentally infected with hRSV. BALB/cJ mice 6–8 wk old were treated for 24 h with CoPP, SnPP, or vehicle control then inoculated intranasally either with mock or hRSV (1×10^6 PFU). hRSV disease progression was monitored by (**A**) determining values for animal weight loss over 7 d [$*p < 0.05$, Student t test was applied between CoPP + hRSV (black open circle) and hRSV (gray open triangle)]. (**B**) Lung homogenates of each experimental group of infected mice were collected at day 4 postinfection and quantified for viral copy number assessing hRSV-N RNA per 5000 copies of β-actin of by qPCR. White bars represent the N RNA copy numbers for MOCK controls and black bars represent hRSV infected mice. (**C**) The BALF from vehicle, CoPP-, and SnPP-treated mice were titrated on HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed as PFUs per milliliter). Data shown are mean ± SEM from three independent experiments, each with three mice per group ($n = 3$). Data were analyzed by one-way ANOVA and multiple comparisons against the vehicle control were performed for statistical analyses ($*p < 0.05$, $**p < 0.001$).

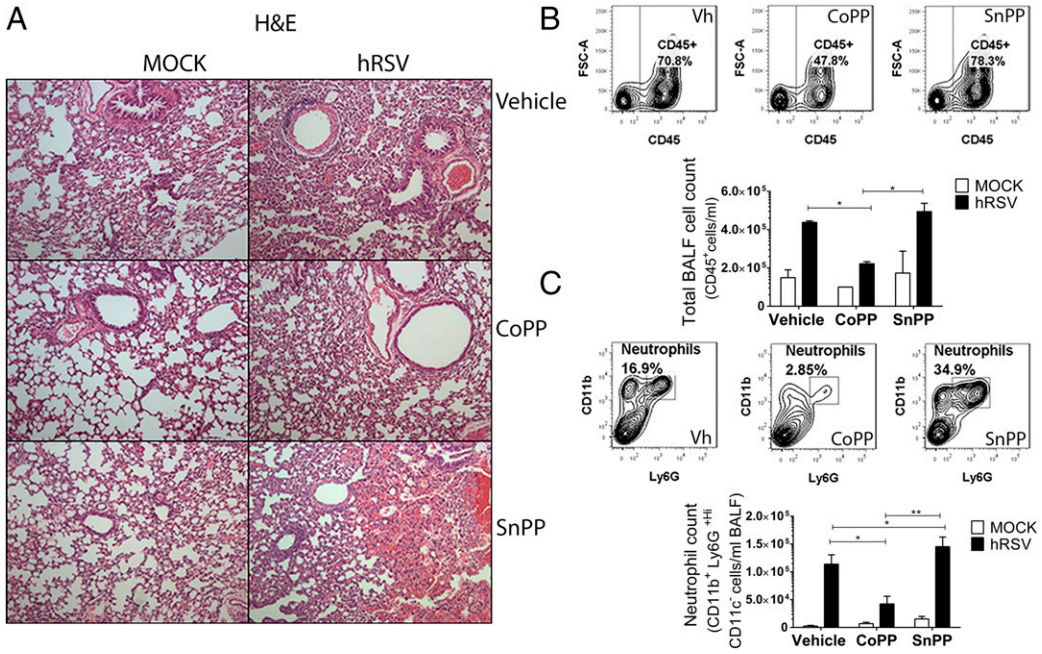

FIGURE 4. HO-1 induction reduces lung inflammation and inflammatory cell infiltration during hRSV infection. BALB/cJ mice 6–8 wk old were treated for 24 h with CoPP, SnPP, or vehicle and then inoculated intranasally, either with mock or hRSV (1×10^6 PFU). Mice were euthanized and BALF analyzed by flow cytometry. Lung sections were stained with H&E. (**A**) Histopathology analyses of lung sections from vehicle (upper panel), CoPP- (middle panel), and SnPP-treated (bottom panel) and hRSV-infected mice, H&E, original magnification ×10. (**B**) Representative dot plots of flow cytometry analysis for vehicle, CoPP-, and SnPP-treated mice (upper panel) and absolute number cell count (bottom panel) of total $CD45^+$ infiltration at day 4 postinfection in BALF for the mentioned conditions. (**C**) Representative dot plots of flow cytometry analysis for vehicle, CoPP-, and SnPP-treated mice (upper panel) and absolute number cell count (bottom panel) for neutrophil cell infiltration at day 4 postinfection in BALF (MHC-II^- $CD11c^-$ $CD11b^+$ Ly-6G/Ly-$6C^+$) for the mentioned conditions. Data shown are mean ± SEM from three independent experiments, each with three mice per group. Values were analyzed by one-way ANOVA and Bonferroni posttest (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

by the inhibition of HCV replication after activation of this enzyme (34). Therefore, we evaluated whether CoPP-mediated HO-1 induction could activate an antiviral type I IFN response in vivo during hRSV infection. Expression of IFN-α/β was measured in lungs from both hRSV-infected and control mice. As shown in Fig. 6, CoPP treatment enhanced mRNA levels of IFN-α (Fig. 6A) and IFN-β (Fig. 6B) in the lungs of hRSV-infected mice at day 4 postinfection. In addition, CoPP treatment also increased IFN-α/β mRNA expression in the lungs of mock (uninfected) control animals. Interestingly, SnPP treatment failed to modulate this type I IFN response. These data suggest that CoPP by itself induces an antiviral state in airway cells and that the upregulation of IFN-α/β requires HO-1 activity.

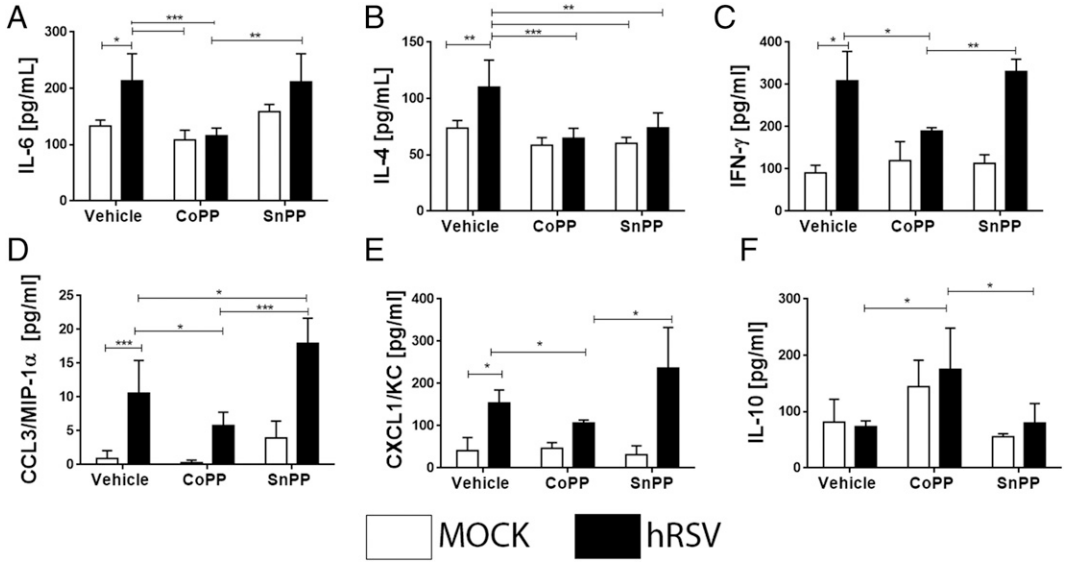

FIGURE 5. HO-1 inhibits hRSV-induced proinflammatory cytokine and chemokine responses in the airways of mice experimentally infected with hRSV. BALF samples were obtained from mice treated with vehicle, CoPP, or SnPP to measure concentrations of proinflammatory cytokines (**A**) IL-6, (**B**) IL-4, (**C**) IFN-γ, (**D**) CCL3/MIP-1α, (**E**) CXCL1/KC (an IL-8 homolog), and (**F**) IL-10 by ELISA. Data shown are mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA and Bonferroni posttest (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

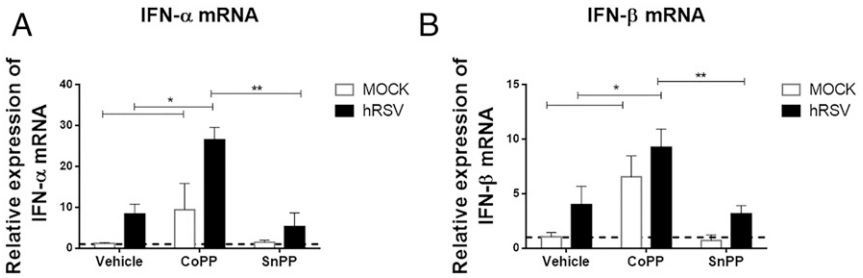

FIGURE 6. HO-1 induction promotes the upregulation of type I IFNs. BALB/cJ mice 6–8 wk old were treated for 24 h with CoPP, SnPP, or vehicle then inoculated intranasally, either with mock or hRSV (1×10^6 PFU). RNA from lungs of each experimental group was collected at day 4 and analyzed by qPCR for IFN-α/β mRNA levels. (**A**) IFN-α relative mRNA expression levels for vehicle and CoPP or SnPP pharmacological-treated experimental groups. (**B**) IFN-β relative mRNA expression levels for vehicle and CoPP or SnPP pharmacological-treated experimental groups. IFN-α/β relative expression was normalized to β-actin levels and calculated using the 2-ΔΔCt method (mock was used as a reference control). Data shown are mean ± SEM from three independent experiments, each with three mice per group ($n = 3$). Values were analyzed by one-way ANOVA and Bonferroni posttest ($*p < 0.05$, $**p < 0.01$, $***p < 0.0001$).

HO-1 induction slightly decreases T cell activation and function during hRSV infection

In addition to the important immunomodulatory properties of HO-1 on the innate immune response, this enzyme also exerts immunomodulatory effects on T cell–mediated adaptive response, by impairing T cell activation, proliferation, and their effector functions (16, 35). To examine whether HO-1 induction affects T cell responses, single-cell suspensions were obtained from mediastinal lymph nodes of each experimental group and were stimulated for 72 h with ultraviolet-hRSV or with anti-CD3ε/CD28 (1 μg/ml), or left untreated. T cell activation in single-cell suspensions was determined by CD69 expression in $CD4^+$ and $CD8^+$ T cells as a parameter for early activation (36). A significant increase in CD69 expression was observed in both $CD4^+$ (Fig. 7A) and $CD8^+$ (Fig. 7B) T cells stimulated with a polyclonal stimulus (anti-CD3ε/CD28) for all treatments. However, CoPP treatment led to a reduction of CD69 expression on anti-CD3ε/CD28-stimulated cells (Fig. 7A, 7B). No significant changes were observed in ultraviolet-hRSV stimulation, as evidenced by a non-significant increase in CD69 expression, which had been previously described by our group (36). In agreement with these data, a significant amount of IFN-γ was secreted in response to anti-CD3ε/CD28 stimulation with a mild decrease for the CoPP treatment (Fig. 7C).

Transgenic expression of human HO-1 in MHC-II^+ cells modulates the pulmonary disease caused by hRSV

To confirm the HO-1 protective role against hRSV infection through CoPP administration, conditional transgenic mice overexpressing the *HMOX-1* gene in MHC-II^+ cells (tTA-tHO-1) were instilled intranasally either with hRSV (1×10^6 PFU) or mock. Then 48 h before infection, 800 μg/ml DOX and 36 mg/ml sucrose were added to drinking water to induce HO-1 expression in these transgenic mice. To address whether the treatment with DOX induces the expression of exogenous human HO-1 in MHC-II^+ cells in the conditional transgenic mice tTA-tHO-1 (Supplemental Fig. 3A), qPCR analyses of the human HO-1 gene were performed from lung tissue obtained from mice of each experimental group (Supplemental Fig. 3B). The detection of human HO-1 in sorted pulmonary MHC-II^+ cells further validated this model (Supplemental Fig. 3C). Disease parameters were evaluated as described above, in this transgenic mouse model expressing human HO-1. Consistent with the effects observed in CoPP treatment, the selective expression of human HO-1 induced by DOX resulted in an accelerated kinetic of body weight recovery at days 3 and 4 postinfection, as compared with littermate controls and untreated transgenic mice (Fig. 8A). No significant unspecific effects were observed for DOX treatment in uninfected mice (Fig. 8A). These results are consistent with the observations described above, as at day 4 postinfection. We found a decreased neutrophil infiltration in the airways of tTA-HO-1 mice treated with DOX, as compared with littermates treated with DOX and infected with hRSV (Fig. 8C). Furthermore, DOX-induced transgenic mice also showed reduced viral loads in the lungs, as compared with infected littermates at day 4 postinfection with hRSV (Fig. 8B). Finally, lung histological analyses were performed to evaluate whether the transgenic expression of HO-1 could prevent lung inflammation. As shown in Fig. 8D, transgenic mice treated with DOX displayed significantly less inflammation than hRSV-infected littermate controls and untreated transgenic mice (Fig. 8D). Therefore, these data suggest that exogenous expression of human HO-1 in MHC-II^+ cells decreases neutrophil infiltration and lung inflammation in the conditional tTA-HO-1 transgenic mice.

Transgenic expression of human HO-1 in MHC-II^+ cells modulates T cell function during hRSV infection

Next, we assessed whether exogenous expression of HO-1 in MHC-II^+ cells in conditional transgenic mice could affect the function of APCs and their ability to process and present Ags to T cells. First, we evaluated the maturing capacity of DCs from tTA-HO-1. tTA-HO-1 DCs were treated with vehicle, CoPP, or DOX for 2 h. Cells were washed, and LPS (1 μg/ml) was added to the medium to induce maturation (22). After 16 h, DC maturation was measured by surface expression of CD80, CD40, and CD86 molecules by flow cytometry. Overall, all assessed surface markers were upregulated in LPS-pulsed DCs, suggesting that HO-1 had no effect in the maturation process of DCs in conditional transgenic mice after HO-1 induction by DOX (Supplemental Fig. 4A). To evaluate whether the ability of DCs to process and present Ags to T cells could be altered by the transgenic expression of HO-1, OVA-pulsed mature DCs from tTA-HO-1 mice were cocultured with OT-II $CD4^+$ T cells, which recognize the OVA-derived peptide OVA323–339/I-Ab complex as a cognate ligand. Thus, LPS-DCs treated with CoPP showed an impairment in T cell activation after 48 h of coculture, suggesting that CoPP pretreated DCs were unable to prime OT-II T cells (Supplemental Fig. 4B). On the contrary, mature DCs from the conditional transgenic mice that were treated with DOX were capable of processing and presenting OVA-derived peptides, resulting in OT-II T cell priming. However, the activation of OT-II T cells in the coculture with DCs from conditional transgenic mice treated with DOX was lower

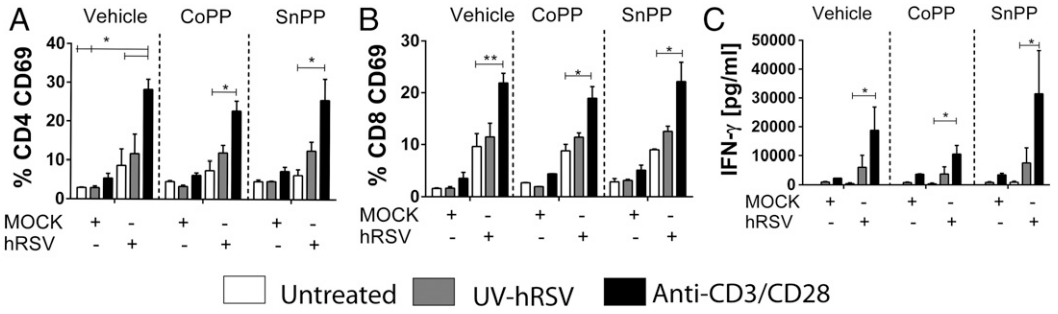

FIGURE 7. HO-1 induction slightly decreases T cell response during hRSV infection. Single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell response in infected mice treated with vehicle, CoPP, or SnPP. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28, or left unstimulated for 72 h. Flow cytometry detection of CD69 expression by $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells derived from each group of mice treated without stimuli, or with hRSV or anti-CD3ε/CD28 Abs. (**C**) IFN-γ secretion detected by ELISA in the supernatant of lymph nodes at 72 h poststimulation with hRSV-or anti-CD3ε/CD28. Data shown are mean ± SEM from three independent experiments.

compared with cocultures between vehicle-treated DCs and OT-II cells (Supplemental Fig. 4B), suggesting that the expression of HO-1 in DCs from conditional transgenic mice slightly affects the T cell priming. Furthermore, to assess whether expression of HO-1 in MHC-II^+ cells affects T cells priming in vivo during hRSV infection, littermate, untreated or DOX-treated tTA-tHO-1 mice were infected with hRSV and 4 d postinfection, single-cell suspensions were obtained from mediastinal lymph nodes and stimulated for 72 h with ultraviolet-hRSV or with anti-CD3ε/CD28 (1 μg/ml) or left untreated. To evaluate T cell activation in the cells suspensions, CD69 expression was measured in $CD4^+$ T cells. No significant increases in CD4 or CD69 expression were observed for cell suspensions from anti-CD3ε/CD28-stimulated DOX-induced transgenic mice as compared with unstimulated DOX-induced transgenic mice, suggesting that T cell activation is affected by HO-1 overexpression in MHC-II^+ cells (Fig. 9A). In agreement with these data, no significant IFN-γ secretion could be measured in supernatants of cell suspensions derived from DOX-induced transgenic mice (Fig. 9B), suggesting that T cell function can also be modulated by HO-1 overexpression in MHC-II^+ cells.

Discussion

HO-1 is a stress-inducible enzyme that catalyzes heme degradation into carbon monoxide, biliverdin, and free iron (37). HO-1 induction has been involved in several pathophysiological conditions, and HO-1 is generally regarded as a cytoprotective enzyme (38–40). Recently, there has been a growing interest about the regulatory role that HO-1 may play in immunity and tolerance (41, 42). Several studies support the beneficial effects that HO-1 and its products can have during inflammatory processes (42–44). However, little is known about the contribution of HO-1 expression during viral infections in vivo. Previous evidence described that gene overexpression of HO-1 can attenuate disease severity of influenza infection in mice (45). Moreover, induction of HO-1 inhibited influenza replication by activating the type I IFN system with the subsequent induction of IFN-stimulated genes (46). Further, HO-1–deficient (*ho-1*$^{-/-}$) mice displayed decreased survival rates after influenza infection, as compared with wild-type mice (47).

In this study, we evaluated whether the induction (or inhibition) of HO-1 could affect the development of the disease caused by hRSV in mice. Our findings indicate that preadministration of CoPP

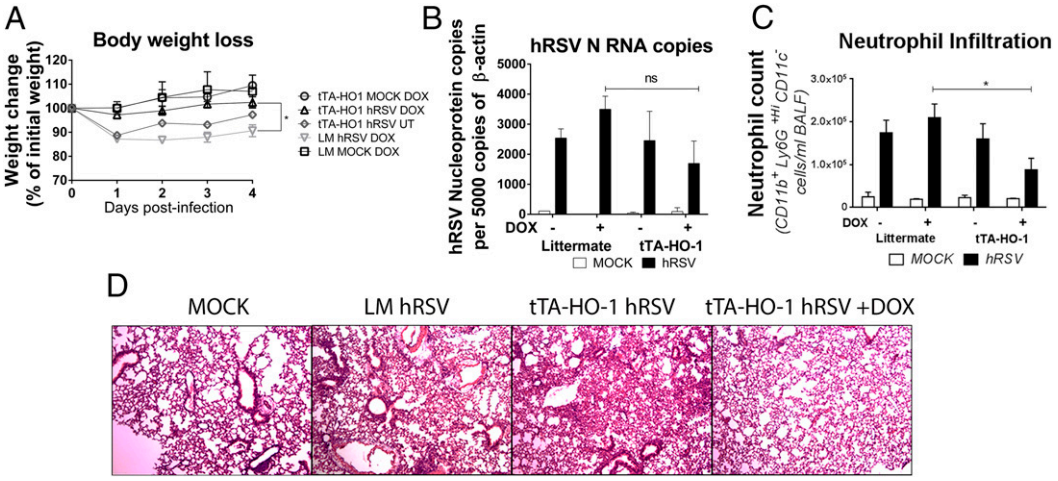

FIGURE 8. HO-1 overexpression in the MHC-II^+ cell subset reduces disease symptoms in rtTA-HO-1 hRSV-infected mice. DOX and sucrose were added to the drinking water of littermate or conditional transgenic mice (tTA-HO-1). (**A**) Mice were inoculated intranasally, either with hRSV or mock (noninfected supernatant) and hRSV disease progression was monitored by analyzing animal weight loss during 4 d (*$p < 0.05$, Student t test between LM DOX hRSV + tTA-HO-1 DOX hRSV values). (**B**) Lung homogenates of each experimental group of mice were collected at day 4 postinfection, and quantified for viral RNA by qPCR, using primers targeting the hRSV-N gene. Data in the graph show N-RNA copy numbers per 5000 copies of β-actin. (**C**) Neutrophil absolute cell count infiltration (MHC$^-$II$^-$ CD11c$^-$ CD11b$^+$ Ly-6G/Ly-6C$^+$) at day 4 in BALF for mock and hRSV-infected mice for each experimental group. (**D**) Histopathology analyses of lung sections for each experimental group (H&E, original magnification ×10). Data shown are from two independent experiments, each with three mice per group ($n = 2$). Mann–Whitney U test (*$p < 0.05$, **$p < 0.01$).

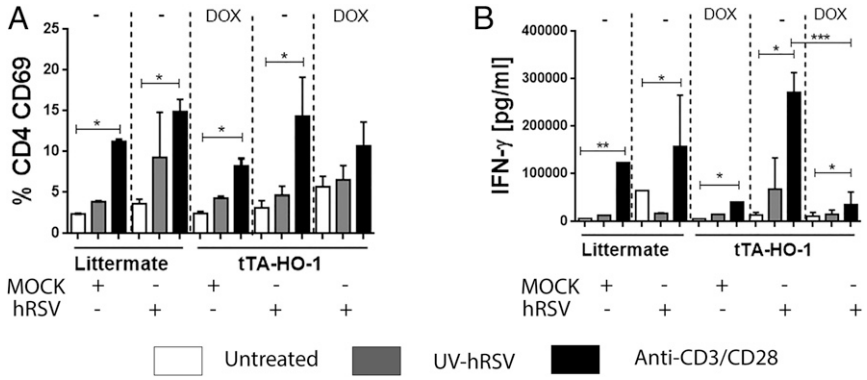

FIGURE 9. Exogenous HO-1 expression in MHC-II$^+$ cells of conditional transgenic mice impairs T-cell function during hRSV infection. DOX and sucrose were added to the drinking water of transgenic mice (tTA-HO-1) infected with hRSV or mock (non-infected supernatant). LM mice were included as a control. Single-cell suspensions were obtained from mediastinal lymph nodes to evaluate T cell response, in the conditional transgenic mice, to hRSV infection. The collected cells were stimulated with ultraviolet-inactivated hRSV or anti-CD3ε/CD28 or left unstimulated for 72 h. (**A**) Flow cytometry detection of CD69 expression on CD4$^+$ T cells derived from mice, treated without stimuli, or with hRSV or anti-CD3ε/CD28 Abs. (**B**) IFN-γ secretion detected by ELISA in the supernatant of lymph nodes at 72 h poststimulation with hRSV or anti-CD3ε/CD28. Data shown are mean ± SEM from three independent experiments.

can promote a significantly faster body weight recovery after hRSV infection, as compared with control mice. Similar results were observed in transgenic tTA-HO-1 mice that overexpress human HO-1 in MHC-II$^+$ cells. However, inhibition of HO-1 activity mediated by SnPP preadministration did not increase body weight loss as a parameter of disease, suggesting that HO-1 enzymatic activity is not necessarily the unique element involved in the protective effect observed in the body weight recovery induced by CoPP. In addition, we found that CoPP preadministration reduced viral mRNA and the titers of infective viral particles in the lungs of hRSV-infected mice, an outcome that coincided with an increase in HO-1 expression in lungs of mice at day 4 postinfection (Supplemental Fig. 2). Interestingly, we observed that SnPP pretreatment caused a slight increase in viral loads in the lungs during the same period of time, however, viral progeny (PFU) was not raised. These data suggest that the HO-1 enzymatic activity or its products are not necessarily involved in the modulation of viral replication and clearance. The increased expression of HO-1, observed in Epcam-positive cells from the lungs of CoPP-pretreated mice, is consistent with the notion that a reduction in viral loads could be related to the promotion of an antiviral response, mediated by HO-1 in airway epithelial cells. This concept is supported by the observation that although CoPP reduced viral loads and virus particle production in hRSV-infected A549 cells, SnPP-mediated inhibition of HO-1 activity increased both parameters. However, the initial steps of the life cycle of the virus, such as hRSV binding and entry, were not affected by HO-1 induction. Furthermore, we observed that CoPP treatment induced an upregulation of IFN-α/β in the lungs of hRSV-infected mice, indicating that HO-1 plays an important role during the development of the antiviral type I IFN response in the airways. All these data suggest that HO-1 induction could be promoting an antiviral state that prevents or limits viral replication and propagation of new virus particles.

In contrast, during acute lung infections, the host inflammatory response requires tight regulation (48) to promote pathogen clearance without evoking an exaggerated inflammatory response that could damage the infected airways. Our results indicate that HO-1 upregulation results in the suppression of lung inflammation, associated with a decrease in the inflammatory cell infiltration and an inhibition of proinflammatory cytokine or chemokine secretion during hRSV infection. Several studies have reported that hRSV infection actively induces activation of NF-κB in the lung tissue, leading to the secretion of proinflammatory mediators (49, 50). Because HO-1 has been found to inhibit NF-κB activity (51), we hypothesized that HO-1 induction decreases lung inflammation through the inhibition of NF-κB, which results in lower levels of proinflammatory cytokines and chemokines. Furthermore, HO-1 induction increases IL-10 secretion in BALF, supporting an anti-inflammatory role for HO-1 during hRSV infection.

Consistent with the observations described in this study for hRSV, decreased neutrophil infiltration and lung injury were described during influenza virus H1N1 infection as a result of adenoviral-mediated HO-1 gene transfer (45). These findings support the notion that HO-1 activity may be important for modulating lung inflammation during viral pulmonary pathologies (47, 52). This idea was further supported by the observation that mice deficient for Nrf2 (*nrf2* $^{-/-}$), a transcription factor controlling HO-1 gene expression, displayed a significantly reduced hRSV clearance, a higher bronchopulmonary inflammation, and a reduced body weight gain as compared with control mice (53).

The results observed for pharmacological induction of HO-1 mediated by CoPP are similar. The conditional expression of HO-1 in MHC-II$^+$ subsets in vivo promoted a reduction in lung disease in hRSV-infected tTA-HO-1 transgenic mice. These data suggest that the HO-1 enzyme is responsible for the protective effect of CoPP treatment. Importantly, although the MHC-II molecule is constitutively expressed by APCs, its expression is not limited to immune cells. In fact, intestinal and pulmonary epithelial cells can also express this molecule (54). Then, the conditional transgenic model used in this work suggests that the upregulation of HO-1 in epithelial cells and immune cells expressing MHC-II can contribute to suppressing the inflammation triggered by the hRSV infection.

It is important to mention that HO-1 was highly induced in DCs exposed to hRSV and it has been described that infection of DCs by hRSV impairs the ability of prime T cells (55), suggesting that HO-1 induction in response to hRSV could contribute to this process. Furthermore, HO-1 upregulation mediated by CoPP treatment or the exogenous expression of human HO-1 in MHC-II$^+$ cells in the conditional transgenic model slightly affects T cell activation and function, indicating that Ag processing and presentation by DCs was impaired by HO-1, in agreement with previous data supporting a role of HO-1 during adaptive immunity (35).

Treatment based in HO-1 upregulation is currently used as a therapeutic approach. The systemic hemin therapy, an HO-1 inducer, has been approved by the Food and Drug Administration to treat acute intermittent porphyria (56). Hemin also has been used to treat thalassemia intermedia, myelodysplastic syndrome, and liver allograft failure in erythropoietic protoporphyria (56). However, this strategy has not been explored for the treatment of infectious diseases.

In summary, the current study demonstrates that hRSV infection can be modulated by the expression of HO-1 both in vitro and in vivo. Thus, HO-1 activity may play a critical role during hRSV infection. HO-1 induction could protect the host from the pulmonary pathology developed upon hRSV infection, by reducing viral replication and lung inflammation, thus favoring disease resolution. Therefore, our results shed light on the potential role of the therapeutic induction of HO-1 in this viral pneumonia and suggest new avenues for the immunomodulatory treatment of hRSV-infected patients.

Acknowledgments

We thank Dr. Pedro Piedra (Baylor College of Medicine), Dr. George Kollias, and Dr. Christophe Benoist for kindly providing the A549 cells, pIi-TTA-TetO-HO-1, and pIi-TTA transgenic mice, respectively. We also thank María José Altamirano for breeding the mouse colonies that were used in this work.

Disclosures

The authors have no financial conflicts of interest.

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Cellular & Molecular Immunology (2017) 14, 1–3

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RESEARCH HIGHLIGHT

THEMIS, the new kid on the block for T-cell development

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Cellular & Molecular Immunology advance online publication, 29 May 2017; doi:10.1038/cmi.2017.34

Understanding T-cell development is a major goal of the immunology field and is crucial for the elucidation of the mechanisms behind self-tolerance and the occurrence of autoimmune disorders. T-cell development is a complex process that begins in the bone marrow or fetal liver, where lymphoid progenitors arise. Then, these progenitor cells migrate to the thymus to start the commitment into the T-cell lineage.[1,2] Thymocyte development has been described as a series of well-documented differentiation steps characterized according to the cell-surface expression of CD4 and CD8 co-receptors.[1] In the initial step, thymocytes are double negative (DN; $CD4^-$ $CD8^-$). In the second step, they become double positive (DP; $CD4^+$ $CD8^+$) before finally maturing into single-positive T cells (SP; CD4 or CD8).[2]

Thymocyte selection is essential for lineage commitment. This phenomenon occurs at the DP stage. The T-cell receptor (TCR) interacts with self-peptide–MHC ligands on the surface of cortical thymic epithelial cells.[1] Then, thymocytes are selected according to the nature of TCR signaling induced by the self-peptide–MHC ligands. While it is thought that signaling derived from weak TCR/MHC-peptide interactions leads to delayed apoptosis (death by neglect), strong TCR signaling promotes acute apoptosis (negative selection). An optimal intermediate activation of TCR signaling leads to positive selection and continues the maturation process to the CD4SP or CD8 stage.[1]

The thymocyte-expressed molecule involved in selection (Themis) is an important regulator of thymocyte positive selection,[3] and mice lacking Themis display a defect in the selection process mediated by the positive selection checkpoints, resulting in reduced numbers of SP cells and mature peripheral T cells.[4]

Themis is expressed exclusively in the T-cell lineage, with the highest expression levels found in DP thymocytes.[4–6] Themis is a member of a small gene family conserved throughout vertebrate evolution and is tyrosine-phosphorylated by Lck (and possibly ZAP70) immediately after TCR crosslinking.[5,7,8] Themis constitutively binds to GRB2[6,7,9] by means of a polyproline region that interacts with the C-terminal SH3 domain of GRB2.[3] Furthermore, Themis also co-immunoprecipitates with other molecules of the LAT/SLP76 signaling module.[7]

Themis contains two novel cysteine-based CABIT (cysteine-containing, all beta in Themis) domains,[10] a bipartite type nuclear localization sequence and a proline-rich sequence.[10] The CABIT domain is a recently designated domain structure conserved among metazoans, which has been predicted by means of multiple-sequence alignments to adopt an all-beta-strand structure with at least 12 strands,[11] suggesting either an extended beta-sandwich-like fold or a dyad of six-stranded beta-barrel units.[10] In mammals, the CABIT domains are conserved among three Themis family proteins (Themis/Themis1, ICB1/Themis2 and 9130404H23Rik/Themis3), harboring two tandemly repeated CABIT domains (CABIT1 and CABIT2) and two Fam59 proteins (Fam59a and b) containing one CABIT domain.[10] Although several proteins containing CABIT domains have been identified, their function is still unknown. The identification of the biological function of the CABIT domain is extremely important to understand the contribution of Themis in the positive selection process during T-cell development.

In a recent issue of *Nature Immunology*, Choi *et al.*[12] demonstrated a critical function of Themis during T-cell development and identified a biological function for the CABIT domain during positive selection and T-cell development. They showed that Themis, through CABIT, negatively regulates the activity of the PTP SHP-1 in DP thymocytes and enhances TCR signaling in response to low-affinity self-pMHC. As a result, positive selection is promoted (Figure 1). The authors demonstrated that the CABIT domain modulates Themis by interacting directly with the PTP domain of SHP-1, using cell-free *in vitro* protein-binding assays. This observation is consistent with previous findings made by

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Received: 14 April 2017; Accepted: 18 April 2017

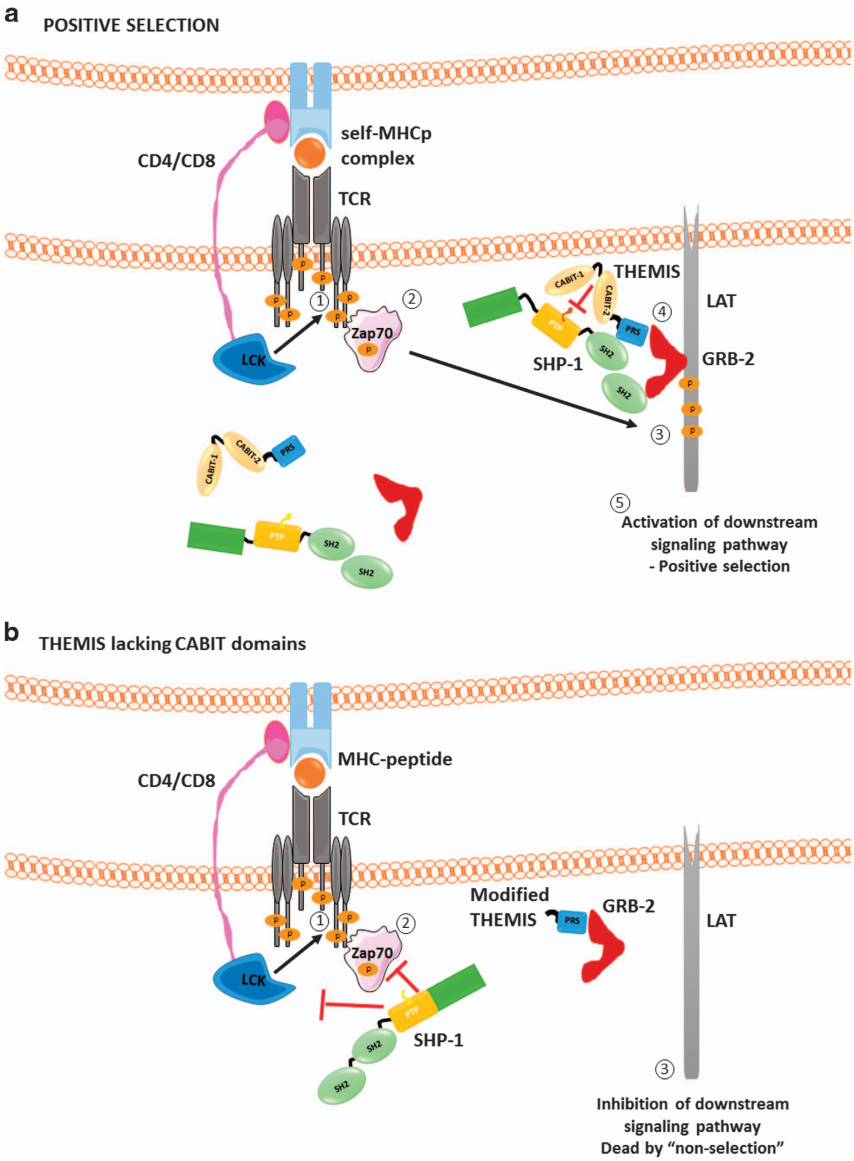

Figure 1 Model of the function of Themis in positive selection. (**a**) In Themis$^{+/+}$ DP thymocytes: (1) binding of the TCR on these cells to the self-peptide and MHC complexes causes phosphorylation of ITAM domains on the CD3 co-receptor by Lck; (2) ZAP70 is recruited and phosphorylated; (3) LAT is phosphorylated by ZAP70; (4) LAT recruits GRB2, Themis and SHP-1; and (5) downstream signaling pathways are activated that are sufficient to support positive selection. (**b**) In thymocytes without a functional Themis: (1) binding of the TCR to the self-peptide and MHC complexes causes phosphorylation of ITAM domains of CD3 by Lck kinase; (2) ZAP70 is recruited and phosphorylated; and (3) ZAP70 and Lck are blocked by the action of SHP-1, which results in the failure of positive selection and death by non-selection.

Paster *et al.*,[13] who showed that Themis constitutively associates with the phosphatases SHP-1 and SHP-2.[13] In addition, Choi *et al.* demonstrated that Themis directly regulates the catalytic activity of SHP-1 through the CABIT domain, which binds to the phosphatase domain, promoting or stabilizing oxidation of the catalytic cysteine residue of SHP-1, leading to inhibition of the tyrosine phosphatase activity of SHP-1.

Choi *et al.* also identified a key role for ROS in thymocyte selection, which agrees with data previously published by Moon *et al.*[14] and Jin *et al.*[15] suggesting that ROS might play a role during positive selection and the maturation of SP thymocytes. Here, Jin *et al.*[15] published data suggesting that the transition from pre- to post-selected DP stages is accompanied by an increase in ROS and transient expression of a variety of redox regulators, such as the thioredoxin (Trx) 1/thioredoxin reductase (TrxR)1 system.[15]

Importantly, Choi *et al.* determined the importance of Themis *in vivo* by demonstrating that the deletion of SHP-1 in the *Themis*$^{-/-}$ *Ptpn6*$^{fl/fl}$ *Cd4*-Cre mice alleviated the developmental block in *Themis*$^{-/-}$ thymocytes. Furthermore, these results, together with the profound block in the positive selection

exhibited by Themis$^{-/-}$ thymocytes, suggested that this protein might be responsible for the selective sensitivity of DP thymocytes to TCR engagement. These findings are in agreement with a previous report by Lesourne *et al.*[4] showing that although DP thymocytes highly express Themis, expression is downregulated as thymocytes transition to the SP stage and become less responsive to low-affinity self ligands. Such a property is thought necessary for the prevention of autoimmunity.[16] That activity, coupled with the stage-specific regulation of Themis during T-cell development, provides an explanation for the unusual sensitivity of DP thymocytes to TCR stimulation, a feature that is essential for positive selection. Previous independent studies had already identified the same gene, *Themis*, whose elimination has a profound effect on T-cell development. Collectively, these authors had provided a comprehensive investigation into the function of Themis but without defining the mechanism of action. The critical finding from the work of Choi *et al.* was the identification of one of the possible mechanisms of action of Themis in the process of positive selection. However, there are several questions that remain to be addressed. Finally, the findings made by Choi *et al.* contribute to the understanding of the process of tolerance of T cells to self and to defining some of the causes of the development of autoimmune diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors are supported by CONICYT/FONDECYT no 3150559, CONICYT no 21130507, FONDECYT 1150862 and the Millennium Institute on Immunology and Immunotherapy, P09/016-f.

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Potenciales consecuencias neurocognitivas de infección por virus respiratorio sincicial humano

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Potential neurocognitive consequences of infection by human respiratory syncytial virus

Human respiratory syncytial virus (RSV) infection remains as a major cause of morbidity and mortality among pediatric population. Immune response is poor and unable to establish a long term effective protection against this virus. Of particular interest has been the description of extrapulmonary manifestations of RSV infection in liver, kidney, endocrine system, heart and brain, associated to infection of peripheral blood. In the central nervous system (CNS), recent studies in animals have suggested long term neurocognitive impairment due to a direct damage from the virus. This was prevented in rats by a recombinant BCG vaccine expressing a nucleoprotein N of RSV that produces an effective immune response against the virus, not allowing its dissemination to the CNS. These findings in animal models highlight the importance of conducting more specific studies in children affected with severe infection by RSV**.** Therefore, our group is currently conducting an assessment of the possible long-term cognitive impairment in children under 2 years. The results of this study could be a strong argument to continue looking for an effective method for protecting against RSV infection.

Key words: Human respiratory syncytial virus, encephalitis, encephalopathy, learning.
Palabras clave: Virus respiratorio sincicial humano, encefalitis, encefalopatía, aprendizaje.

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Los autores no tienen conflictos de interés con este manuscrito.
Financiamiento: Proyecto Interdisciplina 14/2013, Vicerectoría de Investigación, Pontificia Universidad Católica de Chile. Instituto Milenio en Inmunología e Inmunoterapia P09/016-F. Fondo de Desarrollo Científico y Tecnológico proyecto número 1150862. JE y KB son becarias de doctorado de CONICYT.

Recibido: 28 de marzo de 2016
Aceptado: 23 de junio de 2016

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Introducción

El virus respiratorio sincicial humano (VRS) es el principal agente causal de infecciones respiratorias agudas bajas (IRAB) en niños bajo dos años de edad (lactantes) en el mundo[1]. Una revisión sistemática reciente estimó que el VRS causó 3,4 millones de IRAB graves que requirieron hospitalización en niños bajo 5 años de edad, y entre 66.000 y 119.000 muertes en países en desarrollo el año 2005, los que concentran 99% de la mortalidad por infecciones respiratorias[2]. Estos antecedentes muestran que la infección por VRS es uno de los principales problemas de salud pública en el mundo.

El VRS afecta a 70% de los niños durante el primer año de vida, y al cumplir dos años, prácticamente 100% de la población ha tenido contacto con el virus. Su incidencia más alta se alcanza entre los dos y cinco meses de edad, con brotes epidémicos durante los meses invernales[3,4]. Las manifestaciones clínicas de la infección por VRS varían desde cuadros asintomáticos hasta infecciones letales, dependiendo de la asociación a factores de riesgo como prematurez (gestación menor a 35 semanas), edad bajo seis meses, displasia broncopulmonar, cardiopatías congénitas e inmunosupresión[5-11].

En Chile el VRS es la principal causa de IRAB en lactantes, con una tasa de hospitalización de 2% de los lactantes infectados[5]. La epidemia en el invierno chileno depende del virus predominante cada año, observándose que el VRS grupo B tiende a producir un aumento de casos más precoz[5], y el grupo A se ha asociado con mayor gravedad de la infección[12]. En niños sin factores de riesgo, la mortalidad por IRAB en Chile es muy baja (0,1%). Además, en un estudio reciente de co-infección de VRS con rinovirus, realizado en el área norte de Santiago, no se observó mayor gravedad en la presentación clínica[5,13].

Patogenia y respuesta inmune de la infección por VRS

La infección por VRS ocurre a través de la inhalación del virus por vía respiratoria o a través del contacto directo con la mucosa ocular[14]. Un aspecto relevante de la infección causada por este virus es la re-infección. Se ha descrito en estudios epidemiológicos que aproximadamente 36% de los individuos puede reinfectarse durante el mismo brote estacional[15-17]. Este fenómeno se debe a una respuesta inmunológica ineficiente para la eliminación del virus, que sólo establece protección parcial contra infecciones posteriores[18]. Estudios en humanos han demostrado que la respuesta inicial es mediada por células epiteliales, las que promueven el

reclutamiento de células inmunes al sito de la infección. La inmunopatología mediada por VRS se caracteriza por la producción de citoquinas pro-inflamatorias que atraen a células mononucleares, principalmente neutrófilos y linfocitos, al espacio peribronquial. Se describe una respuesta inflamatoria exacerbada debido a un desbalance de linfocitos Th_2 patogénicos, además de una reducción en la actividad de linfocitos T citotóxicos[19,20].

Otro factor relevante en la patología causada por VRS es el estado inmunológico del individuo; se ha descrito que hace variar la presentación clínica. En pacientes inmunocompetentes la presentación clínica más común es obstrucción de la vía aérea, sin afectación importante a nivel alveolar. Por el contrario, en inmunodeficientes se ha observado mayor frecuencia de neumonía, con infiltrados celulares en el parénquima pulmonar y menor frecuencia de sibilancias[21].

Manifestaciones extra-pulmonares de infección por VRS grave

Durante la última década se han reportado diversas manifestaciones extra-pulmonares asociadas a la infección por VRS, entre las cuales están alteraciones hepáticas[22,23], renales[24], endocrinas[22,25], cardiovasculares[26,28]y del sistema nervioso central (SNC)[22,29-31]. El compromiso de estos dos últimos sistemas podría explicar la asociación del virus con muerte súbita en lactantes menores[22,26].

Se ha descrito el compromiso hemodinámico con hipotensión arterial, necesidad de fármacos vasoactivos, daño miocárdico con elevación de enzimas cardíacas y arritmias ventriculares y supraventriculares. La presencia del virus en miocardio ha sido demostrada con métodos de cultivo y reacción de polimerasa en cadena (RPC)[26].

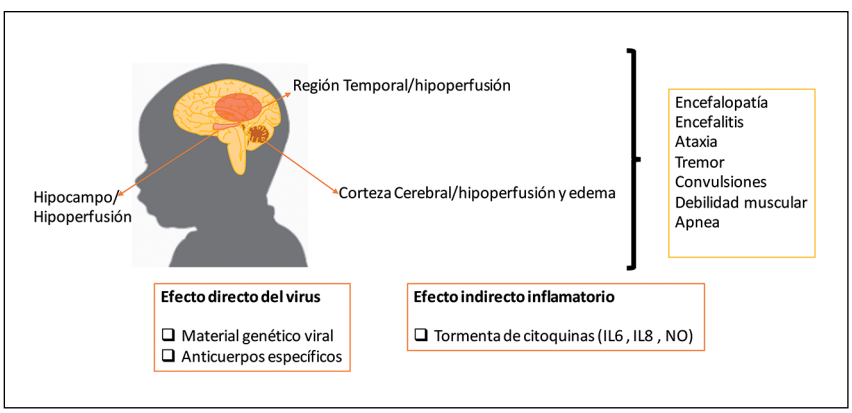

Figura 1. Hallazgos clínicos de alteraciones en el sistema nervioso central asociadas a la infección con VRS. La figura muestra las zonas del cerebro que estarían afectadas por la infección con VRS, así como la sintomatología clínica asociada. Además se destacan los efectos directos e indirectos del VRS en el SNC.

Alrededor de 50% de los niños que requieren apoyo ventilatorio derivado de bronquiolitis grave presentan elevación de transaminasas, hecho que se asoció a un mayor número de días en ventilación mecánica. Al igual que en el compromiso cardíaco, el virus logró ser cultivado desde una muestra de biopsia hepática en un paciente inmunocompetente[26].

La infección por VRS también ha sido asociada con manifestaciones renales. Se ha encontrado material genético y antígenos virales por RPC en muestra de orina de un paciente pediátrico con síndrome nefrótico, que tuviera una buena respuesta a corticosteroides[24].

El efecto endocrino más importante es la hiponatremia producida por secreción inapropiada de hormona antidiurética, descrita en 33% de los pacientes que ingresaron a unidad de cuidados intensivos con infección grave por VRS. Las convulsiones por hiponatremia se asociaron a la administración de soluciones hipotónicas de mantención. Otras alteraciones hormonales descritas en pacientes en ventilación mecánica son concentraciones aumentadas de prolactina y hormona de crecimiento, y una disminución de leptina e IGF-1[26].

La diseminación sistémica de VRS ha sido asociada a la infección de células inmunes en el torrente sanguíneo. Reportes en modelos animales (ratones) y humanos (lactantes) han demostrado la detección de este patógeno en sangre, mediante técnica de RT-PCR[32,33]. Esta evidencia sugiere que VRS es capaz de diseminarse a órganos distantes utilizando la vía hematógena[32].

Compromiso de sistema nervioso central

Alrededor de 2% de los casos de bronquiolitis por VRS reportan síntomas derivados de compromiso del SNC[30,31]. Estudios en humanos han encontrado la presencia de ARN viral y anticuerpos específicos contra VRS en líquido cefalorraquídeo (LCR)[34]. Las áreas del SNC que se han descrito afectadas por la infección por VRS incluyen hipocampo, troncoencéfalo, corteza cerebelar y región temporal (Figura 1)[35-37]. Estos hallazgos han sido asociados a síntomas neurológicos como convulsiones, apneas centrales, letargia, alteraciones de la deglución, estrabismo, anormalidades del tono y encefalopatía[22,29-31,35,38,39].

La etiología de la encefalopatía causada por la infección con VRS es aún desconocida. Estudios en muestras de LCR de pacientes con complicaciones neurológicas han mostrado concentraciones elevadas de citoquinas pro-inflamatorias, tales como IL-6, IL-8 y óxido nítrico, sugiriendo que una exacerbada producción de estos mediadores inflamatorios podría estar involucrada en la encefalopatía causada por VRS[30,36]. Morichi y cols., correlacionan además las concentraciones elevadas de IL-6 y *brain-derived neurotrophic factor* (BDNF) con

secuelas neurológicas evaluadas por puntaje estandarizado (*Pediatric Cerebral Performance Categories*) a los seis meses de ocurrida el alta, en una serie pequeña de 11 pacientes con encefalopatía por VRS[40]. Otra investigación reciente de Erez y cols., apoya la hipótesis inflamatoria como causa del compromiso de SNC debido a que las manifestaciones clínicas se presentan aún en ausencia de ARN viral en el LCR[41]. En dicho estudio, se estudiaron lactantes con infección confirmada por VRS a quienes se realizó punción lumbar por síndrome febril en niños bajo 6 semanas de edad, por sospecha de infección bacteriana o compromiso de SNC. Se separó a los pacientes en dos grupos de acuerdo a la presencia o ausencia de manifestaciones neurológicas en la presentación clínica (apneas, convulsiones y encefalopatía). Se evaluó presencia de ARN viral en el LCR mediante RPC para VRS, virus influenza, parainfluenza, metapneumovirus humano, enterovirus, adenovirus y parechovirus[41]. Todas las muestras resultaron negativas para los agentes infecciosos estudiados, en ambos grupos de pacientes. Los autores sugieren que probablemente hay mecanismos inmunológicos involucrados en las manifestaciones neurológicas asociadas a VRS, que en esta serie correspondieron a apnea y encefalopatía (compromiso de conciencia).

Con respecto a la gravedad, es importante destacar que sólo 50% del grupo de pacientes con síntomas neurológicos, y ninguno en el grupo control, requirió ventilación mecánica durante la hospitalización[41]. Esta información es relevante debido a que en la mayoría de los estudios que han reportado presencia de VRS en LCR se han incluido pacientes con bronquiolitis grave.

Actualmente el número de casos reportados de encefalopatía asociada a infección grave por VRS ha ido en aumento, recalcando la importancia de conocer más en detalle este fenómeno. No se conoce aún con exactitud la fisiopatología que explica esta presentación clínica, su mecanismo de entrada al SNC, localización y forma de diseminación[30]. Nuestro grupo, (Espinoza J. y cols.), evaluó las características del compromiso neurológico en modelos animales de infección por VRS (ratones Balb/cJ y ratas Sprague-Dawley), con resultados que

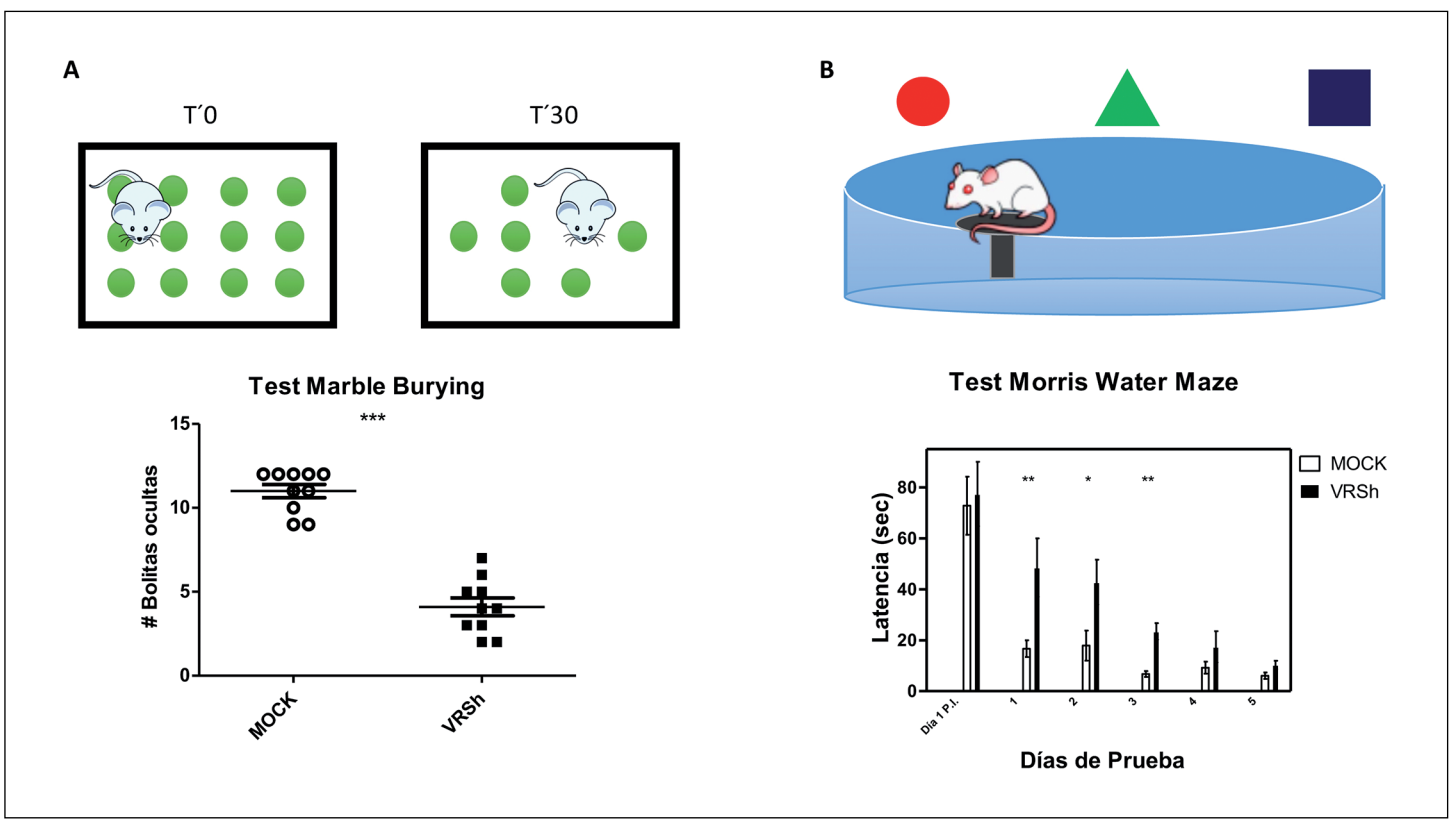

Figura 2. Alteraciones conductuales y de aprendizaje espacial causada por el VRS. La figura muestra los principales hallazgos de Espinoza y cols., con respecto a las alteraciones en el SNC en modelos animales. En **A** se observa un esquema explicativo del test de Marble Buriyng y la gráfica muestra los resultados obtenidos en este test 30 días posteriores a la infección con el VRS. En **B** se muestra un esquema correspondiente al test de Morris Water Maze, el que evalúa el aprendizaje espacial. En el gráfico se puede observar los resultados obtenidos 30 días después de finalizar la infección por VRS.

apoyan la infección directa por parte del virus en el SNC y que permiten entender los mecanismos por los cuales se produce[42]. Después de inoculación nasal de VRS se realizó un detallado monitoreo de la progresión de la enfermedad, encontrándose un patrón de compromiso focal en el SNC, con mayor presencia de ARN y proteínas del virus en las áreas relacionadas a la producción de LCR, bulbo olfatorio y en aquellas con mayor irrigación sanguínea. Estos datos sugieren que el virus posee un neurotropismo e ingresa al SNC por dos vías distintas: hematógena, contenido en leucocitos infectados, y a través de nervio olfatorio[42].

Alteraciones cognitivas y de conducta posteriores a la infección aguda por VRS

En el estudio realizado por Espinoza J. y cols., además se encontraron proteínas virales en la zona del hipocampo, la que es muy importante para procesos cognitivos[42]. Por este motivo, se evaluó si los animales infectados con VRS presentaron algún tipo de alteración en aprendizaje y conducta. Los animales fueron evaluados a los 30 días después de la infección con VRS en términos del aprendizaje espacial utilizando el test de Morris Water Maze (MWM)[42]. Los resultados obtenidos en este test mostraron que las ratas que fueron infectadas con VRS presentan problemas cognitivos con capacidad de aprendizaje alterada (Figura 2). Por otra parte, se evaluó la conducta, tanto de ratas como ratones, utilizando el test de Marble Burying (MB) a 30 días de terminada la infección con VRS. Los resultados mostraron que los animales infectados presentaban alteraciones en el comportamiento al ser comparados con animales control[42]. Estas alteraciones lograron ser prevenidas utilizando inmunización con vacuna recombinante BCG que expresa la nucleoproteína N de VRS, la cual es capaz de inducir inmunidad efectiva, impidiendo la diseminación del virus hacia el SNC y, en consecuencia, evitando las alteraciones anteriormente descritas[42].

Perspectivas futuras para investigación y desarrollo de vacuna contra VRS

Los antecedentes mencionados en este artículo dan cuenta de la importancia de estudiar en humanos si el VRS es capaz de causar alteraciones cognitivas como las descritas en animales. Otro aspecto relevante a destacar es la necesidad de desarrollar una vacuna que sea efectiva y de ese modo prevenir las graves consecuencias neurológicas que puede provocar la infección por VRS. No se han dado a conocer, a la fecha de envío de este artículo, estudios en los cuales se evalúe si el VRS es capaz de producir alteraciones en el aprendizaje a largo plazo. A raíz de estos hallazgos en animales, en nuestro grupo surgió el interés de realizar una investigación, actualmente en curso, que busca caracterizar cualitativa y cuantitativamente si la infección grave por VRS produce consecuencias cognitivas en niños, en una evaluación prospectiva.

Antes de cumplir un año, los niños han avanzado notablemente en el desarrollo de destrezas cognitivas cruciales para la vida, tales como la lengua materna[43,44], cognición numérica[45,46] y cognición social[47]. Estudios de nuestro grupo en esta área han contribuido a caracterizar el rol de la biología y la experiencia en el logro de estas habilidades, tanto en niños sanos como en riesgo cognitivo[44,48,49]. Desde esta perspectiva, el estudio en desarrollo evalúa el impacto de la infección grave por VRS en la adquisición de hitos del desarrollo lingüístico, numérico y social por medio de pruebas estandarizadas realizadas 1, 3 y 6 meses después de haberse resuelto el cuadro clínico. Para estimar la especificidad del compromiso cognitivo asociado a VRS se realizará una comparación con niños sanos no afectados por VRS grave.

Esta área de estudio, sin duda, es de alto interés para las ciencias médicas y para la sociedad en general, debido a las potenciales consecuencias en el neurodesarrollo de una enfermedad de alto impacto epidemiológico y para la cual no existe en la actualidad un método costo-efectivo para tratar o prevenir la infección. Conocer si VRS produce alteraciones neurocognitivas en humanos podría permitir además el desarrollo de protocolos de diagnóstico e intervención precoz para tratar oportunamente los problemas derivados de la infección grave por este virus.

Agradecimientos. Los autores agradecen el apoyo del Proyecto Interdisciplina de la Vice-Rectoría de Investigación UC, el Instituto Milenio en Inmunología e Inmunoterapia, Proyecto FONDECYT 1150862 y de la Comisión Nacional de Investigación Científica y Tecnológica de Chile.

Resumen

La infección por virus respiratorio sincicial humano (VRS) es una de las principales causas de morbimortalidad en población pediátrica. La respuesta inmune generada contra VRS es poco eficiente para su eliminación y logra establecer sólo protección parcial contra infecciones posteriores. De especial interés en los últimos años ha sido la descripción de manifestaciones extra-pulmonares de la infección por VRS en hígado, riñón, sistema endocrino, corazón y cerebro. A nivel de sistema nervioso central (SNC), estudios recientes en modelos animales han sugerido problemas neurocognitivos a largo plazo derivados de un daño directo del virus en el cerebro. Este

daño logró ser prevenido con vacuna experimental BCG recombinante, que expresa la nucleoproteína N de VRS e induce inmunidad efectiva, impidiendo la diseminación del virus hacia el SNC. Estos hallazgos en modelo animal han dado cuenta de la importancia de efectuar estudios más detallados en niños afectados por VRS grave. Por tal motivo, actualmente se está realizando una evaluación de la posible alteración cognitiva a largo plazo en niños bajo dos años de edad por parte de nuestro grupo. Los resultados de este estudio podrían significar un argumento muy importante para continuar en la búsqueda de un método efectivo de protección contra esta infección.

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The American Journal of Pathology, Vol. 187, No. 3, March 2017

The American Journal of
PATHOLOGY
ajp.amjpathol.org

MINI-REVIEW

Modulation of Antiviral Immunity by Heme Oxygenase-1

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Accepted for publication November 15, 2016.

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Heme oxygenase-1 (HO-1) is a stress-inducible, anti-inflammatory, and cytoprotective enzyme expressed in most cell types in the organism. Under several stress stimuli, HO-1 expression and activity is up-regulated to catalyze the rate-limiting enzymatic step of heme degradation into carbon monoxide, free iron, and biliverdin. Besides its effects on cell metabolism, HO-1 is also capable of modulating host innate and adaptive immune responses in response to sepsis, transplantation, and autoimmunity, and preventing oxidative damage associated with inflammation. In addition, recent studies have reported that HO-1 can exert a significant antiviral activity against a wide variety of viruses, including HIV, hepatitis C virus, hepatitis B virus, enterovirus 71, influenza virus, respiratory syncytial virus, dengue virus, and Ebola virus, among others. Herein, we address the current understanding of the functional significance of HO-1 against a variety of viruses and its potential as a therapeutic strategy to prevent and control viral infections. Furthermore, we review the most important features of the immunoregulatory functions for this enzyme. *(Am J Pathol 2017, 187: 487–493; http://dx.doi.org/10.1016/j.ajpath.2016.11.011)*

HOs and Heme Degradation

Heme oxygenases (HOs) are metabolic enzymes that catalyze the oxidative degradation of heme groups (preferably heme b and c and hematoheme) to produce carbon monoxide, free iron, and biliverdin, with the latter rapidly converted into bilirubin by biliverdin reductase.[1] To date, three isoenzymes of HO have been characterized: although the HO-1 form is a ubiquitously expressed after induction, HO-2 expression is constitutive, yet mostly limited to certain organs, such as the brain, testes, and the vascular system.[2,3] Although an additional form, termed HO-3, has been described in rats, in humans it is nonfunctional, suggesting that the *Hmox3* gene is likely a pseudogene derived from HO-2 transcripts.[4]

Under homeostasis, heme essentially exists as a prosthetic group in numerous hemoproteins, such as hemoglobin, myoglobin, cytochrome *c*, cytochrome p450, nitric oxide synthases, and guanylate cyclase and is nonhazardous for cells.[3] However, under pathological conditions, such covalently bound heme may be released from these hemoproteins causing oxidative damage, because ferrous iron from these molecules may promote the generation of reactive oxygen species (ROS) and lipid peroxidation.[5] Hence, proper heme degradation from such proteins is essential to avoid pro-oxidizing environments in cells, which is mediated by HOs. More importatnt, heme group degradation by HO-1 is the main source of iron in the body and plays a key role in iron recycling/homeostasis.[1]

Considering that HO-1 is a critical mediator in cellular responses to injury and plays important biological roles as a cytoprotective enzyme, the function of this isoform has been thoroughly studied and characterized.[6] The cytoprotective

Supported by The National Fund for Scientific and Technological Development grants 1158262, 1140011, and 3150559, FONDEF grant D11/1080, International Centre for Genetic Engineering and Biotechnology (ICGEB) grant CRP-ICGEB CRP/CHI14-01, and the Millennium Institute of Immunology and Immunotherapy grant P09/P016-F. J.A.E. is National Commission for Scientific and technology of Chile fellow number 21130507.

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2016.11.011

function of HO-1 also can be mediated by its enzymatic products. HO-1/carbon monoxide promotes antiapoptotic effects through the activation of the p38 mitogen-activated protein kinase signaling transduction pathway.[7]

Furthermore, biliverdin and bilirubin exert cytoprotective antioxidant effects in rat hepatocytes.[8] The physiological importance of HO-1 is emphasized by the fact that most mice that lack the *Hmox1* gene (knockout mice) die before birth, whereas partial *Hmox1* deficiency is related to malformed placental vasculature.[1] In humans, the absence of functional HO-1 is rarely seen, with only few cases of *Hmox1* deficiency reported showing severe damage of endothelial cells, hemolysis, disseminated intravascular coagulation, and a chronic inflammatory state.[9] These observations support essential roles for HO-1 activity and devastating consequences in its absence.

Regulation of HO-1 Expression

Basal expression of HO-1 is weak in mammalian tissues, with the exception of the liver and spleen, where this gene is strongly expressed.[10] Nevertheless, the expression of HO-1 can be strongly up-regulated in response to various stimuli related to cellular stress and pro-oxidant signals, such as free heme, lipopolysaccharide, ROS, nitric oxide, cytokines, growth factors, certain protoporphyrins, heavy metals, UV, hyperthermia, hypoxia, hyperoxia, and infection.[5,11]

The expression of HO-1 is essentially regulated at the transcriptional level by the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor E2-related factor 2 (Nrf2) and the transcription regulator protein BACH1 (Bach1)/transcription factor Maf (MAF) systems.[12] In a steady state, the expression of HO-1 is suppressed by the repressor protein Bach1, which heterodimerizes with MAF to bind DNA, specifically at an antioxidant responsive element in the *Hmox1* gene promoter (Figure 1A).[13] At the same time, Nrf2 forms a complex with Keap1 in the cytoplasm, with Keap1 promoting the proteasomal degradation of Nrf2 (Figure 1A).[13]

Upon cellular stress, such as during proinflammatory stimuli or the presence of heme analogs, the Nrf2 protein dissociates from Keap1 and is phosphorylated by protein kinases, which induce its translocation to the nucleus. During this process, the Bach1-MAF complex dissociates and free MAF now interacts with Nrf2 to form a transcriptionally active complex that promotes the transcription of *Hmox1* (Figure 1B).[13]

In addition, HO-1 expression is also regulated in a post-transcriptional manner by miRNAs.[14] Indeed, studies performed in the context of an intravascular hemolysis model, showed that miR-377 and miR-217 interact with the *Hmox1*−3′-untranslated region, resulting in the reduction of HO-1 protein expression and, hence, reduced enzymatic activity.[15] Furthermore, miR-122, which is a miRNA highly expressed in the human liver, also down-regulates HO-1 expression. Experiments in Huh-7 cells transfected with an antagomir of miR-122 showed that Bach1 expression is promoted by miR-122, leading to HO-1 down-regulation.[16,17] A negative regulation of HO-1 also was described for miR-200c in renal proximal tubular epithelial cells and for miR-378 in lung cancer cells, both having binding sites within the 3′-untranslated region sequence of *Hmox1*.[14]

On the other hand, an up-regulation of HO-1 expression has been described for miR-155, which promotes the degradation of the mRNA of the Bach1 repressor and, thus, indirectly increases the expression of HO-1 in endothelial cells and macrophages by enhancing Nrf2 activity.[18] Similar effects have been described for miR-196 and let-7 in human

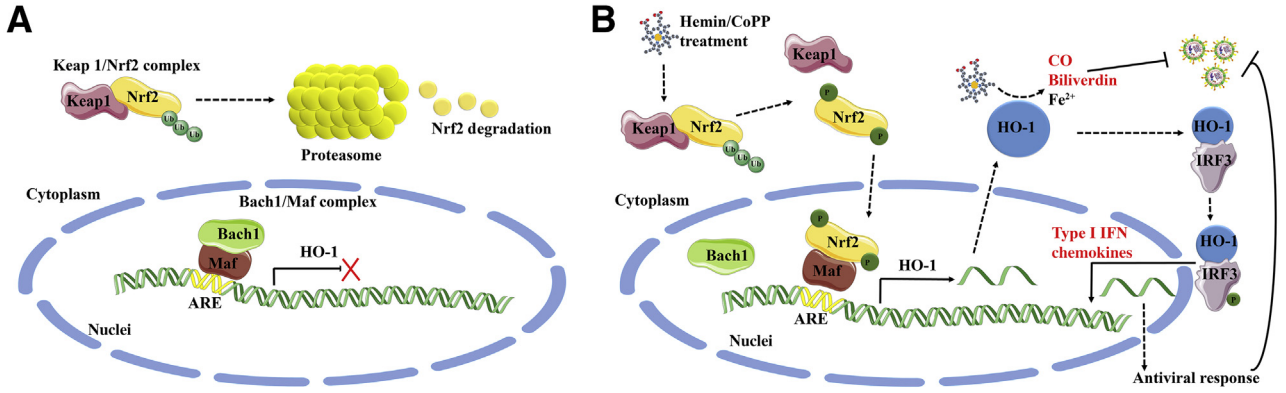

Figure 1 Regulation of HO-1 expression and induction of antiviral responses by heme analogs. HO-1 expression is tightly regulated at the transcriptional level by the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor E2-related factor 2 (Nrf2) and the transcription regulator protein BACH1 (Bach1)/transcription factor Maf (Maf). **A:** Schemes for the suppression of *Hmox1* gene expression mediated by the binding of Nrf2 to Keap1 in the cytoplasm, which induces the proteosomal degradation of Nrf2. In the nucleus, the binding of the complex Bach1/MAF to an antioxidant responsive element (ARE) in the *Hmox1* gene promotor site blocks the transcription of HO-1. **B:** Schemes for the induction of HO-1 mediated by heme analogs that produce the dissociation of Keap1 and Nrf2. Then, Nrf2 is phosphorylated and translocated to the nucleus. The Bach1-MAF complex is dissociated, releasing MAF that interacts with Nrf2, promoting the transcription of *Hmox1*. Expression of HO-1 can promote antiviral activity directly by its products biliverdin and carbon monoxide that affect viral replication by the inhibition of the function of viral proteases and reducing reactive oxygen species production, respectively. Besides, indirectly, HO-1 promotes an antiviral state by the interaction of the HO-1 enzyme with interferon (IFN) regulatory factor-3 (IRF3), promoting the activation of type I IFN responses.

hepatocytes.[19] An important feature of the modulation of HO-1 expression by miRNA is that it can be cell type specific, because some miRNAs are only expressed in particular cell types.[14]

Polymorphisms in the HO-1 promotor also have been described to modulate HO-1 gene expression. Indeed, the $(GT)_n$ repeat length polymorphism has been reported to exert an important functional regulation of HO-1.[20] Depending on the length of the $(GT)_n$ repeat, different outcomes are possible. For instance, short (<25) $(GT)_n$ repeats significantly up-regulate HO-1 transcription and activity in response to inflammatory stimuli and are associated with a protective phenotype, by reducing the risk of ischemic cerebrovascular events,[21,22] rheumatoid arthritis,[23] or chronic pulmonary emphysema.[24] On the contrary, long $(GT)_n$ repeats led to a lower transcriptional activity of HO-1 and have been reported to be associated with higher susceptibility to the development of malignant tumors[25] and coronary artery atherosclerosis.[26]

In addition, the single-nucleotide polymorphism 413 A>T in the *Hmox1* gene promoter has been associated with high transcription levels of HO-1.[20] Considering the essential role of HO-1 in the metabolism of heme and homeostasis of iron, as well as its cytoprotective effects, it is important to identify the processes involved in the regulation of HO-1 gene expression and its enzymatic activity.

Modulatory Effects of HO-1 on the Immune Response

Elevated levels of HO-1 expression and activity result in anti-inflammatory, antioxidant, anti-apoptotic, and antiproliferative properties that exert tissue-protective effects under proinflammatory conditions.[5,27] Furthermore, modulatory effects on the immune system have also been described for this stress-induced enzyme, suggesting that HO-1 can be considered an interesting target for clinical research in infectious and autoimmune diseases, as well as oncology, transplantation, and obstetrics (fetal tolerance).[27]

A role for HO-1 in immune regulation has been sustained by the observation that deficiencies in HO-1 expression can induce chronic inflammatory statuses in both mice and humans. Subjects that experience HO-1 deficiencies present common features, such as increased counts in blood leukocytes, high amounts of type 1 helper T cell cytokines [eg, IL-1β, IL-6, interferon (IFN)-γ, and tumor necrosis factor-α], high serum levels of IgM, increased amounts of polymorphonuclear (PMN) cells and monocytes/macrophages in the spleen, as well as in nonlymphoid tissues and widespread oxidative tissue injury elicited by these cells.[6,13,28]

In addition, HO-1 knockout mice are highly vulnerable to experimental sepsis induced by classic proinflammatory mediators, such as endotoxins.[29] Interestingly, the association between sepsis shock and reduced HO-1 expression is mediated by the pattern recognition receptor Toll-like receptor 4.[30] Noteworthy, under cellular stress, the free heme produced is recognized by Toll-like receptor 4, promoting the production of low levels of proinflammatory cytokines by innate immune cells, such as macrophages and dendritic cells (DCs).[28] More important, macrophages exposed to free heme display a reduced sensitivity to Toll-like receptor 4 agonists, such as lipopolysaccharide.[28,30,31] Similar immunomodulatory effects have been observed in DCs after inducing HO-1, particularly related with the production of carbon monoxide, which has been shown to cause a conformational change in the Toll-like receptor 4/MD2 complex affecting its function.[30]

Moreover, HO-1 induction has been reported to enhance the polarization of macrophages to an M2 phenotype, while suppressing the M1 phenotype.[29] Other innate immune cells are also modulated by the induction of HO-1. For instance, it has been reported that HO-1 decreases PMN cell chemotaxis and activation, reducing oxidative tissue damage.[32] In the same study, mice treated with hemin, a pharmacological HO-1 inducer, showed reduced PMN influx, whereas the addition of tin-protophorphyrin-IX, a competitive inhibitor of HO-1 enzyme, augmented PMN migration.[32] Furthermore, pharmacological induction of HO-1 also inhibits the activity of the p47 phox, p67 phox, and gp91 phox subunits of NADPH oxidase, thus resulting in reduced ROS production by activated PMN cells and macrophages.[28] These events further contribute to limit oxidative tissue injury. In addition, the immune function of HO-1 in myeloid cells has been studied with the conditional ablation of *Homx1*, which evidenced that this enzyme is required for IFN-β production and the activation of the transcription factor IFN regulatory factor-3.[33] In addition, this study showed that IFN regulatory factor-3 activation and subsequent IFN-β production is severely impaired in HO-1−deficient macrophages infected with Sendai virus and that this enzyme is fundamental for triggering antiviral responses against Sendai virus infection.[33,34]

Immature mouse DCs express small amounts of HO-1; however, its expression is induced on activation.[35] Interestingly, both rat and human immature DCs express HO-1 constitutively and down-regulate its expression after their activation.[36] Regardless of this observation, pharmacological induction of HO-1 expression and activity impairs mouse, rat, and human DC activation and their immunogenicity.[13,36−38] Similar effects can be obtained by pharmacologically delivering carbon monoxide by carbon monoxide−releasing molecules[30] or biliverdin/bilirubin,[39] suggesting that the immunosuppressive effects associated with HO-1 pharmacological induction are mediated by the HO-1 enzymatic product carbon monoxide and/or biliverdin/bilirubin. However, it is important to consider that the pharmacological inducers of HO-1 used in these studies may promote immunosuppressive effects in DCs, independently of HO-1 activity.[40]

Besides the important immunomodulatory properties of HO-1 over the innate immune response, several lines of evidence also suggest that HO-1 exerts immunomodulatory effects on adaptive immunity, by impairing T-cell activation, T-cell proliferation, and/or effector functions.[28] The immunomodulatory effects of HO-1 have been described in several studies, such as allograft survival models.[27,41,42] Spleenocytes from mice that overexpress HO-1 have shown that both $CD4^+$ and $CD8^+$ T cells display lower proliferation indexes after allogeneic stimulation than control animals and display increased allograft survival, which is mediated by the inhibition of infiltrating inflammatory cells and $CD4^+$ T cells.[41] Indeed, the pharmacological induction of HO-1 in wild-type mice inhibits $CD8^+$ T-cell activation and type 1 helper T cell–mediated cytokine production, reducing the lymphoproliferative alloresponse and differentiation of citolytic T lymphocytes.[43] Similar observations have been reported in experiments using human peripheral blood mononuclear cells, with $CD4^+$ T cells expressing HO-1 and Jurkat T cells overexpressing HO-1, which displayed lower proliferative responses.[44] The antiproliferative mechanisms mediated by HO-1 in $CD4^+$ T cells have been associated with the inhibition of extracellular signal regulated kinase activation, particularly by carbon monoxide, resulting in the suppression of proliferation and IL-2 secretion by $CD4^+$ T cells.[45] Modulation of T-cell activation by HO-1 has also been observed by its enzymatic products, namely carbon monoxide and biliverdin/bilirubin.[45]

Antiviral Activity Mediated by HO-1 Induction

Several pieces of evidence propose beneficial effects for HO-1 and its products over the modulation of inflammatory responses. However, much less is known on the role of HO-1 during infectious diseases.[46] Considering that HO-1 elicits anti-inflammatory effects, it is conceivable that inducing the activity of this enzyme could interfere with the ability of the immune system to combat and eliminate infectious agents. Nevertheless, HO-1 has been demonstrated to produce the opposite effect and at present has been reported to display significant antiviral properties that are summarized in Table 1 and Figure 1B.[46,58]

HO-1 pharmacological induction with cobalt-protoporphyrin-IX (CoPP) has been reported to elicit a direct antiviral effect against hepatitis B virus (HBV) infection by suppressing viral replication and decreasing liver damage in a mouse model of acute HBV infection.[47] These results are supported by experiments performed in stably transfected hepatitis B virus core hepatoma cells and in a transgenic mouse model of chronic HBV infection.[47] The mechanism behind the antiviral effect of HO-1 in HBV infection has been suggested to be mediated by

Table 1 Immunomodulatory and Antiviral Properties of HO-1 and Its Enzymatic Products

Virus	Component	Direct antiviral mechanism	Indirect antiviral effect: cytoprotective response and immune modulation	Reference
HBV	CoPP/heme HO-1 enzyme	Reduction of the stability of the HBV core protein. Reduction of the nuclear localization of covalently closed HBV circular DNA	Decreased in liver damage. Impaired viral persistence.	47
HCV	Biliverdin Iron HO-1 enzyme	Inhibition of non-structural 3/4A protease activity. Inhibition of the activity of the non-structural 5B RNA-dependent RNA polymerase of HCV.	Decreased oxidative liver damage. Activation of an IFN antiviral response by biliverdin.	48–51
HIV	Biliverdin/bilirubin Heme/hemin HO-1 enzyme	Inhibition of HIV protease activity	NA	52,53
Influenza	HO-1 enzyme induction	Not reported	Reduction of infiltration of inflammatory cells in the lungs. Decreased apoptosis of epithelial cells. Activation of type 1 IFN response.	54–56
EV71	Carbon monoxide HO-1 enzyme	Not reported	Decreased NADPH oxidase/ROS activity.	57
RSV	CoPP/heme HO-1 enzyme	Not reported	Decreased lung inflammation.	Unpublished results
EBOV	CoPP/heme HO-1 enzyme	Not reported	NA	58
DENV	Biliverdin	Inhibition of the DENV non-structural 2B/non-structural 3 protease	Activation of host antiviral IFN response.	59,60

CoPP, cobalt-protoporphyrin-IX; DENV, dengue virus; EBOV, Ebola virus; EV71, enterovirus 71; HBV, hepatitis B virus; HCV, hepatitis C virus; HO-1, heme oxygenase-1; IFN, interferon; NA, not available; ROS, reactive oxygen species; RSV, respiratory syncytial virus.

reducing the stability of the HBV core protein and, thus, blockade of nuclear refilling of covalently closed HBV circular DNA.[47] Experiments using a siRNA directed against HO-1 showed that this effect depended on the expression level of HO-1.[47]

Furthermore, overexpression or induction of HO-1 also results in reduced hepatitis C virus (HCV) replication and decreased oxidative liver damage.[48] Experiments performed in hepatoma cell lines incubated in the presence of the enzymatic products of HO-1 revealed that biliverdin can directly interfere with HCV replication by triggering the expression of antiviral interferons, such as interferon α2 and α17.[49] On the other hand, bilirubin derived from HO-1 has been described to interfere with activity of the nonstructural 3/4A protease of HCV.[50] Furthermore, HO-1−derived iron has been reported to inhibit the activity of the non-structural 5B RNA-dependent RNA polymerase of HCV.[51]

In addition, HO-1 activity has also been reported to suppress the replication of HIV. Treatment with hemin, which increases HO-1 expression, has been shown to protect monocytes against HIV infection with numerous clinical and antiretroviral drug-resistant virus isolates.[52] Protection was evidenced by achieving almost undetectable levels of viral RNA and cells free of HIV-1 p24 protein in a dose-dependent manner.[52] Furthermore, hemin treatment of humanized, nonobese diabetic severe combined immunodeficiency mice significantly inhibited HIV replication.[52] Treatment with tin-protoporphyrin-IX, a competitive inhibitor of HO-1 activity, attenuated the effect mentioned above, suggesting a fundamental role for HO-1 in the modulation of HIV infection.[52] Finally, bilirubin derived from HO-1 has been reported to inhibit the protease activity of HIV, which negatively affects virus replication.[53]

The beneficial properties of HO-1 expression have also been reported for viruses that produce lung disease, such as influenza virus infection. Mice that overexpress HO-1 in the lungs display less inflammatory cell infiltration into the lungs and decreased apoptosis of respiratory epithelial cells, as compared to control mice, suggesting that HO-1 expression prevents an exacerbated immune response in this tissue and subsequent damage.[54] These observations are supported by the worsened disease observed in HO-1$^{-/-}$ mice infected with influenza, as compared to control mice.[55] In addition, rupestonic acid derivatives have been reported to have an anti-influenza virus activity, which recently were attributed to the induction of HO-1 and the subsequent activation of type I IFN expression, as well as the induction of IFN-stimulated genes, possibly in a HO-1 enzymatic activity−independent manner.[56]

Unpublished data from our group suggest similar results for lung disease caused by the human respiratory syncytial virus. We observe that CoPP treatment reduces viral replication and decreases lung inflammation in BALB/c mice after human respiratory syncytial virus challenge (data not shown). These results are supported by a transgenic model of overexpression of human HO-1 in myeloid cells, which displays a protected phenotype against human respiratory syncytial virus disease.

The antiviral properties of HO-1 have also been assessed for Ebola virus (EBOV). Cell culture studies showed that HO-1 suppresses EBOV infection.[58] The up-regulation of HO-1, mediated by CoPP, decreased EBOV replication in Vero cells and Huh 7,0 VP30 cells. CoPP administration, both before and after infection, considerably reduced EBOV replication in both cell lines, demonstrating that HO-1 presents antiviral properties against EBOV that are mediated by intracellular mechanisms and not by a virucidal effect of CoPP over the virus.[58] Although CoPP treatment showed impaired transcription/translation of EBOV gene products, the precise mechanism mediating the blockade of virus replication within these cells was not reported.

More important, induction of HO-1 activity has been shown to have beneficial effects over cells infected with viruses that trigger the production of reactive oxygen species and activation of NADPH oxidase, such as enterovirus 71.[57] Herein, the overexpression of HO-1 decreased NADPH oxidase/ROS production that is induced by enterovirus 71 and hence reduced the replication of this pathogen. This effect was abolished if cells were pretreated with zinc−protoporphyrin IX, a HO-1 activity inhibitor. Furthermore, in this study, carbon monoxide was shown to reduce ROS production and enterovirus 71 replication, which was recovered if cells were treated with hemoglobin acting as a carbon monoxide scavenger or after the treatment with KT5823, a cGMP-dependent protein kinase inhibitor.[57] These findings suggest that HO-1 may induce several cytoprotective and antiviral effects on cells infected with enterovirus 71, which could lead to reduced tissue damage and eventually disease resolution.

Moreover, HO-1 also exerts antiviral activity against dengue virus (DENV). Experiments performed in Huh-7 cells infected with DENV and treated with CoPP or hemin showed a reduction of DENV protein synthesis and RNA replication. These effects were attenuated after the treatment with tin-protophorphyrin-IX, which promoted DENV replication.[59] More important, the anti-DENV effect exerted by HO-1 was mediated by biliverdin, which trigged the host antiviral IFN response by noncompetitively inhibiting the DENV non-structural 2B/non-structural 3 protease.[59] In addition, it was reported that HO-1 induction in ICR suckling mice infected with DENV delayed the mortality induced by DENV infection.[60] Furthermore, similar antiviral effects were obtained when treating animals with andrographolide, which inhibited DENV both *in vitro* and *in vivo* by inducing HO-1 expression.[59]

Concluding Remarks

HO-1 is a cytoprotective host enzyme with several anti-inflammatory and antioxidant properties, which additionally plays important roles in the modulation of innate and

adaptive immune responses. More important, HO-1 and its products carbon monoxide and biliverdin/bilirubin have been reported to provide beneficial effects for the host during diseases, with considerable contributions on inflammatory processes. However, much less is known about the effects of HO-1 and its products during infection with pathogens, specifically viral infections. Noteworthy, recent reports have described numerous antiviral effects for HO-1 against a broad spectrum of viruses (namely, against enterovirus 71, HCV, HBV, HIV, influenza virus, DENV and EBOV, among others). In many cases, the mechanisms of action of HO-1 products over these viruses have been identified, evidencing direct effects on virus components or cellular processes that interfere with virus replication. An important consideration is that HO-1 expression and activity can be induced by numerous drugs that are already approved by the US Food and Drug Administration for human use. Thus, this enzyme is an attractive therapeutic target for combating a broad spectrum of viral infections, including chronic viral infection caused by pathogens that are considered major health burdens worldwide. Alternatively, HO-1 induction may be elicited to prevent annual outbreaks by respiratory viruses, such as influenza and respiratory syncytial virus infection. We expect to see in the upcoming years numerous new studies that evaluate the effects of HO-1 on virus replication and disease.

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Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A single, low dose of a cGMP recombinant BCG vaccine elicits protective T cell immunity against the human respiratory syncytial virus infection and prevents lung pathology in mice

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ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form 9 December 2016
Accepted 21 December 2016
Available online xxxx

Keywords:
Human respiratory syncytial virus
Pulmonary inflammation
Viral infection
T cells
Th1
Th17
Bacillus Calmette et Guerin
Recombinant vaccine

ABSTRACT

Human respiratory syncytial virus (hRSV) is a major health burden worldwide, causing the majority of hospitalizations in children under two years old due to bronchiolitis and pneumonia. HRSV causes year-to-year outbreaks of disease, which also affects the elderly and immunocompromised adults. Furthermore, both hRSV morbidity and epidemics are explained by a consistently high rate of re-infections that take place throughout the patient life. Although significant efforts have been invested worldwide, currently there are no licensed vaccines to prevent hRSV infection. Here, we describe that a recombinant Bacillus Calmette-Guerin (BCG) vaccine expressing the nucleoprotein (N) of hRSV formulated under current good manufacture practices (cGMP rBCG-N-hRSV) confers protective immunity to the virus in mice. Our results show that a single dose of the GMP rBCG-N-hRSV vaccine retains its capacity to protect mice against a challenge with a disease-causing infection of 1×10^7 plaque-forming units (PFUs) of the hRSV A2 clinical strain 13018-8. Compared to unimmunized infected controls, vaccinated mice displayed reduced weight loss and less infiltration of neutrophils within the airways, as well as reduced viral loads in bronchoalveolar lavages, parameters that are characteristic of hRSV infection in mice. Also, *ex vivo* re-stimulation of splenic T cells at 28 days post-immunization activated a repertoire of T cells secreting IFN-γ and IL-17, which further suggest that the rBCG-N-hRSV vaccine induced a mixed, $CD8^+$ and $CD4^+$ T cell response capable of both restraining viral spread and preventing damage of the lungs. All these features support the notion that rBCG-N-hRSV is a promising candidate vaccine to be used in humans to prevent the disease caused by hRSV in the susceptible population.

1. Introduction

For more than fifty years, the human respiratory syncytial virus (hRSV) has been recognized as the single most important cause of infant hospitalizations due to acute lower respiratory tract infections, and also an important pathogen in the elderly and the immunocompromised individuals [1–5]. As such, hRSV has a worldwide impact in the public health and the economy of both developing and developed countries [6–8]. Furthermore, despite significant resources have been invested in researching hRSV biology, there is still no licensed vaccine for the prophylaxis of hRSV disease in children and other susceptible population [9].

HRSV circulates in the community establishing seasonal outbreaks of disease, which are based on repetitive events of infections that can occur even during the same outbreak [1,2]. Reinfections are thought to derive from the poor induction of adaptive immunological memory after the resolution of naturally acquired infections. Indeed, the identification of a scarce population of circulating B cells capable of secreting neutralizing

Abbreviations: BAL, bronchoalveolar lavage; BCG, Bacillus Calmette et Guerin; CFUs, colony-forming units; cGMP, current Good Manufacturing Practices; hRSV, human respiratory syncytial virus; FI-hRSV, formalin-inactivated hRSV; HI-hRSV, heat-inactivated hRSV; FACS, fluorescence activated cell sorting.

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http://dx.doi.org/10.1016/j.vaccine.2016.12.048
0264-410X/

antibodies against the virus [10], as well as the demonstration that adults remain susceptible to few infectious hRSV particles [11], support the notion of a limited acquired immunity to hRSV infections throughout lifetime. Moreover, this is further supported by independent studies demonstrating the deleterious effects of hRSV infection over the capacity of DCs to activate and expand naïve T cells [12,13]. Furthermore, we have recently shown that compared to memory cells, naïve $CD4^+$ T cells have an increased susceptibility to the inhibition delivered by the hRSV nucleoprotein [14], suggesting that (1) this mechanism may be instrumental for impairing acquired immunity to the virus, and (2) in order to promote strong antiviral immunity, a good vaccine candidate must elicit specific memory T cell responses able to overcome the restrictions imposed by hRSV over naïve T cell activation.

Memory $CD4^+$ T cells are instrumental in the host defense against invading viruses [15]. Activated memory cells differentiate into helper T cells (T_H) that among others, exert direct perforin-mediated cytotoxicity and promote the activation of other key immune cells required for virus clearance, including DCs, macrophages, $CD8^+$ CTLs, and B cells [15]. In the context of hRSV natural infections, it is known that human lungs display aberrant T cell responses described as two possible scenarios: a poor infiltration of $CD8^+$ CTLs [16], or conversely, by a more marked infiltration of $CD4^+$ T cells [17]. In mice, hRSV infection elicits a T_H2 immune response, which is poor antiviral and highly pro-inflammatory. Cytokines secreted by T_H2 cells mediate the recruitment of a variety of inflammatory cells, including neutrophils, monocytes, $CD11b^+$-inflammatory DCs, plasmacytoid DCs and eosinophils into the bronchoalveolar spaces of humans and mice (known as bronchopneumonia), and into the tissue interstitium generating thickening of the alveolar walls (known as interstitial pneumonia) [18–21].

The need of a balanced T-cell response to hRSV has been further stressed by the study of the formalin-inactivated hRSV (FI-hRSV)-vaccine-enhanced disease (VED) first reported in children [22,23]. The FI-hRSV-VED was successfully replicated in the mouse model of infection, yielding compelling evidence of the role of suboptimal $CD4^+$ T-cell responses in hRSV pathogenesis [24]. FI-hRSV immunization elicits a T_H2 response that mediates lung damage through the massive recruitment of eosinophils and neutrophils into infected lungs [25]. Importantly, several reports indicate that hRSV-specific $CD8^+$ T cells successfully prevent FI-hRSV-VED, supporting the notion that $CD8^+$ T cells are both important regulators of hRSV pulmonary pathology, and attractive elements for rationale vaccine design [26,27]. Along these lines, we recently demonstrated that a recombinant BCG expressing the hRSV nucleoprotein (N) (herein rBCG-N-hRSV) elicits IFN-γ-secreting $CD4^+$ (T_H1) T cells and $CD8^+$ CTLs that promoted viral clearance and prevented lung damage in vaccinated mice [12,28]. Hence, our results demonstrated that induction of memory T cell responses by rBCG-N-hRSV immunization was an efficient strategy to prevent hRSV lower respiratory tract infections [12,28]. Nevertheless, that initial approximation used extremely high vaccination doses (10^8 CFUs of rBCG-N-hRSV), raising concerns regarding its immunogenicity in infants, which are immunized with BCG at significant lower doses ($1–4 \times 10^5$ CFUs). Here, in order to move forward into the characterization of this promising vaccine for clinical use, we studied the safety and immunogenicity of a low dose of rBCG-N-hRSV formulated under current Good Manufacture Practices (cGMP) standards. Using the mouse model of infection, we observed that a single, human dose of $1–4 \times 10^5$ CFUs of cGMP rBCG-N-hRSV elicited an acquired immunity able to prevent lung damage, while promoting a significant clearance of infectious viral particles from the airways. We also observed that the immunization with rBCG-N-hRSV prevented the CNS alterations caused by hRSV described previously [29]. Moreover, our results suggest that the transfer of as few as 1.5×10^6 hRSV-specific T cells, are sufficient to restrain viral dissemination in the lungs and to protect recipient mice from hRSV respiratory disease. Therefore, the rBCG-N-hRSV vaccine prototype appears as a safe and immunogenic tool for the prophylaxis of hRSV in susceptible individuals.

2. Material and methods

2.1. Mice

BALB/cJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained under SPF conditions at the Pontificia Universidad Católica de Chile animal facility. Mice were used according to the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2011).

2.2. Virus preparation & titration

The hRSV serogroup A2 strain 13018-8 (obtained from the Public Health Institute of Chile) was propagated and titrated over HEp-2 cell monolayers (ATCC-CCL-23) as previously described [14]. Heat-inactivation of hRSV (56 °C/30 min) was performed in a thermo block (Thomas Scientific). Supernatants of uninfected HEp-2 cells were prepared in parallel to virus stocks and used in all experiments as mock controls.

2.3. Current good manufacturing practices (cGMPs) BCG strains

The rBCG-N-hRSV (Danish 1331 strain) was produced following cGMP standards at the AERAS Global TB Vaccine Foundation (Rockville, MD USA). As negative control for hRSV immunizations, a non-recombinant, cGMP quality, wild type Danish 1331 BCG strain (herein BCG-WT) was used (Statens Serum Institute, Copenhagen, Denmark).

2.4. Mice immunization and challenge

Six to eight weeks old BALB/cJ mice were immunized by a sub dermal (s.d.) injection of 3×10^5 colony-forming units (CFUs) of wild type BCG (BCG-WT) or rBCG-N-hRSV [28] (no vaccine boost was performed). Twenty-one days after immunization mice were infected intranasally with 1×10^7 PFUs of hRSV A2 clinical strain 13018-8 [28]. To evaluate possible side effects of rBCG-N-hRSV immunization we recorded clinical parameters daily (mouse body weights, injection site appearance) (Fig. 1/Table 1). To evaluate long-term protection, a group of mice was vaccinated as explained above and challenged at day 50th post-immunization with the same strain of hRSV. Following infection, mouse body weights were recorded daily until the end of the experiment at day 7th post-infection.

2.5. Ex vivo T cell stimulation

Twenty-eight days after immunization, spleens from rBCG-N-hRSV and BCG-WT-immunized mice were collected, disaggregated in sterile RPMI 1640 media using a 70-μm cell-strainer, treated for Red Blood Cells (RBC) lysis, and resuspended in fresh, supplemented RPMI 1640 media (10 mM HEPES, 10% FBS, 50 μM 2-mercaptoethanol). Immediately after collection, T cells were incubated for 72 h in the presence of recombinant hRSV nucleoprotein (N; 10 μg/mL), heat-inactivated hRSV (HI-hRSV, at a final multiplicity-of-infection (MOI) of 5 plaque-forming units (PFUs)/-cell), or irrelevant antigens (human metapneumovirus, hMPV, at MOI 5 and mock). Heat inactivation of virions produces the physical disruption of the viral particle and the viral protein denatura-

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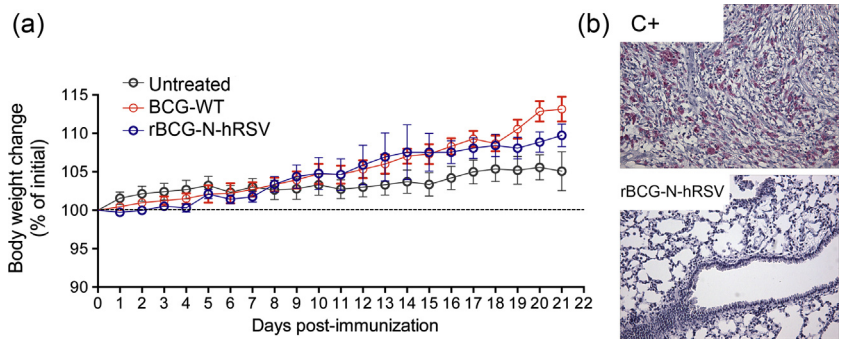

Fig. 1. Immunization with a single, low dose of rBCG-N-hRSV is safe in mice, causing neither significant body weight loss nor clinical signs of disease. (a) In order to evaluate the safety of the rBCG-N-hRSV vaccine, 6–8 weeks-old BALB/cJ mice were immunized and examined daily for 21 days. Body weights were recorded daily after a single sub dermal injection of 3×10^5 CFUs of either rBCG-N-hRSV or BCG-WT. As control, a group of untreated mice was added. (b) Ziehl-Neelsen (acid-fast) stain of mouse lung sections collected at day 28th post-immunization with rBCG-N-hRSV cGMP. As positive control (C^+), lungs from an animal instilled intranasally with BCG-WT (positive bacilli is shown as pink-violet) and sacrificed 14 days post-infection were used (10× magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Clinical evaluation of mice immunized with rBCG-N-hRSV and controls.

Parameter	rBCG-N-hRSV vaccinees	BCG-WT vaccinees	Untreated
Mice mortality (%)	0	0	0
Granuloma formation (%) (No. mice/total mice)	100 (15/15)	36 (4/11)	0
Mucopurulent discharge from injection site (%)	0	0	0
Localized erythema (%)	0	0	0

tion, including the abundantly expressed N protein in viral particles [14]. After 72 h, the production of IFN-γ and IL-17 was evaluated by ELISA according to manufacturer instructions.

2.6. T cell adoptive transfers

Mice were immunized with 3×10^5 CFUs of either BCG-WT or rBCG-N-hRSV and 10 days later boosted with an equal dose of the relevant BCG vaccine. Twenty-one days post-immunization, T cells were expanded *in vitro*, and transferred to naïve mice as described previously [28]. A total of $1–1.5 \times 10^6$ purified pan T cells ($CD4^+/CD8^+$) were injected intravenously into syngeneic recipient mice. Twenty-four hours later mice were challenged with 1×10^7 PFUs of hRSV.

2.7. FACS and lung histology

Seven dpi mice were terminally anesthetized for collection of bronchoalveolar lavages (BALs, i.n. instillation of 700 µl of 2% FBS-10 mM HEPES-PBS) and lungs. After BAL collection, the right lung was processed as previously described [28] (for a complete description please refer to supplementary Material and Methods). Briefly, tissue samples were homogenized after 30 min incubation at 37 °C with collagenase IV (1 mg/mL) in sterile RPMI media. Lung homogenates were passed through a 70-µm cell-strainer, treated for RBC lysis, and resuspended in 2% FBS-PBS. Then, 3×10^6 lung cells were stained for the determination of distinct leukocyte populations as detailed in supplementary Material and Methods and Supplementary Fig. 1. For intracellular staining of $CD8^+$ T cells from mediastinal lymph nodes, cell suspensions were incubated with Brefeldin A (5 mg/ml) for 5 h. After incubation cells were recovered, washed and stained for surface T-cell markers (anti-CD45 BV510; anti-TCR FITC; anti-CD4 APC-H7, and anti-CD8alpha PECy7; all from BD Biosciences). Then, cells were fixed with 2% PFA in PBS for 10 min at RT, permeabilized using PBS supplemented with 0.1% Saponin 0.1% BSA, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 40 mM HEPES, and stained for 30 min with an anti-mouse IFN-γ BUV737 antibody (BD Biosciences). After washes with the same permeabilization buffer, cells were resuspended in PBS and immediately acquired using a BD LSRFortessa X-20 flow cytometer.

2.8. Quantitation of plaque-forming units (PFUs)

HEp-2 cell monolayers at 70% confluence were inoculated with BALs collected from vaccinated and control mice and incubated for 1 h at 37 °C. Then, the supernatants were replaced by DMEM 1% FBS and after 48 h the plaque forming units (PFUs) were quantified by immunocytochemistry, using a primary anti-N and anti-F hRSV-specific antibodies and a secondary anti-mouse IgG HRP-linked antibody (as described elsewhere [14]).

2.9. Delayed type hypersensitivity (DTH) reaction assays

Eighty days after vaccination, mice were injected in their hind footpads with 20 µl of either PBS pH 7.4 (vehicle, injected in the left footpad) or 20 µg (in 20 µl) of *Mycobacterium tuberculosis* purified protein derivative (PPD Batch RT 50 from the Statens Serum Institute, Denmark) (injected in the right footpad). The width of each hind foot was measured with a high-resolution (0.01 mm), digital caliper (iGaging-IP54). Measurements were recorded at the level of the posterior walking pad at 24 and 48 h post-injection, and compared with the measures obtained prior to vehicle/PPD injections (see Supplementary Fig. 2).

3. Results

3.1. A single, low dose of rBCG-N-hRSV prevents hRSV interstitial pneumonia with no observable adverse effects

To evaluate the protective capacity of a single low dose of rBCG-N-hRSV prepared under cGMP, mice of 6–8 weeks of age were immunized subdermally with 3×10^5 colony-forming units (CFUs). Mice immunized with an equivalent single dose of the conventional BCG (BCG-WT) were used as controls. Also, a group of

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animals were left unimmunized as positive controls for hRSV pathology.

As a primary safety measurement, we recorded the body weights of BCGs-vaccinated and control animals. We found that the rBCG-N-hRSV vaccine prototype was as safe as the conventional BCG vaccine, as evidenced by no weight loss (Fig. 1a) and the absence of other clinical manifestations of skin or systemic disease (Table 1). Indeed, mouse clinical scores remained 0 throughout the 21 days observation period (data not shown). Interestingly, compared with BCG-WT vaccinees, rBCG-N-hRSV immunized mice showed an increased frequency of granuloma formation, suggesting that the rBCG-N-hRSV vaccine is more immunogenic than BCG-WT (Table 1). Importantly, as compared to BCG-WT, the rBCG-N-hRSV did not display increased growth kinetics (data not shown), suggesting that the differences found in granuloma formation may be related to an intrinsic increased immunogenicity of our rBCG-N-hRSV cGMP formulation. Further, the increased frequency of granuloma did not correlate with the dissemination of rBCG to other tissues, such as lungs, suggesting that the former feature is not related to an increased bacilli virulence (Fig. 1b).

Twenty-one days post-immunization, rBCG-N-hRSV and control animals were infected with the hRSV A2 clinical strain 13018-8. Body weight loss was recorded daily until the 7th day post-infection (dpi) as a measurement of disease development in all experimental animals. As expected, mice infected with hRSV, either vaccinated with BCG-WT or unimmunized, showed a significant weight loss (approximately 10%) that was sustained until the end of experiments (Fig. 2a). Interestingly, rBCG-N-hRSV immunized animals showed initially a brief loss of body weight that was rapidly recovered by day 3 post-infection (Fig. 2a), suggesting that vaccination with rBCG-N-hRSV was efficient in controlling disease development in hRSV-challenged mice. Consistent with this observation, FACS analyses of BALs showed a significant infiltration of neutrophils (Ly-6G^{hi} CD11b^{hi} cells) in the airways of hRSV-infected mice, but not in mock controls or mice immunized with rBCG-N-hRSV ($p = 0.0003$) (Fig. 2b). Strikingly, compared to unimmunized mice, BCG-WT vaccinees showed a trend towards reduction in neutrophil counts in BALs (Fig. 2b), having a significant difference when compared to rBCG-N-hRSV immunized animals ($p = 0.0323$). The reduced neutrophil infiltration in BCG-WT- immunized mice suggests a non-specific, yet limited

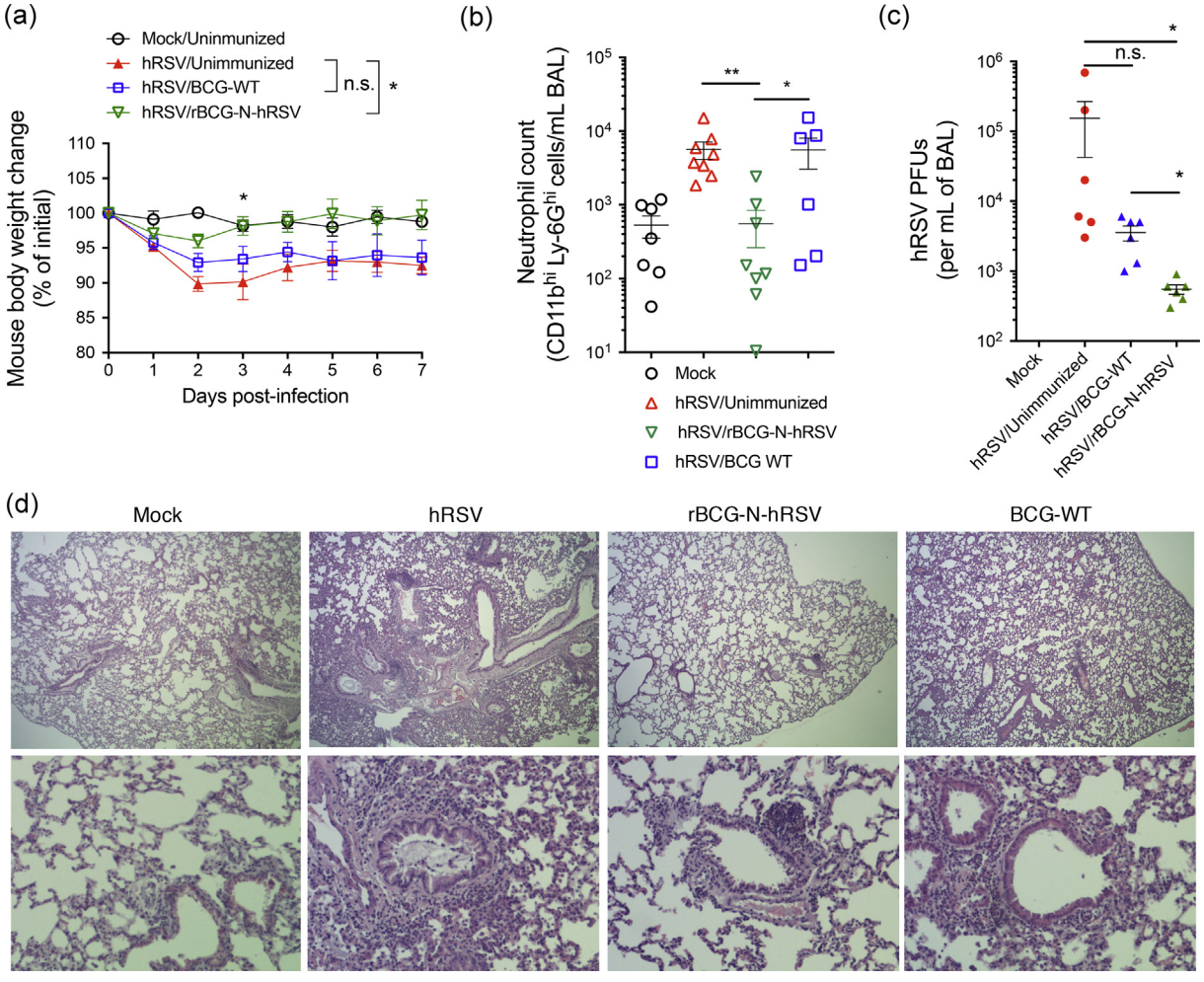

Fig. 2. Immunization with a single, low dose of a rBCG-N-hRSV protects mice from pulmonary pathology and promotes virus clearance from the respiratory tract. (a) Body weight loss of mice unimmunized (red triangles) and immunized with 3×10^5 CFUs of either BCG-WT (blue squares) or rBCG-N-hRSV (green inverted triangles), and then challenged with 1×10^6 PFUs of hRSV A2, strain 13018-8. Mice instilled intranasally with non-infectious supernatants of HEp-2 cells were used as Mock controls (dark grey circles). (b) Flow cytometry analyses for the detection of neutrophils (Ly-6G^{hi} and CD11b^{hi}) in bronchoalveolar lavages (BALs) of mice. (c) The BALs of rBCG-N-hRSV immunized and control animals were titrated over HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed as PFUs/mL). (d) Histopathology analyses of lungs from rBCG-N-hRSV vaccinated and control mice. *Upper panel:* representative High Power Field (HPF) images of 4-μm lung sections stained with Hematoxylin and Eosin (H&E, 4× magnification). *Lower panel:* Magnification images of lung sections shown in upper panels. Note the marked inflammation of the lung interstitium. In (b) and (c), data was analysed by Mann-Whitney test, in which hRSV and BCG-WT groups were compared to rBCG-N-hRSV, separately (b) $p = 0.0003$ (**) and 0.0323 (*), and (c) $p = 0.0022$, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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protective effect, for BCG immunization in the defence against hRSV bronchopneumonia.

Further, to determine the amount of infective viral particles in the respiratory tract of immunized and control animals we quantified hRSV PFUs in the BALs, which cover the entire lower airways recovering viral particles from bronchi, bronchiole and alveoli. We selected this approach, because hRSV does not infect interstitial pulmonary cells, and in our hands BAL titration yielded more consistent results across different experiments (Data not shown). Consistent with the reduction in airway inflammation, we observed a significant reduction in viral loads in BALs from rBCG-N-hRSV immunized mice, as compared to BCG-WT-immunized or hRSV-infected naive mice (Kruskal-Wallis test, $p < 0.0001$; Mann-Whitney test, $p = 0.0022$) (Fig. 2c). As expected, naïve mice and BCG-WT immunized mice displayed similar viral loads (Kruskal-Wallis test, $p < 0.0001$; non-significant difference), suggesting that the observed lesser inflammation in the lungs of BCG-WT immunized mice after hRSV infection would correlate with regulation of lung inflammation, rather than specific control of viral spread in the airways (Fig. 2c).

Finally, to corroborate that the reduced weight loss and less PFUs in the airways of rBCG-N-hRSV immunized mice is due to less lung pathology after infection, we performed lung histopathological analyses in vaccinated and control mice. Consistent with body weight loss, flow cytometry analyses and viral titration data, we observed that rBCG-N-hRSV immunized mice displayed significantly less pulmonary inflammation than their hRSV-infected controls (Fig. 2d). Furthermore, rBCG-N-hRSV immunized animals displayed reduced inflammatory infiltrate in both airways and lung interstitium, suggesting that immunization with rBCG-N-hRSV protects mice against bronchopneumonia and interstitial pneumonia (Fig. 2b and d). Consistent with BAL analyses, BCG-WT-immunized mice developed a lower degree of inflammatory cell infiltration within aerial spaces as compared to hRSV-infected control mice (Fig. 2d). However, BCG-WT-immunized mice developed a significantly more pronounced interstitial inflammation of the lungs as compared with mice immunized with rBCG-N-hRSV (Fig. 2d). These data suggest that the non-specific protective effect observed of BCG-WT immunization is limited. Therefore, BCG-WT immunization regulates the development of pulmonary pathology by decreasing the infiltration of inflammatory cells in bronchoalveolar spaces, but not in the lung interstitium. Thus, our data suggest that immunization with rBCG-N-hRSV elicits a protective immune response without the development of VED.

To evaluate whether the virus-specific protection elicited by rBCG-N-hRSV persists over time, vaccinated mice were challenged after 50 days of the initial immunization. Unimmunized mice (hRSV infected) and mice immunized with BCG-WT displayed a significant body weight loss as compared to uninfected controls. As expected, rBCG-N-hRSV immunized mice presented significant lower weight loss and showed an improved recovery of their body weight by day 7th post-infection (Supplementary Fig. 3a). Moreover, compared with BCG-WT immunized controls, rBCG-N-hRSV immunized mice presented significant lower infiltration of neutrophils and reduced amounts of viral particles within the airways, showing a specific response in the observed long-term protection (Supplementary Fig. 3b and c). Although no significant differences in the $CD8^+IFN\text{-}\gamma^+$ populations were found in both BCG-WT and rBCG-N-hRSV immunized and challenged mice (Supplementary Fig. 3d), our previous analyses showed that in rBCG-N-hRSV vaccinees such repertoire presents a tendency to be a better responder to denatured N protein (Supplementary Fig. 4b). Altogether these findings suggest that rBCG-N-hRSV promotes long-term protection to hRSV infection in a specific manner.

3.2. Immunization with rBCG-N-hRSV induces a TH1/TH17 memory T cell response

Considering that we have previously shown that high doses of the non-cGMP rBCG-N-hRSV vaccine promoted a strong antiviral T_H1-based $CD4^+$ and $CD8^+$ T cell immunity [12,28], we evaluated quantitatively and qualitatively the T-cell response elicited by a low dose of cGMP rBCG-N-hRSV immunization upon infection with hRSV. As previously shown for pulmonary T cell responses against hRSV [30], we observed a significant increase in the T cell recruitment in lungs of hRSV-infected mice at day 7, as compared to day 5 post-infection using flow cytometry (Supplementary Fig. 4a). Therefore, we evaluated by flow cytometry the amount and phenotype of T cell in the lungs of rBCG-N-hRSV immunized and control mice at day 7 post-infection. Flow cytometry analyses showed a significant reduction in the number of activated memory $CD8^+$ T cells ($CD44^+CD69^+$) in the BALs of rBCG-N-hRSV vaccinated animals despite no significant differences in total $CD8^+$ T cells were observed among infected groups (Supplementary Fig. 5a and b). Although a significant increase of total $CD4^+$ T cells was found in all infected groups as compared to mock controls (Supplementary Fig. 5c), no significant differences in the numbers of activated memory $CD4^+$ T cells were observed (Supplementary Fig. 5d). Nevertheless, compared to unimmunized hRSV-infected mice controls, rBCG-N-hRSV and BCG-WT immunized mice showed a significant decrease in the percentage of $CD44^+CD69^+CD4^+$ T cells (around 5%) (Supplementary Fig. 5c and d, right table). Since T cells are

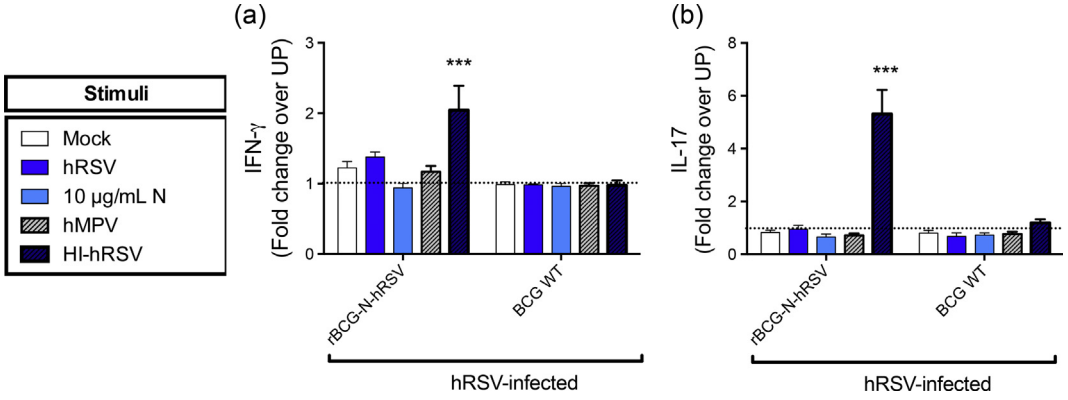

Fig. 3. rBCG-N-hRSV elicits virus-specific type 1 and 17 helper T cell responses. The spleens of mice sacrificed at day seventh post-infection were collected, and the resulting splenocytes were then stimulated with different antigens, including mock, recombinant nucleoprotein, hMPV (as irrelevant antigen), infectious hRSV, and heat-inactivated hRSV (HI-hRSV) (57 °C for 30 min). Seventy-two hours post-stimulation the supernatants of activated T cells were harvested and analysed by ELISA for the measurement of (a) IFN-γ (T_H1 cytokine) and (b) IL-17A (T_H17 cytokine). One-Way ANOVA and multiple comparisons against the untreated control were performed for statistical analyses (*** = $p < 0.001$, n.s. = non-significant).

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recognized as important pathogenic components in experimental hRSV infection, these data suggest that immunization with rBCG-N-hRSV elicits a well-regulated T-cell response that promotes viral clearance and prevents lung pathology. As previously described, an accelerated kinetic of recruitment and egress of hRSV-specific T cells from the lungs of rBCG-N-hRSV vaccinated mice may explain the reduced numbers of activated memory $CD8^+$ T cells detected at 7th day post infection [28]. On the other hand, a reduced activation of memory $CD4^+$ T cells may be one of the mechanisms explaining the reduced pathology observed in BCG-WT-immunized animals. Consistent with this notion, we observed BCG-WT immunized animals displayed a significant decreased secretion of IL-6 and an increased secretion of the immunomodulatory cytokine IL-10 (Supplementary Fig. 6b and d).

Secondly, we sought to both evaluate whether rBCG-N-hRSV immunization elicits T-cell memory and determine the phenotype of these cells upon recall. After 7 days post-infection, the spleens of immunized and controls mice were harvested for *ex vivo* T cell stimulation with hRSV-derived antigens (adding irrelevant antigens as controls). After 72 h of stimulation, cell-culture supernatants were analysed for the detection of pivotal T helper cytokines: IFN-γ (T_H1), IL-4 (T_H2) and IL-17A (T_H17) by ELISA (Fig. 3). Interestingly, immunization with single, low doses of rBCG-N-hRSV elicited a mixed T_H1/T_H17 T cell response, characterized by the secretion of both IFN-γ and IL-17A in response to viral antigens (Fig. 3a and b). Consistent with this notion, we have observed that stimulation with denatured N protein induced the synthesis of IFN-γ by $CD8^+$ T cells from mediastinal lymph node of rBCG-N-hRSV immunized mice, but not from BCG-WT-immunized controls (Supplementary Fig. 4b). As expected, no cytokine secretion was observed in cells stimulated with hMPV, demonstrating the specificity of the recall response (Fig. 3a and b). For all groups, we observed a non-significant secretion of IL-2 and IL-4 in response to viral antigens (Data not shown). Interestingly, no significant T cell activation was observed in cultures stimulated with soluble, recombinant N protein (Fig. 3a and b), which suggests that this protein is inhibiting T cell activation, as previously reported by our group [14]. Because we observed a consistent memory response upon recall with hRSV antigens, our data suggest that the rBCG-N-hRSV delivers N to APCs without interfering with the priming of naïve T cells.

3.3. rBCG-N-hRSV prevents cognitive long term CNS alteration in immunized mice

Central nervous system (CNS) disorders that include seizures, apnea and ataxia have been associated with severe hRSV ARTIs in children (reviewed in [31]). Consistently with these observations, we have previously described that hRSV can induce CNS alterations in mice, characterized by behavioural and learning deficits that last up to 2 months post infection [29]. Importantly, these symptoms are subclinical, meaning that animals only showed cognitive deficiencies with no other significant signs of neurologic damage, such as seizures or paralysis. Therefore, we sought to evaluate whether the rBCG-N-hRSV vaccine prototype could protect mice from CNS ailments caused by hRSV infection. Mice vaccinated with rBCG-N-hRSV were challenged at 21 days post-immunization with hRSV and six months later evaluated for cognitive disorders, using the Marble Burying (MB) behaviour test. As shown in Fig. 4, hRSV-infected mice displayed a behavioural impairment that persisted until six months post-infection, which was significantly extended as we had previously observed [29]. As expected, rBCG-N-hRSV immunized mice showed normal MB behaviour, suggesting that immunization with rBCG-N-hRSV protects against hRSV-induced long-term CNS alterations. An incomplete protection was observed

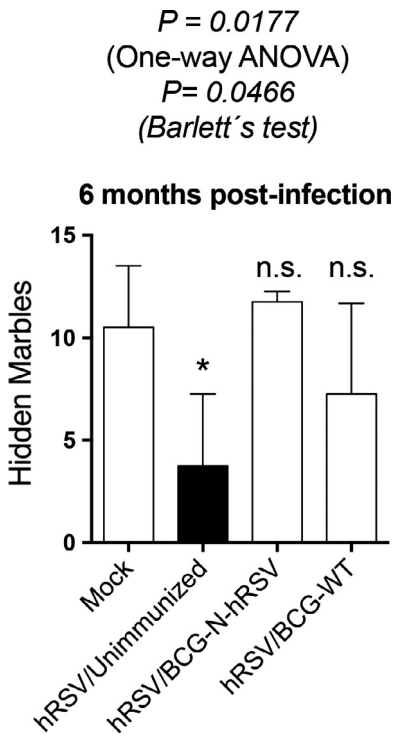

Fig. 4. Immunization with rBCG-N-hRSV prevents long-term behavioural impairment in hRSV-infected mice. Limbic behaviour was evaluated in vaccinated and control mice after 6 months of hRSV challenge. Twelve glass marbles were evenly placed ~4 cm apart in each cage prior to animal placing. After 30 min, the number of bedding-buried marbles was counted. Data in graph shows the quantification of hidden marbles for both vaccinated and unvaccinated mice (including mock controls). One-Way ANOVA and multiple comparisons against the mock control were performed for statistical analyses (* = $P < 0.05$, n.s. = non-significant).

in BCG-WT immunized mice, suggesting that CNS alterations may be linked to the severity of respiratory disease caused by hRSV.

3.4. rBCG-N-hRSV elicits an hRSV-specific T-cell repertoire that protects naïve mice upon adoptive transfer

In order to evaluate the real contribution of T cells in the protection elicited by the rBCG-N-hRSV vaccine, we first evaluated whether the transfer of purified $CD8^+$ and $CD4^+$ T cells from rBCG-N-hRSV immunized and control animals protects naïve mice. Since rBCG-N-hRSV vaccinated mice showed circulating hRSV-specific IgGs only after 7 days post-infection, including total IgGs, IgG1 and IgG2a (with no significant statistical differences to naïve, hRSV-infected mice) (Supplementary Fig. 7a–c), we hypothesized that T cells are the primary mediators of protection in rBCG-N-hRSV vaccinees. Hence, in order to evaluate the protective capacity of the memory T cell repertoire elicited by rBCG-N-hRSV vaccination, we then performed adoptive transfer experiments using a total of $1–1.5 \times 10^6$ *ex* vivo activated pan T cells (i.e. including $CD4^+$ and $CD8^+$), which were transferred to naïve mice 24 h prior to virus challenge. As expected, we observed that the adoptive transfer of as few as $1–1.5 \times 10^6$ pan T cells (i.e. $CD4^+$ and $CD8^+$) to naïve mice prevented body weight loss, lung inflammation and the replication of hRSV in the lungs of recipient mice (Fig. 5a–c). No significant protection was observed in mice receiving the same amount of pan T cells originated from BCG-WT-immunized donors (Fig. 5a–c). Finally, to corroborate these findings we determined lung damage in T cell-recipient and control mice. Consistent with our flow cytometry data, histopathological analyses of lung sections showed that mice that received pan T cells from rBCG-N-hRSV immunized donors developed a minor

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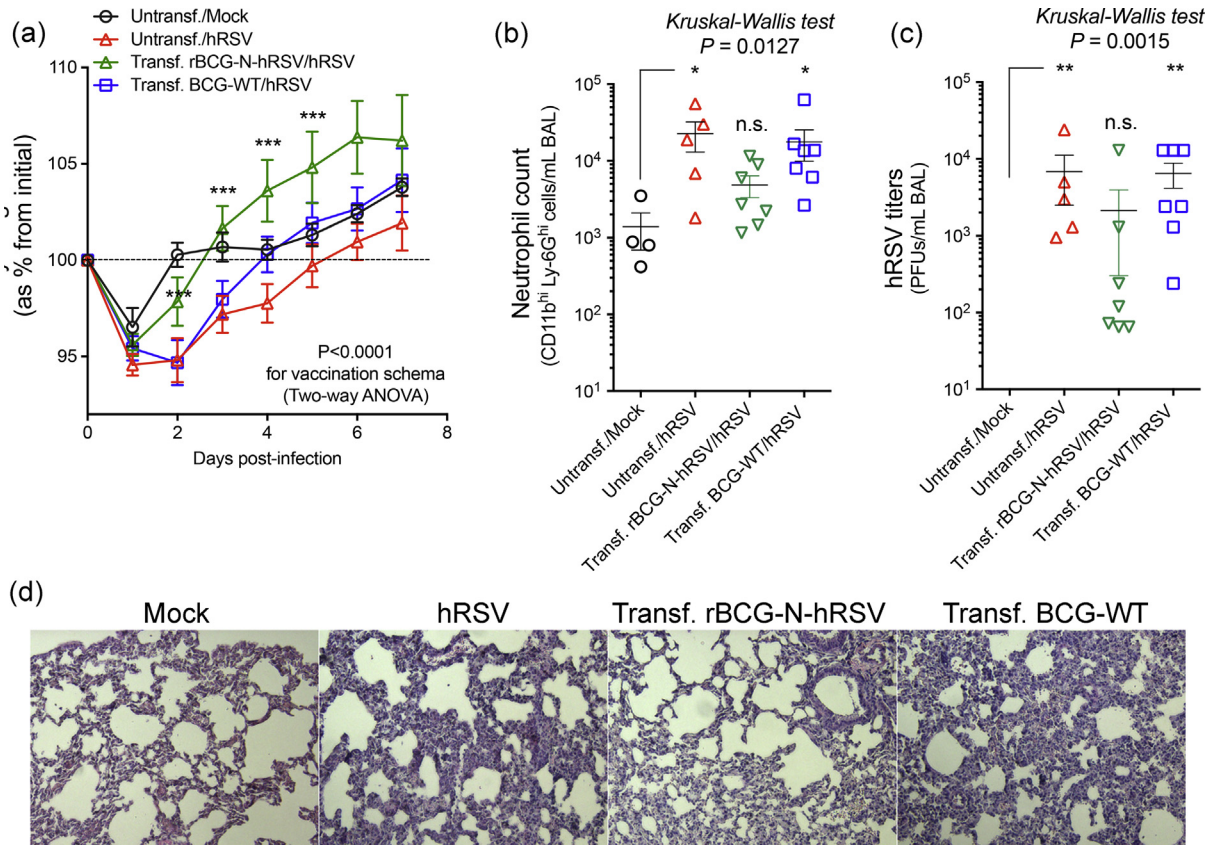

Fig. 5. Immunization with rBCG-N-hRSV generates a repertoire of memory T cells that protect recipient, naïve mice from hRSV pneumonia. Naïve BALB/cJ mice were adoptively transferred with 1–1.5 × 10^6 pan T cells from mice immunized either with rBCG-N-hRSV or BCG-WT, which were subsequently challenged with 1 × 10^6 PFUs of hRSV A2, strain 13018-8 (24 h post-transfer). (a) Body weight loss of untransferred (red triangles) and T-cell recipient, naïve mice. The graph shows data for mice transferred with T cells form BCG-WT (blue squares) or rBCG-N-hRSV (green inverted triangles). Mice instilled intranasally with non-infectious supernatants of HEp-2 cells were used as Mock controls (dark grey circles). (b) Flow cytometry analyses for the detection of neutrophils (Ly-6G^{hi} and CD11b^{hi}) in bronchoalveolar lavages (BALs) of mice showed in a. (c) At 6 days post-infection, the BALs of mice adoptively transferred as explained in (a) were titrated over HEp-2 monolayers for the quantification of infectious viral particles in the airways (expressed PFUs/mL). (d) Histopathology analyses of lungs from pan T cell recipient and control mice. Images of 5-μm lung sections stained with Hematoxylin and Eosin (H&E, 40× magnification). Note the marked inflammation of the lung interstitium in mice transferred with pan T cells derived from BCG-WT-immunized animals as well as naïve, hRSV-infected mice. In (a), data was analysed using multiple *t* test, with a false discovery rate of 1% ($P < 0.00001$). In (b) and (c), data was analysed using Kruskal-Wallis test and multiple comparisons to the mock group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lung pathology as compared to those receiving pan T cells from BCG-WT-immunized donors, which developed an important inflammation of the lung interstitium (Fig. 5d). Furthermore, the observed lung pathology of mice that received pan T cells from BCG-WT-immunized donors was similar to that developed by non-transferred, hRSV-infected controls (Fig. 5d). These data suggest that immunization of rBCG-N-hRSV elicits a highly efficient T-cell memory repertoire capable of restraining viral dissemination and pathology in the lungs. These data also suggest that the non-specific protective effect observed in mice immunized with BCG-WT (Fig. 2) may be due to the modulation of the lung immune milieu.

3.5. rBCG-N-hRSV induces an anti-mycobacterial T-cell immunity equivalent to its conventional BCG counterpart

Worldwide, the BCG vaccine has been administered to newborns for decades for the prevention of infections caused by bacteria of the *Mycobacterium tuberculosis* complex [32]. Furthermore, BCG immunization is instrumental in preventing the more severe extrapulmonary presentations of childhood tuberculosis, such as tuberculosis meningitis and military disease [33]. Thus, in order to evaluate whether the rBCG-N-hRSV vaccine induce an anti-mycobacterial T-cell memory comparable to that induced by BCG-WT, we performed a delayed type hypersensitivity (DTH) assay in immunized mice using mycobacterial antigens (Fig. 6a). A *Mycobacterium tuberculosis* purified protein derivative (PPD RT50, from Statens Serum Institute, Denmark, *Mtb* PPD) was used to recall the T cell response generated in mice immunized with rBCG-N-hRSV and conventional BCG (Fig. 6a). We observed a significant increase in foot swelling in BCG-immunized animals of both BCG-WT and rBCG-N-hRSV immunized mice (Fig. 6b and c). No significant swelling was observed in naïve mice injected with the same amount of PPD, demonstrating the specificity of the DTH assay (Fig. 6b and c, and lower panel pictures). As expected, no significant swelling was recorded in the left foot of animals, demonstrating the null stimulation provided by the vehicle control.

Finally, to compare in more detail the anti-mycobacterial cellular immunity elicited by rBCG-N-hRSV and its wild-type counterpart, we analysed the IFN-γ and IL-17A response of splenocyte cultures stimulated for 72 h with increasing concentrations of *Mtb* PPD. As controls, no antigen (UP) and anti-CD3ε (1 μg/mL, as soluble agonist) were used for proper analysis of the T cell response. Consistent with the DTH response, splenocyte cultures of both rBCG-N-hRSV and BCG-WT vaccinees showed significant secretion of IFN-γ and IL-17A in response to *Mtb* PPD (Fig. 6c and d). Furthermore, there were no significant difference

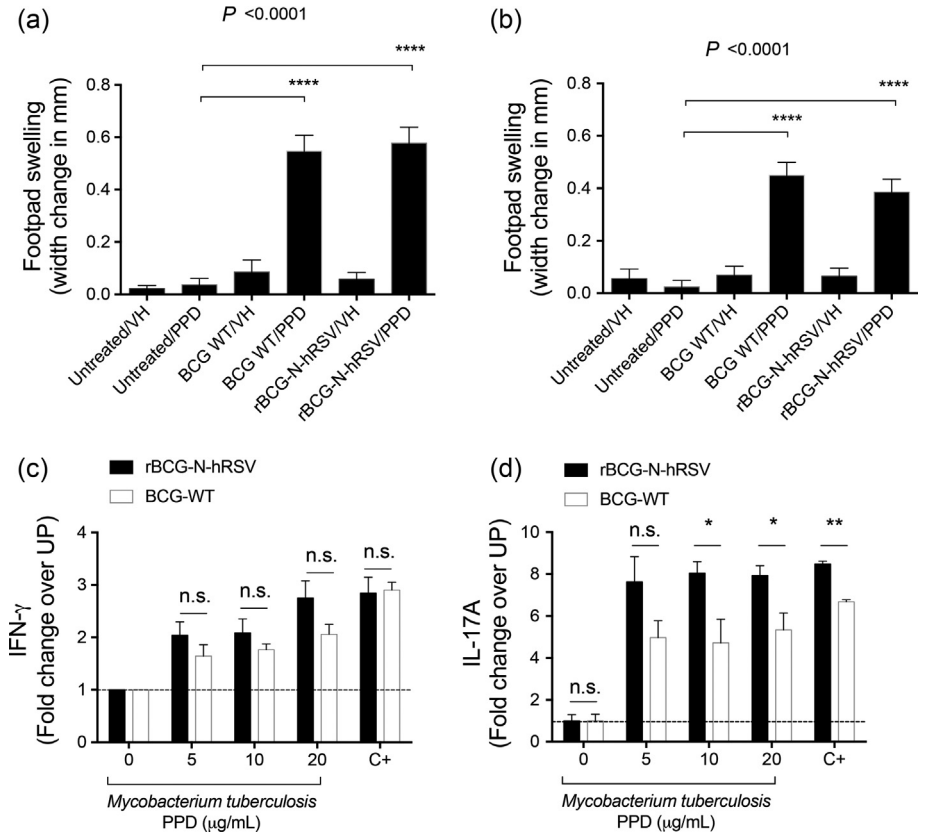

Fig. 6. Immunization with rBCG-N-hRSV elicits equivalent mycobacterial-specific T cell responses than conventional BCG vaccines. (a) and (b) Eighty days post-immunization, BCG-WT and rBCG-N-hRSV immunized mice were inoculated with 20 μg of *M. tuberculosis* PPD in the right footpad. As vehicle control, the left footpad was inoculated with an equivalent volume (20 μl) of phosphate buffered saline (PBS). The bar graphs show the absolute increase in foot swelling (as millimetres of width) after 24 h (a), and 48 h (b) of PPD administration. Foot swelling was determined comparing the foot width at day 0 (pre-injection) with foot widths at day 1 and 2 after PPD administration. (c and d) 21-days post-vaccination, rBCG-N-hRSV and BCG-WT immunized animals were sacrificed and their splenocytes recovered and stimulated *ex vivo* with increasing concentrations of *M. tuberculosis* PPD. After 72 h of stimulation, the presence of IFN-γ (c) and IL-17A (d) in cell-culture supernatants of stimulated splenocytes was analysed by ELISA. Bar represents data from at least two independent experiments in (a)–(c). N = 30 animals in A and B, N = 10 animals in (c) and (d). In (a) and (b), data was analysed using One-Way ANOVA and multiple comparison to the Untreated-PPD group. In (c) and (d), data was analysed by Two-Way ANOVA ($P < 0.00001$).

in the levels of IFN-γ and IL-17A secreted by splenocytes of BCG-WT and rBCG-N-hRSV vaccinees (Fig. 6c and d). Altogether, the data suggests that the rBCG-N-hRSV vaccine promotes an anti-mycobacterial T-cell response phenotypically and quantitatively similar to that induced by the conventional BCG vaccine, implying that the rBCG-N-hRSV vaccine is a promising candidate for replacing current BCGs and prevent hRSV infection in newborns.

4. Discussion

Despite hRSV was first identified more than fifty years ago, to date there is no licensed ant-hRSV vaccine approved for human use. Worldwide, identifying a good vaccine candidate has become a priority in the field of hRSV. Most vaccines studied have been focused in the development of neutralizing humoral immunity against hRSV [9], however in the present study we sought to evaluate whether a T-cell based immunization approach with single vaccine administration is sufficient to induce protective immunity. Under the rationale that the BCG vector would elicit a T_H1 immune response against hRSV antigens expressed heterologously, we developed a recombinant BCG expressing the hRSV nucleoprotein (N) [12,28]. As expected, we observed that in fact the rBCG-N-hRSV vaccine was competent at eliciting a T_H1 antiviral immune response capable of clearing the virus from the respiratory tract, and at the same time, in preventing the inflammatory hyper-responsiveness and lung damage developed in unimmunized mice challenged with hRSV [12,28].

Nevertheless, the high doses of recombinant vaccine used to test its protective capacity in the first instance raised concerns regarding the conservation of its immunogenicity at 1000 times lower doses. This is extremely relevant, because infants, who are the target of this vaccine prototype, are immunized by a unique, maximum dose of 4×10^5 CFUs of BCG [34]. On the other hand, adults and children >1 year of age display increased tolerability to BCG, being immunized with a maximum dose of 8×10^5 CFUs of BCG [34]. Hence, considering these antecedents we developed a cGMP vaccine prototype of the rBCG-N-hRSV, which is suitable for human trials. Nevertheless, prior to advance further in the clinical characterization of the vaccine, we sought to evaluate whether a low dose of rBCG-N-hRSV is safe in mice, and confers protection against experimental hRSV infection. Beyond demonstrating that this vaccine is safe and immunogenic, as evidenced by no observable adverse effects in mice and a consistent protection conferred either by direct vaccination or adoptive transfer of vaccine-elicited T cells, our data suggest that the rBCG-N-hRSV vaccine is a suitable candidate to replace conventional BCG vaccination, as a bivalent vaccine against hRSV and *Mycobacterium tuberculosis*. Therefore, this work represents an important milestone for the study of this vaccine candidate in future Phase I clinical trials.

Although we have recently demonstrated that the nucleoprotein (N) of hRSV inhibits the assembly of activating immunological synapses between naïve T cells and DCs [14], N represents an

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excellent T-cell antigen. In part, this is due to the high degree of amino acid sequence identity between hRSV isolates (~92%), becoming a promising antigen for the development of hRSV-specific memory T cells. Furthermore, data derived from the vaccination studies described in this manuscript suggest that delivery of hRSV-N protein in a bacterial vector, such as BCG, avoids the dampening effects of N over the priming of virus-specific T cells. It is likely that APCs, such as DCs and macrophages, can access to the N protein through the engulfment of recombinant bacteria expressing N in their cytoplasm, thereby remaining unavailable to interact with host membranes. These findings encourage the development of vectored vaccines that deliver enclosed antigens as similar approaches to circumvent the counterproductive effects of virulence determinants of other pathogens, which may have nevertheless a high immunogenic potential as T cell antigens.

Further research is needed to define the mechanisms underlying the non-specific anti-viral protective response observed in the lungs of BCG-WT-immunized mice. In hRSV-infected animals, non-specific protection due to BCG-WT immunization was accompanied by (1) a significantly reduced secretion of IL-6 and an increased secretion of IL-10 in BALs; (2) reduced activation of $CD44^{+}CD69^{+}CD4^{+}$ T cells (Supplementary Figs. S5d and 3) no such unspecific protection was induced by the adoptive transfer of T cells derived from BCG-WT immunized mice (Fig. 5). The specific protection elicited by rBCG-N-hRSV is further supported by the observation that after 50 days of vaccination no protection was observed in BCG-WT immunized animals (Supplementary Fig. 3). Importantly, the short-term unspecific protection observed in BCG-WT immunized mice seems to be associated with the half-life of alveolar macrophages (30 days [35], pointing to a potential role of these cells in the establishment of a protective respiratory immunological milieu after BCG immunization. This notion is further supported by previous observations showing that alveolar macrophages play a protective role in hRSV pneumonia [36], however further research is needed to investigate the effects of BCG vaccination in the phenotype and function of mouse alveolar macrophages. These observations would suggest that the non-specific and modulatory effect conferred by BCG immunization is due to a T cell-independent modulation of the lung immunological milieu. Several reports suggest that BCG administration improves immunity to allergens and to a variety of antigens, derived from infectious agents, such as influenza, for periods of time ranging from 14 days to 12 weeks [37,38]. Consistently with our data, it has been suggested that BCG skews pathological inflammatory responses into a more regulated immune phenotype. Indeed, BCG can skew T cell differentiation from T_H2 to T_H1 by down-modulating signaling of thymic stromal lymphopoietin (TSLP) in DCs [39]. Therefore, BCG-WT may modulate the pathogenic immune response elicited by hRSV infection by the impairment of TSLP signaling, which is an instrumental cytokine promoting lung pathogenesis during infection by this virus [40–42]. Further research is required to determine whether BCG-WT immunization interferes with the normal signaling of the TSLP pathway by modifying the response of lung alveolar macrophages and DCs to hRSV.

Finally, the hRSV-specific T cell immunity generated by the rBCG-N-hRSV recombinant vaccine can be efficiently recalled up to 50 days post-immunization (Supplementary Fig. 3). Due to the inhibitory effect of nucleoprotein in the T cell activation previously shown [14], denaturated N protein or HI-hRSV were used to stimulate T cells from vaccinated mice (Supplementary Fig. 4). Whereas denatured N protein or whole denatured viral particles promote IFN-γ, the soluble nucleoprotein does not. These results confirm that soluble nucleoprotein with their intact structure is required to exert that inhibitory effect. The long-term T cell immunity observed for hRSV in our infection experiments and for mycobacterial antigens in our DTH assays (Fig. 6), highlights the usefulness of BCG in the promotion of long-lasting T cell immunity to antigens of interests. Future research should focus in determining the breath of the T cell repertoire elicited by a single immunization with rBCG-N-hRSV in mice and humans.

5. Conclusions

- As BCG-WT, the rBCG-N-hRSV vaccine is safe, showing no side effects in mice.
- The rBCG-N-hRSV vaccine elicits a T_H1/T_H17 T cell repertoire that efficiently mediates virus clearance and prevents the inflammatory pathology in the lungs.
- The rBCG-N-hRSV vaccine elicits an anti-mycobacterial T-cell repertoire that is similar to that induced by BCG-WT.
- The rBCG-N-hRSV formulation elicits a long-lasting antiviral immunity that in mice persists up to 50 days after a single vaccine administration.

Conflict of interest

A patent for the rBCG-N-hRSV has been filled and issued in several countries.

Acknowledgements

This work was supported by grants CONICYT/FONDECYT POSTDOCTORADO No. 3140455 and No. 3160249, DOCTORADO CONICYT No. 21130507, FONDECYT 1070352; FONDEF D061008; FONDEF D11I1080; and the Millennium Institute on Immunology and Immunotherapy (P09/016-F).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.12.048.

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Please cite this article in press as: Céspedes PF et al. A single, low dose of a cGMP recombinant BCG vaccine elicits protective T cell immunity against the human respiratory syncytial virus infection and prevents lung pathology in mice. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2016.12.048

VIRULENCE
2017, VOL. 8, NO. 6, 685–704
https://doi.org/10.1080/21505594.2016.1265725

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REVIEW

Aberrant T cell immunity triggered by human Respiratory Syncytial Virus and human Metapneumovirus infection

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ABSTRACT

Human Respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are the two major etiological viral agents of lower respiratory tract diseases, affecting mainly infants, young children and the elderly. Although the infection of both viruses trigger an antiviral immune response that mediate viral clearance and disease resolution in immunocompetent individuals, the promotion of long-term immunity appears to be deficient and reinfection are common throughout life. A possible explanation for this phenomenon is that hRSV and hMPV, can induce aberrant T cell responses, which leads to exacerbated lung inflammation and poor T and B cell memory immunity. The modulation of immune response exerted by both viruses include different strategies such as, impairment of immunological synapse mediated by viral proteins or soluble factors, and the induction of pro-inflammatory cytokines by epithelial cells, among others. All these viral strategies contribute to the alteration of the adaptive immunity in order to increase the susceptibility to reinfections.

In this review, we discuss current research related to the mechanisms underlying the impairment of T and B cell immune responses induced by hRSV and hMPV infection. In addition, we described the role each virulence factor involved in immune modulation caused by these viruses.

ARTICLE HISTORY
Received 3 February 2016
Revised 22 November 2016
Accepted 23 November 2016

KEYWORDS
adaptive immunity; hMPV; hRSV; immunological synapse; T cells

Introduction

The human Respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV), which belong to the Pneumoviridae family,[1] are the main viral etiological agents of severe lower respiratory tract infection (LRTI), especially in infants, children and the elderly.[2,3] HRSV and hMPV can lead to bronchiolitis and pneumonia, and have also been implicated in the development of recurrent wheezing and asthma.[2-6]

HRSV is the second most common etiological pathogen for pneumonia after influenza virus around the world.[7] Furthermore, it has been reported worldwide between 66,000 to 239,000 deaths per year of children less than five years of age, who suffered LRTI caused by hRSV.[5,8]

HRSV was first isolated in 1956 from a colony of chimpanzees that presented various symptoms, including coughing, sneezing, and purulent nasal discharge.[9,10] The illness quickly spread from sick chimpanzees to other monkeys, indicating the presence of a highly contagious pathogen that was originally denominated the chimpanzee coryza agent.[10] In 1957, Chanock and Finberg isolated a similar agent from throat swab samples of infants with a severe respiratory disease,[11,12] which was found to be identical to the one reported by Blount et al in chimpanzees.[10] The isolated pathogen induced syncytia formation, via the virus fusion protein (F) on permissive cell types in cultures.[12,13] Therefore, this pathogen was renamed as a hRSV.

On the other hand, hMPV was first isolated in 2001 from young Dutch children with a respiratory tract disease.[14] HMPV is a member of the Pneumoviridae family and Metapneumovirus genus.[1] Genomic and phylogenic analyses suggest that hMPV diverged from avian MPV, a virus that causes serious respiratory diseases in chickens.[15-17] Infection caused by hMPV presents a

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similar symptomatology as other respiratory viruses such as hRSV, parainfluenza virus and influenza,[18] whereby the diagnosis is difficult. Symptoms of hMPV include rhinorrhea, cough, acute otitis media, fever, and, less frequently, conjunctivitis, rash, diarrhea and vomiting.[19] Frequently, risk population, including young children and the elderly, infected with hMPV require hospitalization.[20] Moreover, mortality as a result of hMPV can reach up to 10% in the elderly.[3,20]

Recurrent infections with hRSV or hMPV are common in children[21,22] and adults.[21-24] Studies in healthy young adults subjected to experimental challenge or natural infection, showed mild upper respiratory symptoms for both viruses infection.[23,25] However, reports performed in humans and mouse dendritic cells (DCs) have described that both viruses infect these cells affecting DCs capacity of promote an adequate immunological memory due to their interference with naïve T cell priming.[26-29]

The ineffectiveness of the natural infection to induce long-term immunity has hampered vaccine generation and currently there is no licensed vaccine available to prevent the bronchiolitis and pneumonia caused either by hRSV or hMPV.[30-34] However, several candidate vaccines are in different stages of development for preventing the diseases caused by these viral agents. Candidate vaccines for hRSV and hMPV employ different approaches, including a chimeric virus (hMPV),[35] live attenuated virus (hRSV),[36] and purified proteins (hRSV),[37] among others. Furthermore, recombinant Bacillus Calmette-Guérin (rBCG) vaccines have been developed using hRSV and hMPV antigens to provide a protective T_H1 response in mice.[31,38,39] In addition, antibody-mediated immunity is relevant for protection against both viruses.[40,41] For example, Palivizumab, an IgG1 humanized anti-F monoclonal antibody, is safe and effective when given in a prophylactic manner to children at risk of severe hRSV infection.[42] Thus, the ideal vaccine for either hRSV or hMPV should provide both antibody and T cell-mediated immune protection.

In this review, we describe the most recent findings relative to the mechanisms employed by hRSV and hMPV to evade the host immune system. Further, we will discuss the virulence factors of each virus that contribute at modulating the adaptive immune response.

Adaptive immunity triggered by hRSV

The Humoral response against hRSV infection

The humoral immune response plays a major role in protecting humans from hRSV infections.[43-45] Indeed, high titers of pre-existent mucosal IgG are correlated with reduced viral loads in hRSV-infected infants.[46] Similar correlation have been obtained for nasal IgA levels in naturally infection and in experimental challenges in healthy adults.[47,48] On the other hand, it has been described in mice that antibodies play a major role during reinfection, even more than in the first infection, giving a principal role to T cells in the clearance of the virus during the first hRSV challenge.[49]

Interestingly, prophylactic treatment with Palivizumab reduces severe hRSV-mediated LRTI,[42,50] and consequently diminish hRSV-associated hospitalization of premature infants, children with congenital heart disease (CHD) and children with cystic fibrosis (CF),[50] suggesting that a neutralizing antibody of high affinity and titer is enough to confer a clinical protection against hRSV disease. However, in most adults antibody titers are underneath the levels needed to reach a complete airway protection, despite repeated infection occurring throughout life.[51] Experimental challenges in healthy adults have shown that serum and nasal IgA titers increase after infection but those levels are poorly maintained.[47] Similar results have been reported for neutralizing antibodies levels in a birth cohort followed-up over three hRSV epidemics.[52] These antecedents suggest that acute production of short-life antibody-secreting cells (ACS) is not impaired. However, a defect occurs in long-lived plasma cells that arise from the ASC population, which normally should migrate to bone marrow and respiratory mucosa.[47] This phenomenon might explain, at least in part, as to why the specific hRSV-IgA generated humoral response is not sufficient to provide protection upon reinfection.

On other hand, several pieces of evidences suggest that the production of virus-specific antibodies plays an important role in the regulation of hRSV-specific T cell responses.[53] The interaction between antibodies and T cell responses was associated with the ratio of neutralizing and non-neutralizing antibodies. Wherein, for hRSV infection, a higher ratio of neutralizing versus non-neutralizing antibodies enhanced the balance of $CD4^+$/$CD8^+$ T cells *in vitro* that respond specifically to the virus in humans PBMC, as well as *in vivo* assays in mice model.[54] Interestingly, the infection of mice with hRSV immune-complexes increase the immune response against the virus, particularly promoting a TH1 response by $CD4^+$ T cells and IgG2c response by B cells.[55] Higher amounts of non-neutralizing antibodies might enhance infection and could cause immune complex deposition, leading to enhanced respiratory disease.[56] Considering the whole body of data described above, it is possible to hypothesize that hRSV infection can modulate the humoral response to impair recurrent reinfection and indirectly affect T cell activation.

The cellular immune response against hRSV infection

Both memory $CD4^+$ and $CD8^+$ T cells contribute significantly at achieving protective immunity upon hRSV infection.[57-59] This applies especially in children with defective T cell responses, who exhibit severe hRSV infection and prolonged virus shedding.[60] Supporting this observation, T cell depletion assays in BALB/c mice results in higher hRSV replication upon infection, while the adoptive transfer of virus-specific memory T cells enhances virus clearance in recipient mice.[61] Furthermore, it has been demonstrated that transfer of hRSV-N-specific T cells also contribute to reduce viral immunopathology.[38,39] Moreover, memory T cells appear to be clinically important in protecting from severe diseases caused by hRSV reinfections. This notion is supported by the fact that minor symptoms are observed in populations of older children and young adults infected with hRSV, despite of defective responses in IgA B cell memory and in hRSV-specific serum.[47,62]

Recently, it has been demonstrated that tissue-resident memory (Trm) T cells are relevant to the capacity of the host to rapidly limiting the spread of pathogens in tissues.[63,64] Thus, hRSV-specific $CD4^+$ and $CD8^+$ Trm T cells could provide immediate immunological protection against hRSV infections. In fact, analyses of hRSV-specific $CD8^+$ memory T cells have shown that these cells mostly remain in lungs and a minority of these cells circulates in peripheral blood from healthy individuals.[65,66] Moreover, increased activated hRSV-specific airway Trm T cell frequencies were observed in bronchoalveolar lavage fluid (BALF) from healthy adults inoculated with hRSV, which coincided with a reduction in the viral load.[59]

hRSV-mediated lung pathology in mice is not completely dissected and primary reports attributed this effect to T cells, specially $CD8^+$ T[67,68] but in humans, it has mostly been associated with a large influx of neutrophils in the lungs of patients with bronchiolitis, as well as in fatal cases of infants.[69-71] It is suggested that neutrophils recruitment induced by hRSV infection promote lung damage through the generation of reactive oxygen species and extracellular traps (NETs).[72,73]

Nevertheless, a recent study using experimental hRSV infection of adults in which a 65% of individuals presented inflammation symptoms, has shown that the virus replicate in the lower respiratory tract, inducing cellular infiltration of $CD8^+$ T cells to the airways.[59] Consistent with this notion, there is evidence that $CD8^+$ T cells can cause immunopathology in infants when a high amount of $CD8^+$ T cell encounter a large number of hRSV particles in the tissue.[74] However, the drawback of these studies is that no other cell types were evaluated, therefore it is not possible to rule out the neutrophils contribution to the pathology. In addition, another study showed that T cell responses are reduced or absent in exacerbated lungs of fatal cases of infants infected with hRSV, who had a severe LRTI caused by this virus.[71] In these tissues a positive staining for macrophages and neutrophils was observed.[71] Thus, in more severe cases of infantile viral LRTI caused by hRSV infection, lung inflammation appears to be due to a pronounced infiltration of neutrophils and macrophages.

$CD4^+$ T cell response against hRSV and mechanisms of evasion used by the virus

An adequate $CD4^+$ T cell response can efficiently aid at reducing viral load upon hRSV infection.[39] Indeed, it has been reported that adoptive transfer of $CD4^+$ T cells from immunized mice with a prototype vaccine consisting in a recombinant rBCG expressing hRSV N protein (rBCG-N-hRSV), resulted in a significant reduced viral load in the lungs after infection in recipient mice, thus providing a protective T_H1 antiviral response.[38] These data suggest that $CD4^+$ T cells itself stimulated with the proper antigens in can significantly contribute to hRSV clearance.

However, in infants and children naturally infections, an inefficient adaptive immune response occurs, which is characterized by 1) a skewed T_H2 immune response;.[75,76] 2) a deficient anti-viral T_H1 response and 3) a low secretion of IFN-γ and TNF-α in peripheral blood mononuclear cells[77] and in nasopharyngeal aspirates,[78] respectively. Nevertheless, hRSV-infected infants suffering from bronchiolitis present higher levels of TNF-α in BALF at day 1 of intubation, as compared with controls. This observation suggest that a mixed T_H1/T_H2 response is generated at the first stage of the disease.[79] Consistently with this observation, a mixed T_H1/T_H2 response was also observed in the lung of hRSV-infected mice, although with a significant increase of IL-13, which is known to induce airway hyperreactivity.[80,81]

The predominant T_H2 immune response observed in infants and children could be due to the capacity of hRSV to polarize the adaptive immune response from a protective T_H1 phenotype to a T_H2-type response.[76] However, whether a pathogenic T_H2 immune response significantly contributes to disease in at-risk human groups remains to be demonstrated.

Importantly, *in vitro* studies have described that hRSV-infected mouse DCs are unable to properly activate T cells,[27] due to an impairment of immunological synapse formation. This study evaluated the formation and functionality of the immunological synapse between

OT-II $CD4^+$ T cells with hRSV-infected DCs, pulsed with OVA peptide,[27] showing a lack of sustained immunological synapse as well as reduced secretion of cytokines from OT-II cells, as compared with uninfected controls. This phenomenon was observed by using naïve T cells, in contrast, it has been described that memory/effector T cells have different responses, which is less affected by hRSV-infected DCs.[82] This phenomenon, could explain why healthy adults infected with hRSV can clear the virus and not in young children who have no memory T cells.

TH2 polarization also is induced by Tymic-stromal lymphoprotein (TSLP), an epithelial cell-derived cytokine that signals through the TSLP receptor (TSLPR).[83] This cytokine potently activates myeloid DCs (mDCs), since these cells are known to express high levels of the TSLPR.[84] Then, TSLP-stimulated DCs upregulate OX40 ligand (L) cell surface expression and produce T_H2 cell-attracting chemokines, including CCL17 and CCL22.[84] Indeed, TSLP from *in vitro* hRSV-stimulated rat AECs induced the functional maturation of mDCs and enhanced the surface expression of the thymus-activation-regulated chemokine (TARC) and OX40L on DCs,[85] which induce mainly inflammatory T_H2-polarized immune responses.[86] Experiments performed in BALB/c mice infected by hRSV shown elevated levels of TSLP protein in lungs compare with uninfected control.[80] The role of TSLP in the promotion of Th2 response under hRSV infection was evaluated using a TSLPR-deficient ($TSLPR^{-/-}$) mice. These studies shown a decrease hRSV-mediated immunopathology in this model after infection. In addition, analysis of supernatant fluids of re-stimulated mediastinal lymph nodes (MLN) of $TSLPR^{-/-}$ mice infected with hRSV show a significant decrease of IL-13 and IL-5 production, as compare with WT mice, but both mice produced equivalent levels of IFN-γ and IL-17A. These results support, the notion that TSLP is required to TH2 polarization.[80] In other studies, it has been shown that the TSLP-OX40L –OX40 axis contributes to the hRSV-induced airway hyperresponsiveness (AHR) and inflammation after reinfection of mice that were initially infected as neonates. Further, administration of an anti-OX 40L antibody treatment during primary infection as neonates prevented the enhancement of the AHR upon reinfection 5 weeks later.[86] Moreover, treatment with the anti-OX40L during primary infection in newborn BALB/c mice reduced the TH2 cytokines IL-5 and IL-13 in BALF upon reinfection.[86]

The pathogenic role of $CD4^+$ T during re-infection with hRSV in adult mice, after being infected as neonates, can be explained by the induction of an exaggerated T_H2 response, demonstrated by the cytokine profile observed in these animals.[87] This could be explained, at least in part, by the upregulation of the IL-4Rα. Specific deletion of the IL-4Rα. gene in $CD4^+$ T cells abolished hRSV-induced airway AHR and lung damage upon reinfection with hRSV.[87] However, these results are controversial because several reports have shown that $CD4^+$ T cells are required for the clearance of the virus and to promote antibody response against hRSV.[60,88-90] In this sense, it seems that dysregulation of $CD4^+$ T cells promote harmful Th profiles but these cells are still required for hRSV clearance.

Additionally, an IL-17-mediated T_H17 response is a possible third type of immune response associated with the respiratory pathogenesis induced by $CD4^+$ T cells during hRSV infection.[91,92] Indeed, binding of IL-17 to its receptor promotes an inflammatory response and increased viral loads in the lungs of infected BALB/c mice.[92] Stimulation of T cells with hRSV-infected human bronchial epithelial cells (HBEC) induced the production of IFN-γ, IL-4, and IL-17, suggesting that hRSV can activate these three T_H cell subsets.[93] Consistent with the latter, stimulation of PBMC from healthy donors with hRSV infected A549 cells induced the production of IFN-γ and IL-4.[94] In other studies, the concentration of IL-17 in nasopharyngeal aspirates of children during hRSV infection was higher at the moment of discharge of the hospital.[95] However, the data are controversial since the latter was only valid for infants that do not require ventilation.[95]

In addition to these mechanisms used by hRSV to avoid the $CD4^+$ T other studies have demonstrated that IL-25 and IL-17RB are expressed in lungs of hRSV-infected BALB/c mice and their expressions correlate with potentially pathogenic cytokines, such as IL-13 (T_H2), IL-5 (T_H2) and IFN-γ (T_H1), promoting the hRSV-mediated lung disease.[91]

Finally, using a mouse model, it has been demonstrated that antibody-mediated depletion of neutrophils decreased the number of IL-13 producing $CD4^+$ T cells, as well as TNF-α and mucin production, when compare with the isotype-control treated group upon hRSV infection, suggesting that an interaction of neutrophils and $CD4^+$ T cells occurs during hRSV infection.[96] On the other hand, has been described that neutrophils can have a APC-like phenotype expressing costimulatory molecules and activating $CD4^+$ T cells polarizing to a TH1/TH17 phenotype in inflammatory mouse model, but for hRSV infections this phenomenon has not been demonstrated yet.[97,98] This could be an important unexplored area because has been described that hRSV can infect human neutrophils. Indeed, it has been possible to measure F, G and N proteins and viral RNA on those cells,[99] suggesting that neutrophils can uptake virus in the

airways of infected children and then present antigens to T cells, but further evidence is necessary to support this idea.

Taken together, data derived from *in vivo* and *in vitro* studies suggest that hRSV infection in the lung can induce different mechanisms to prevent an efficient $CD4^+$ T cell proliferation and differentiation into protective antiviral memory or effector cells of the host.

$CD8^+$ T cell response against hRSV and mechanisms of evasion used by the virus

It is well documented that $CD8^+$ T cells are pivotal at controlling respiratory viral infections, such as the one caused by hRSV.[57] Indeed, data from an adoptive transfer model demonstrated that transfused hRSV H2-K_d-restricted Cytotoxic T lymphocytes (CTLs) specific for the $M2_{82-90}$ ($KdM2_{82}$) epitope, the most predominant one of the M2 protein recognized *in vivo* (dominant epitope)[100] rapidly clear the virus from the lungs of recipient BALB/c mice. However, a significant lung pathology was also observed.[67] Consistent with that observation, BALB/c mice immunized with a DNA vaccine expressing the K^dM2_{82} epitope linked to human β2-microglobulin (β2m) developed an enhanced pulmonary inflammatory response after hRSV challenge, characterized by enhanced weight loss.[101] Likewise, intranasal administration of the K^dM2_{82} epitope combined with Escherichia coli heat-labile toxin (LT)/LTK63 elicited a strong antiviral $CD8^+$ T-cell response in BALB/c mice, but also enhanced lung pathology.[102] This data indicates that the K^dM2_{82} epitope-specific $CD8^+$ response is associated with enhanced disease. In addition, Ruckwardt et al.[103] demonstrated that infection of CB6F1/J mice with a recombinant hRSV containing a mutation in the dominant K^dM2_{82} epitope resulted in an increased response of $CD8^+$ T cells specific for the subdominant epitope D^bM_{187} ($M_{187-195}$) with significantly less clinical disease. In contrast, hRSV containing mutations in this subdominant epitope induced an augmented K^dM2_{82}-specific $CD8^+$ T cell response and increased severity of illness.[103] Consistent with the latter, in CB6F1 hybrid mice, which recognize multiple MHC class I-restricted epitopes, it was described that D^bM_{187} specific $CD8^+$ T cells control hRSV replication more efficiently with less pulmonary inflammation and illness than the K^dM2_{82} specific $CD8^+$ T cells.[104] In addition, another study has shown that after immunization with a recombinant PR8 influenza virus carrying the subdominant hRSV KdF_{85} epitope, KdF_{85}-specific CTLs were induced in BALB/c mice with a significant reduction of the viral load in the lungs upon hRSV challenge,[105] indicating a protective effect against this virus. Taken together, these results from the mouse models suggest that a subdominant epitope-specific $CD8^+$ T cell response could be more beneficial to the host by promoting an effective anti-viral immune response and reduced lung disease, than the dominant ones, which promote a robust anti-viral immune response but also an enhanced lung pathology upon hRSV infection.

Likewise, when $CD8^+$ T cells from rBCG-hRSV-N immunized-BALB/c mice are stimulated with hRSV N peptides, these cells produce significant amounts of IFN-γ, partially protecting recipient mice of hRSV infection.[38] The efficiency of the $CD8^+$ T cell-mediated virus control could depend on how the hRSV antigen is presented by DCs, since less IFN-γ secretion was observed when BALB/c mice were immunized with purified hRSV N or M2 in alum, compare with when mice immunized with rBCG-N-hRSV and rBCG-M2-hRSV.[38] Furthermore, $CD8^+$ T cell-mediated virus control and immunopathology is dependent on IFN-γ production during early infection.[67]

In human, has been described lesser about single epitope immune-modulation, in fact the majority only describe hRSV-derived peptides that induce IFN-γ secretion by stimulated T cells,[59,106,107] but they do not address their possible role during pathology. Table 1, summarizes the hRSV epitopes described so far for HLAs. It is possible that these epitopes are implicated in $CD8^+$ T cells response but further studies are required to demonstrate if these specific T cells response is favorable or harmful for an infected person.

The impairment of CTL function appears to be another immune evasion mechanism evolved by hRSV.[57] How specific hRSV proteins affect these cells will be discussed below, but here additional mechanisms that have not been related yet to specific hRSV proteins. For instance, the upregulation of the programmed-death ligand 1 (PD-L1), which binds PD-1 on $CD8^+$ T cells in human bronchial epithelial cells (BECs), occurs after hRSV infection and it has been shown to cause a

Table 1. hRSV epitopes described for HLA.

Protein	Epitopes	HLA	Reference
N	N peptides	HLA-A*02 and HLA-B*08	106
	(NPKASLLSL [NPK]	HLA-B*07:02	59
	QVMLRWGVL [QVM])	HLA-B*07:02	59
M2	$M2_{(151-159)}$	HLA-A*03	107
	$M2_{(64-72)}$)	HLA- B44	107
NS1	(LSDSTMTNY [LSD])	HLA-A*01:01	59
NS2	$NS2_{41-49}$	HLA-B51	107
	(FLVNYEMKL [FLV])	HLA-A*02:01	59
L	(IPAYRTTNY [IPA])	HLA-B*07:02	59
M	$M_{195-203}$	HLA-B51	107
	$M_{229-237}$	HLA-A1	107
	YLEKESIYY [YLE]	HLA-A*01:01	59
	VTDNKGAFKY [VTD])	HLA-A*01:01	59
G	(KPNIRTTLL [KPN]	HLA-B*07:02	59

functional impairment of CD8$^+$ T cells.[150] Indeed, blocking PD-L1 with a specific-antibody in hRSV-infected BECs, co-cultured with CD8$^+$ T cells, enhances CD8$^+$ T cell effector functions and decreases hRSV gene expression in BECs.[108]

In addition, CD8$^+$ T cells may not only be directly influenced by the impairment of the T cell priming by DCs, but also be indirectly affected by the modulation of cytokine environment, as NK cell-derived IFN-γ production precedes lung CD8$^+$ T cell recruitment,[109] in a mechanism similar to the Influenza A virus.[110] Also, temporally association has been made between CD8$^+$ T cells and neutrophils, where an important influx of neutrophils to the airways in infants occurs before CD8$^+$ T cells activation. This initial neutrophil influx correlates to the expression of the most severe hRSV symptoms suggesting that the inflammatory environmental potentiated by neutrophil influx could modulate CD8$^+$ T cell response.[111]

In summary, CD8$^+$ T cell function appears to be impaired by hRSV through different mechanisms. Nonetheless, these different strategies used by this virus in targeting the CD8$^+$ T cell response may not be that relevant in populations of older children and young adults, since most of them have mild symptoms or are asymptomatic when undergoing hRSV viral infection.

Role of hRSV Proteins in Immunomodulation

The hRSV genome consists of a 15.2 kb long, negative sense RNA, which contains 10 genes encoding for 11 proteins, with two overlapping open reading frames encoding two proteins: M2-1 and M2-2 (Fig. 1).[112,113] Several hRSV proteins play a role in evading the immune system of the host (see Table 2). The hRSV glycoproteins, located in the viral envelope, that have been involved in interfering the immune response of the host are:

HRSV Glycoprotein protein (G): this protein is involved in virus attachment[114] and it also exists as a secreted form, which prevents opsonization and neutralization of hRSV by anti-G specific antibodies.[115]

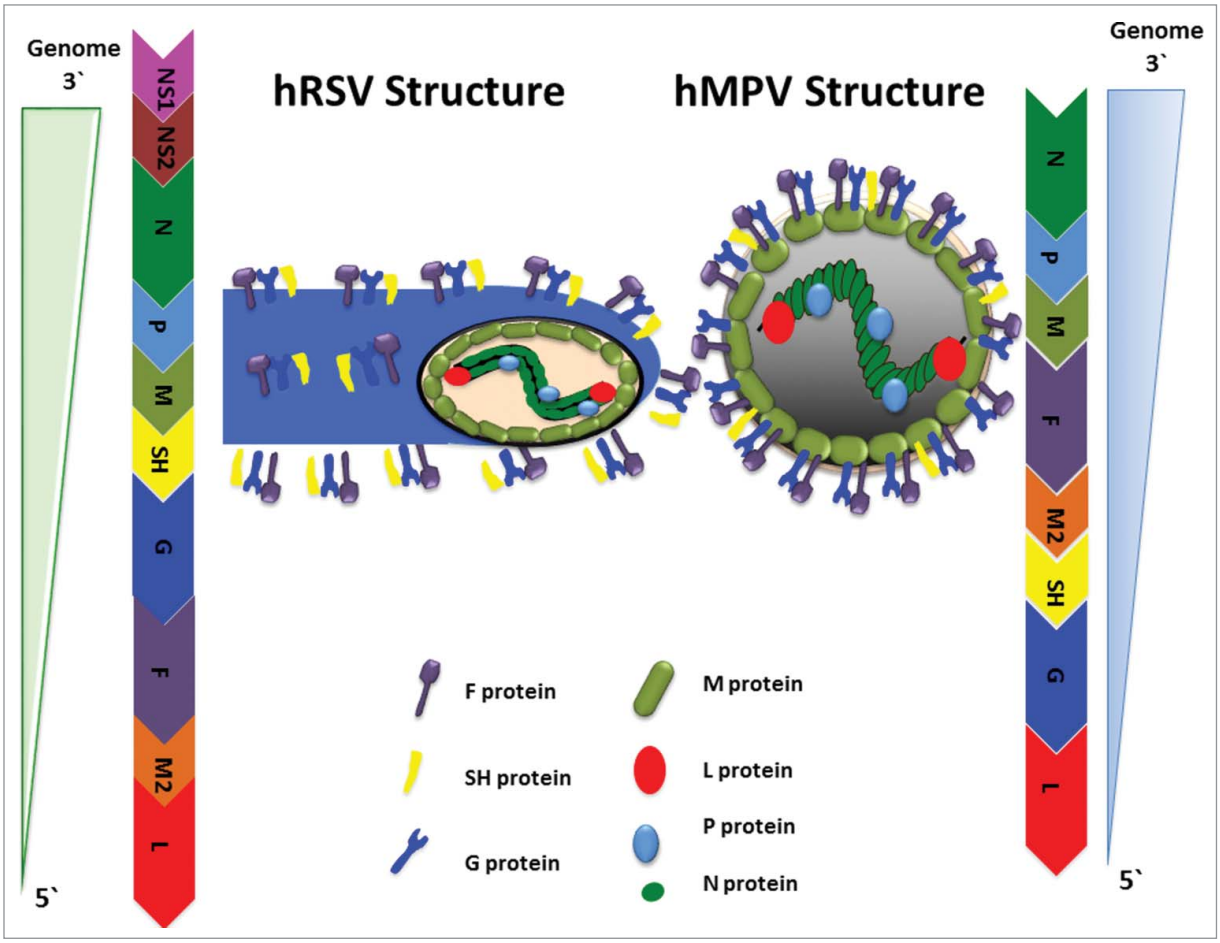

Figure 1. Viral Structure and Genome Organization of hRSV and hMPV. Schematic representations of hRSV and hMPV structures are shown. Both are negative single-stranded RNA (3′ to 5′), enveloped viruses that mainly differ in the number and order of genes in their genomes. These genes encode for P, N, SH, G, F, L, M, and M2 proteins, which are similar for both viruses. The M2 gene has an open reading frame that encodes for the M2-1 and M2-2 proteins. The hRSV genome also contains the non-structural proteins NS1 and NS2, which are absent in hMPV.

Table 2. Function of hRSV and hMPV proteins and their roles modulation of immune response.

Protein/Gene	Protein Function		Role in Immunomodulation		References
	hRSV	hMPV	hRSV	hMPV	
G	Attachment to host cells	Attachment to host cells	-Evasion of the anti-G antibody functions. -Impairment of CX3CR1$^+$ T cell migration and function (CX3C motif).	Inhibition of the type I IFN pathway (RIG-I)	114-116,155,156
F	Fusion of viral particle with host cells		Interfere with the proliferation of T cells by contact.	N/A	4,117
SH	Virioporin	Virioporin	- Inhibition of apoptosis. - Inhibition of TNF-α-mediated NF-κB signaling and NF-κB activation.	-Inhibition of the NF-κB pathway	118,119,157
NS1		N/A	Impairment of the type I IFN pathway. (IRF-3)	N/A	121-127
NS2		N/A	Impairment of the type I IFN pathway	N/A	121-127
M2.1	Transcription anti-terminator factor	Involved in viral replication through its Zinc binding domain	Activation of the NF-kB pathway	N/A	52,53,145,160,161
M2.2	Regulatory factor in replication and in transcription	Regulatory factor in replication and in transcription	N/A	Inhibition of the type I IFN pathway (Interaction with MAVS).	112,128,162,163
N	Assembly of the nucleocapsid and protection of the viral RNA	Interaction with the viral RNA	-Interference with the immune synapse assembly. - Inhibition of the Type I IFN pathway by MAVS interaction	N/A	129-131
M	Viral particle assembly	Viral particle assembly	N/A	It induces: - Maturation of monocyte-derived DCs. - Secretion of pro-inflammatory cytokines.	158,159

Moreover, the secreted form of the G protein has a chemokine-like motif (CX3C) that competes with fractalkine (CX3CL1) in binding to its receptor CX3CR1, thus reducing the CX3CR1$^+$ T cells response.[116]

HRSV Fusion (F) protein: this viral protein is required for the fusion of viral particle with the host cells[4] and also play a direct role in the ability of hRSV to decrease the proliferation of these cells by contact.[117] In fact, when Vero cells express the F protein or are infected with a version of hRSV that only have F protein on its surface, they reduce the proliferation and response to mitogen stimulus.[117]

HRSV small hydrophobic (SH) protein: it is thought that this protein works as an important viroporin during hRSV pathogenesis.[118] This protein also inhibits apoptosis in order to promote viral replication, as recombinant hRSV lacking the SH protein induced a significant cytopathic effect in different cell lines, compare with WT hRSV.[119] In addition, the hRSV SH has been shown to inhibit the NF-$\kappa\beta$ pathway through a decrease in the TNF-α production in mouse fibroblastic cells.[119]

Another studies have demonstrated that glycoproteins affect the innate and adaptive immune response to hRSV. In this study, immunization of BALB/c mice with a recombinant strain of hRSV lacking both G and SH (CP52) increased the number of pulmonary natural killer (NK) cells, as well as the levels of IFN-γ and TNF-α at day 3 p.i. with a concordant reduced expression of the T_H2 related cytokines (IL-4 and IL-6) when compare with the parental RSV strain (B1).[120] However, in the same study it was also shown that during primary infection, RSV-specific MHC II CTL precursor frequencies were delayed in CP52-immunized mice compare with B1-immunized mice in BALF, cervical lymph nodes and spleen at day 5 p.i.[120] Furthermore, during secondary infection, both RSV-specific MHC I and MHC II CTL precursor frequencies were delayed in spleen at day 3 p.i in CP52- immunized mice, as compare with mice immunized with B1 or control Vero cell lysate.[120] Altogether these data suggest that the hRSV G and/or SH proteins play a role in: a) downregulation of specific NK cell response, as mutants lacking these proteins increase pulmonary NK cells; b) polarization toward a T_H2 immune response, as RSV strains deficient in these proteins decrease T_H2 related cytokines.

Beside glycoproteins, hRSV also expresses other proteins involved in the impairment of the host's immune response, which are described below.

HRSV Non-structural (NS) proteins 1 and 2: these proteins impair the type I IFN pathway by targeting the

interferon-regulatory factor 3 (IRF3), thus inhibiting the IFN-α/β antiviral signaling.[121] Additionally, these NS proteins suppress human DC maturation by downregulation of the type I IFN production; inhibit apoptosis; control protein stability; and regulate host cell mRNA.[122-125] Moreover, the NS2 protein induces cell rounding and shedding *in vivo* in hRSV-infected human ciliated cells in the large airways,[124] promoting the reduction of viral titers in the airway mucosa and initiating the obstruction of distal airways.[124] In studies using co-cultures of WT or mutant hRSV-infected human DCs with autologous $CD4^+$ T cells, it has been demonstrated that NS1 protein promotes $CD4^+$ T cell proliferation with a T_H2 immune response.[126] In addition, NS1 was shown to suppress the $CD8^+$ T cell anti-viral response, as there were increased levels of IFN-γ in the supernatant of lymphocytes co-cultured with human DCs infected with hRSV ΔNS1 as compare with lymphocytes co-cultured with DCs infected with WT RSV.[126] In the same study, it was also shown that NS1 suppresses the $CD8^+$ $CD103^+$ T cell activation and proliferation, which is required to accomplish the cytolytic function of these cells at the mucosal epithelium of the respiratory tract.[126] Therefore, the hRSV NS1 protein can impair the efficient anti-viral function of $CD8^+$ T cell in different manners. Further, *in vivo* suppression of the CTL response by hRSV is mediated by NS2, as BALB/c mice infected with a hRSV mutant deficient in the NS2 gene (ΔNS2) or both the NS1 and NS2 genes (ΔNS1/ΔNS2) showed increased pulmonary hRSV-specific CTL responses compare with those of mice infected with WT hRSV or with the virus lacking NS1 (ΔNS1).[127]

HRSV M2–1 protein: This is a transcription anti-termination factor important for the efficient synthesis of full-length viral mRNAs.[112] However, M2–1 has also been shown to activate the NF$\kappa\beta$ pathway as demonstrated by translocation to the nuclei of the NF$\kappa\beta$ factor in A549 cell transfected with a M2–1 encoding vector.[128]

HRSV N protein: This protein is critical for the assembly of the hRSV nucleocapsid and protection of the viral RNA (Fig. 1).[129] Furthermore, the hRSV N protein interfere with the type I IFN pathway by targeting the mitochondrial antiviral signaling protein (MAVS), thus inhibiting the MAVS-dependent antiviral pathway.[130] Moreover, hRSV-infected DCs are unable to activate naïve $CD4^+$ T cells *in vitro* probably due to the impairment of the immunological synapse assembly by the hRSV N protein expressed at the host cell membrane (Fig. 2, upper left box), rendering T cells unresponsive to subsequent TCR engagement..[27,93,131] Specifically, the Golgi apparatus polarization within T cells, an event needed for a proper immunological synapse assembly, is barely detectable in T cells co-cultured with hRSV-infected DCs, in contrast to T cells co-cultured with mock-inoculated DCs.[27] Moreover, impairing this signaling event significantly decreases tyrosine phosphorylation of TCR-associated CD3z-chain tyrosine-based activation motifs (ITAM) by LCK,[132] in naïve T cells stimulated with a cognate antigen.[27] Furthermore, immune synapse assembly inhibition is accompanied by a reduced binding of ICAM-1, suggesting that the N protein interferes with receptor-ligand interactions at the immunological synapse, reducing the TCR clusters that are usually observed within a mature immunological synapse.[131] A putative viral mechanism of this inhibitory effect could be the interaction of the hRSV N protein with an element of the TCR complex, since central clustering of this protein occurs alongside the TCR, even in the absence of pMHC.[131] Further studies are required to define the molecular mechanisms underlying the immune synapse inhibition by RSV infection.

Adaptive Immunity Triggered by hMPV

The humoral response against hMPV infection

It has been described that hMPV-specific antibodies are produced after hMPV infection in the childhood. The most predominant antibodies detected are anti-F antibodies after the first infection and can be detected over the time at least for 20 y evidenced by the fact that 95% of 20 y old individuals are seropositive for the F protein.[133] Likewise, it has been described that individuals with low levels of anti-hMPV antibodies are more susceptible to hMPV infection.[134] Studies in BALB/c mice have demonstrated that passive transfer of hyper-immune hMPV-specific mouse sera to naïve mice decreased virus titer, seven days post-infection, suggesting that hMPV-specific antibodies provide a level of protection from viral challenges.[135] However, the hMPV-specific antibody response appears to be inefficient in mediating viral clearance, since hMPV persists in lungs of infected mice despite the presence of neutralizing antibodies.[136] In fact, it has been observed that in humans hMPV can persist in immunocompromised patients, suggesting a principal role of immune response for control hMPV infection.[137,138] This could be explained by a waning effect on protective hMPV-specific antibodies over time, or the levels of these antibodies may not be sufficient to protect from a re-exposure to hMPV infection.[134] Moreover, in hMPV-infected BALB/c mice, seroconversion and the development of neutralizing anti-hMPV specific antibodies (IgG, IgG1, and IgG2a) are observed post-challenge, but these antibodies do not prevent the persistence of infectious hMPV.[136] In humans, from 257 hMPV-positive individuals, only a 25% remained asymptomatic, whereas 75%

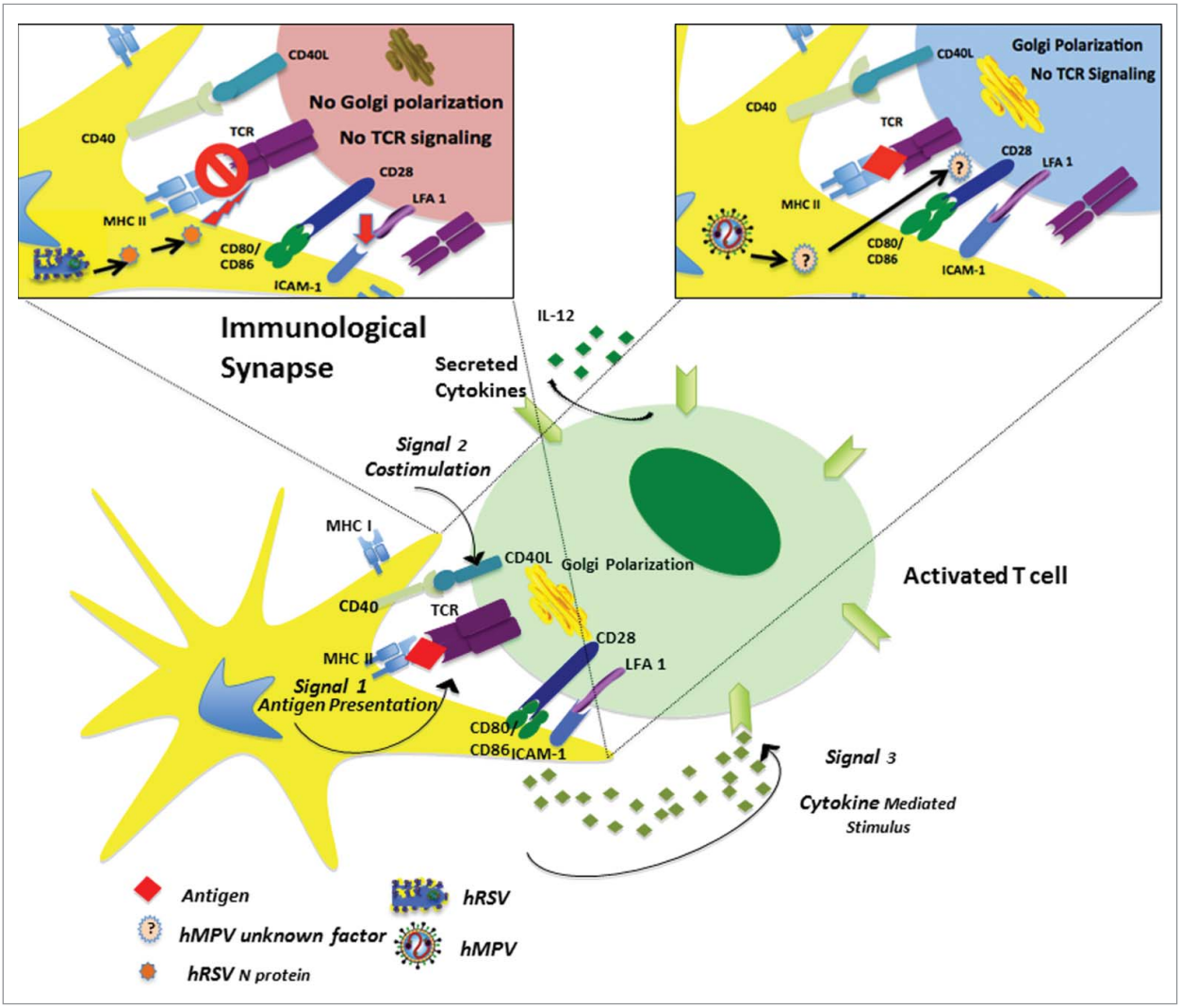

Figure 2. Effect of hRSV and hMPV Infections on T cell Activation. To induce proper T cell activation by DCs presenting a specific antigen, the following three signals are required to establish a mature immunological synapse: i) interaction between MHC molecules (class I or II) and the TCR molecule; ii) co-stimulation of molecules, including CD80, CD86, and CD28; and iii) T cell-polarizing molecules, either soluble or membrane-bound. Additionally, adhesion molecules, such as the intracellular adhesion molecule 1, are also involved. In the amplified diagrams, the possible mechanisms by which hRSV or hMPV are able to impair antigen presentation are shown. In the case of hRSV (upper left box), the virus interferes with the immunological synapse through its N protein, which impairs Golgi polarization and TCR signaling. This inhibitory effect is accompanied by reduced intracellular adhesion molecule 1 binding to the lymphocyte-function associated antigen 1 (red arrow). In the case of hMPV (upper right box), the virus impairs TCR signaling and, consequently, T cell activation through an unknown soluble factor, which does not affect the immunological synapse itself.

presented symptoms, despite their severity.[134] hMPV-infected individuals presented low IgA and IgG titer compare with non-infected individuals, and also lower neutralizing capacity, consistent to what is observed in mice.[134] Therefore, the humoral responses raised against hMPV following natural infection might not be sufficient to reduce re-infection episodes.[22]

The cellular response against hMPV infection

For hMPV, it was recently shown that the presence of virus-specific T cells in airways and in lungs of BALB/c mice, are associated with an effective anti-viral immune response.[139] These data suggest that Trm T cells could also be important for controlling hMPV infection. However, further studies are still required, especially in humans.

$CD4^+$ and $CD8^+$ T cells are required for the clearance of hMPV from infected lungs in hMPV-infected BALB/c mice.[140] Likewise, an efficient anti-viral immune response based on IFN-γ-secreting $CD4^+$ and $CD8^+$ effector and memory T cells is necessary for preventing the spreading of hMPV in airways and to avoiding the development of bronchiolitis and pneumonitis. This is supported by a study, that used a candidate vaccine based on rBCG

expressing hMPV antigens, where vaccination with this hMPV vaccine expressing the M2–1 or P protein protects BALB/c mice from hMPV-mediated lung pathology and reduced viral load in lungs.[31] However, this virus also evades these effective anti-viral immune responses.[26] Moreover, naïve BALB/c mice were shown to develop a biphasic immune response *in vivo* after hMPV infection.[135] During the first week, a T_H1 response initially controls virus replication before polarizing toward a T_H2 immune response that facilitates viral persistence.[135,136] A recent *in vivo* study, though, showed mixed T_H1 and T_H2 responses during the first week of hMPV infection in BALB/c mice.[141] Consistent with the notion observed in the mouse model, in adults undergoing hMPV infection, it has been described that PBMCs from these patients presented cytolitic activity, as evaluated by a Chrome-release assay, which suggest a T_H1 response.[142] However, PBMCs, from healthy adults, stimulated with heat-inactivated hMPV promotes the induction of high levels IL-6 (a T_H2 polarizing cytokine that prevents T_H1 differentiation[143]) and low levels of IFN-γ and CCR5 (T_H1 cytokines) when compare with hRSV-stimulated PBMCs, thus suggesting a polarization toward a T_H2 response.[144] In contrast, other studies demonstrate that infants undergoing hMPV infection presented lower levels of pro-inflammatory cytokines compare with infants undergoing hRSV or influenza infection, including TNF-α and IL-1β, two cytokines related to the chemotaxis of neutrophils in lungs, as well as IL-12, IL-6 and IL-8.[145] Furthermore, in infants undergoing hMPV infection it was observed that a predominant T_H1 response is generated, since it was detected an increase in the IFN-γ/ IL-4 ratio in nasal airway secretions. Taken together, this data indicate that hMPV induces a complex immune response, which might include a mixed T_H1/T_H2 response, similar to what was observed in mice, though further studies are required to evaluate the T cell response in humans upon hMPV infection.

$CD4^+$ T cell response against hMPV and mechanisms of evasion used by the virus

HMPV has also evasion mechanisms to interfere specifically with $CD4^+$ T cell function that do not promote an efficient antiviral immune response and enhances lung pathology. Specifically, depletion of $CD4^+$ T cells in hMPV-infected BALB/c mice reduces lung pathology and airway obstruction, without affecting viral loads.[140] These data demonstrate that the subset of $CD4^+$ T cells contributes to lung pathology but they are not critical for viral clearance.

As has been mentioned above, hMPV can induce a mixed TH1/TH2 response and this type of response is dependent of activation of TSLP pathway, which favors TH2 response over TH1 response that is known is necessary for hMPV clearance. Related to this, hMPV infection has been proven: to promote TSLP expression in both human AECs and mouse lungs; to stimulate OX40L+CD11b+DCs lung infiltration; and to increase the levels of pro-inflammatory TH2 cytokines-producing T cells, including TARC, IL-5 and IL-13, but also TNF-α in BALB/c mice,[141] as previously reported for OX40L on TSLP-activated DCs.[86] Moreover, TSLPR−/− mice showed decreased lung inflammation and hMPV replication, as well as a higher frequency of $CD8^+$ and $CD4^+$ T cells.[141] These findings highlight the possibility that a repertoire of virus-specific T_H and CTLs may incompletely eliminate infected cells within the airways following primary infection, leading to an exacerbated inflammatory response, mainly mediated by the TSLP pathway, thus inducing an aberrant T cell response.[141]

Moreover, hMPV-infected DCs, similarly to hRSV-infected DCs, also impair the activation of $CD4^+$ T cells. Upon stimulation with hMPV-infected and antigen loaded DCs, naïve antigen-specific $CD4^+$ T cells displayed significantly reduced proliferation, expression of surface activation markers, such as CD25 (IL-2 receptor/ IL-2Rα), CD69 and CD71 (transferrin receptor) and IL-2 secretion, as compare with T cells stimulated with uninfected control DCs.[26] However, this inefficiency is not due to a deterioration of the immune synapse assembly since both TCR cluster formation and Golgi polarization still occur (Fig. 2, upper left box).[26] This impairment may contribute to a delayed T_H1 response. Indeed, $CD4^+$ T cells elicit a poor IFN-γ response when activated with hMPV-infected human peripheral blood mononuclear cells *in vitro*,[144] suggesting that these hMPV-infected cells can also inhibit the function of $CD4^+$ T cells to stimulate an efficient antiviral T_H1 immune response in humans. The impairment of T cell immunity could be the result of impaired DC functions by hMPV, possibly through of a soluble factor derived from hMPV-infected DCs, as supernatants from these infected cells impairs T cells activation when stimulated by plate-bound anti-CD3ε and anti-CD28.[26] Thus, hMPV impairs the TCR signaling, without disturbing the immune synapse formation and Golgi polarization in T cells[26] (Fig. 2, upper right box). Consistent with the latter, the SH and/or G proteins reduced $CD4^+$ T cell proliferation in a co-culture assay of hMPV-infected MDDC with $CD4^+$ T cells when compare with T cells co-cultured with ΔSH/G hMPV-infected DCs.[146] Likewise, *in vitro* studies show that human and mouse hMPV-infected DCs lose the capacity to activate and expand naïve T cells, although to a lesser degree than hRSV-infected DCs.[26,146-148]

Furthermore, the neutralizing antibodies detected in mice after hMPV infection, appear to be dependent on

$CD4^+$ T cells, because no detectable neutralizing antibodies were observed in hMPV-infected mice depleted of $CD4^+$ T cells.[140]

$CD8^+$ T cell response against hMPV and mechanisms of evasion used by the virus

In a similar manner as for hRSV, $CD8^+$ T cell response specific to hMPV antigens is critical for an effective viral clearance, as demonstrated in studies using vaccine candidates against hMPV, such as virus-like particles harboring hMPV F or hMPV M antigens.[149] Supporting this notion, hMPV-specific effector $CD8^+$ T cells, in BALB/c mice that lack protective anti-hMPV antibodies and in the absence of $CD4^+$ T cells, no detection of virus load and reduction of lung disease were found upon hMPV infection, suggesting that the cytotoxic activity of $CD8^+$ T cells alone can confer protection against hMPV[140] and highlighting the importance of $CD8^+$ T cells in controlling hMPV infection. Moreover, when specific hMPV M2 CTLs were transferred into RAG-1−/− mice, these lymphocytes protected the host of hMPV challenge.[150] Furthermore, hMPV-specific IFN-γ-producing $CD8^+$ T cells can be found in the mucosa of the airways and in lungs, but not in the lymph nodes or in spleen of hMPV-infected BALB/c mice at 7 d p.i, suggesting the activation of the Trm T cells specific for hMPV epitopes.[139] Nevertheless, hMPV-virus specific CTLs were also induced 21 d p.i in spleen.[139] These data underscore the importance of Trm $CD8^+$ T cells in controlling rapidly hMPV infections. Recently, in human has been described that memory $CD8^+$ T cells are reactive to the majority of hMPV proteins (M, F, G, M2-1, N and SH) and particularly, $CD8^+$ T cells that recognize M and F protein can secrete IFN-γ after 21 months post-infection.[142] Nevertheless, further studies are required in humans to clarify if this $CD8^+$ T cells populations are protective or not, because independently of the presence of this population, hMPV keeps its capacity to generate re-infection episodes.

Recent studies associate the lack of the type I IFN pathway with $CD8^+$ T cell impairment.[151] In fact, hMPV infected-IFNAR−/− mice had a higher peak of early viral replication, less airway dysfunction and lung inflammation, but cleared the virus with the same kinetics as observed in WT mice.[151] Likewise, $CD8^+$ T cells from IFNRA−/− mice expressed similar levels of PD-L1 when compare with $CD8^+$ T cells from WT mice. However, these cells showed an upregulation of the inhibitory receptor TIM-3, thus impairing the $CD8^+$ T cell function.[151] Additionally, $CD8^+$ T cells can be impaired by hMPV in a PD-1 dependent manner, similar to hRSV, as lung $CD8^+$ T cells are impaired in HLA B7.2 transgenic (B7tg) mice and had upregulated PD-1.[152] Conversely, blocking of PD-1 by administration of monoclonal specific antibodies in B7tg mice prevented the $CD8^+$ T cells impairment. Similarly, impairment of $CD8^+$ T cells was prevented on hMPV-infected PD-1−/− mice.[152]

In other studies, an impairment of lung hMPV-specific memory $CD8^+$ T cells was observed in μMT mice, which lack B-cells and are used as a model for hMPV reinfection, which suggest the importance of memory $CD8^+$ T cells in viral clearance during a second infection.[153] Specifically, during reinfection, $CD8^+$ T cells had upregulated several inhibitory receptors, including PD-1.[153] Similarly to it is observed in B7tg mice, blockade of PD-1 in μMT mice restored lung $CD8^+$ T cell effector functions (i.e., degranulation and cytokine production) and enhanced viral clearance.[153] In other studies, immunization of μMT mice with virus-like particles encoding the hMPV F and M proteins, generates hMPV F-specific and M-specific $CD8^+$ T cells in lungs, but their function is impaired, as inhibitory receptors are upregulated on these cells, similar to what is seen in WT C57BL/6 mice under a second hMPV infection.[149]

On the other hand, the depletion of $CD8^+$ T cells in hMPV-infected BALB/c mice resulted in a lower lung pathological score, although to a lesser degree than depletion of only $CD4^+$ T cells.[140] By other part, mice that was depleted of neutrophils showed a significant reduction of TNF-α and IL-13 secreted by $CD8^+$ T cells suggesting that neutrophils modulate the production of these cytokines by $CD8^+$ T cells, [141]In this manner, $CD8^+$ T cells that infiltrate in the airways and secrete TNF-α and IL13 contribute to the lung pathology triggered by hMPV infection in mice.[140,141]

Taken together, $CD8^+$ T cells confer protection against hMPV infection through cytotoxic activities, but these cells may also contribute to lung pathology.[140] Furthermore, the function of these cells can be hampered by evasion mechanisms of the virus. However, further studies in humans are still required.

HMPV molecular characteristics and the role of its viral proteins in immunomodulation

The hMPV genome is comprised of a negative, 13 kb single-stranded RNA with the following eight genes: N-P-M-F-M2-SH-G-L (3′ to 5′).[18] Furthermore, the hMPV mRNA transcribed by the M2 gene contains two overlapping open reading frames that give rise to the M2–1 and M2–2 proteins, in similar manner than RSV (Fig. 1).[18] In contrast to RSV, hMPV lacks the NS1 and NS2 genes, thus the inhibition of the type I IFN pathway is less robust than the one is observed in RSV.[154] The G protein, a glycoprotein involved in the attachment of the viral particle,[155] inhibits the type I IFN pathway by

targeting the retinoic-inducible gene 1 (RIG-I), thus it contributes in inhibiting the antiviral innate response of the host.[156] Likewise, the SH protein, a type 2 transmembrane protein with properties as a viroporin, has been shown to inhibit the NF$\kappa\beta$ pathway, as infection with a recombinant virus lacking the SH gene increased the NF$\kappa\beta$-dependent transcription pathway in BALB/c mice.[157] The M protein, which participates in virus assembly and packaging,[158] stimulates the inflammatory response *in vitro* by inducing the maturation of monocyte-derived DCs and the secretion of inflammatory cytokines by these cells, including IL-8, IL-6, IL-1β and TNF-α (Table 2).[159]

In addition, the M2–1 protein is critical for hMPV replication and pathogenesis through its Zinc binding activity, since a recombinant hMPV carrying mutations in the zinc binging motif was highly attenuated in cotton rats.[160] This is consistent with studies that demonstrate that hMPV lacking the M2–1 gene could not replicate in hamsters.[161] However, is not essential for hMPV infectivity and growth *in vitro* as deletion of the ORF of the M2–1 protein is dispensable for viral replication in VERO cells.[161] In contrast, the hMPV M2–2 protein, defined as a terminator factor, since it inhibits viral replication and transcription,[162] interact with MAVS,[163] a protein that links the cytoplasmic viral sensors RIG-I and the melanoma differentiation associated protein 5 (MDA5) to the downstream TNF receptor-associated factors (TRAFs) and IκB kinases (IKKs), which are known to induce the type I IFN-pathway through the activation of IRF-3 and NFκB.[163] Thus, hMPV M2–2 protein contributes in decreasing efficiently the anti-viral response.

Concluding remarks

Adequate T cell priming is critical for establishing effective anti-viral immune responses.[164,165] Due to the importance of this process several respiratory viruses, including hRSV[27] and hMPV[26] have evolved different mechanisms to impair T cell activation. Indeed, both of these viruses impair the T cell response, causing, at least in part, an aberrant adaptive immune response and poor immunological memory against hRSV[71,166] and hMPV.[136] However, in both cases, memory T cells are produced after infection but the role of these cells are still controversial because reinfection episodes are recurrent in both viruses. By the other hand, the viral mechanism underlying the inhibition of T cell functions for both viruses is different.[26,131] hRSV impairs T cell activation by preventing a mature immune synapse assembly, possibly through the hRSV N protein at the DC plasma membrane.[131] Conversely, hMPV prevents T cell activation,[26] likely though a soluble factor(s) without interfering with the formation of a mature immune synapse and Golgi polarization in the T cell, as the inhibition of the TCR signaling is induced with the supernatants of hMPV-infected DCs.[26]

Interference at the immune synapse level by hRSV and T cell activation by hMPV prevents the T_H1 polarization required to induce an efficient antiviral response.[26,27] Additionally, hRSV infection triggers detrimental inflammation in the airways that is characterized by an exacerbated T_H2 response, thus preventing adequate viral clearance.[167] This exacerbated T_H2-type immune response to hRSV could be mediated by TSLP through activation of the OX40/OX40L interaction, which consequently stimulates an inappropriate subset of T cells.[80] In addition, IL-25, detected in lungs of infected hRSV mice, can also contribute to the hRSV-mediated lung pathology.[91]

Similarly to hRSV, hMPV activates the TSLP pathway to induce a mixture of T_H2- and T_H1-type responses, most likely through OX40/OX40L interactions.[141] This mixed induction causes pathology and promotes viral replication in mice lungs.[141]

Similarly, to T cells response, antibody response is not effective to reduce reinfection and probably this is mediated by the interaction between B and T cells during infection. Where T cells that present a polarization to Th2 in case of hRSV or Th1/Th2 in case of hMPV, modulate B cell response generating non-protective antibodies. Contrary, has been showed that neutralizing antibodies are produced after infection with these viruses but the proportion between neutralizing and non-neutralizing could be mediate a better outcome.

The respective evasion mechanisms induced by each virus could synergistically act to prevent the activation of an effective anti-viral T and B cell response during infection periods. In this sense, is important to advance in human studies with these two viruses and the mechanism behind its immune modulation.

Abbreviations

AEC	Airway epithelial cell
BEC	bronchial epithelial cell
BCG	Bacillus Calmette-Guérin
CTL	cytotoxic T lymphocyte
DC	Dendritic cell
hMPV	Human metapneumovirus
IFN	interferon
M	matrix
mDC	myeloid dendritic cell
N	nucleoprotein
NS	non-structural

pMHC	peptide major histocompatibility complex
hRSV	human Respiratory Syncytial Virus
SH	small hydrophobic
SV	Sendai virus
TCR	T cell receptor
TSLP	Thymic stromal lymphopoietin

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

The authors acknowledge Ms. Virna Salazar for critically reading the manuscript.

Funding

Funding awarded by Grants NO 1158262 and 3150559, from the National Fund for Scientific and Technological Development (FONDECYT) program, Ministry of Education, Chile; Grant NO D11/1080, from the Fund for the Promotion of Scientific and Technological Development (FONDEF), Ministry of Education, Chile; and Grant P09/P016-F, from the Millennium Institute of Immunology and Immunotherapy, Ministry of Economy, Chile.

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Human Respiratory Syncytial Virus: Infection and Pathology

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Semin Respir Crit Care Med 2016;37:522–537.

Abstract

The human respiratory syncytial virus (hRSV) is by far the major cause of acute lower respiratory tract infections (ALRTIs) worldwide in infants and children younger than 2 years. The overwhelming number of hospitalizations due to hRSV-induced ALRTI each year is due, at least in part, to the lack of licensed vaccines against this virus. Thus, hRSV infection is considered a major public health problem and economic burden in most countries. The lung pathology developed in hRSV-infected individuals is characterized by an exacerbated proinflammatory and unbalanced Th2-type immune response. In addition to the adverse effects in airway tissues, hRSV infection can also cause neurologic manifestations in the host, such as seizures and encephalopathy. Although the origins of these extrapulmonary symptoms remain unclear, studies with patients suffering from neurological alterations suggest an involvement of the inflammatory response against hRSV. Furthermore, hRSV has evolved numerous mechanisms to modulate and evade the immune response in the host. Several studies have focused on elucidating the interactions between hRSV virulence factors and the host immune system, to rationally design new vaccines and therapies against this virus. Here, we discuss about the infection, pathology, and immune response triggered by hRSV in the host.

Keywords
- human respiratory syncytial virus
- virulence factors
- innate immune response
- adaptive immune response
- extrapulmonary manifestations
- central nervous system

Acute lower respiratory tract infections (ALRTIs) are the major cause of morbidity and mortality in young children, the elderly, and immunocompromised individuals worldwide.[1] Importantly, the human respiratory syncytial virus (hRSV) is the principal microbial agent known to cause ALRTIs.[2–5] Most clinical manifestations caused by hRSV range from mild symptoms, such as rhinorrhea, cough, congestion, low-grade fever, reduced appetite, and respiratory distress, to severe alveolitis, bronchiolitis, and pneumonia.[6] The heterogeneity of the diseases caused by hRSV depends, among others, on host risk factors, including preterm birth,[7] congenital heart disease,[7,8] chronic lung diseases,[9] and immunosuppression.[10]

hRSV infections are considered highly contagious, affecting nearly 70% of infants before the first year of life and nearly 100% of children by the age of 2.[11] Worldwide, approximately

Issue Theme Respiratory Viral Infections; Guest Editor: Sunit K. Singh, PhD

DOI http://dx.doi.org/10.1055/s-0036-1584799.
ISSN 1069-3424.

34 million new cases of hRSV-associated ALRTI occur in children younger than 5 years and as much as 200,000 deaths are estimated annually.[12] Indeed, hRSV causes a minimum of 3.4 million hospitalizations per year in the United States.[12] Due to the high hospitalization rates and increased health care system costs, hRSV infections are considered a major public health burden globally. For instance, medical costs related to hRSV infection in hospitalized individuals is estimated at 394 million USD annually, just in the United States.[13] Thus, safe and effective vaccines against this virus are urgently needed.

hRSV spreads rapidly and efficiently throughout the population by inhalation of aerosolized droplets of infectious viral particles, or directly through the contact of these droplets with the ocular mucosa.[14,15] One of the most relevant characteristics of hRSV infection is the capacity to produce high reinfection rates in individuals during epidemic outbreaks. For instance, epidemiological studies indicate that approximately 36% of individuals can be reinfected at least once during a given season.[13,16,17] Based on the current evidence, these reinfection episodes may be due to the elicitation of a deficient or hampered cellular and humoral immune memory after a first exposure to the virus.[4,18–20]

Bronchiolitis is one of the most severe illnesses caused by hRSV infection. The term *bronchiolitis* is referred to distal bronchiole inflammation and obstruction. Such an obstruction reduces the airflow into small airways and causes an alteration in the exhalation capacity. This phenomenon can lead to lung hyperexpansion, lung function alterations, increased mucus production, atelectasis, and wheezing.[21,22] Furthermore, infection of alveolar epithelium by hRSV leads to pneumonia, which prevents an efficient gas-exchange process. Such infection triggers distal airway inflammation, leading to severe pulmonary disease.[22] The initial response against hRSV infection is given by the airway epithelial cells (AECs), which promotes the recruitment of effector immune cells at the site of infection. hRSV-associated immunopathology is characterized by the expression of proinflammatory cytokines and the subsequent perivascular/peribronchial infiltration by mononuclear cells, mainly neutrophils and lymphocytes.[21] This exacerbated inflammation is thought to trigger an unbalanced and pathogenic T helper (Th)2 response.

For more than 50 years, most research on hRSV has focused on elucidating the mechanisms involved in respiratory pathology, as well as on the design of vaccines and therapies against hRSV.[23–25] Nowadays, it is known that hRSV is able to migrate from the airways to various tissues in the host, such as heart, kidney, liver, and brain, thereby producing diverse clinical manifestations, including cardiopathy, hepatitis, and encephalitis.[6,26–28] As a consequence, extensive research aimed at understanding the extrapulmonary manifestations of hRSV infection has gained attention in the past few years. For instance, neurological abnormalities associated with hRSV infection in patients with severe bronchiolitis have been detected. Such patients have shown symptoms including seizures, apnea and altered proinflammatory cytokine levels in cerebrospinal fluid (CSF).[26] However, the cellular and molecular bases for the encephalopathy caused by hRSV remain unknown.

hRSV Characteristics

hRSV is an enveloped, negative-sense, singled, stranded RNA virus, which belongs to the Paramyxoviridae family, *Pneumovirus* genus.[29] The virus was first described as a human pathogen in 1957, after being associated with the chimpanzee coryza virus.[30] The viral genome is nonsegmented RNA, 15.2 kb in length, that encodes 10 genes and 11 proteins, particularly because of the M2 gen, which has two open reading frames.[31] The order of the genes within the genome from 3′ to 5′ is NS1-NS2-N-P-M-SH-F-G-M2-L, and these genes are transcribed into 10 monocistronic, capped, methylated, and polyadenylated mRNAs (►**Fig. 1**).[29,31]

Within the viral particle, nine proteins can be found: N, P, M, SH, F, G, M2.1, M2.2, and L.[29,31] Three proteins, F, G, and SH (small hydrophobic), are expressed at the surface of the particle, attached to the virion membrane (►**Fig. 1**).[31,32] Both F and G proteins are the main antigenic proteins against which the majority of the antibodies are raised after hRSV infection.[2,5] The F protein is highly conserved between hRSV serogroups with less than the 10% of sequence diversity between the A and B groups.[33] The F protein is generated from mRNA at the cytoplasm of the host cell and converted to an active protein after cleavage by furin-like protease in the Golgi apparatus. This proteolytic cleavage generates three polypeptides, in which C- and N-terminal (F1 and F2 subunits) polypeptides are linked by two disulfide bonds.[11,34] The active form of the F protein in the viral particles is a trimer in a prefusion conformation.[35] This protein mediates the fusion of the viral envelope with the host membrane by changing its conformation to a postfusion form after interacting with the receptor.[36] Importantly, the F protein can interact with different proteins on the surface of host cells, such as TLR4,[37] ICAM-1,[38] and nucleolin.[39] Particularly, nucleolin has been described as the main hRSV receptor that interacts with the F protein, because it was shown that nonpermissive cells expressing nucleolin became susceptible to hRSV infection.[39] Furthermore, no new virions could be made after infection with a mutant virus lacking this protein.[40] These data suggest that the F protein is one of the most important hRSV proteins contributing to infection and interaction with host cells.

The G protein is responsible for the attachment of the virus to the host cell.[29,31] This protein is highly glycosylated and can interact with heparin[41] and annexin II,[42] based on sugar interactions. Likely, this interaction allows a proper approach between the F protein and nucleolin.[32] In addition, the G protein exists also as a secreted form, which has been shown to be important for capturing antibodies generated by the host against this protein. This soluble form prevents the opsonization and neutralization of the virus by G-specific antibodies.[43] Furthermore, another important feature of this protein is the capacity to impair the function of chemokines and cytokines due to a CX3C chemokine-like motif that can mimic and compete with these molecules for the interaction

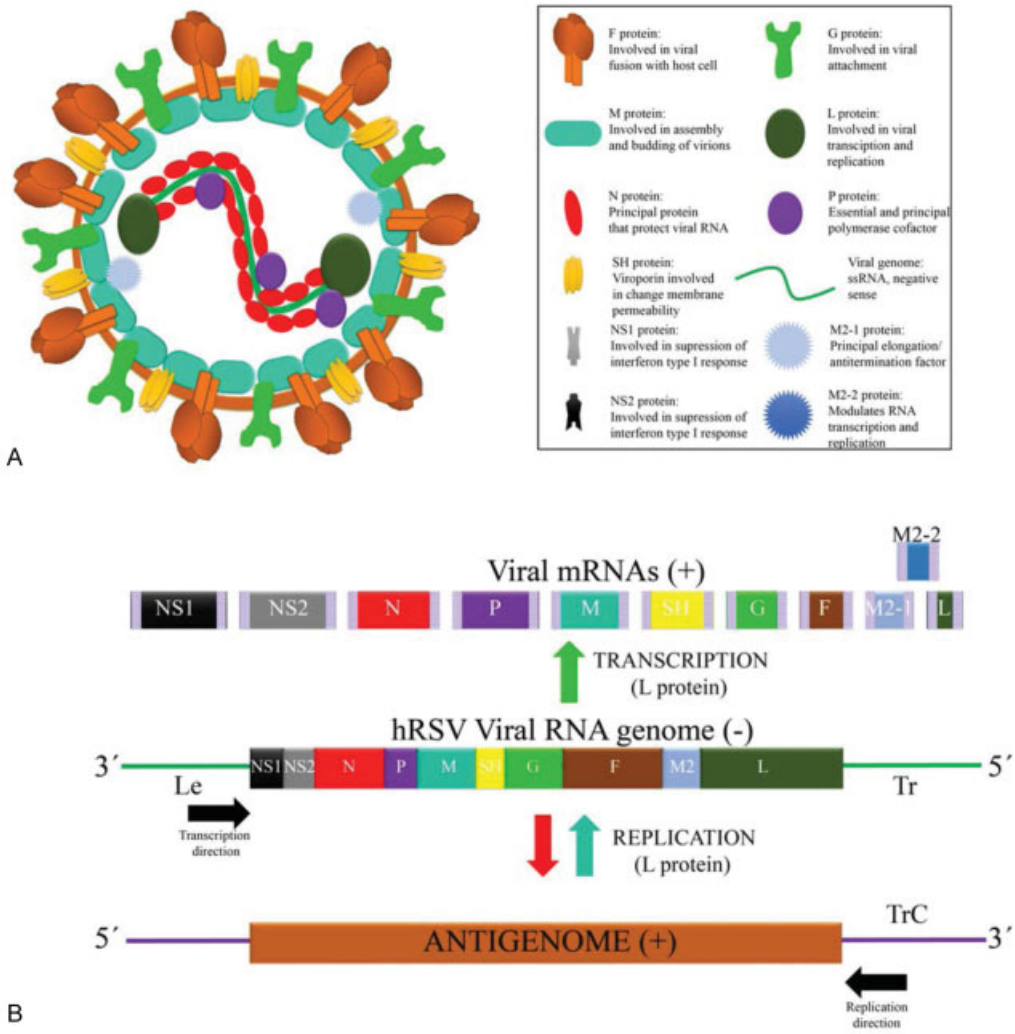

Fig. 1 hRSV virion and genome structure. (**A**) Schematic representation of hRSV virion particle. In the rectangle, each protein are represented with their principal associated function. (**B**) Schematic representation of hRSV genome. Transcription is mediated by L protein which generates 11 viral mRNAs, with cap (vertical bars at the beginning) and polyA (horizontal bars at the ends). One mRNA for each proteins and width of each box represent the quantity of transcription rate of each gene. Replication is mediated by L protein and is necessary for the generation of antigenome product to generate new hRSV RNA. TrC segment in the 3′ is where replication promotor is located.

with their receptors and, thus, modulate $CD8^+$ T-cell responses.[44,45] Moreover, the G protein was shown to display structural homology with tumor necrosis factor (TNF) receptors and is likely to interact with TNF family cytokine members, conducting to a misbalance in the inflammatory response mediated by these molecules.[46] Although G protein is not totally necessary for hRSV infection, it plays an important role at modulating the immune response triggered by hRSV infection.

The SH protein locates at the surface membrane of the virion has been shown to display two different forms that vary in size depending on the hRSV serotype: one of 64 amino acids (serotype A) or 65 amino acids (serotype B).[47,48] SH protein has been described as a viroporin belonging to the family of small/highly hydrophobic viral proteins that are capable of forming ion channels in cellular membranes.[49] In fact, the hRSV-SH protein has been described to allow the entrance of low-molecular-weight compounds and change the permeability of the cellular membrane.[49–51] In addition, the SH protein seems to be involved in the activation of the inflammasome, particularly through signal 2 by the activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), which triggers the cleavage of pro-IL1β and secretion of this cytokine.[50] Surprisingly, the SH protein has not been described to be involved in virus entry into host cells, as a mutant virus lacking the SH protein can infect and replicate inside permissive cells (in vitro) and generate syncytia similar to wild-type virus.[11,48] However, this mutant virus (hRSVΔSH) is attenuated in vivo, which suggests that this protein may work as a virulence factor during hRSV infection.[11,34,47] Other features described for the SH protein include an antiapoptotic effect that promotes viral replication.[48] Indeed, a hRSVΔSH mutant virus led to larger syncytia and more apoptosis rates, as compared with WT hRSV.[48] In addition, studies with the hRSVΔSH mutant virus showed that the SH protein inhibit NFκB by the overexpression of TNF-α[48] Although the functions of the

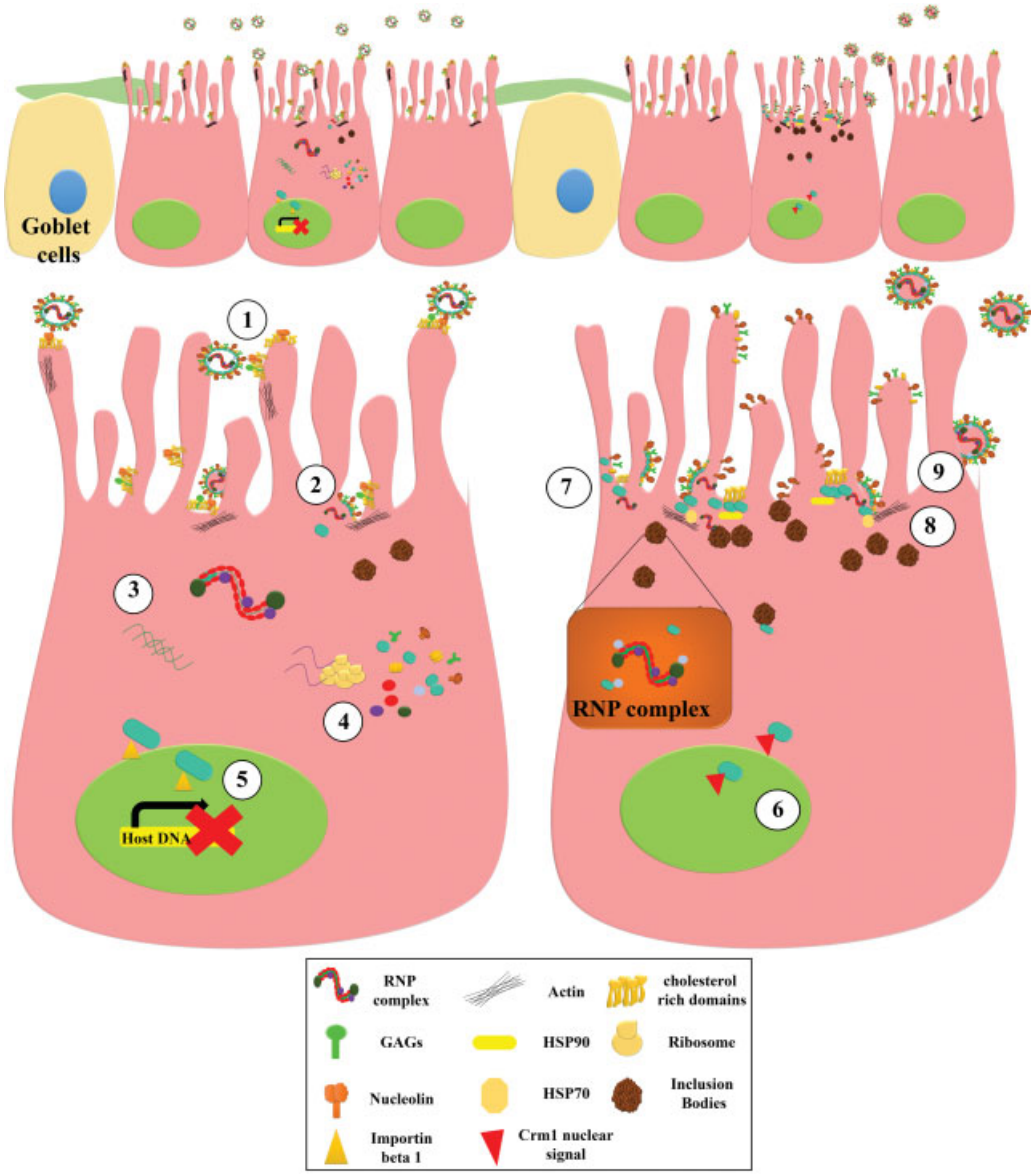

Fig. 2 hRSV infection of airway epithelial cells: (1) G and F proteins interact with host receptors to initiate virus entry. (2) RNP complex is release, by the separation of RNP with M protein. (3) Replication of viral RNA. (4) Transcription and translation of viral proteins. (5) M protein is imported to the nucleus and inhibit host cells transcription. (6) M protein is exported to the nucleus and is transported to cholesterol-rich domains. (7) M protein starts to interact with surface proteins as beginning of assembly. (8) RNP interacts with M protein to finish the assembly process. (9) Budding of nascent virions.

SH protein have not been fully defined, it clearly promotes hRSV replication and dissemination.

Below the virus envelope lie the other viral proteins, namely, proteins N, P, L, M, and M2–1.[29,31] The nucleoprotein N is in close contact with the viral genome and is thought to protect the viral RNA from nucleases and together with P and L proteins constitute the hRSV ribonucleoprotein (RNP), which regulate the transcription and replication of the viral RNA.[52,53] Importantly, the N protein prevents the genomic RNA from forming double-stranded RNA structures, as well as RNA cleavage by host components.[54] Noteworthy, its structure with viral RNA has been recently determined.[55,56] The N protein is generally located within cytoplasmic inclusion bodies, where it interacts with the M2–1, P, and L proteins. During the first hours after infection, the N protein has been shown to associate within these structures with MDA5 and mitochondrial antiviral signaling (MAVS), which contribute to the innate immune response.[57] The sequestering of these molecules by the N protein would cause a poor detection of viral genome by these nucleic acid sensors, which could dampen the antivirus interferon (IFN) response.[57] Importantly, it has been recently described that the N protein can be expressed on the surface of infected epithelial and dendritic cells (DCs).[58] Expression of this protein impairs the capacity of hRSV-infected DCs to activate T cells, probably due to a blockade of the interaction of peptide-MHC (pMHC; MHC, major histocompatibility complex) complexes with the T-cell receptor (TCR).[58] Such novel role for the N protein has provided new insights relative to the localization of this protein and how hRSV can interfere with the induction of

protective T-cell responses, which is often impaired by hRSV infection.[3,19,58]

The viral phosphoprotein P has been described as a cofactor of the RNP complex and the most important for the L protein. Indeed, the P protein can interact with N protein, allowing it to access the L protein.[52,59] The P protein is highly stable as a tetramer and the C-terminal domain (P_{CTD} from the residue 161 to the residue 241) is critical for the interaction with the L and N proteins.[60–62] Consistent with this notion, the phosphorylation of the P protein has been shown to play an important role in the pathogenesis mediated by the virus, as a virus lacking the five phosphorylation sites in this protein shows reduced replication in vivo in mice and cotton rats, as well as in vitro in HEp-2 sites.[63] However, this recombinant virus can replicate normally in Vero cells, thereby suggesting that the phosphorylation of the P protein is necessary for an efficient viral replication.[63]

The RNA-dependent RNA polymerase (RdRp) L protein is the lesser expressed of all viral proteins in the infected host cells. The principal role of this protein is the replication and transcription of the viral genome, regulated and supported by the RNP complex.[64] Because hRSV is a negative sense RNA virus, the L protein transcripts the genome directly into mRNA for the expression of each hRSV gene.[59] In this process, the L protein recognizes a promoter region in the 3′ extreme of the negative RNA strand and starts the transcription of each gene. Accumulating evidence suggests that transcription is modulated by the N protein.[52,53,59] During the replication process, the L protein copies the complete virus genome from a negative sense RNA into a positive sense RNA, which is called an *antigenome*. This RNA is then used as a template to generate new negative sense RNA, which finally will be encapsided in the virions.[59,65] A typical characteristic of the L protein is that in the process of transcription, it generates a gradient of gene expression, from 3′ to 5′, producing more mRNAs of the genes 3′ as compared with those 5′ in the genome.[66,67]

The matrix protein M promotes viral assembly and is essential for hRSV replication.[68] Early after infection, the M protein is located in the nucleus, where it is able to decrease the transcriptional activity of the host cells genes.[69–71] Another role of matrix protein is to arrest the cell cycle in the G1 phase, as shown in A549 cells. It also arrests the G1 and G2/M phases in human bronchial epithelial cells.[72] These actions, which are p53-dependent, increase hRSV replication.[72] In addition, the M protein is directly related to the maturation of viral filaments.[73] In this line, it has been described that hRSV strains that are null for the M protein show significantly lesser infective progeny particles.[73] Moreover, not only lesser new viral particles are generated but also protein trafficking is affected, particularly with the N protein being concentrated in cytoplasmic inclusion bodies, before virus budding. This phenomenon suggests that M protein is important for triggering the trafficking of viral proteins to the budding site.[73] Other studies show that the M protein is expressed in inclusion bodies and interact with the M2–1 protein, as a means to interact with the RNP complex.[74] The M protein is also capable of inhibiting viral transcription and interact with hRSV G and F proteins to signal the assembly of the virions.[75,76]

The M2–1 protein is involved in the transcription process as part of the RNP complex, and acts as an antitermination/elongation factor promoting the transcription of all hRSV genes, aiding the L protein to proceed with transcription of viral genes.[77] Interestingly, it was also showed that NS1 and NS2 genes can be transcribed by the L protein independently of the hRSV M2–1 protein, suggesting that several transcription mechanisms for viral genes exist.[77] To exert its antitermination functions, M2–1 needs to form as tetramers.[78] Importantly, without this oligomerization, the protein cannot function correctly, which is supported by using a mutant virus for M2–1 protein that cannot generate tetramers.[78] As M2–1 is part of the RNP complex, this protein can interact with different protein of this complex.[74,78–81] The interaction with the N protein is particularly mediated through interactions with the viral RNA, as treatment with RNAses disrupts this binding.[80] Another function of this protein is the activation of nuclear factor-κB (NF-κB) and its association with the RelA protein.[82] On the other hand, the M2–2 protein is involved in the regulation of transcription to the replication by the virus polymerase.[83,84] This effect was discovered by studying an ΔM2–2 virus, in which the accumulation of mRNA was higher in cells infected with this mutant virus, as compared with WT virus.[84] In addition, viral titers were reduced over 1,000 times in the first 5 days and over 10 times after 7 to 8 days when the ΔM2–2 virus was used.[84] Thus, these two proteins play a critical role in the regulation of transcription and replication of hRSV RNA.

Besides the structural proteins mentioned earlier, the hRSV genome also encodes two nonstructural proteins, namely, NS1 and NS2, both with the capacity to interfere with host type 1 IFN innate response. This process negatively modulates DCs maturation and T-cell responses.[85,86] The NS1 protein interferes with the activation of the IFN gene promotor by inhibiting the phosphorylation of interferon regulatory factor 3 (IRF-3).[87] NS2 also can interfere with the activation of IRF-3 by its interaction with retinoic acid–inducible gene 1 (RIG-I), inhibiting the activation of IFN response genes (IFNRs).[88,89] The NS1 protein is able to interrupt the signaling of JAK/STAT pathways that are activated by IFN receptor pathways, particularly through the degradation of STAT-2.[88,89] Both proteins, NS1 and NS2, are able to promote phosphoinositide 3-kinase (PI3K) pathways promoting the survival of infected cells, increasing viral yield.[90] In this line, interference with the type 1 IFN response by NS1 and NS2 proteins blocks DCs maturation.[86] Concomitantly, ΔNS1/NS2 and ΔNS1 viruses are able to increase the expression of maturation markers on DCs compared with WT hRSV.[86] Furthermore, this effect in DCs could interfere with their capacity to activate T cells.[85,86] Indeed, human DCs infected with a ΔNS1 virus show increased activation and proliferation of $CD8+$ T, increase the activation and proliferation of Th17 protective cells, and decrease the activation of $IL\text{-}4^{+}$ $CD4^{+}$ T cells, which are related to increased hRSV pathogenesis.[85] In addition, a recent study showed that the expression of NS1 and NS2 proteins by human bronchial epithelial cells

decreases the polarization of T cells toward Th1, Th2, and Th17 phenotypes by the NS1 protein, and Th2 and Th17 polarization by the NS2 protein.[91] Thus, these two nonstructural proteins are very important virulence factors that directly affect the immune response of the host.

Viral Infection Cycle

The infection of target cells, such as airways epithelial cells (AECs), starts with the attachment of virions to the cell surface aided by the G protein, which interacts with heparan sulfates and chondroitin sulfate B glycosaminoglycans (GAGs).[92] After this interaction, which helps the virus approach the membrane of the cell to be infected, the F protein contacts its receptor, nucleolin (►**Fig. 2**).[39] The entry of hRSV has been described to occur particularly in cholesterol-rich microdomains on the cell surface.[93] Further, the fusion membranes require the participation of Pak-1 in the rearrangement of actin filaments.[93] Entry via endocytosis has been discarded because the use of Dynasore shows that, despite dynamin-endocytic process is inhibited, viral fusion still occurs.[93] Therefore, the fusion of the hRSV membrane with the host cell membrane depends on the interactions of hRSV G and F proteins with their receptor and the rearrangement of actin filaments close to cholesterol-rich microdomains (►**Fig. 2**).[32,93]

The fusion of the viral and cell membranes triggers the release of the viral nucleocapsid content into cytoplasm. Here, the nucleocapsid is dissociated from the RNP complex and repetitions of the M protein, which is mediated by the phosphorylation of the P protein (►**Fig. 2**).[94] Importantly, this process is mediated not only by viral proteins but also by host cell enzymes, such as glycogen synthase kinase-3 (GSK-3) β and protein phosphatase 2A (PP2A).[94] The transcription process mediated by the function of L protein mainly occurs in cellular inclusion bodies together with N and P proteins.[95] As mentioned earlier, the L proteins associated with the P protein are able to recognize the promotor region on the 3′ of viral RNA, and initiate transcription of viral genes.[59,67] The polymerase initiates transcription in gene start (GS) regions carrying out mRNA capping and methylating the 5′ of the messenger.[59,67] Then, the L polymerase recognizes a gene end signal at the end of the mRNA and carries out polyadenylation.[59,67] This process goes on again after recognition of a new GS sequence downstream of a previously transcribed gene. It is known that the minimal proteins required for transcription are N, P, and L. The M2–1 protein also appears to be important because of its ability to interact with all RNP proteins,[78–81] included with the M protein.[74] Furthermore, host proteins are also involved in the transcription process; for instance, profilin, an actin-modulatory protein, is required for an optimal transcription.[96] Additionally, host heat shock proteins (HSPs) are also involved in this process, particularly HSP90 and HSP70. Both proteins are expressed in lipid rafts and are associated with the viral RNP complex. Recently, it was described that HSP90 is critical for the stability and functionality of the L polymerase and that HSP70 is necessary for efficient RNA synthesis.[97,98] When the viral genome is replicated, the L polymerase recognizes the TrC promoter region at the 3′ of the antigenome and generates genomic hRSV-RNA.[99] This new RNA strand is immediately encapsulated by the N protein.[59]

Virus assembly, after viral RNA transcription and replication, depends on the M protein localization and occurs at cholesterol-rich domains.[73,100] As described earlier, at the beginning of the infection cycle, the M protein is transported to the nucleus by the interaction with importin-β1, where M protein can interfere with cellular transcription (►**Fig. 2**).[69,70] The M protein is exported from the nucleus to the cytoplasm by a Crm1-dependant nuclear signal so that it localizes to lipids rafts.[71] When the M protein is associated with these domains, the assembly and budding process begins and involves interactions with surface proteins F, G, and SH.[101] Accordingly, a recent report showed that the F and G proteins are expressed on the surface of ciliated cells.[102] Thereafter, the interaction of the F protein with the M proteins promotes assembly of the new virions (►**Fig. 2**).[103] On the other hand, the M2–1 protein has been shown to bind to the M protein, promoting its assembly with the RNP complex.[74] The formation of filaments that contain the virions is regulated by the hydroxymethylglutaryl coenzyme A reductase enzyme, which mediates changes in F-actin to generate viral filamentous projection that are involved in cell-to-cell transmission.[104] Finally, the budding process is not regulated by the endosomal sorting complex required for transport machinery, as occurs for other enveloped RNA viruses. In its place, hRSV budding is controlled by the RAB11 family interacting protein 2 (FIP2),[105] which has been described as a novel pathway for this type of process. Taken together, the hRSV-infective cycle depends of three main processes: (1) hRSV protein localization, where inclusion bodies and rich cholesterol sites are principal places where the hRSV proteins can be founded; (2) hRSV protein interaction, the particular interaction between RNP complex and accessory proteins for replication and transcription and the interaction of M protein to surface protein triggering the virion budding; and (3) interaction of host cell proteins with hRSV proteins and structures, principally how host cells help in the release of nucleocapsid at the beginning of the process and how a novel process of budding depends of host proteins (►**Fig. 2**).

Innate Immune System against Respiratory Syncytial Virus

Upon infection, AECs, DCs, and macrophages play a key role in the innate response against hRSV in the lungs.[106] Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), retinoic acid–inducible gene I (RIG-I)-like receptor (RLR) family members, and NOD-like receptors (NLR) are activated following hRSV infection.[107] TLRs have been shown to be fundamental for the recognition of hRSV.[108–110] TLR4/TLR6 triggers a signaling cascade that activates innate immune responses by enhancing the production of TNF-α, interleukin (IL)-6, CCL2, and CCL5.[110] HRSV is also sensed by endosomal TLR3 and TLR7, which triggers CCL5, IFN-α, and IFN-β production by TRIF-mediated- and MyD88 pathways,

respectively.[108,109] The NLRP3 inflammasome, which belongs to the NOD-like receptor family, senses the SH protein of hRSV and triggers pro-IL-β cleavage and secretion of IL-1β cytokine.[50]

After PRRs are activated, NF-κB, IRFs, and ATF-2/cJun are promoted.[111] As a consequence, the expression of type 1 IFNs and the production of inflammatory cytokines, such as IL-8 (IL-8/CXCL8), IL-4, IL-5, IL-6, and IL-10, as well as chemokines and adhesion molecules are triggered.[112,113] Such signaling cascades also prompt the recruitment of immune cells, such as eosinophils, monocytes, and neutrophils to the lungs.[114] As a result, exacerbated Th2-mediated airway inflammation is triggered, which contributes to lung damage (►**Fig. 3**).

hRSV also induces the secretion of both surfactant proteins A and D (SP-A and SP-D) in the airways.[115] These proteins play an important role in the regulation of the immune response in the lung.[116,117] Indeed, SP-A and SP-D can also stimulate macrophage activation by increasing chemotaxis, phagocytosis, and increase cytokines secretion.[118] Interestingly, SP-D is able to bind hRSV G protein, thereby inhibiting hRSV infection in vivo and in vitro.[119] Recently, hRSV infection of AECs has been shown to be involved in the production of thymic stromal lymphopoietin (TSLP) and epithelial cell-derived IL-7, an IL-7-like cytokine.[120,121] Interestingly, these cytokines, together with IL-25 and IL-33, are related with acute exacerbations of asthma and Th2 inflammatory responses triggered by viruses.[122,123] Importantly, Qiao et al showed the induction of functional maturation of myeloid DCs (mDCs) in hRSV-infected AECs through Th2-polarizing molecules, such as thymus activation-regulated chemokine (TARC/CCL17) and OX40 ligand (OX40L) activation.[121] Indeed, they suggested that mDCs activation was mediated by TSLP, as TSLP-targeted siRNA abrogated mDCs activation.[121]

Both hRSV NS1 and NS2 proteins inhibit the secretion of type 1 IFNs in host cells by decreasing the levels of TNF receptor-associated factor 3.[124] Furthermore, survival of infected epithelial cells is achieved, thanks to NS1 and NS2, which activate the PI3K pathway as previously mentioned.[108,125] Consistent with this notion, suppression of these proteins resulted in accelerated apoptosis in hRSV-infected cells and consequently reduction in the virus yield.[90] Importantly, the hRSV nucleoprotein could also attenuate the IFN response, as colocalization of this protein with RIG-1 and MAVS protein were found 6 hours postinfection.[57]

Adaptive Immune Response against Respiratory Syncytial Virus

T cells play an important role in hRSV infection. $CD4^+$ and $CD8^+$ T cells have been shown to play pivotal roles in both hRSV clearance and pathogenesis. Such a dichotomy in the role of virus-specific T cells has been observed for infection, and also lung damage after challenge with the virus.[126] For instance, T cells expanded in mice experimentally infected with hRSV have been shown to be essential for the clearance of the hRSV, although this immune response causes an exacerbated activation of the immune system within the airways.[127] Similarly, mice immunized with a vaccine

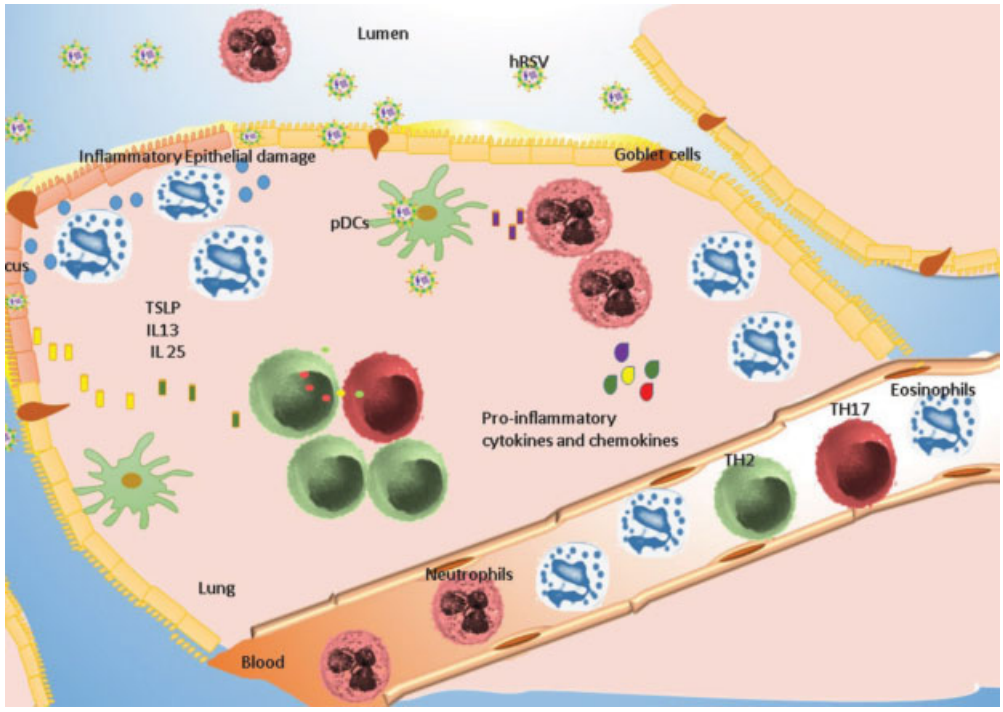

Fig. 3 Immune response trigged by hRSV infection in respiratory airways. hRSV reaches the lower respiratory tract and is recognized for respiratory epithelial cells by pattern recognition receptors (PRRs) expressed leading to the secretion of innate cytokines and chemokines such as TSLP, IL-13, and IL-25. Inflammatory cytokines and chemokines promote the recruitment of innate immune cells into the lungs, such as eosinophils, neutrophils, and monocytes. The inflammatory environment induced by the innate immune cell recruitment and mucus production together with an excessive Th2 and Th17 response generate destruction of the respiratory epithelium and the obstruction of distal bronchiolar airways.

consisting of formalin-inactivated virus suffered "vaccine-enhanced disease."[128] Pathology was observed as an exacerbated increase in the immunological response of vaccinated mice to the virus upon challenge, which was manifested by increased eosinophil infiltration and Th2-like responses in the lungs.[128] Importantly, in this scenario, T cells were described as a critical cell subset mediating the "vaccine-enhanced disease."[129] Furthermore, Th17 cells have also been shown to contribute to hRSV airway pathology in human newborns.[130] On the contrary, mice immunized with BCG expressing either the hRSV nucleoprotein (BCG-N) or M2 protein (BCG-M2) showed a significant recruitment of IFN-γ-producing T cells in the lungs, promoting a Th1-response, which was protective and led to virus clearance without detrimental inflammation.[131]

Cytotoxic $CD8^+$ T cells (CTLs) are usually responsible for viral clearance by recognizing the F and N proteins of the hRSV.[132] However, hRSV-specific $CD8^+$T cells have also been shown to play a role in a detrimental immune response. Consistent with this notion, depletion of $CD8^+$ T cells reduced the severity of hRSV-induced disease during primary and secondary infection.[133] Such detrimental responses have been suggested to occur because $CD8^+$ T cells play a role in the regulation and activation of the $CD4^+$ T cells toward Th2 polarized phenotypes.[134] On the other hand, a reduction of intracellular granzyme B content, diminished secretion of IFN-γ, and impairment of perforin expression have been observed in $CD8^+$ T cells in the lungs of hRSV-infected individuals.[135,136]

Inefficient T cells against hRSV have been reported in infected individuals.[137,138] Such suppression of T-cell activation is thought to be due to an impairment of DC-T cell immunological synapse assembly, which has been observed by a decrease in Golgi polarization in T cells cultured with hRSV-infected DCs[3] (►**Fig. 4**). This impairment of immunological synapse assembly also causes improper TCR engagement, leading to the failure of antigen-specific T cells priming.[3] Interestingly, these authors reported that DC-derived soluble factor mediators were not involved in this suppression.[3] By contrast, alteration of cytokine secretion surrounding the DC-T cell environment by hRSV have been shown to modulate T cell response.[139] Furthermore, hRSV has also been observed to alter the quantity of surface cognate peptide-MHC, impairing T cell activation.[140] Likewise, secretion of Th1-like cytokines is reduced in hRSV-infected DCs and may decrease cytotoxic T cell activity.[3] Taken together, immunological synapse is a fine system that could be exploited by hRSV at multiple levels. Thereafter, further research is needed to elicit mechanisms of hRSV to interfere in the immunological synapse.

Regulatory T cells (Tregs), characterized by the expression of forkhead box transcription factor (Foxp3), have emerged as key cells in preventing hRSV inflammatory-associated disease.[141,142] This notion is supported by studies in which depletion of $CD4^+FOXP3^+CD25^+$ cells prior to infection results in increased hRSV-associated pathology.[141] Likewise, these mice display enhanced weight loss, cellular influx in the lungs, and high eosinophils in the airway after infection.[142] Interestingly, this was associated with an increase of IL-13^+ T cells and enhanced expression of the Th2-like transcription factor GATA-3 in the airways.[142] Taken together, Tregs play an advantageous role against hRSV infection by downregulating unfavorable proinflammatory cytokines, thereby reducing lung damage.

In addition to the role of T cells in hRSV infection, macroautophagy in DCs has emerged as a key process contributing to proper antiviral adaptive responses against hRSV.[143,144] Mice deficient for autophagy processes (beclin +/− mice) display higher weight loss, elevated Th2 cytokine production, and eosinophil infiltration in the lungs.[143] Indeed, DCs with impaired autophagy machinery presents an amelioration of IFN-γ and IL-17 stimulation in $CD4^+$ T cell cocultures.[143]

Extrapulmonary Manifestations Caused by Severe hRSV Infection

Despite human AECs are the main target for hRSV, several reports have shown that this virus can also infect immune cells, such as macrophages, monocytes, DCs, and B lymphocytes.[3,145–147] Moreover, endothelial and neuronal

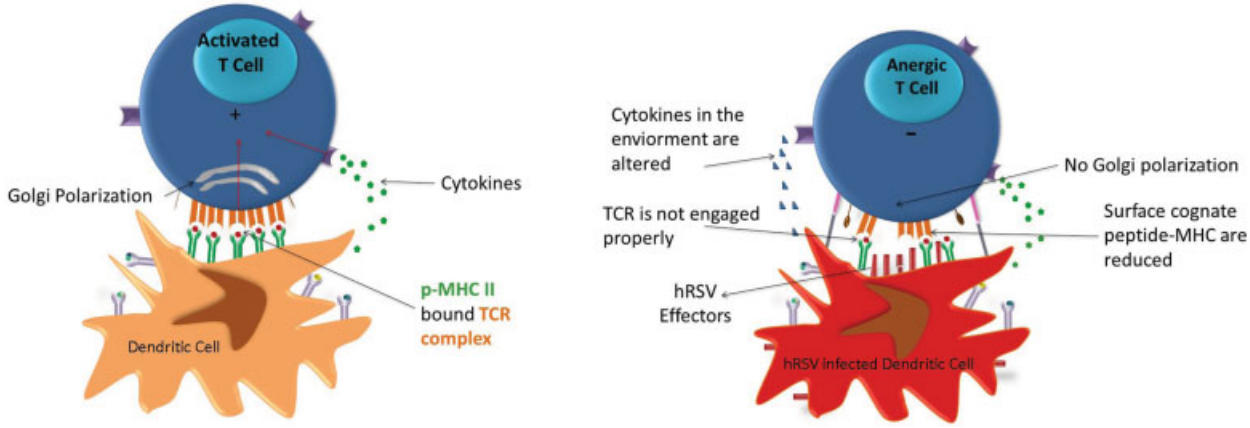

Fig. 4 hRSV blocks the DC-T-cell synapse assembly. hRSV elicits immune host system by impairment T-cell activation. The DC-T-cell synapse assembly is interfered by decreasing Golgi polarization, altering the cytokines secreted in the environment, reducing the surface cognate peptide MHC and impairing the TCR engagement.

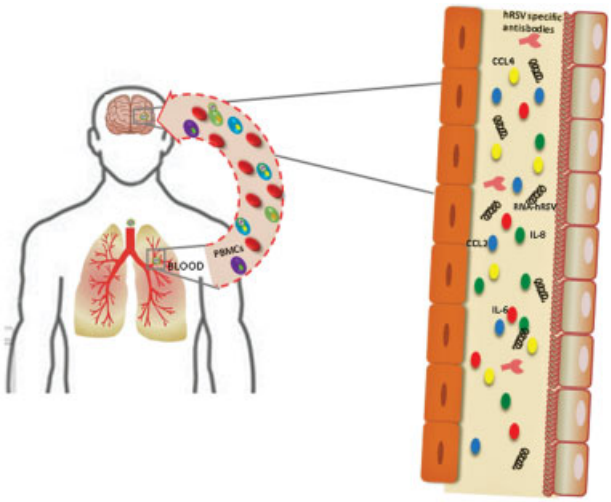

Fig. 5 Model for hRSV spread from the lungs to the CNS. The figure shows the possible hRSV spread from the lungs to CNS through the hematogenous pathway.

cells have also been shown to be infected by this pathogen in vitro.[113,148] The infection of nonepithelial cells by hRSV has been related to the expression of the hRSV receptor nucleolin, as well as other surface molecules, such as GAGs and TLR4, which interact with hRSV proteins.[145] Importantly, infected immune cells are detectable in systemic blood, as shown in infected infants by RT-PCR (reverse trancriptase-polymerase chain reaction) (►**Fig. 5**)[149,150] and in PBMCs in BALB/c mice.[146] Such evidence supports the notion that hRSV is able to spread through the hematogenous pathway, thereby reaching distant organs (►**Fig. 5**).

hRSV infections in peripheral lungs have been associated with severe bronchiolitis in hospitalized children.[26,151] For instance, myocardial disease has been extensively associated with severe hRSV bronquiolitis in infants who do not necessarily carry congenital heart diseases.[151–153] Consistent with this notion, elevated levels of cardiac troponin T (cTnT), a sensitive and specific marker of myocardial damage, have also been detected in severe hRSV-infected infants with hypotension (low blood pressure).[152,154] The first report of myocardial failure during an hRSV-driven bronchiolitis was described in 1972 and ended with a fatal case of interstitial myocarditis, an inflammation of the myocardium.[155] Importantly, cardiac alterations during hRSV infection can range from arrhythmias or irregular heartbeat to mechanical dysfunction.[153,156] Noteworthy, hRSV-RNA has been detected in the myocardium by PCR in a case report of myocarditis, thereby suggesting that such alteration can be a direct effect of viral infection.[157] Additional evidence of the cardiovascular manifestation of hRSV infection has shown that 76.5% of positive patients for severe hRSV bronchiolitis present sinoatrial blocking, characterized by interference in the passage of impulses from the sinoatrial node, and this manifestation is common in patients with elevated viral load (≥100,000 copies per mL).[27] In addition, clinical manifestations of pericardial effusion, an abnormal amount of fluid in the pericardial space, were associated with severe bronchiolitis in a 1-month-old infant (►**Table 1**).[158]

Hepatic alterations have also been related to hRSV infection, as evidenced by the detection of elevated levels of transaminase in patients with hRSV-associated bronchiolitis.[159] Additionally,

Table 1 Extrapulmonary complications associated with hRSV infection

Complications due to hRSV infection	Clinical manifestations	Findings	References
Cardiovascular	Heart block Ventricular tachycardia Ventricular fibrillation Myocarditis Pericardial effusion	hRSV-RNA in a patient with myocarditis and a correlation between viral load and sinoatrial blocks	27,158,177–179
Hepatic	Hepatitis	hRSV particles were detected in culture of liver from an immunocompromised patient and elevated transaminase levels	26,28
Endocrine	Hyponatremia	Patients with hyponatremia and hRSV bronchiolitis show elevated ADH levels	26,56
Renal	Steroid-responsive simple nephrotic syndrome (SRSNS)	hRSV-RNA and antigens were detected by RT-PCR and alkaline phosphoesterase–anti-alkaline phosphoesterase enzyme-linked assay (APAAP) in the urines, respectively	161
Neurological	Apneas Status epilepticus Seizures Encephalopathy Encephalitis Strabismus	hRSV-RNA by RT-PCR antibodies and elevated proinflammatory cytokines in CSF, such as IL-6	6,26,164,165,167–170,180,181

Abbreviations: ADH, antidiuretic hormone; CSF, cerebrospinal fluid; hRSV, human respiratory syncytial virus; RT-PCR, reverse trancriptase-polimerase chain reaction.

severe hepatitis characterized by elevated alanine aminotransferase levels up to 3,000 IU/L have also been described and further associated with impairment in coagulation.[152] Moreover, high levels of transaminases were associated with severe-hRSV disease in a study comprising 54 children who needed mechanical ventilation.[159] Here, hRSV liver infection was confirmed for one of the hRSV-infected immunocompetent infants in a liver biopsy.[160] Additionally, a case of adipose hepatic infiltration has been reported in a fatal case of Reye syndrome associated with hRSV infection,[26] as well as in developed hepatitis during hRSV infection (►**Table 1**).[28]

On the other hand, alterations of the endocrine system have also been associated with hRSV infection, as evidenced by a clinical study that showed that 33% of patients with severe hRSV bronchiolitis who were under intensive care manifested low sodium concentration in the blood, condition named *hyponatremia*,[26,56] and that 11% presented sodium levels below 130 mM/L in the serum.[56] Patients with hyponatremia and hRSV bronchiolitis have also been shown to display elevated levels of the antidiuretic hormone (ADH).[56] Furthermore, it has been reported that ADH levels are significantly higher in patients with bronchiolitis, as compared with patients with apneas or upper respiratory tract infections with hRSV.[56] Further, increased ADH levels have been associated with higher carbon dioxide arterial partial pressure and excessive expansion of the lungs or hyperinflation which was visible in chest X-rays (►**Table 1**).[56]

Renal manifestations have also been described in hRSV infections. hRSV-RNA and viral antigens have been detected by RT-PCR and alkaline phosphoesterase–anti-alkaline phosphoesterase enzyme-linked assay (APAAP) in urine samples of children with active, steroid responsive, simple nephrotic syndrome (SRSNS) (►**Table 1**).[161,162] Consistent with this notion, Liu et al evaluated the association of hRSV infection with nephropathy in a rat model. In this study, hRSV-infected rats showed a gradual increase in proteinuria and alterations in tubular epithelial cells with slight inflammatory cell infiltration in the renal interstitium. Also, both hRSV-RNA and viral titers were detected in the renal tissue with a peak at 8 days postinfection. These data suggest that hRSV can cause nephrotic syndrome, although minimal in an hRSV-infected animal model.[162]

Neurological Manifestations of hRSV Infection

Epidemiological data suggest that hRSV infection may cause neurological complications in 1.2 to 1.8% of the cases.[6,163] These neurological alterations include seizures, central apnea, lethargy, feeding or swallowing difficulties, tone abnormalities, strabismus, abnormalities in the CSF, and encephalopathy.[26,163–165]

Acute encephalopathies resulting from viral infection have been classified into three mayor types: metabolic error, cytokine storm, and excitotoxicity.[166] The etiology of the encephalopathies induced by hRSV infection remain unclear. However, an association between the overproduction of inflammatory cytokines and free radicals in the CSF has been extensively associated with neurological complications. For instance, IL-6, IL-8, and nitrogen oxide are increased in the CSFs of hRSV-infected patients, thereby suggesting that a cytokine storm may be involved in the pathogenesis of hRSV encephalopathy (►**Fig. 5** and ►**Table 1**).[163,166,167] Indeed, increased protein levels, cell infiltration, and low glucose levels have been observed in the CSF of hRSV-infected patients.[26] Interestingly, CSF abnormalities have also been detected in infants with no apparent neurological symptoms, suggesting that hRSV can induce subclinical neurological alterations.[168] Furthermore, brain imaging of patients with acute hRSV encephalopathy is also unclear. These findings include diffuse edema, an abnormal shifts of fluid in various compartments of the brain parenchyma, that involves the entire cerebral cortex and subsequent diffuse brain atrophy, which results in loss of neurons and the connection between them.[163]

Central apnea and seizures are the most frequent neurological alterations described in clinical reports.[169] Nevertheless, the frequency of neurological complications can reach up to 40% in children younger than 2 years with critical consequences due to severe hRSV infection.[6] Supporting evidence for an association between neurological alterations and hRSV infection is the detection of viral RNA and specific antibodies against hRSV in CSF of patients with neurological alterations (►**Fig. 5**). Furthermore, several studies have shown that encephalopathy-associated hRSV patients display altered cytokine profiles, as compared with patients infected with hRSV and bronchiolitis, but without neurological alterations.[167,170]

Central apnea can occur in up to 21% of children admitted into the clinic after hRSV infection. It has been proposed that hRSV infection can cause disruptions in neural control pathways by reducing nonadrenergic and noncholinergic inhibitory responses, which eventually may cause the central apnea symptom.[171,172] Further, hRSV causes abnormal cholinergic responses in an animal model, which suggests that hRSV can directly alter specific central nervous system (CNS) responses[173] by participating in CNS inflammation. An alternative explanation could be the significantly prolonged laryngeal chemo reflex observed in sleeping infants with hRSV bronchiolitis, as compared with those infected with hRSV but that do not develop central apnea.[172]

Other extrapulmonary clinical manifestations by hRSV, besides seizures, include recurrent neurological abnormalities. Such abnormalities have been described in two different types: generalized tonic–clonic and partial seizures with altered consciousness and focal motor features or eye deviation (►**Table 1**).[165,168] Similar to hRSV encephalopathy, seizures have also been related to the overproduction of cytokines and free radicals.[163,167] Importantly, hyponatremia can also contribute to seizures.[165,168] The direct infection of cranial nerves can also result in less common neurological alteration as reported for hRSV infection–induced strabismus in the form of esotropia.[165]

Taken together, extrapulmonary manifestations occurring in severe cases of hRSV infections are not isolated cases. Thus, there is an urgent need to evaluate the occurrence of these

events in hospitalized children with hRSV bronchiolitis and to study the possible consequences of acute neurological abnormalities due to the infection with this virus. Furthermore, it is imperative to determine whether these extrapulmonary effects are due to direct effects of tissues with hRSV or by inflammatory mediators dispersed from the airways or responding immune cells.

Possible Mechanisms behind the Neurological Alterations Caused by hRSV

The neurological complications observed upon hRSV infection[167] have encouraged researchers to understand the mechanisms involved in CNS dysfunction. Studies performed in BALB/c mice and Sprague Dawley rats have detected hRSV-RNA and viral proteins in the brain of animals previously infected intranasally with this pathogen.[174] Studies have found that immune cells are associated with hRSV in peripheral blood from hRSV-infected patients. Consistent with this notion, hRSV-infected immune cells would migrate to the CNS by the hematogenous pathway and trespass the blood–brain barrier.[174] An unexpected and important finding regarding the access of hRSV to the CNS was the description that impairment in cognitive function is observed after pulmonary disease was resolved in mice and rats.[174] Indeed, our group recently described that mice and rats infected with hRSV have a deficient performance in tests that evaluate these abilities. hRSV-infected mice performed significantly worse than noninfected mice, both in the Marble Burying (MB) and Morris Water Maze (MWM) tests, several weeks after viral challenge.[174] The MB test consists in measuring the ability of rodents to dig and hide marbles, which is controlled by hippocampal function.[175] In addition, the MWM evaluates the animal's ability for spatial learning through spatial localization of relevant visual cues that are subsequently processed, consolidated, retained, and then retrieved in the brain to successfully navigate and thereby locate a hidden platform to escape from the water.[176] In both tasks, hRSV-infected animals showed significant alterations in behavioral and learning processes, as compared with control animals. Moreover, electrophysiological assays suggested that impaired cognitive function was due to a failure to efficiently induce long-term potentiation responses in the stratum radiatum in the hippocampus area. Our study supports the previously proposed idea that hRSV can alter CNS function. Accordingly, hRSV has been shown to infect primary neuronal cells in vitro, as well as neural processes innervating the lungs.[148]

The association of an exacerbated immune response against hRSV together with hRSV-induced cognitive impairment is supported by the observation that a vaccine that induces protective T cell immunity prevents virus spread into the CNS, as well as neurological alterations caused by infection.[174] A possible explanation is that hRSV may enter the CNS associated with leucocytes or freely, triggering an elevated secretion of proinflammatory cytokines that affect normal neuronal function.

In summary, hRSV infection can cause important extrapulmonary symptoms, which can lead to important and long-lasting health sequelae in children affected by this virus. Therefore, significant research efforts are required for the generation of vaccines and therapies to prevent or treat the infection caused by this virus in the most susceptible population.

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Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

Modulation of antigen processing by haem-oxygenase 1. Implications on inflammation and tolerance

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doi:10.1111/imm.12605
Received 25 January 2016; revised 25 February 2016; accepted 29 February 2016.
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Summary

Haem-oxygenase-1 (HO-1) is an enzyme responsible for the degradation of haem that can suppress inflammation, through the production of carbon monoxide (CO). It has been shown in several experimental models that genetic and pharmacological induction of HO-1, as well as non-toxic administration of CO, can reduce inflammatory diseases, such as endotoxic shock, type 1 diabetes and graft rejection. Recently, it was shown that the HO-1/CO system can alter the function of antigen-presenting cells (APCs) and reduce T-cell priming, which can be beneficial during immune-driven inflammatory diseases. The molecular mechanisms by which the HO-1 and CO reduce both APC- and T-cell-driven immunity are just beginning to be elucidated. In this article we discuss recent findings related to the immune regulatory capacity of HO-1 and CO at the level of recognition of pathogen-associated molecular patterns and T-cell priming by APCs. Finally, we propose a possible regulatory role for HO-1 and CO over the recently described mitochondria-dependent immunity. These concepts could contribute to the design of new therapeutic tools for inflammation-based diseases.

Keywords: antigen presentation; carbon monoxide; cytokine; dendritic cells; haem-oxygenase 1.

Biological function of HO-1 activity

Several proteins, such as myoglobins, cytochromes and haemoblogins use the haem group as a cofactor.[1] As is the case for most cellular components, these proteins are degraded after being damaged or aged.[2,3] Because haem is a pro-oxidant molecule that can participate in the formation of oxidative radicals, leading to oxidative-toxic injury

that results in cell death, degradation of haem is required after the turnover of haem-containing protein.[4] Therefore, animal cells contain a specific set of haem-degrading enzymes, known as haem-oxygenases (HOs).[2,5–7] Depending on the tissue, three different HO isoforms can be expressed (HO-1, HO-2 and HO-3). HO-1 is mainly expressed in hepatic,[8] endothelial,[9] myeloid[10,11] and respiratory epithelial cells.[12] HO-2 is expressed in testis, brain and vascular system.[7,13–15] Although HO-3 is constitutively expressed, it has no catalytic activity, and genetic studies in rats have shown that the *Hmox3* gene is an HO-2-derived pseudogene.[16]

The HOs degrade the haem group into Fe^{3+}, biliverdin and carbon monoxide (CO). Although Fe^{3+} and CO are conserved and employed as second physiological signals, biliverdin is rapidly converted into bilirubin by the biliverdin reductase system.[2,7] Due to their functions, HOs are known as shock-stress-protecting enzymes.[4]

As a consequence of the high biological impact recently described for HO enzymatic activity, HO-1 function has been the most studied and characterized HO enzyme. An HO-1 deficiency leads to haem accumulation, causing several health burdens to the host.[17,18] The first patient suffering from HO-1 deficiency was reported in 1999.[19] In addition to all the metabolic, vascular and endothelial alterations, this patient suffered from an acute inflammatory state.[18,19] Lymph node swelling and leukocytosis were observed in this patient, which was in agreement with an advanced health deterioration and subsequent death.[19] Accordingly, HO-1 knockout (KO) mice display similar alterations, such as splenomegaly, lymph node swelling, altered $CD4^+$ T-cell numbers and an enhanced T-cell activation state.[18,20] Furthermore, HO-1 KO mice showed an unexpected increased susceptibility to lipopolysaccharide (LPS) -endotoxic shock.[21] Splenocytes from HO-1 KO mice also showed an augmented secretion of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α. Consistent with these observations, monocytes from patients suffering from the autoimmune disease systemic lupus erythematosus, which manifests as exacerbated general inflammation, showed a reduced expression of HO-1.[22] Similar results were seen in patients with multiple sclerosis, who displayed reduced levels of HO-1 in peripheral blood mononuclear cells during disease exacerbation.[23] These results suggest that HO-1, in addition to a pro-homeostatic function, can contribute to modulating the inflammatory response in the host.

Because antigen-presenting cells (APCs), such as dendritic cells (DCs), monocytes and macrophages express high levels of HO-1, the function and anti-inflammatory capacity of this molecule have been extensively studied.[24–26] A better understanding of the biology and function for HO-1 within these APCs could contribute to the design of improved anti-inflammatory therapies. In this article, we review recent findings for the role of HO-1 in the modulation of immunity.

Regulation of HO-1 gene expression

Regulation of HO-1 gene expression (*Hmox1*) is driven by pro-inflammatory and pro-oxidant molecules, such as pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs, e.g. haem group). These PAMPs and DAMPs activate signal transduction pathways that can modify intracellular equilibrium causing cell stress by activation of stress response genes.[27–29] These pathways include mitogen-activated protein kinases (MAPK) and the c-Jun N-terminal kinases (JNK).[30,31] HO-1 expression is regulated by the Keap1/Nrf2 and the Bach-1/Maf systems. During cellular stress or an inflammatory response,[32,33] nuclear erythroid 2-related factor-2 (Nrf2) dissociates from kelch-like erythroid cell–derived protein with CNC homology (ECH)-associated protein 1 (Keap1), the molecule that retains Nrf2 at the cytoplasm (Fig. 1a). Activated kinases from the MAPK and JNK pathways catalyse the phosphorylation of Nrf2, allowing translocation to the nucleus and binding to antioxidant response elements (ARE) in the *Hmox1* promoter site[34] (Fig. 1b, c). In addition to pro-inflammatory stimuli, the anti-inflammatory cytokine IL-10 can also induce the transcription of the *Hmox1* gene through the p38–MAPK pathway to suppress the PAMP-mediated and pro-oxidant molecule-mediated inflammatory responses.[35,36] Because HO-1 expression also induces IL-10 production, it is likely that a positive feedback loop takes place between IL-10 and HO-1 expression in the responding cells. On the other hand, the haem-binding protein Bach-1 has been shown to form a heterodimer with small Maf proteins and represses *Hmox1* transcription by competing with Nrf2 for the binding to AREs[37,38] (Fig. 1). Only during stress responses, Bach-1 dissociates from V-maf musculoaponeurotic fibrosarcoma oncogene homologues (Mafs), allowing Nrf2 to heterodimerize with these molecules (Fig. 1b, c). Therefore, there is a constant competition between Nrf2 and Bach-1 for the binding to small Maf proteins at the ARE. Recently, it was shown that IL-10 and other anti-inflammatory molecules can regulate the Bach-1/Maf system by reducing the expression of miR-155, a Bach-1 repressor molecule.[39,40] Hence, it is thought that micro RNAs can also contribute to the balance between cellular homeostasis and inflammatory response by modulating the access of Nrf2 to the *Hmox1* gene promoter. Consistently with this notion, Nrf2 KO mice develop several pathological manifestations including an enhanced proliferative response of $CD4^+$ T cells and a lupus-like syndrome with the presence of antinuclear antibodies, intravascular deposition of immune complexes, glomerulonephritis and decreased survival rates, showing a similar phenotype to HO-1 KO mice.[41]

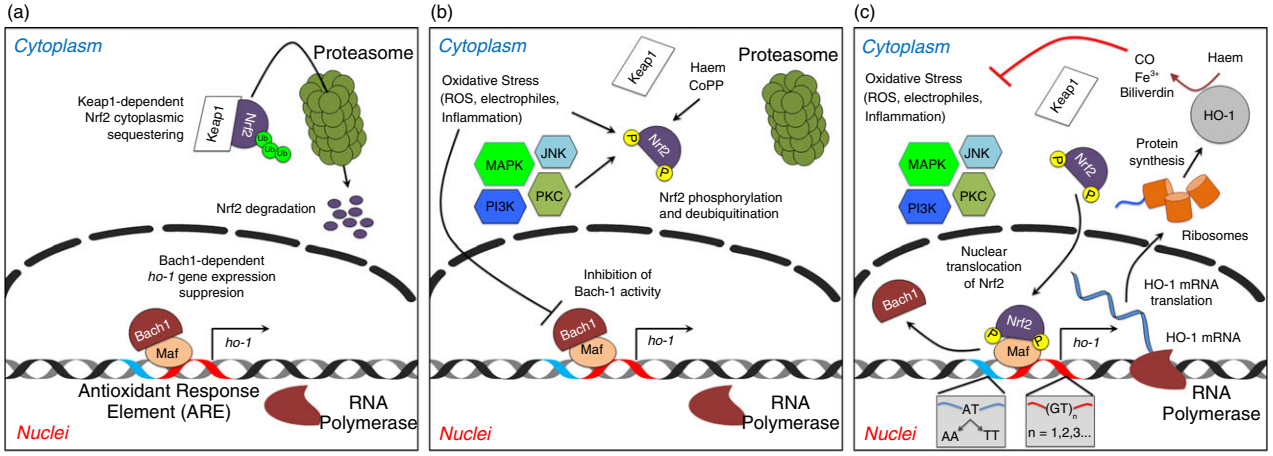

Figure 1. *Hmox1* gene expression and its regulation. (a) In resting state, Nrf2 remains as bound to Keap1 in the cytoplasm. Nrf2 is constantly being ubiquitinated and targeted for proteasomal degradation. During this process, Bach-1 heterodimerizes with small Mafs proteins at Antioxidant Response Elements (ARE) in the *Hmox1* gene promoter site. (b) During cell stress caused either by pathogen-associated molecular pattern (PAMPs) and/or pro-oxidant molecules, Nrf2 is released from Keap1, increasing its stability and reducing its proteasome-dependent degradation. Different kinases access to phosphorylate Nrf2 and activate its translocation to the nucleus. The binding of Bach-1 to small Mafs is compromised by the same pro-inflammatory and pro-oxidants signals. (c) Phosphorylated Nrf2 migrates to the nucleus and displaces Bach-1. Heterodimers Nrf2/Maf induce the activation of the *Hmox1* gene promoter site and the recruitment of the RNA polymerase. The *Hmox1* gene transcription begins. Depending on the $(GT)_n$ (n = number of repetitions) and the 413A > T (AT -> AA; AT -> TT) polymorphisms present in the promoter site, the amount of mRNA haem oxygenase 1 can vary.

Recent studies have shown that the expression of the *Hmox1* gene can be modulated by the presence of certain microsatellites located in the gene promoter $(GT)_n$.[42] Individuals with short GT repetitions have been shown to have a reduced risk of suffering rheumatoid arthritis[43] or chronic pulmonary emphysema,[44] with a favourable outcome to sepsis,[45] as well as other diseases,[42] due to an increased HO-1 promoter activity.[29] In addition, the single nucleotide polymorphism 413 A > T in the *Hmox1* gene promoter has been associated with increased transcription levels of HO-1 transcription.[46–48] The presence of this polymorphism correlated with an augmented incidence of hypertension in women.[46] Interestingly, individuals harboring this genetic alteration manifested lower incidence of acute kidney injury[48] and ischaemic heart disease,[47] suggesting a differential protective capacity for the 413 A > T polymorphism. Hence, although the activities of Nrf2 and Bach-1 are essential to regulate the transcription of *Hmox1* gene and protein quantity, specific DNA modifications in the promoter region also contribute to regulating these processes.

Pharmacological modulation of HO-1 expression using metalloporphyrins is an interesting experimental approach to study the role of this enzyme in several biological processes.[49] Cobalt protoporphyrin IX (CoPP) is a haem group homologue that induces the up-regulation of *Hmox1* gene expression by promoting the degradation of Bach1 protein and decreasing degradation of Nrf2 protein.[50] Contrary to CoPP, Tin protoporphyrin IX (SnPP), a metalloporphyrin formed by a chelate of tin with the porphyrin ring, is one of the most efficient inhibitors of HO-1 activity at the catalytic site.[51,52] This molecule works as a competitive substrate for the haem group, although it enhances the synthesis of new enzyme without catalytic activity.[51]

Blockade of pro-inflammatory receptors by HO-1: the case of TLR4/MD2

As described above, HO-1 is up-regulated after PAMP recognition in APCs.[29,32,33] Because PAMPs mainly signal through Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs), it has been suggested that these recognition pathways can play a central role in the induction of HO-1 expression.[32,53] Therefore, the contribution of HO-1 activity to the function of immune cells expressing TLR and NOD receptors, such as DCs and macrophages,[53–56] has been extensively studied. It has been shown that HO-1 over-expression inhibits the secretion of inflammatory cytokines after an LPS challenge in DCs.[11,27,35,57,58] These studies underscored CO as the most important product responsible for the immune suppressive capacity of HO-1.[27,59] CO can block the interferon regulatory factor 3/inducible protein 1 and the JNK/ inducible protein 1 inflammatory pathways in DCs[27] and macrophages,[10] respectively. Reduced signalling led to impaired *de novo* expression of different acute inflammatory cytokines, such as IL-6.[60] However, the molecular mechanisms used by CO to modify these pathways still remain unknown. Recent studies have

provided insight as to how this gas produced by HO-1 can block pro-inflammatory pathways.[21,27,57,61] Two different groups have shown that CO directly interferes with the normal surface expression of the LPS-recognizing receptor in DCs,[62] neutrophils[62] and macrophages.[63] This receptor consists of a complex formed by the TLR4 and the myeloid differentiation factor 2 (hereafter TLR4/MD2). These studies suggested that CO modifies the native conformation of the TLR4/MD2 complex without altering the surface expression of either individual TLR4 or MD2. As a result, CO could impair a key step required for the proper conformational assembly of the TLR4/MD2 complex on the surface of APCs. This notion was further supported by the observation that CO exposure reduced the MD2-dependent glycosylation of TLR4 induced by LPS and inhibited both the transport and surface expression of these two molecules in hepatic cells.[64] Hence, a decrease in the expression of the TLR4/MD2 complex can reduce the sensitivity to LPS stimulation, reducing and dampening inflammation. Although DCs and macrophages did not up-regulate TLR4 after LPS stimulation, reduced TLR4 glycosylation can explain the capacity of CO to impair subsequent LPS stimulation in monocytes (Fig. 2). Hence, by reducing the MD2-dependent TLR4 glycosylation, CO would cause an absence of functionally assembled TLR4/MD2 on the surface of the monocyte without altering the total amount of individual TLR4 and MD2 over time. However, additional studies are required to demonstrate this hypothesis.

Due to the contribution of TLR4/MD2-dependent inflammation to LPS-mediated septic shock, a potential protective capacity for HO-1 and CO has been explored.[62] CO-treated animals displayed reduced sensitivity to LPS-induced shock.[35,62,64] Several explanations are possible, but most of them rely on the ability of CO to reduce the secretion of pro-inflammatory molecules and to increase the production of immune suppressive cytokines, such as IL-10.[11,35] The role of IL-10 has been widely studied during inflammatory diseases because, in addition to inducing HO-1 expression as mentioned previously, this cytokine can efficiently suppress both innate and adaptive immunity.[35] Hence, the HO-1–CO system can reduce inflammation *in vivo* by, for instance, promoting the secretion of IL-10.[35,65] CO-mediated protection has been observed in some inflammatory pathologies, such as acute pancreatitis,[63] haemorragic shock,[66] Alzheimer's amyloid-β1-42-induced toxicity,[67] ischaemia/reperfusion,[68] autoimmunity[20] and graft rejection.[69] Because these pathologies are mainly mediated by innate or adaptive immune responses, it remains unknown whether CO can ameliorate disease progression by blocking the same or different inflammatory pathways in either innate or adaptive immune cells. Because the innate and adaptive immune responses can be linked by professional APCs that express HO-1 (DCs and macrophages), this enzyme's activity could be considered as a mechanism to down-modulate APC function and reduce detrimental innate and adaptive immune responses.

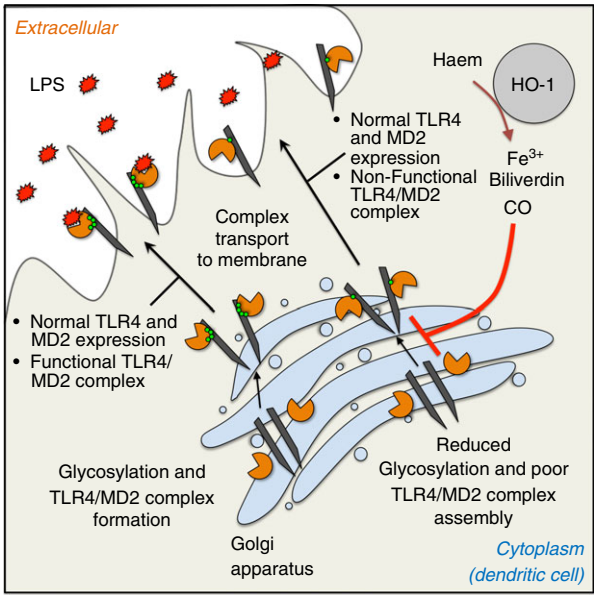

Figure 2. Carbon monoxide (CO) reduces the expression of the Toll like receptor 4/ myeloid differentiation factor 2 (TLR4/MD2) complex receptor in the surface of myeloid cells. TLR4 and MD2 form a glycosylation-dependent complex in the Golgi apparatus, which is then transported to the cell surface. Once in the surface, this complex can recognize lipopolysaccharide (LPS) and trigger an intracellular inflammatory cascade, which will lead to the activation of innate immune responses. After haem oxygenase 1 (HO-1) expression and CO production, a blockade in the glycosylation is produced in the Golgi apparatus and the standard generation and assembly of the TLR4/MD2 complex is compromised. Then, a non-well assembled complex is transported to the cell surface. Normal levels of TLR4 and MD2 are placed in the cell surface but its geometric association lack of effectiveness to recognize LPS. The innate immune response is compromised and reduced.

The HO-1–CO system as a modulator of antigen presentation by DCs

Dendritic cells are professional APCs that reside strategically in tissues that are normally exposed to foreign antigens and infectious agents.[70] PAMPs induce the maturation of DCs and, in conjunction with the capture of surrounding antigens, promote the migration of mature DCs to secondary lymphoid organs where they prime antigen-specific T cells (naive and memory).[71] Because of the key role that DCs play during the innate and adaptive immune responses, a DC deficiency can cause significant immune suppression and an increased susceptibility to infections.[72,73]

Haem-oxygenase 1 is constitutively expressed by DCs and can be up-regulated after PAMPs stimulation.[11,74]

However, HO-1 over-expression is not observed at early times during maturation. Hence, it seems that this enzyme can contribute to the recovery of DCs after a long period of inflammatory stress. Recent studies have shown that HO-1 induction by a haem homologue, CoPP, reduces maturation in human and rat DCs, by decreasing the expression of surface maturation markers as well as the secretion of inflammatory cytokines.[11,27] Furthermore, LPS-mediated reactive oxygen species (ROS) production also was blocked by HO-1 activity, which is consistent with an early defined role for this enzyme during oxidative stress. In agreement with these results, inhibition of the basal HO-1 activity by SnPP enhanced maturation of murine DCs after stimulation of the p38–MAPK, cAMP-responsive element binding protein and the activating transcription factor 1 pathways.[75] Reduced DC maturation due to CoPP-induced HO-1 expression abolished activation of allogeneic T cells.[11,27,76] These observations were consistent with data showing an augmented priming of antigen-specific T cells by DCs in which HO-1 was inhibited by SnPP.[75] Similar results have been obtained with the HO-1 inhibitory molecule Tin mesoporphyrin, which increased the capacity of cytomegalovirus$_{pp65}$-peptide-pulsed peripheral blood mononuclear cells to prime virus-specific naive T cells.[77] Consistently with the ability of HO-1 to down-modulate the capacity of DCs to prime T cells, improved graft acceptance and reduced leucocyte infiltration were observed in an allogeneic aorta rat transplantation model after virus-mediated *Hmox1* gene transfer.[59] Notably, both reduced leucocyte recruitment and tissue acceptance were reproduced in CO-treated animals,[59,78] suggesting that this gas was the molecule responsible for the HO-1-mediated inhibition of T-cell activation. The immune suppressive role proposed for CO has been corroborated by other studies showing that HO-1 expression and CO production reduce MHC-II expression in DCs. In addition, a deficiency of this enzyme increased the susceptibility to neuro-inflammation in a murine experimental autoimmune encephalomyelitis model by inducing both increased accumulation of effector T cells and central nervous system damage.[79]

Either CoPP-treated or CO-treated human and murine DCs retained their capacity to secrete IL-10, despite losing their ability to produce IL-12p70,[11,76] a finding that was reproduced in murine cells.[27,62] However, although human DCs show reduced surface expression of maturation markers after CoPP/CO incubation, these molecules were not altered in murine DCs. These data suggest that the effect of HO-1 activity could vary among species.[27,80] However, the precise explanation as to why the HO-1–CO system shows different pattern of responses between human/rat and mouse cells remains unknown.

Because DCs are professional APCs, the capacity of HO-1 to regulate antigen presentation has been an intensive area of research.[80] In the murine system, it was shown that both the CoPP-mediated induction of HO-1 and CO were able to reduce the presentation of foreign antigens to naive $CD4^+$ or $CD8^+$ T cells (Fig. 3a). Furthermore, the effect of CO relied on a reduced capacity of both mature DCs and macrophages to target soluble extracellular antigens to intracellular lysosomal compartments. Conversely, HO-1 activity and CO treatment had no effect on the processing and presentation of larger sized antigens, as those contained in 3-μm latex beads.[80]

These data support a model in which the HO-1–CO system can discriminate between small and large antigens, by selectively inhibiting intracellular processing routes for the small soluble antigens.[76] Along these lines, it seems that size is a crucial parameter for defining the intracellular processing pathway for an antigen. This notion is supported by recent studies showing that when extracellular large-volume antigens make contact with cells, an endoplasmic reticulum (ER) -assisted phagocytosis occurs.[81,82] As part of this process, the ER supports phagosome formation by providing membrane fragments that form a structure known as the ERgosome (ER + phagosome). This compartment does not seem to require to be transported to the perinuclear zone for antigen-processing because, at early times, it is enriched with lysosomal markers, proteasomal machinery and MHC molecules, all derived from cytoplasmic vesicles.[83,84] Hence, presentation on MHC-II and cross-presentation on MHC-I of antigens attached to large bodies occurred regardless of CO treatment. In agreement with this observation is the fact that CO was unable to inhibit proteasome-dependent cross-presentation of intracellular soluble antigens, which suggested that only the endosome-to-lysosome route for extracellular soluble antigens can be targeted by CO.[80]

Despite the HO-1–CO system only inhibiting the endosome-to-lysosome route, this pathway contributes to the onset of several antigen-specific T-cell-dependent pathologies. Hence, CO-mediated blockade of this pathway could be evaluated to interfere with the onset and progression of several inflammatory detrimental responses. Moreover, understanding how HO-1 controls antigen-dependent, as well as antigen-independent induction of immunity by DCs can contribute to designing new therapies to prevent and treat inflammatory diseases.

Regulation of mitochondrial function by CO: implications for immunity

Recent studies have shown that mitochondria play a key role in the initiation of the antiviral innate immune response.[85,86] It has been observed that this organelle participates as a signalling platform for the activation of mitochondrial antiviral signalling protein, which ends mainly in the priming of the interferon response.[86,87] Furthermore, mitochondria can contribute to the processing

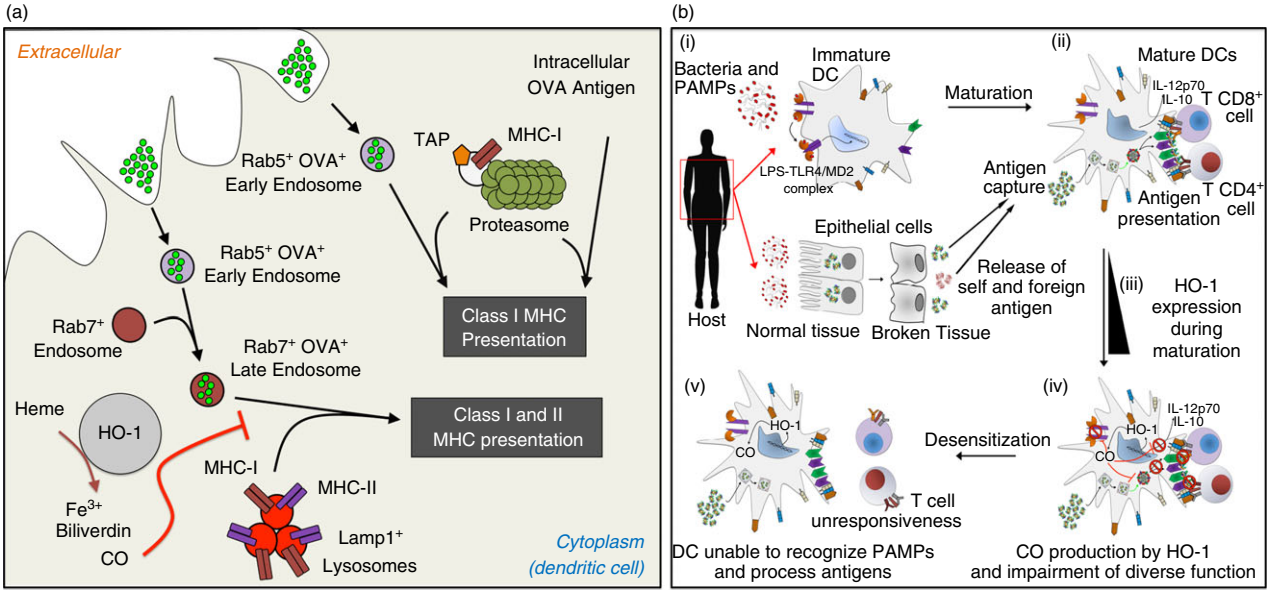

Figure 3. Carbon monoxide (CO) impairs the endosome-to-lysosome pathway to soluble antigens in myeloid cells. (a) After the extracellular antigen is captured, it fuses with Rab5$^+$ early endosomes. After that, these vesicles can fuse with proteasome/MHC-I/TAP-containing endosomes, which drive cross-presentation. In parallel, antigen-containing Rab5$^+$ vesicles can fuse with Rab7$^+$ endosomes to form late endosomes and then, sequentially, they can fuse with lysosomes (Lamp1$^+$). These lysosomes harbour a full repertoire of MHC molecules that receive and present the small peptides obtained after the antigen is processed by lysosomal proteases. Once haem oxygenase 1 (HO-1) is over-expressed and CO is produced, there is an interference in the fusion between antigen-containing late endosomes and lysosomes so compromising the correct antigen processing and antigen presentation to T cells. No effect of CO over cross-presentation has been observed. (b) (i) Under local presence of pathogen-associated molecular pattern (PAMPs); either by soluble molecules or presence of pathogens, dendritic cells (DCs) become activated. After binding the Toll like receptor 4/ myeloid differentiation factor 2 (TLR4/MD2) complex, LPS induces DC maturation by up-regulation of co-stimulatory molecules and secretion of cytokines. In addition, PAMPs cause local tissue damage and release of self- and non-self-antigens. (ii) Resident DCs capture soluble antigens presenting them to local T cells (something also observed in autoimmunity and graft rejection). Antigen-containing mature DCs can travel to secondary lymphoid organs and activate antigen-specific naive T cells. (iii) After PAMPs exposure [or treatment with cobalt protoporphyrin IX (CoPP), for example], DCs over-express HO-1, degrade haem-group and produce CO. This process will modulate the immunogenicity of DCs recovering their initial homeostasis. (iv) CO-producing mature DCs will lose their capacity to process antigens through the endosome-to-lysosome pathway. In addition, DCs reduce their secretion of cytokines. (v) Finally, mature DC-dependent innate and adaptive immune inflammation is suppressed. Tissue homeostasis is recovered and pathologies caused by PAMPs and either foreign or self-antigens are restricted.

of intracellular bacteria by enhancing ROS production after TLR signalling in macrophages.[88] Thus, innate immune cells, such as APCs, employ mitochondria to exert some of their immune functions.

Because APCs require internal regulators to control inflammatory pathways and to restore homeostasis, it is possible that mitochondria could decrease immunity. Traditionally, mitochondria have been considered as cellular organelles associated with energetic, metabolic and genetic roles, whose oxidative function is carried out mainly by cytochrome-containing complexes.[89–91] Cytochromes are proteins that use Fe^{2+}-to-Fe^{3+} porphyritic groups as co-factors, which facilitate the transit of mobile electrons (high oxide-redox potential). Based on their chemical properties, the porphyritic group and the Fe^{2+} also show high affinity for exposed electrons from diatomic gases, such as O_2, NO and CO.[92,93] Hence, these gases in mitochondria can control both cytochrome-dependent oxidative phosphorylation and ROS production. Experiments using soluble cell-free/purified mitochondria have shown that CO binds to complex I and III in the respiratory chain.[94,95] CO affinity for these molecules prevents electron transport, reducing the mitochondrial membrane potential ($\Delta\Psi$), ATP and mitochondrial ROS generation (mROS, O_2^-). Importantly, non-toxic doses of CO have been used in these *in vitro* experiments to avoid mitochondrial disorganization and destruction.[95] Because HOs are the only enzymes in mammal cells that produce CO at non-toxic levels, it is likely that they could play a relevant role at modulating both mitochondrial function and at controlling immune cells, such as APCs. However, this hypothesis remains to be evaluated.

Because the HO-1-CO system interferes with the initiation of the APC-dependent adaptive immune response, it is likely that a CO-dependent mitochondrial blockade could reduce T-cell priming.[96] Consistent with this

notion, it was shown that Kupffer cells require both a functional respiratory chain and high levels of mROS to prime antigen-specific $CD4^+$ T cells.[97] These data agree with a recent study showing that mitochondrial stability is required to process and present soluble antigens by B cells to T cells in an ATP-dependent manner.[98] However, it remains unclear how and where mitochondria could be regulating antigen-dependent immunity and whether endogenously produced CO can regulate these processes. Recent data from our laboratory propose a role for mitochondria in the transport and processing of antigen-containing vesicles.[99] These observations suggest that the HO-1–CO system inhibits this pathway by dropping down mitochondrial ATP production without impairing the glycolysis-dependent DC maturation.[99] As a consequence, CO impairs mitochondrial function in DCs up to a point of down-modulating antigen-specific T-cell priming. Because DCs link the innate and the adaptive immune responses, their important function could be modulated by targeting the inflammatory activity of mitochondria by using molecules that regulate ROS and ATP production. It is likely that HO-1 activity and CO can act as natural regulators of the mitochondria-dependent inflammatory pathway in APCs by blocking the maturation of antigen-containing endosomes.

Regarding the interaction between mitochondria and HO-1, studies performed in human alveolar and bronchial epithelial cells have shown the translocation of HO-1 to the mitochondrial compartment after the exposure to cigarette smoke, LPS and haemin.[100] The localization of HO-1 at mitochondria *in vivo* has also been reported using the model of gastric mucosal tissue injury induced by non-steroidal anti-inflammatory drugs.[101] This phenomenon resulted in the prevention of non-steroidal anti-inflammatory drug-induced mitochondrial dysfunction and oxidative stress, gastric mucosal cell apoptosis and gastric mucosal injury. The proposed mechanism is the stabilization of complex I-driven mitochondrial respiratory control and the transmembrane potential. These processes have been recently proposed as a novel cytoprotective effect of HO-1.[101] Mitochondrial HO-1 translocation was also observed in macrophage RAW-264.7 cells after the treatment with $CoCl_2$ or exposure to hypoxia. However, in this case the in-organ localization of HO-1 caused mitochondrial dysfunction.[102]

Taken together, these findings suggest that the anti-inflammatory effects of HO-1 could be in part explained by the suppression of the antigen-dependent immunity, which is directly associated with mitochondrial function. In addition, HO-1-mediated mitochondrial protection after the translocation of this enzyme to that organelle reduces cytotoxicity and massive cell death, reducing DAMPs release. These findings also uncover new therapeutic targets to control the HO-1–CO system as a manner to approach diseases caused by the adaptive immune response.

The HO-1–CO system reduces pathologies caused by the immune response

HO-1 reduces innate immunity-mediated inflammatory diseases

Several pathologies are associated with the effector function of the innate immune system.[103] Fast and acute inflammatory conditions, such as fever, organ swelling and septic shock are mainly mediated by the rapid recruitment of monocytes and neutrophils to the site of infection.[104] Cellular recruitment is associated with a gradient of inflammatory cytokines that are secreted either at the infection site or at damaged tissues (Fig. 3b(i)–(ii)).[105–107] Hence, interference with the secretion of these pro-inflammatory cytokines is likely to reduce the pathology. Expression of HO-1 and the subsequent CO production can contribute to controlling several innate immunity-driven inflammatory pathologies.[108] For instance, CO can protect from the permeability induced by LPS in epithelial tissues by reducing inflammatory cytokine secretion and preventing the down-regulation of tight junction proteins, such as ZO-1 and occludin.[109] Furthermore, it has been recently shown that an IL-10-dependent HO-1 induction decreases both the recruitment of innate inflammatory cells and the secretion of inflammatory cytokines in a septic shock animal model.[35] Hence, blockade of myeloid cell-derived inflammatory cytokines by the HO-1–CO system can work as a mechanism to reduce the sensitivity to stimulation by PAMPs (Fig. 3b(iii)–(iv)).

The HO-1–CO system reduces adaptive immunity-dependent inflammation. Implications in tolerance during transplantation and pregnancy

Activated T cells can contribute to several immune-based pathologies. T cells become activated after recognizing antigens as peptide–MHC complexes (pMHC) on the surface of APCs. The pMHCs are generated by APCs as a result of the processing and presentation of internalized antigens, either foreign or self, as is the case of pathogen infections or autoimmune disorders, respectively.

Furthermore, allospecific T cells are primed by APCs expressing MHC molecules at variant from the host.[110–112] Such an allogeneic recognition can lead to organ rejection in patients who have received a transplant to treat illnesses, such as kidney failure. During transplant rejection, DCs stimulate T cells through direct, indirect or semi-direct pathways of allorecognition.[111,112] In the direct pathway, donor DCs can migrate out of the grafted tissue and present intact donor MHC/peptide complexes to allospecific T cells (which recognize non-self antigens).[111,113] In the indirect pathway, recipient DCs process donor alloantigens (foreign/non-self antigens) and present

them to autologous reactive T cells.[111,113] Finally, through the semi-direct pathway of allorecognition, recipient T cells recognize intact donor MHC/peptide complexes that have been transferred to the surface of recipient DCs by a process known as 'nibbling'.[114] From these three pathways, the direct route is associated with early graft rejection and has been classified as the most powerful mechanism of rejection.[115] However, this mechanism decreases with time because donor DCs mainly die by senescence. On the other hand, recipient DCs arise as the most potent factor able to activate host T cells during the time. Hence, the indirect pathway arises as the major cause of chronic graft rejection after presentation and recognition of alloantigens.[112,113,115]

These mechanisms of allo-recognition pose an important challenge to improve graft acceptance, so new specific approaches are required to prevent T-cell activation by donor or recipient DCs after transplantation. Graft acceptance could be promoted either by suppressing *de novo* activation of T cells or inducing T-cell antigen-unresponsiveness. A promising approach to achieve these goals is the generation of antigen-specific regulatory T cells.[116–118] This technique contributes to specific protection and graft survival. Although regulatory T cells show antigen-specificity, these cells are efficient at suppressing locally reactive effector T cells. Whether the regulatory T-cell approach can produce as a side effect local immune suppression leading to pathogen spread remains to be defined. A combination of different strategies, for example regulatory T cells together with DCs that induce T-cell unresponsiveness, might be a solution. However, these alternatives must be evaluated, both in animal models and in clinical studies.

It was recently shown that *in vivo Hmox1* gene transfer using adenoviral vectors improves long-term heart graft survival in a myeloid cell (DC) -dependent fashion.[59,78] Also, in another model of graft survival, injection of pigs with a lentiviral vector encoding for *Hmox1* gene ameliorated the outcome of a Duchenne muscular dystrophy therapy with myogenic cell precursors.[58] Similar results were obtained in diabetes,[119,120] renal transplantation[121,122] and human cardiac stem cell transference[123] in which all the over-expression of HO-1 was induced with CoPP. This notion was further supported by the observation that regulatory T-cell-dependent suppression of activated T cells required the expression of HO-1 in APCs.[124] Similar observations were made in mouse models for inflammatory diseases, such as in lactobacillus-mediated infection where HO-1 activity was required to efficiently produce mesenteric $Foxp3^+$ $CD25^+$ $CD4^+$ T cells.[125] Moreover, transfer of wild-type regulatory T cells into HO-1 heterozygous mice restored the ratios between regulatory and effector T cells and reduced inflammation in a model of necrotizing enterocolitis, supporting a possible direct role for HO-1 in the generation and function of regulatory T cells.[126] Hence, the HO-1–CO system arises as a powerful candidate to restrict antigen presentation to T cells *in vivo* and also to impair the activation of lymphocytes in transplantation and autoimmunity. Furthermore, the generation of regulatory T cells during bacteria-dependent inflammation and graft acceptance seems to depend on the HO-1 activity.

Because these pathologies are mainly mediated by the presentation of extracellular antigens that have been processed by the endosome-to-lysosome pathway, the mechanism by which CO could be reducing the activation of T cells can be associated with a blockade of this route (Fig. 3b(v)). Moreover, as seen in rat and human models of organ transplantation, reduced DC maturation by HO-1 can impair the expression of both MHCs and co-stimulatory molecules. As a result, the priming of MHC mismatched T cells would be reduced. Hence, due to its impact on antigen presentation, modulation of the HO-1–CO system *in vitro* either by pharmacological or gene therapy could work as efficient strategies to induce antigen-specific tolerogenic DCs that are useful for cell transfer-based therapy during autoimmune diseases.

In addition, the HO-1–CO system has also been characterized as protective during pregnancy.[127,128] During embryo development, new cells and antigens are produced. To protect the developing fetus from being attacked by the adaptive immune response of the mother, a tolerant immune state must be established in the fetal–maternal interface. Consistent with this notion, it has been shown that HO-1 expression can prevent natural abortion in a well-established mouse model.[129] HO-1 upregulation induces BCL-associated athanogene-1 (Bag-1) and neuropilin-1, two markers associated with the development of regulatory T cells and the induction of tolerance.[130] Furthermore, recent studies showed that HO-1 regulates regulatory T-cell-mediated protection against abortion in mice because this enzyme reduced DC maturation and regulatory T-cell expansion.[131] It is thought that reduced DC maturation avoided the priming of effector T cells in the mother, which protected embryo from the immune response. These mechanisms were supported by recent data suggesting that progesterone regulates both the expansion of regulatory $CD8^+$ $CD122^+$ T cells and the establishment of fetal tolerance after inducing expression of HO-1 in the placenta.[132] In addition, the progesterone–HO-1 system reduced the expansion of cytotoxic $CD8^+$ T cells that recognize non-self antigens expressed by the embryo.[132] Consistently, a correlation was shown for pregnant women between current miscarriage and a reduced amount of regulatory T cells, despite having regular numbers of circulating DCs.[133] These data suggest that HO-1 regulates DC activity and the capacity to induce T-cell-mediated tolerance.

Because DCs are crucial for the establishment of peripheral tolerance, their contributions to tolerance dur-

ing pregnancy and to protecting the new embryo from immune recognition have been extensively studied. It has been shown that uterine DCs display an immature/IL-10-secreting phenotype during pregnancy and that maturation and IL-12 secretion are directly associated with abortion in mice.[134,135] Indeed, absence of IL-10 during pregnancy leads to an altered phenotype for DCs, macrophages, effector and regulatory T cells, which contribute to LPS-mediated abortion.[136] These data suggest that production of IL-10 by APCs controls T-cell responses after recognition/presentation of embryo-derived antigens. In addition, uterine DCs might be crucial for embryo implantation because they accumulate before pregnancy during the oestrous cycle[137] and their remotion definitely affects the success of implantation.[138]

Because mature uterine DCs release several inflammatory mediators, activation of these cells by PAMPs could promote inflammation in mother tissues and impair embryo development. Hence, it has been shown that bacterial infection is an important risk factor that can trigger abortion because, in addition to the inflammatory condition, pathogens down-regulate HO-1 expression in the placenta.[139,140] Up-regulation of HO-1 by CoPP increased fetus development and augmented cell survival of the placental tissue.[128] This process is likely to be promoted by the capacity of HO-1/IL-10-expressing DCs to induce regulatory T-cell expansion after antigen presentation, which contributes to placental stability. It has also been shown that HO-1 regulates placental development.[141] Consistently with this notion, the amount of apoptotic placental cells was reduced when HO-1 expression was promoted by transduction with an adenoviral *Hmox1* gene transfer system.[129] Another study showed decreased placenta size and weight, as well as impaired cell viability in mice heterozygous ($Hmox1^{+/-}$) for HO-1.[141,142] Along these lines, it has been shown that both $Hmox1^{+/-}$ and $Hmox1^{-/-}$ mice show an impairment in uterine natural killer (uNK) -dependent maternal spiral arteries remodeling during implantation because of reduced numbers of uNK.[143,144] Interestingly, treatement of $Hmox1^{+/-}$ mice with low doses of CO improved both uNK proliferation, spiral arteries remodelling and also stabilized blood presure, suggesting that this gas plays a key role during uNK-dependent intrauterine growth.[143,144] Similar data were obtained in a trophoblastic stem cell line that resembles the development of trophoblasts into giant cells, which showed that HO-1 inhibition compromises cell viability and their ability to differentiate.[145] In agreement, trophoblastic tissue from women suffering spontaneous abortion displayed reduced HO-1 expression levels compared with healthy pregnancies.[142] It is noteworthy that CO administration during implantation and the early placentation window improved cell survival and increased Bag-1 expression in the same manner as shown by the expression of HO-1, suggesting that the main mediator for this enzyme in placenta stability is CO.[146] Hence, the HO-1–CO system not only plays an important role in the regulation and tolerance associated with the immune system, but also implies a direct function in the placental tissue development that will protect the embryo during the interaction with tissues of the mother.

Altogether, these results suggest that the HO-1–CO system can prevent and reduce the outcome of inflammatory pathologies related to innate and adaptive immunity (Fig. 3b). In the case of innate immunity, both CO-mediated cytokine secretion suppression and reduced cell recruitment to the site of PAMPs accumulation arise as the most important regulatory mechanism used by HO-1. In the case of adaptive immunity, CO-mediated suppression of both fusion between antigen-containing endosomes and lysosomes, antigen surface presentation and impairment of DC maturation is associated with reduced T-cell priming, lower tissue destruction and an establishment of tolerance.

Concluding remarks

HO-1 is expressed in APCs, such as DCs and macrophages. The production of CO by this enzyme mediates the suppression of different inflammatory pathways. HO-1 and CO reduce the capacity of APCs to recognize PAMPs and suppress both pro-inflammatory cytokine secretion and antigen presentation. These modulatory processes uncouple the whole immune network by blocking the activation of either antigen-specific or allogeneic T cells. HO-1–CO-mediated reduction of immunity controls the onset and progression of several diseases, such as sepsis, organ rejection and autoimmunity. The exploitation of the molecular targets of the HO-1–CO system, such as mitochondria, arises as a promising alternative to create anti-inflammatory therapies. More research is needed to evaluate whether by inhibiting mitochondria until non-toxic levels, as CO does it, can lead to improving life quality in patients suffering from inflammatory ailments.

Ackowledgements

This study was supported by the following grants: FONDO NACIONAL DE CIENCIA Y TECNOLOGIA DE CHILE (FONDECYT numbers 1110397, 1131012, 1140010 and 1110604), Millennium Institute on Immunology and Immunotherapy P09/016-F and Grant 'Nouvelles Equipes-nouvelles thématiques' from the La Région Pays De La Loire. SAR and JAE are supported by the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT). LJC is a Latin American Pew Fellow. AMK is a Chaire De La Région Pays De La Loire, Chercheur Étranger D'excellence, France.

Disclosures

The authors have declared that no conflict of interest exists.

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Expert Opinion on Investigational Drugs

ISSN: 1354-3784 (Print) 1744-7658 (Online) Journal homepage: http://www.tandfonline.com/loi/ieid20

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To cite this article: Claudia A Rivera, Roberto S Gómez, Rodrigo A Díaz, Pablo F Céspedes, Janyra A Espinoza, Pablo A González, Claudia A Riedel, Susan M Bueno & Alexis M Kalergis (2015): Novel therapies and vaccines against the human respiratory syncytial virus, Expert Opinion on Investigational Drugs, DOI: 10.1517/13543784.2015.1099626

To link to this article: http://dx.doi.org/10.1517/13543784.2015.1099626

Published online: 12 Oct 2015.

EXPERT OPINION

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Introduction: Human respiratory syncytial virus (hRSV) is the leading cause of acute lower respiratory tract infections worldwide in infants, as well as an important pathogen affecting the elderly and immunocompromised individuals. Despite more than a half a century of research, no licensed vaccines are available and only palivizumab has been approved to use in humans, mostly recommended or limited to high risk infants. Therefore, novel therapeutic and preventive drugs need to be developed to fight this major human pathogen.

Areas covered: This review discusses current therapeutic approaches in preclinical and clinical stages, aimed at controlling or preventing hRSV infection. These methods include passive immunization, experimental drugs, vaccine candidates and maternal immunization.

Expert opinion: Based on the results of various immunization strategies and therapeutic approaches, it is likely that the most effective strategy against hRSV will be a prophylactic tool aimed at developing a strong antiviral T-cell response capable of both, promoting the generation of hRSV-specific high affinity antibodies and leading the protective immunity required to prevent the disease caused by this virus. Alternatively, if prophylactic strategies fail, antiviral drugs and novel passive immunity strategies could significantly contribute to reducing hospitalization rates in susceptible individuals.

Keywords: antibody therapy, hRSV, T-cell immunity, vaccines

Expert Opin. Investig. Drugs [Early Online]

1. Introduction

Worldwide, the human respiratory syncytial virus (hRSV) is considered the most important etiological agent of acute lower respiratory tract infections (ALRTIs) in infants and young children. This pathogen infects more than 70% of infants before their first year of life, and nearly 100% of children by age of 2.[1] It has been estimated that globally each year, 33.8 million children younger than 5 years will present episodes of ALRTI, including bronchiolitis, pneumonia, tracheitis and acute bronchitis associated to hRSV.[2] Furthermore, 3.4 million will develop severe symptoms that require hospital admission.[2] The same study also estimated that hRSV causes the death of 66,000 – 199,000 children each year around the globe and that the incidence of hRSV-associated ALRTI in developing countries is more than twice that of industrialized countries.[2] Severe symptoms related to hRSV infection include apnea, periodic breathing and feeding difficulties in infants younger than 1 year.[3] In older children and adults the most common symptoms are wheezing, rhinorrhea, cough, nasal congestion and fever, similar to symptoms produced by other respiratory viral infections. Despite being an important

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pathogen for pediatric populations, hRSV can also produce severe complications in the elderly and high-risk adults, by producing high morbidity, prolonged hospitalizations and high mortality rates, similar to seasonal influenza.[4] Furthermore, hRSV has become increasingly important as a major public health burden in industrialized countries with aging populations.[4]

hRSV belongs to the Paramyxoviridae family and is classified within the Pneumovirinae subfamily and the *Pneumovirus* genus. It is an enveloped non-segmented, negative sense, single-stranded RNA virus.[5] The genome of the virus is 15.2 kb in length with 10 genes that encode for 11 proteins, as the M2-1 and M2-2 proteins, two RNA synthesis factors, are translated from one mRNA with two overlapping open reading frames.[5] Eight of the 11 viral genes encode structural proteins, which comprise three transmembrane glycoproteins: the fusion (F) protein, which mediates syncytia formation and hRSV penetration, the attachment glycoprotein (G) protein and the small hydrophobic (SH) protein.[6] Whereas F mediates syncytia formation and hRSV-cell membranes fusion, which is essential for virus penetration into target cells, G mediates the attachment of viral particles to glycosaminoglycans present in the surface of epithelial cells, such as heparan sulfate.[6,7] On the other hand, SH has been proposed as a cation-selective ion channel similar to a viroporin that interferes with the permeability of infected cells.[8] The F and G proteins are the major antigens located on virion surface and induce host antibodies against hRSV.[9] While F is highly conserved between virus isolates, G displays significant sequence variability among two main hRSV antigenic subgroups, A and B.[9] Underneath the virion envelope lays the matrix (M) protein, involved in viral particle assembly. The M2-1 protein, the nucleoprotein (N), the RNA-dependent RNA polymerase (L) and the phosphoprotein (P), a co-factor for the polymerase, compose the nucleocapsid that protects the viral genome.[10] Finally NS1 and NS2, two nonstructural proteins found in infected cells, but not in the viral particle, inhibit the production of type I interferon.[5] Importantly, recently it has been demonstrated that N is expressed on the surface of infected cells and impairs the communication between infected cells, such as dendritic cells, and T cells impairing their activation upon antigen encounter.[11]

hRSV transmission takes place by direct contact of small droplets containing infective virions that enter the host through eyes or nose gaining access to the upper respiratory tract.[12] Infection of host cells starts with the activity of G and F that allow attachment and entry of hRSV to target cells.[13] After the fusion of viral envelope with the host cellular membrane, the viral ribonucleoprotein transcriptase–replicase (TR) complex is released into the cytosol where the transcription and replication of the hRSV genome initiates.[14] The TR complex transcribes the viral genome into two different RNAs of positive polarity: the antigenome and the 10 different monocistronic mRNAs needed for viral protein synthesis. The antigenome, in turn, serves as a template for the synthesis of new genome copies. The M2-2 protein modulates the latter two processes.[15] In polarized cells, viral assembly and budding take place at the apical surface and can yield pleomorphic or spherical virions in the form of filaments up to 10 μm in length, or 150 – 250 nm diameter spheres, the former apparently being the predominant morphology for this virus.[16]

The main cellular targets for hRSV are the ciliated epithelial cells and phagocytic cells, such as dendritic cells, inhabiting the airways.[15,17] The immune response triggered by hRSV infection of these cells is characterized by an exacerbated inflammatory response that is orchestrated by hRSV-infected airway epithelial cells through the secretion of pro-inflammatory cytokines, which create an inflammatory environment.[15] The cytokines produced in this first stage of hRSV infection are, among others, TSLP, IL-33, IL-4, IL-5, IL-6, IL-10 and IL-13. The presence of these pro-inflammatory molecules prevents the activation of an effective antiviral T-cell immunity.[18,19] In turn, these soluble factors recruit monocytes, T cells, neutrophils and eosinophils into the infected airways.[18,19] The inflammation described above contributes to the persistence of the virus and tissue destruction, thereby exacerbating the pathology. During the innate immune response, macrophages and dendritic cells (DCs) also arrive at the site of infection.[20] Although DCs present a mature phenotype upon hRSV infection, these cells fail at effectively activating virus-specific T cells capable of clearing hRSV.[20] Recruited DCs also fail at inducing the T-cell polarization required for an efficient adaptive antiviral immunity that can prevent hRSV spreading. This notion is supported by a recent study showing that the hRSV nucleoprotein can directly impair the formation of the immunological synapse between DCs and T cells.[11,21] The adaptive immune response to hRSV is also characterized by naïve T cells polarized toward Th2 and Th17 phenotypes, leading to a low production of IFN-γ and exacerbated epithelial tissue inflammation.[22,23] Also, hRSV triggers IgG1, IgG3 and IgE antibody secretion by B cells, which are associated with T-helper type-2 immune response (Th2), which are detrimental and fail to neutralize the virus.[22,24,25] Infected individuals exhibited lower titers of anti-F and anti-G IgG and IgA antibodies in the serum and nasal secretions, respectively, as compared to uninfected control subjects. Therefore, reduced IgG and IgA titers correlated with an augmented risk of hRSV infection.[26,27] Indeed, reinfections with hRSV are common in most individuals due to a weak antiviral immune response triggered by this pathogen.[28] Importantly, although immune memory can be generated upon hRSV challenge, such a response usually fails at protecting against re-infection in subsequent exposures to the virus.[28,29] As a result, hRSV-induced disease can be acquired continuously throughout an individual's life, especially in young children and the elderly.[29]

In the early 1960s, a formulation based on formalin-inactivated hRSV (FI-hRSV) was engineered as a first vaccine candidate against this virus.[30] Clinical trials in children showed that after a natural infection with hRSV, FI-hRSV-immunized children exhibited exacerbated pulmonary disease with 80% requiring hospitalization.[30] Accumulating evidence generated after decades of research has suggested that FI-hRSV vaccination induced an unbalanced Th1/Th2 immune response, biased toward a Th2 response that promoted mucus hyper-secretion and airway hyper-reactivity.[31] These symptoms were accompanied by significant airway obstruction and loss of weight in animal models, stimulated by TNF-α, a Th1 profile-associated cytokine.[31] Moreover, the FI-hRSV vaccine was not able to promote specific $CD8^+$ memory T-cell responses.[31] Although a large recruitment eosinophil into the airways was induced by the FI-hRSV vaccine, these cells were not rendered responsible for the pathology associated with the formalin-inactivated vaccine approach.[31] Rather, exacerbated disease was due to low affinity non-neutralizing antibodies induced by formalin-modified hRSV epitopes, which accumulated as immune-complexes in the lungs leading to excessive tissue inflammation.[32,33]

Since the failure of FI-hRSV in conferring protection against hRSV infection, numerous experimental strategies to prevent and treat hRSV infection have been evaluated.[34] Although a few preventive strategies have shown effectiveness, these remain far from affordable and are limited to narrow groups of individuals at risk. Broad-spectrum, safe and affordable prophylactics and therapies are urgently needed.[34] We describe below current approaches for the prophylaxis and treatment against hRSV infection focusing on passive immunization, experimental drugs and vaccine candidates for eliciting adaptive immunity.

2. Passive immunity as current therapy against hRSV

The hRSV F protein is an essential component for hRSV infection of epithelial cells [35] and was recently shown to interact with nucleolin, one of the main receptors for the virus.[36] The F protein has also been described as one of the major antigenic proteins of hRSV, together with the G protein. For this reason, many of the prophylactic and therapeutic strategies have focused on generating neutralizing antibodies against this protein, with the purpose of blocking virus fusion and entry into host cells. Consistently with this notion, the most successful therapy against hRSV to date consists of the use of a humanized anti-F monoclonal antibody named palivizumab. However, although this molecule has been approved for use in humans since 1998, it is prescribed rather in a limited manner and mainly for high-risk infants.[37] Prevention with palivizumab requires monthly intramuscular injections in high-risk infants during hRSV outbreaks [38,39] and may decrease up to 55% the rate of hospitalization as compared to placebo.[40,41]

Despite the effectiveness of palivizumab, this drug has drawbacks, such as high costs per patient. Further, to be effective this humanized antibody requires monthly injections during 4 – 5 months and children may need to be hospitalized, despite receiving the treatment.[40,41] For this reason, the generation of new and improved strategies to block hRSV infection has been pursued by researchers and pharmaceutical companies.[42–44]. For instance, another monoclonal antibody designed for the treatment of hRSV infection is motavizumab, a modified antibody derived from palivizumab.[43] Motavizumab, also named MEDI-524 or Numax, was generated by modifying the complementarity determining regions (CDRs) of palivizumab to improve the affinity for the F protein by decreasing the dissociation constant and increasing the association rate.[42] Motavizumab has shown to perform better than palivizumab in animal models, leading to significantly lower viral loads even when administrated 24 h before hRSV challenge.[44] Moreover, treatment with motavizumab decreased disease severity in mice by down-modulating several inflammatory cytokines after hRSV challenge, such as IL-1α, TNF-α and IL-12p70 at day 1 and IFN-γ on days 1 and 5 after infection.[44] In the cotton rat infection model, motavizumab decreased hRSV viral titers 100-fold as compared to palivizumab.[44] Further, only motavizumab was able to inhibit upper respiratory viral replication. Due to the promising results obtained in animal models, this antibody was later evaluated in multiple clinical trials.[45–50] Initially, healthy children (<2 years) were inoculated with motavizumab at different doses: 3, 15 or 30 mg/kg during one or two cold seasons to evaluate their clinical outcomes and any possible side or adverse effects.[45,46] These clinical trials showed appropriate safety, tolerability, similar pharmacokinetics to other monoclonal antibodies already in use, as well as low levels of immunogenicity against motavizumab.[45,46] Later, efforts to evaluate motavizumab's efficacy and safety were performed in phase II trials in high-risk children and compared to palivizumab.[48,49] Five monthly doses of 15 mg/ml of motavizumab and/or palivizumab were injected to children younger than 2 years and adverse events or severe adverse events were evaluated up to 30 days after the last dose. Importantly, no significant differences were observed for the safety profiles of passively immunized children. However, the efficacy obtained with both antibodies was equivalent, consisting of 1.9 and 2.6% hospitalization rates for hRSV-infected patients receiving motavizumab and palivizumab, respectively.[49] A phase III clinical trial comparing palivizumab with motavizumab in high-risk children also failed at evidencing significant differences between patients receiving either one of these humanized antibodies.[47,49] Both groups showed similar rates of medical attention due to lower respiratory tract infections and hospitalizations.[47,49] Importantly, skin rashes were observed more frequently when motavizumab

was administrated (10.2%) as compared to palivizumab (7.2%), suggesting higher frequency of adverse symptoms associated with motavizumab.[47] Moreover, a recent phase II clinical trial performed in healthy children that were hospitalized due to hRSV-induced lower respiratory tract infections showed that motavizumab neither decreases viral loads nor the severity of the disease, as compared to placebo.[50] Further, motavizumab-treated children displayed similar viral loads in nasal samples at two different doses (30 or 100 mg/kg).[50] These data are at variance with previous studies showing that motavizumab recipients showed decreased viral loads in nasal washes, and suggest that administration of motavizumab after hRSV infection is not optimal for preventing disease in recipients. Further, it is important to take into consideration that skin rashes due to motavizumab administration were observed in all studies.[45–50] As a result, after requesting additional clinical data for motavizumab, the Biological License Application for this was withdrawn from the Food and Drug Administration (FDA) and no further clinical trials have been performed afterwards.

Nevertheless, molecular modifications on antibodies are still considered as an attractive alternative to generate new prophylactic treatments for hRSV.[51] Due to the adverse effects caused by motavizumab, molecular modifications are in progress on this antibody to enhance neutralizing capacity and reduce adverse effects. Motavizumab-YTE or MED-557 is currently undergoing four clinical trials to evaluate safety.[52] NCT01562938, NCT00578682, NCT01475305 and NCT01455402 are variants of motavizumab with specific changes in the Fc portion of the antibody that enhance binding to the neonatal Fc receptor (FcRn) and increase serum half-life, as well as lung bio-availability up to four-fold as compared to the original motavizumab in nonhuman primates models [52] (Figure 1). In addition, a clinical trial in healthy adults with motavizumab-YTE has been reported to exhibit decreased clearance of this antibody (71 – 86%) and increased serum half-life 2- to 4-fold (up to 100 days) as compared to motavizumab.[52] More importantly, the neutralizing activity for this antibody has been shown up to 240 days, which is equivalent to a 2.7-fold increase as compared to motavizumab.[52] The promising preliminary data obtained with this new prophylactic antibody support an enhanced serum half-life and neutralizing activity with only a single injection, which could be considered as a significant therapeutic improvement over palivizumab.

The impact of hRSV on public health has led to continuous efforts at identifying compounds that could effectively decrease hospitalization rates and disease severity associated with infection with this virus. Along these lines, the generation of antibodies with improved capacity to block the F protein is considered highly relevant as a strategy to decrease the rate of severe symptoms and hospitalization due to hRSV. Particularly, new insights on the conformations of the F protein have led to new approaches to block the activity of this protein, especially during its pre-fusion conformation.[53] Indeed, it has been described that neutralizing antibodies, such as D25 or MEDI-8897—which are specific for the pre-fusion conformation of the F protein—have been shown to be more efficient than palivizumab at decreasing hRSV infection [53] (Figure 1). Interestingly, MEDI-8897 binds only to the pre-fusion conformation of F protein, particularly to the antigenic site zero (Ø), which is advantageous for improving virus neutralization.[53] MEDI-8897 has been tested in two clinical trials and the FDA has recently given this drug a Fast Track designation for continuing assessment.

Before palivizumab was available, the use of intravenous IgG from hyper-immune individuals (hRSV-IGIV) was accepted as a strategy to control hRSV infection.[54] hRSV-IGIV has shown promising results in animal models and also in patients, with one simple dose showing rapid peak in plasma and a decrease of hRSV titers in cotton rats.[55] However, with the approval of palivizumab, the interest for RSV-IGIV as a prophylaxis treatment for high-risk children was decreased, mainly due to reproducibility and safety concerns. Recently, a new RSV-IGIV (RI-001) has been evaluated in clinical trials (NCT00632463). However, due to low recruitment of immunocompromised patients, only 21 individuals in total (7 high dose, 7 low dose and 7 placebo) RI-001 has hampered obtaining conclusive results. Nevertheless, the strategy of hyper-immune serum continues to be used and may contribute to decrease hRSV dissemination, particularly in immunocompromised patients (NCT00632463).

Another strategy aimed at blocking the activity of the F protein consists of antibody-like compounds, namely nanobodies, which have demonstrated promising results.[56] The main feature of these molecules is that they are small in size and have increased access to somewhat hidden epitopes that regular antibodies cannot neutralize. Indeed, nanobodies against the F protein have shown a hRSV neutralizing capacity up to 4000-fold higher than conventional antibodies in epithelial cells.[56] These molecules have shown an efficient capacity to reduce viral loads in the lungs and to prevent detrimental *in vivo* inflammation in mouse models.[56] A promising nanobody aimed at decreasing hRSV hospitalization is known as ALX-0171, which consists of a trivalent structure composed of VH/H chains of camelid immunoglobulins specific for the F protein (Figure 1). This molecule has shown to be efficient at reducing hRSV infection and viral loads both in cotton rats and lamb infection models for hRSV.[57,58] In the lamb model, different administration routes were evaluated for ALX-0171, showing that nebulization (inhalation) was the most efficient way of delivery of this drug.[58] Three clinical trials have already been carried out for ALX-0171 (NCT01875926, NCT01909843, NCT01483911), in which safety and pharmacokinetics studies have demonstrated promising results. However, data of these trials have not been yet published. A fourth phase I clinical trial is not recruiting participants (NCT02309320).

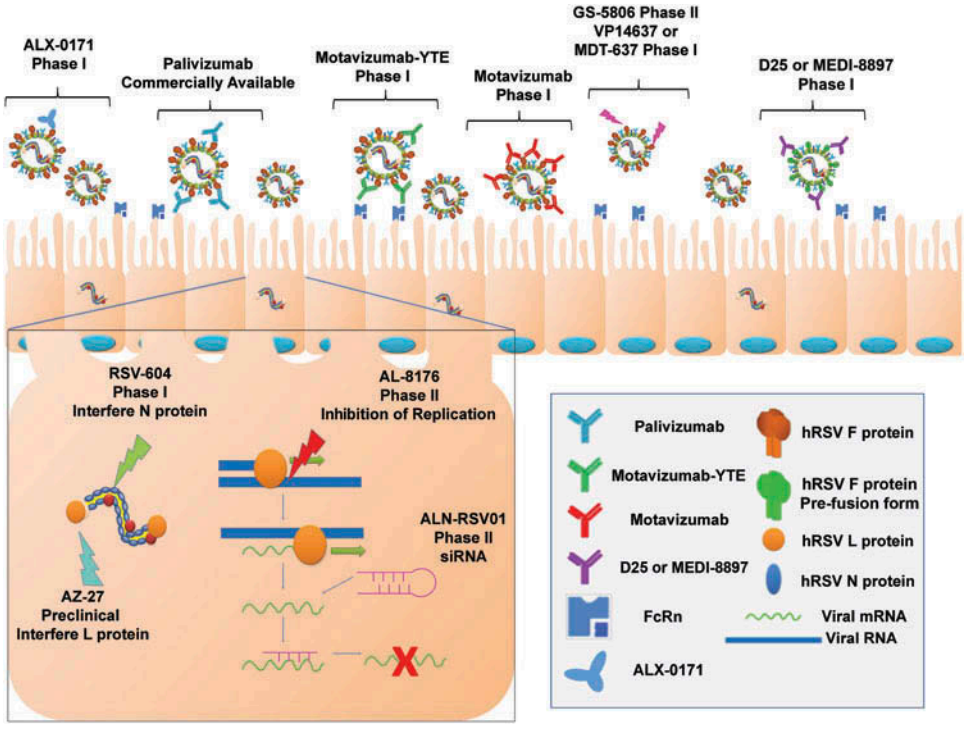

Figure 1. Passive immunity and therapeutic drugs against (Human respiratory syncytial virus) hRSV infection. Monoclonal antibodies have been used to target F protein and inhibit the infection of hRSV on host cells. Palivizumab (blue) is the unique antibody treatment that has been approved for human use in high-risk children. Motavizumab (red) was engineered from palivizumab to display increased binding capacity to the F protein (Phase I). Motavizumab-YTE (orange) is a motavizumab-derived antibody that has the capacity to bind more efficiently to FcRn receptors expressed in lung epithelial cells and have a longer half-life (Phase I). Moreover, D25-MEDI-8897 (purple) is a new generation antibody that has the capacity to bind only to the pre-fusion conformation of the F protein (Phase I). Finally, ALX-0171 is a nanobody composed of three VH/H camelid immunoglobulin chains specific for the F protein (Phase I). Additionally, several drugs have been developed to interfere with different steps of the viral infection cycle. One strategy is the blockade of the virus F protein to prevent virus entry: two drugs have been evaluated to achieve this goal: GS-5806 (Phase II) and VP14637 (Phase I). Viral replication is another drug target, such as the nucleoside analogue AL-8176 (Phase II) and the inhibitor of the viral polymerase L AZ-27 (Preclinical). ALN-RSV01, a small interfering RNA (siRNA), acts against the N protein mRNA by preventing translation and virus assembly (Phase II). RSV604 is a benzodiazepine that targets the N protein as well, although the mechanism of action is still unknown (Phase I). All these drugs are in clinical studies and could become alternative treatments that replace ribavirin as an antiviral approach against hRSV.

hRSV: Human respiratory syncytial virus; siRNA: small interfering RNA.

In summary, significant efforts have been made during the past 20 years for the development of anti-hRSV antibodies, mainly with F protein specificity. Some of these molecules have achieved a significant reduction of hRSV-caused hospitalization in high-risk newborns, from RSV-IGIV to palivizumab, which is currently the only approved treatment against this virus. Furthermore, new variants based on molecular modifications of palivizumab (motavizumab/motavizumab-YTE) or that block more efficiently antigenic sites in the F protein (zero Ø by the D25 antibody) have shown promising results. Alternatively, antibodies against the hRSV G protein may also reduce the hRSV-induced immunopathology, although this strategy has only been tested in animal models.[59,60] Indeed, up to date no clinical trials have been carried out in infants or adults to evaluate this prophylactic approach. Taken together, anti-F antibodies remain the most commonly evaluated and used strategy to prevent hRSV disease in high-risk infants. Whether other viral targets may be as effective as blocking the F protein remains to be determined.

3. Active immunity and vaccine development against hRSV

3.1. Strategies to target hRSV proteins

In spite of decades of research and the medical and economic burden caused by hRSV, to date there are no licensed hRSV

vaccines available. Several obstacles have hampered the development of an effective vaccine against hRSV, such as: (1) the failed clinical trials carried out in the early 1960s with formalin-inactivated virus, (2) potentially different requirements for each vaccine in a particular target population, (3) animal models that do not fully recapitulate the infection that occurs in humans and (4) an elusive protective immune response against this virus.[61] However, because hRSV remains a high priority pathogen for clinicians worldwide, several efforts to develop a safe and efficacious vaccine against hRSV are in progress. The hRSV F protein has been the major target for neutralizing antibodies against the virus, as this protein shows significant conservation between both hRSV serotypes A and B. Therefore, several vaccine candidates have focused on this protein as the main antigenic target [34] (FIGURE 2 and TABLE 1). One such approach against hRSV F is a nanoparticle vaccine consisting of a modified version of full length F produced in Sf9 insect cells using a recombinant baculovirus.[62] This vaccination strategy demonstrated protection against viral replication in cotton rats, inducing high titers of neutralizing antibodies, with no significant side effects.[63,64] Evaluated in a phase I clinical trial, this nanoparticle vaccine was shown to induce the generation of neutralizing anti-F antibodies, was well tolerated and showed no side effects. These data support the use of this formulation as a promising inducer of protective immunity against hRSV. Phase I clinical trials in seropositive children and phase II clinical trials with >60-year-old adults, as well as with third-trimester pregnant women, are currently being carried out with this formulation (NCT02266628, NCT01704365, NCT01709019, NCT02247726, NCT01960686 and NCT02296463). Another study, using three different rodent models including BALB/c mice, cotton rats and Sprague Dawley rats, showed that hRSV soluble F protein conjugated to a Toll-like receptor 4 agonist named glucopyranosyl lipid A (GLA) in a stable emulsion provided protection against viral challenge [65] (FIGURE 2 and TABLE 1). This vaccine induced serum neutralizing responses and a Th1-biased cellular F-specific response including both $CD4^+$ and $CD8^+$ T cells that produced IFN-γ.[65] MEDI 7510, a related approach based on an F protein having a post-fusion conformation, takes into consideration the detrimental cellular immune response observed in the elderly. This type of response is characterized by Th2-biased specific anti-hRSV T-cell immunity,[66,67] which could be prevented by the use of the Th1-promoting adjuvant GLA.[68] Currently, phase Ib and phase II trials are being carried out to assess the safety, immunogenicity and efficacy of this approach in adults that are above 60 years (NCT02289820, NCT02508194). A similar strategy named

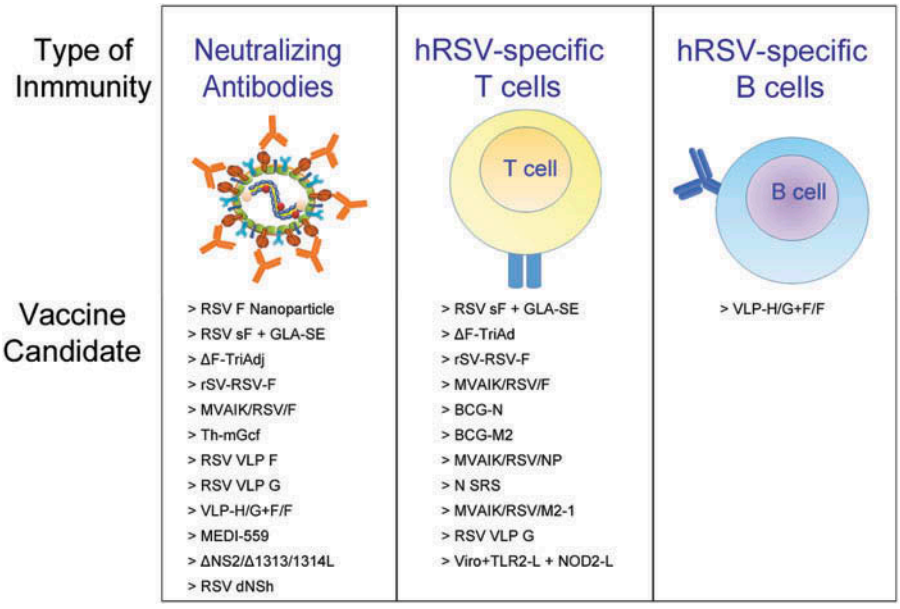

Figure 2. Type of immunity developed by vaccine candidates against hRSV. Several strategies are needed to effectively counteract a viral infection including the production of neutralizing antibodies, virus-specific T cells and virus-specific B cells. The principal focus of vaccine candidates mentioned in this review is the production of antibodies against hRSV-F protein and hRSV-G protein, which reduces the infection by hRSV (12 vaccine candidates). Generation of specific T cells is essential for the proper clearance of the virus, as shown by 11 vaccine candidates that are capable to generate this kind of response in the host and efficiently prevent RSV infection. Finally, VLP-H/G+F/F vaccine has the ability to generate a memory B-cell response against hRSV as it has been demonstrated in preclinical assays.

Table 1. Vaccine candidates against hRSV.

Vaccine strategy	Vaccine name	Model evaluated	Effects after vaccination	Evaluation of enhanced disease*	Preclinical or clinical phase
Target: F protein	RSV F Nanoparticle	Cotton rats Human	Reduction of lung viral titer Generation of Palivizumab-like neutralizing antibodies	No sign of pathology or inflammatory response in lungs	Phase II
	RSV sF + GLA-SE	Balb/c mice Cotton rats Sprague Dawley rats	Reduction of lung viral titer Increase in neutralizing antibodies Induction of Th1 response on T cells	Not evaluated	Preclinical
	ΔF-TriAdj	Balb/c mice Cotton rats	Reduction of lung viral titer Increase in neutralizing antibodies and IgA titer in lungs Generation of memory T cells and hRSV-specific T cells	Low sign of alveolitis and peribronchiolitis	Preclinical
	rSV-RSV-F	Cotton rats African green monkeys	Reduction of lung viral titer Increase in neutralizing antibodies Induction of IFN-γ-producing T cells	No enhanced immunopathology	Preclinical
	MVAIK/RSV/F	Cotton rats	Reduction of lung viral titer Generation of neutralizing antibodies Generation of antigen-specific CD8 T cells IFN-γ producer	Very mild inflammation in lungs	Preclinical
Target: N protein	BCG-N	Balb/c mice	Reduced lung viral load Th1 response polarization on T cells	Not evaluated	Preclinical
	N SRS	Balb/c mice	Reduced lung viral load Induction of IFN-γ-producing T CD4 cells and hRSV-specific T cells Th1/Th2 humoral response Infiltration of neutrophils in BALF and lymphocytes and neutrophils in lungs	Not evaluated	Preclinical
	MVAIK/RSV/NP	Cotton rats	Reduction of lung viral titer Low generation of neutralizing antibodies Generation of antigen-specific CD8 T cells IFN-γ producer	No infiltration of inflammatory cells in lungs after a second dose	Preclinical
Target: G protein	PR8/RSV.HA-G	Balb/c mice	Reduction of lung viral titer Induction of Th1 humoral response No generation of T cell response	Not evaluated	Preclinical
	G nanoparticles	Balb/c mice	Reduction of lung viral titer Generation of strong CD8 T cell response Th1/Th2 humoral and cellular response	Not evaluated	Preclinical
	Th-mGcf	Balb/c mice	Reduction of lung viral titer Induction of hRSV-specific antibodies	Not evaluated	Preclinical
	MVAIK/RSV/G	Cotton rats	Low generation of neutralizing antibodies	Moderate interstitial pneumonia in lungs	Preclinical
Target: M2-1 protein	MVAIK/RSV/M2-1	Cotton rats	Reduction of lung viral titer No generation of neutralizing antibodies Generation of antigen-specific CD8 T cells IFN-γ producer	No infiltration of inflammatory cells in lungs after a second dose	Preclinical
VLP/Virosomes	RSV VLP F	Balb/c mice	Reduction of lung viral titer Generation of neutralizing antibodies	Not evaluated	Preclinical
	RSV VLP G	Balb/c mice	Reduction of lung viral titer Generation of neutralizing antibodies Mucus production and eosinophilia in lungs Induction of CD8 T cells IL-2 and IFN-γ producer	Not evaluated	Preclinical
	VLP-H/G + F/F	Balb/c mice	Reduction of lung viral titer Generation of neutralizing antibodies persisting for 14 months Generation of antigen-specific memory B cells and germinal center B cells	Not evaluated	Preclinical
	Viro+TLR2-L + NOD2-L	Balb/c mice	Reduction of lung viral titer Induction of serum and mucosal antibodies Th1-biased immune response	Not evaluated	Preclinical

(*continued*)

Table 1. Vaccine candidates against hRSV. (continued).

Vaccine strategy	Vaccine name	Model evaluated	Effects after vaccination	Evaluation of enhanced disease*	Preclinical or clinical phase
	RSV-MPLA	Balb/c mice Cotton rats	Reduction of lung viral titer Increase on IgG and IgA antibodies Th1-biased immune response	No sign of alveolar, peribronchial or perivascular infiltration in lungs	Preclinical
Attenuated virus	MEDI-559	Human	Generation of neutralizing antibodies	Non-enhanced disease after natural hRSV infection, but increase medically attended LRTIs‡	Phase I/2a
	ΔNS2/Δ1313/1314L	Juvenile chimpanzee	Generation of neutralizing antibodies	Not evaluated	Phase I
	RSV dNSh	Balb/c mice	Generation of neutralizing antibodies	Not evaluated	Preclinical

Source: ClinicalTrials.gov
*Enhanced disease parameter is considered only when is evaluated in Cotton rats model
‡Evaluated in human.
hRSV: Human respiratory syncytial virus; LRTIs: lower respiratory tract infections

ΔF/TriAdj, based on a truncated F protein (ΔF) formulated with polyI:C, a TLR3 agonist, polyphosphazene, a synthetic polymer that enhances antibody responses and an immune defense regulator (IDR) peptide, provided protection against hRSV challenge in BALB/c mice and cotton rats [69] (Figure 2 and Table 1). This formulation was able to induce the production of hRSV F-specific IgG, IgA and virus-neutralizing antibodies and promoted a cytolytic $CD8^+$ T-cell response against hRSV.[69] Long-term clinical trials performed with these vaccine candidates have demonstrated that immunity against hRSV lasts for at least 1 year. To assess potential interference of maternal antibodies with these vaccine formulations, pregnant ewes were vaccinated and later newborn lambs vaccinated with three doses of ΔF/TriAdj.[70] The results showed that the vaccinated animals induced similarly high-affinity F-specific IgG, virus neutralizing antibodies and IFN-γ-secreting cells, indicating that immunization was effective even in the presence of maternal antibodies.[70]

A mouse virus (Sendai virus, SV) recombinant for parainfluenza virus type 1 (PIV-1), which has already been tested before as a candidate vaccine against PIV-1 in humans, was modified to express a full length F-hRSV protein derived from an hRSV subtype A virus.[71] Immunization with this vaccine demonstrated protection in cotton rats after challenge with either hRSV group A or B [71] (Figure 2 and Table 1). Vaccination with this formulation induced high titers of hRSV-neutralizing antibodies and IFN-γ-producing T cells.[71] The rSV-hRSV-F formulation also demonstrated protection in African green monkeys without any associated clinically relevant adverse effects.[72] Furthermore, the contribution of maternal antibodies to vaccination efficacy was assessed in the cotton rat model.[73] It was observed that passively transferred antibodies onto the animals before immunization with rSV-hRSV-F did not interfere with the capacity of the vaccine to induce the *de novo* generation of virus neutralizing antibodies as well as a cellular response that protects against hRSV challenge.

Another important target considered for the development of vaccines against hRSV is the G protein. In this regard, a recombinant influenza A virus harboring chimeric hemagglutinins (HAs) conjugated to a conserved-domain of the G protein (PR8/RSV.HA-G) was shown to protect against hRSV challenge by significantly reducing viral titers in the lungs and inducing a favorable Th1-type antibody response detectable in serum samples.[74] This was also accompanied by an increase in anti-G specific IgG titers in bronchoalveolar lavage fluid samples after infection.[74] Nonetheless, this vaccine candidate failed at producing a strong hRSV-specific T-cell response. As the conserved-domain of G protein was inserted into the HA protein without affecting the role that this protein plays in replication or immunogenicity, the recombinant influenza may act as an attenuated virus providing dual protection against influenza virus and hRSV.

Another study targeting the G protein considered the fact that mice receiving G-specific type-2 helper T cells suffered neutrophil recruitment and intense eosinophilia in the lungs.[75] Taking this into consideration, a modified G protein fragment (Gcf) containing two amino acid changes to prevent $CD4^+$ T-cell activation was fused to a T-cell epitope from the F protein to broaden the hRSV-specific T-cell responses [76] (Figure 2, left panel and Table 1). Th-mGcf combined with a cholera toxin adjuvant significantly reduced viral titers in the lungs, induced an hRSV-specific humoral response and prevented lung immunopathology, as well as eosinophil recruitment.[76] hRSV A2-derived Th-mGcf was also able to provide cross-protection against hRSV belonging to the B subtype.[76]

The hRSV G protein includes a conserved CX3C chemokine-like motif that binds to the chemokine receptor CX3CR1 expressed on several cell types and has been proposed to modulate the pathogenesis induced by hRSV infection.[77] The effect of the CX3C motif on the immune response after hRSV infection was evaluated by mutating or blocking this motif with a monoclonal antibody in a two-chamber *in vitro* model using human airway epithelial cells and human immune cells (A549 and PBMCs).[77] This study suggests that the CX3C chemokine motif contributes to diminish IFN-I/III production by human airway epithelial cells (A549) and by innate and adaptive immune cells. Furthermore, antibody blockade of the CX3C chemokine motif showed a reduction of viral titers in lungs, as well as reduced cellular inflammatory responses when administered at early times after infection.[77]

Along these lines, a nanoparticle vaccine was designed based on G protein polypeptides that included the CX3C chemokine motif.[78] In immunized BALB/c mice, no pulmonary eosinophilia could be detected after hRSV challenge in BALs. In this study, vaccinated mice showed a faster weight recovery, reduced Th2-type cytokines in bronchoalveolar lavage and stronger $CD8^+$ T-cell responses than did control mice.[78] Further, this vaccine also induced a balanced Th1/Th2 humoral and cellular immune response characterized by IgG1 and IgG2a anti-hRSV antibodies, as well as IFN-γ and IL-4 secreting T cells.[78]

Despite the fact that the hRSV N is one of the main targets for cytotoxic T cells and highly conserved among hRSV isolates, this protein has been poorly studied as an antigenic target in vaccines against this virus. This scenario is probably due to the rationale that a protective hRSV vaccine requires the production of high amounts of neutralizing antibodies. The N protein assembled as sub-nucleocapsid ring structures (N SRS) together with a mucosal adjuvant LT(R192G) has been shown to produce both antigen-specific $CD8^+$ T cells and IFN-γ-producing $CD4^+$ T cells, as well as mixed Th1/Th2 humoral responses mainly with IgG1 antibodies [79] (Table 1). N SRS plus LT(R192G) also induced the generation of local IgA antibodies in BALs, although these antibodies failed at neutralizing the virus.[79] Although eosinophils were not detected in BALs after viral challenge, an increase in the percentage of neutrophils was identified in these samples and inflammatory infiltration of lymphocytes and neutrophils was detected in the lungs.[79]

Another study assessed recombinant measles viruses expressing the F and G proteins (MVAIK/RSV/F and MVAIK/RSV/G, respectively), which showed in a cotton rat model that only the formulation expressing F protein was able to produce an antibody response against both subtypes of hRSV, with an evident neutralizing capacity [80] (Figure 2 and Table 1). Although both vaccine candidates decreased viral titers in the lungs, infiltration of inflammatory cells in lungs was detected soon after hRSV infection. New recombinant measles viruses have been developed afterwards, including the viral nucleoprotein (N) and M2-1 protein (MVAIK/RSV/N and MVAIK/RSV/M2-1).[81] Cotton rats immunized and boosted with these vaccines showed a significant decrease in lung viral titers, with little to no infiltration of inflammatory cells in the lungs. While MVAIK/RSV/N produced a weak neutralizing antibody response, MVAIK/RSV/M2-1 was not able to induce neutralizing antibodies against hRSV.[81] However, both recombinant measles viruses and MVAIK/RSV/F induced important amounts of antigen-specific $CD8T^+$ cells that produce IFN-γ [81] (Table 1). These results support the notion that not only neutralizing antibodies are required to prevent hRSV infection, but the presence of IFN-γ-producing T cells is key for viral clearance.

It has been proposed for a while that the pathologic Th2-type inflammatory immune response induced by hRSV infection could be ameliorated by the induction of a balanced antiviral Th1-type response [82] (Figure 2 and Table 1). Based on this notion, the current *Mycobacterium tuberculosis* vaccine bacillus Calmette–Guérin (BCG) consisting of attenuated *M. bovis* is a recognized inductor of Th1 immunity in infants, and has been used as a recombinant vector against several viruses, such as measles and rotavirus with favorable results.[83,84] Recombinant BCG strains expressing either the hRSV N or the M2 proteins have been shown to prevent infiltration of inflammatory cells in the airways, decrease viral loads in the lungs and induce the expansion of hRSV-specific IFN-γ secreting T cells [85,86] (Figure 2 and Table 1). Adoptive transfer of antigen-specific $CD4^+$ and $CD8^+$ T cells from BCG-N- or BCG-M2-immunized mice led to a significant reduction of hRSV pathology equivalent to uninfected mice, suggesting that Th1 cellular immunity generated by those recombinant BCG strains was completely protective.[85,86]

3.2. Virosomes, VLPs and attenuated virus as strategies for vaccine development

Virus-like particles (VLPs) are formed by the assembly of multiple copies of viral proteins that when expressed in a recombinant assemble spontaneously into virus-size

complexes without the need of viral genome [87] and display potent immune-stimulatory properties that do not usually require combinations with adjuvants.[88] Thus, these particles resemble infectious viruses, but cannot replicate.[87] Current vaccines that use this strategy are the papillomavirus and the hepatitis B virus VLP-based vaccines, which have been licensed for human use.

VLPs consisting of a core of the influenza virus matrix (M1) protein and full length hRSV F or G proteins on their surface have been developed and tested for their effectiveness as potential vaccines against hRSV [89] (Figure 2 and Table 1). In a mouse model, the VLP-F formulation significantly decreased viral loads and hRSV titers in the lungs after challenge, while the VLP-G formulation resulted in an immunopathology equivalent to FI-hRSV, characterized by excessive mucus production and eosinophilia in the lungs. [89] Despite these observations, the VLP-F and VLP-G vaccinated mice displayed IgG2a-dominant hRSV-specific immunoglobulins in serum and lung extracts. Although the VLP-G formulation was able to elicit virus-neutralizing antibodies, the VLP-F version induced a better humoral response.[90] When the cellular response was evaluated, only VLP-G immunized mice showed a significant increase in IL-2 and IFN-γ producing $CD8^+$ T cells.[90] A mixed VLP-F plus VLP-G immunized group was also assessed, which showed an increase in $CD8^+$ IFN-γ and IL-2 producing T cells.[90] Humoral responses of the combined formulation were similar to that observed for VLP-F, as well as undetectable eosinophilia in the lungs, suggesting that such a combined immunization procedure induces better protection against hRSV challenge than each formulation separately.[90] Another study analyzed VLPs prepared from avian cells that contained the nucleocapsid and membrane proteins of Newcastle Disease Virus (NDV), as well as chimeric proteins composed of the ectodomains of hRSV G fused to the cytoplasmic domain of the NDV hemagglutinin-neuraminidase (NDV-HN) protein and hRSV F fused to the transmembrane domains of NDV F protein (VLP-H/G + F/F) [91] (Figure 2 and Table 1). Immunization with a single dose of these VLPs without any adjuvant elicited a significant decrease in hRSV lung titers after viral challenge and no signs of pulmonary inflammation after infection. Vaccination with such formulation also induced anti-hRSV F and G protein immunoglobulins with high IgG2a/IgG1 ratios and robust virus neutralizing antibodies titers that lasted for 14 months. [91,92] To better understand B-cell responses induced by immunization with VLPs, adoptive transfers of enriched splenic B cells obtained from VLP-immunized mice have been performed into immunodeficient $rag^{-/-}$ mice 14 months after vaccination.[92] These studies have shown that recipient mice displayed important anti-F and anti-G IgG titers, suggesting that vaccination with VLP-H/G+F/F elicited the generation of antigen-specific memory B cells in donor-immunized mice.[92] VLPs also stimulated the production of germinal center B cells. Experiments with T cell-deficient C57BL/6-TCRβα knockout mice allowed determining that VLP-H/G+F/F elicits T cell-dependent B cells to generate such IgG responses.[92]

Virosomes are another form of VLPs used as vaccine candidates, which consist of reconstituted viral envelopes mimicking the original virus in its native form with no viral genome.[93,94] Lipophilic adjuvants or receptor ligands can be conjugated to these formulations to enhance their immunogenicity.[93,94] In this regard, incorporation of TLR2/NOD2 ligands in virosomes has shown to stimulate the production of mucosal and serum IgG responses that display a balanced Th1/Th2 phenotype and lead to significantly less hRSV lung titers with practically no signs of enhanced respiratory disease in the BALB/c mice.[95] On the other hand, monophosphoryl lipid A (MPLA) conjugated virosomes have shown to elicit increased IgG and IgA levels against hRSV with neutralizing capacity [96] (Table 1). Virosomes conjugated to this latter TLR4 ligand were shown to significantly decrease viral titers in the lungs of hRSV-challenged mice, which exhibited high IFN-γ levels and low IL-5 levels in lung and spleen, representative of Th1-type immune response.[96] Protection was also assessed in cotton rats, which showed favorable results after infection with no signs of enhanced disease.[97]

Virus attenuation is the most common approach in vaccine design against these pathogens and concomitantly the most advanced strategy in clinical settings for vaccine development against hRSV. However, developing a successful formulation is not trivial, as it must exhibit an adequate balance between attenuation, immunogenicity, safety and efficacy.[98] A novel strategy for attenuating viruses is targeting nonessential virus genes involved in virulence with deoptimization of codon usage.[99] This strategy consists of developing viruses that incorporate in their genome least used codons, which will translate into reduced virulence. Such mutant viruses were recently designed by deoptimizing the NS1 and NS2 genes simultaneously (dNSh), wherein every NS1 and NS2 codon is the least frequently used for that amino acid in human cells.[99] In normal human bronchial epithelial cells (NHBE), hRSV dNSh virus displayed a more limited growth phenotype as compared to hRSV-A2. [99] In vaccinated mice, the deoptimized virus was significantly attenuated displaying similar protection levels against hRSV challenge than did immunization with hRSV-A2 (Table 1). It also showed to be slightly more immunogenic than the parental virus, which correlated with a significant increase in virus neutralizing antibodies.[99] hRSV dNSh also induced a significant decrease in the degradation of STAT2, a member of the type-I IFN pathway and a known target of the NS1 and NS2 proteins.[100] It also produced decreased levels of activated NF-κB, which is induced by NS1 and NS2 early during hRSV infection to promote survival signals within infected cells and the secretion of pro-inflammatory cytokines.[99] These data suggest that deoptimization of nonessential virulence genes could be a

promising strategy to generate live-attenuated vaccines against hRSV. The development of other attenuated viruses that are genetically stable at physiological temperature include the deletion of the NS2 gene, a mutation at 1313 position in the L polymerase protein and an I1314L substitution in the same L protein (ΔNS2/Δ1313/1314L).[98] Both mutations and substitutions derive from compensatory mutations that can be generated by the virus. The ΔNS2/Δ1313/1314L virus strain was tested in juvenile chimpanzees and showed highly attenuated phenotypes and low levels of virus replication.[98] This vaccine also elicited a four-fold increase in the titers of virus neutralizing antibodies, thus becoming a good candidate for further evaluation in a phase I clinical trial, which is currently under development (NCT01893554). RA2cp248/404/1030ΔSH is another example of a live attenuated virus vaccine candidate that has shown promising results in clinical trials.[101] This vaccine candidate was adequately tolerated by hRSV-seronegative children, exhibited high levels of attenuation in adults and seropositive children and use of second doses showed restricted replication. However, after this second dose only 44% of 1- to 2-month-old infants developed a significant increase in anti-hRSV IgG and IgA titers.[101] On the other hand, MEDI-559, a vaccine candidate derivative of rA2cp248/404/1030ΔSH, which only differs by 39 silent nucleotide substitutions, retains virus attenuation in lungs of cotton rats and in hRSV seronegative children.[102] Here, it has demonstrated to be immunogenic by virus microneutralization assays [102] (Figure 2 and Table 1). Although no evidence of enhanced disease was reported, an increase in medically attended lower respiratory illness was observed for MEDI-559 immunized children when compared to placebo, therefore additional safety studies would be needed.[103]

3.3. A novel approach for hRSV vaccine development: maternal immunization

Recently, in the United States palivizumab has been recommended as a prophylactic only for preterm infants born before the 29th week of gestation, premature infants with chronic lung disease born under 32 weeks of gestation and infants below 1 year with hemodynamically significant heart disease.[104] Thus, it is not recommended for children at their second year of life.[104] Due to these limitations, maternal immunization, which has already been implemented for pertussis and influenza in the United States and other countries, appears as a promising strategy to protect young infants from hRSV infection.[105] The transfer of anti-hRSV antibodies transplacentally or through the milk has been proposed as a strategy to protect infants within 3 – 6 months old and afterwards.[106] However, to date only a few studies have assessed this approach, being maternal immunization with FI-hRSV one example of these.[107] This study showed that passively transferred antibodies decreased viral loads in the lungs of mouse neonates and induced neutralizing antibodies without exhibiting any signs of vaccine-enhanced disease.[107]

Despite the fact that the levels of maternal transferred antibodies are inversely associated with hRSV-induced acute lower respiratory tract infections,[108] high titers of hRSV-specific antibodies can also induce an immunosuppressive effect in infants, thus becoming a major obstacle for the development of a protective humoral response for children.[108–111] Therefore, vaccine candidates should include in their studies models for assessing maternal antibody transfer and their potential interference with the development of protective immune response in infants. Vaccination in pregnant women should also consider reduced reactogenicity in order to minimize potential adverse effects to the mother and fetus.[105] Furthermore, such a strategy should consider the seasonality of hRSV outbreaks for the optimal generation of maternally derived antibodies that exert maximum effects at birth.[106]

4. Development of therapeutic drugs to treat hRSV infection

Although numerous drugs have shown some effectiveness at reducing hRSV infection, to date only ribavirin has been approved for use in humans against this virus.[112] Ribavirin is a guanosine analog that interferes with the replication of hRSV, as well as of other RNA viruses. However, ribavirin is seldom used against hRSV, in part because it has shown poor cost/effectiveness and some side effects.[113,114] In this regard, the development of new drugs to counteract hRSV infection for the general population and high-risk groups, such as newborns, elderly and immunocompromised patients, is highly needed.

Several drugs have been identified for treating hRSV infection, either by preventing the entry of the virus into target cells or by preventing replication or assembly of viral particles. Drugs that prevent the entry of the virus are mainly focused on blocking the fusion protein and some are being assessed in clinical trials.[115–118] Among the drugs that prevent hRSV replication are those that target the viral polymerase L, which prevents gene expression and genome replication.[119,120] Other drugs identified against hRSV target the nucleoprotein.[121] Importantly, many of these drugs, described in detail below, have made significant progress in clinical trials with promising results.

Recently, a virus entry blocker termed GS-5806 was shown to display hRSV fusion inhibition when taken orally.[116] A phase I clinical study has further shown that treatment with GS-5806 results in a significant reduction of viral loads in the lungs as compared to placebo groups.[116] Furthermore, treatment with this drug has shown to decrease mucus production and other clinical manifestations associated with hRSV infection (Figure 1). A phase IIb clinical

study is currently recruiting hRSV-positive adults to evaluate the pharmacokinetics, antiviral effects, safety and tolerability of GS-5806 (NCT02135614). Also, within this group of drugs is an imidazoisoindolone derivative named BTA9881, which works as an inhibitor of hRSV fusion. Regretfully, this drug displayed a poor safety profile and was discontinued, despite good bioavailability after oral ingestion and adequate pharmacokinetics.[122] Another example of antiviral drugs is VP14637, also known as MDT-637, a drug to be delivered as a powder by inhalation to the upper and lower respiratory tract. Preclinical studies have shown that this drug works as a more potent inhibitor of hRSV replication in HEp-2 cells than ribavirin, and also displays lower toxicity [115,122] (FIGURE 1). Furthermore, studies in cotton rats have shown that VP14637 can decrease viral loads and reduce the histopathology observed in the lungs of cotton rats challenged with hRSV.[118] However, phase I clinical studies on this molecule have not yielded conclusive results yet.

In order to prevent viral replication, the nucleoside analogue AL-8176 (viral polymerase inhibitor) has been evaluated in clinical trials, showing a significant reduction in viral loads in the lungs and an improvement in the symptom score in patients, as compared to placebo (https://clinicaltrials.gov). AZ-27, another hRSV L protein inhibitor, has shown promising results *in vitro* in HEp-2 cells and other cell lines by significantly reducing viral titers after hRSV infection.[123] However, clinical studies need to be performed with this latter drug to test effectiveness in patients.

Inhibition of viral assembly has been assessed with RSV604, an antiviral benzodiazepine that is likely to target the hRSV-nucleoprotein,[119] although the mechanism of inhibition remains unknown. It was observed in *in vitro* studies that RSV604 is capable of inhibiting hRSV replication and phase I clinical trials have shown an adequate safety for this drug. Although more studies are needed, RSV604 seems to be a promising antiviral drug for the treatment of hRSV infection.

RV568 is a narrow spectrum kinase inhibitor (NSKI) that has been evaluated in clinical trials for the protection of patients after hRSV exposure. The results of these phase I studies have shown decrease up to 46% in nasal IL-8, a proinflammatory cytokine produced in response to hRSV in treated patients as compared to placebo.[124] Unfortunately, no significant differences were found for IL-6, another cytokine secreted in response to hRSV, neither in viral loads, clinical symptoms or infectivity.[124]

Another strategy used as therapy to treat hRSV infection is the development of small interfering RNAs (siRNA), which can target specific viral proteins. ALN-RSV01 is an siRNA able to bind directly to the mRNA of the hRSV nucleocapsid protein and has been shown to display antiviral activity both *in vitro* and *in vivo* in murine models.[121,125] A phase II study with 101 healthy patients showed that ALN-RSV01 is safe and adverse effects were similar in severity and frequency to those in the placebo group. In another phase II study, significant differences were observed for its antiviral action when compared to placebo-controlled trial, supporting the notion that this drug is a promising approach for hRSV treatment.[125]

5. Conclusion

Since the first attempt to generate a protective vaccine against hRSV 50 years ago, which threatened the lives of vaccinated children, significant research efforts have been made for developing effective prophylactic and therapeutic approaches against hRSV. Most approaches have aimed at preventing the entry of hRSV into cells by inducing neutralizing antibodies against the F and G proteins. In addition, strategies to block hRSV proteins involved in either virus replication or assembly, such as L or N, which have also been assessed, but to a lesser extent. Importantly, these latter approaches have yielded promising results, thus highlighting the need to diversify viral targets. Furthermore, vaccines have been shown to be cost-effective for respiratory diseases and would undoubtedly contribute enormously to decrease hRSV morbidity and mortality if developed and implemented. Such a formulation should induce not only a potent humoral immune response characterized by neutralizing antibodies, but also the differentiation of virus-specific effector T-cell responses able to clear hRSV from the respiratory tract by preventing excessive lung damage.

6. Expert opinion

Although several immunization and therapeutic drugs have been designed to prevent or ameliorate hRSV-caused disease, none of them has shown enough protection or safety in humans to reach infants and the elderly, the most susceptible populations. It is likely that a combined immunization strategy consisting of maternal immunization to promote passive humoral immunization followed by infant immunization with vaccines that induce strong Th1 immunity (T cells and antibodies) would be the most effective approach to control the burden of disease caused by this virus in children. This notion is supported by the fact that newborns develop nonoptimal humoral responses to hRSV as compared to children older than 3 months, possibly because of a limited expansion of their B-cell repertoire as a consequence of reduced somatic mutations in genes encoding for hRSV-specific antibodies.[126] Although it has been proposed that maternal immunization could achieve passive transfer of palivizumab-like antibodies to the fetus, important potential caveats have questioned suitability of this approach for preventing hRSV infection in children younger than 6 months. For instance, transfer of maternal antibodies could have an immunosuppressive effect in infants. [110,111] Thus, vaccine candidates designed for pregnant women should guarantee that no interference would take

place between maternal antibodies and the development of active infant humoral immunity. Additionally, several factors can affect the transfer of antibodies of the right isotype and affinity from mother to the fetus, as well as their half-life in the circulation.

Because of their essential role in promoting B-cell responses, as well as their direct effects in mediating hRSV clearance independent of B cells,[85,86] memory T cells should be in all cases considered as an essential component for a protective vaccine approach. This is particularly important, considering that hRSV has evolved molecular mechanisms to impair naïve, but not memory T-cell activation and function,[11,127] which dampens the establishment of protective immune memory and leads to ineffective airway inflammation. Therefore, a robust T-cell response is key for preventing severe disease caused by hRSV and to promote high affinity/neutralizing antibodies, as well as cytotoxic T cells that clear the virus. Along these lines, considering that for more than 90 years BCG vaccination has shown to be safe and to induce Th1 immunity in newborns despite the Th2 bias of neonatal immune responses,[128,129] the use of BCG strains that are recombinant for hRSV antigens appears as a promising new alternative for the prevention of lower respiratory tract diseases and nervous system sequelae due to infections by hRSV in infancy and childhood. Such notion has been supported by our observation that immunization with recombinant BCGs elicits a protective Th1 cellular response that counteracts the respiratory disease caused by hRSV,[85,86] and successfully prevents neurological alterations commonly associated with the experimental infection of mice.[130]

On the other hand, considering that the elderly are an important population at risk for severe hRSV infections and that these individuals exhibit immune-senescence, characterized by decreased cellular immunity, vaccines that promote long-lasting T-cell responses could dramatically improve protection against this virus in this population. However, because diminished immune responses are usually observed in the elderly after vaccination, as compared to young adults,[66] it might be difficult to promote such long-term protective T-cell responses against hRSV. Thus, approaches focused on treatment with antiviral drugs that directly block hRSV infection might be more effective than vaccines at preventing community-acquired hRSV pneumonias in this susceptible group.

Despite the fact that most antiviral drugs display either considerable adverse effects or nonoptimal pharmaceutical properties, they nevertheless provide therapeutic intervention opportunities that could perhaps enhance characteristics conferred by vaccines alone. Considering that the immune responses generated by infants to naturally acquired hRSV infections are frequently poor and ineffective, antiviral drugs could counteract viral mechanisms by interfering with the host adaptive immune response which leads to disease. Thus, next generation antivirals should be designed not only to treat ongoing hRSV infections, but also to target hRSV virulence determinants that impair the host immune response in order to allow the natural establishment of protective immunological memory after hRSV infections. However, important efforts need to be invested to improve the safety of these types of treatments.

On the other hand, passive immunity strategies against hRSV have mainly focused on the development of anti-F antibodies. Although this approach has translated into the only drug currently licensed to decrease hRSV hospitalizations in high-risk infants, it has drawbacks. Indeed, palivizumab is extremely expensive and requires continuous intravenous administrations. Thus, modifications to this drug are being assessed to improve efficacy. Alternatives include the design of specific neutralizing antibodies that target the pre-fusion conformation of the F protein, which could be better than palivizumab at decreasing hRSV infection. Additionally, small antibody-like compounds (e.g. nanobodies) targeting the F protein could improve access to epitopes within the protein than other antibodies with poor neutralizing capacity. Moreover, the development of antibodies against new targets, such as the G protein, which has shown good results in animal models, may also be a valid alternative in the future. These latter approaches have highlighted the fact that antibody-based strategies continue to be an attractive alternative for both prophylaxis and treatment of hRSV infections and the reduction of hRSV-associated hospitalizations.

Finally, as discussed throughout this review, important progress has been made in the last decades in developing affordable and effective drugs and vaccines against hRSV. However, important efforts are still needed to overcome certain obstacles that have delayed the identification of new solutions. Particularly, the development of an optimal hRSV animal model is critical for assessing the effectiveness of drugs and vaccines, as well as their safety in the context of both hRSV infection and the establishment of immunity. Indeed, several concerns have been raised on the wide use of the cotton rat model in the study of candidate vaccines and drugs, mostly because very limited information is available for this animal regarding the dynamics of B-cell and T-cell responses and the duration of T-cell immunity. Other concerns also exist on the similarity of immune components with human orthologs, such as major histocompatibility complex antigenic-binding residues and the diversity of cotton rat immunoglobulins, which are known to differ in response to hRSV as compared to the human.[131] However, because hRSV is a global priority concern that promotes important research efforts, we believe that it will be a matter of time before cost-effective therapies and new prophylactic strategies will be available to the general population.

Declaration of interest

The authors are supported by grants: Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) no 1110518, FONDECYT no 1070352, FONDECYT no 1085281, FONDECYT no 1100926, FONDECYT no 3070018, FONDECYT no 3100090, Comisión Nacional de Investigación Científica y Tecnológica (CONICYT)/ FONDECYT POSTDOCTORADO no 3140455, FONDECYT no 11075060, FONDECYT no 1110397 and FONDECYT no 1140011. CONICYT Capital Humano Avanzado en la Academia no 791100015, Vicerrectoría de Investigación de la Pontificia Universidad Católica de Chile No 04/2010 and Millennium Institute on Immunology and Immunotherapy (NoP09/016-F). AM Kalergis is a Chaire De La Région Pays De La Loire De Chercheur Étranger D'excellence and a Directeur de Recherche via a Contract Durée Determinée (CDD-DR) at INSERM. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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* **Recent study that demonstrated that hRSV nucleoprotein prevents the immunological synapse assembly.**

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Current Opinion in Immunology

Inflammatory damage on respiratory and nervous systems due to hRSV infection

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The exacerbated inflammatory response elicited by human Respiratory Syncytial Virus (hRSV) in the lungs of infected patients causes a major health burden in the pediatric and elderly population. Since the discovery of hRSV, the exacerbated host immune-inflammatory response triggered by this virus has been extensively studied. In this article, we review the effects on the airways caused by immune cells and cytokines/chemokines secreted during hRSV infection. While molecules such as interferons contribute at controlling viral infection, IL-17 and others produce damage to the hRSV-infected lung. In addition to affecting the airways, hRSV infection can cause significant neurologic abnormalities in the host, such as seizures and encephalopathy. Although the origin of these symptoms remains unclear, studies from patients suffering neurological alteration suggest an involvement of the inflammatory response against hRSV.

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Current Opinion in Immunology 2015, **36**:14–21

This review comes from a themed issue on **Host pathogens**

Edited by **Peter A Barry** and **Guido Silvestri**

For a complete overview see the Issue and the Editorial

Available online 27th May 2015

http://dx.doi.org/10.1016/j.coi.2015.05.003

Introduction

Prime infection by the human Respiratory Syncytial Virus (hRSV) occurs early during infancy causing a broad range of pulmonary manifestations, from acute upper respiratory to lower respiratory tract symptoms, such as bronchiolitis and pneumonia [1]. hRSV pathogenesis has also been associated with the development of airway hyperreactivity, mucus production and lung inflammation in cases of severe infections [2]. The heterogeneity of hRSV pathogenesis has been attributed to host factors, including premature birth, very young or old age, immunosuppression and chronic lung and heart illnesses [3]. However, most children with hRSV that develop severe disease are immunocompetent and have no risk factors associated, except for being under two years of age [4].

hRSV bronchiolitis is caused by the obstruction and inflammation of distal bronchioles, reducing the airflow in the small airways [5•]. Airway obstruction alters the exhalation capacity leading to lung hyperexpansion, increased respiratory rates, reduced lung function, upregulated mucus production, atelectasis and wheezing [5•,6•]. Most of these symptoms have been considered as common with infections caused by other respiratory viruses, such as Influenza (Flu), Adenovirus (hADV) and Human Metapneumovirus (hMPV) [7–9]. Two events can cause viral pneumonia: direct infection of the alveolar epithelium and distal airway inflammation induced by the viral infection. Both events promote injury and oedema, which block the gas-exchange process, leading to severe pulmonary disease [6•].

In response to hRSV infection, airway epithelial cells (AECs) initiate the antiviral response to promote the recruitment of effector cells at the site of infection. The presence of proinflammatory cytokines and the perivascular/peribronchial infiltration with immune cells (mainly neutrophils and lymphocytes) are associated with the pathogenesis in lungs caused by hRSV infection [5•].

Infection by hRSV can also cause diverse alteration in organs different from lungs, which are denominated extrapulmonary manifestations [10]. Neurological abnormalities are the most important extrapulmonary manifestations observed in patients with severe bronchiolitis due to hRSV infection [10,11]. Studies of patients that present symptoms, such as seizures and apnea, have shown altered pro-inflammatory cytokine levels in the cerebrospinal fluid (CSF) [10]. However, the nature of the encephalopathy caused by hRSV remains controversial.

Indeed, infection with other respiratory viruses, such as hMPV, Flu and hADV, has been associated with neurological dysfunction [12–15]. In addition, seizures are a common symptom associated to the neurotropic strain of Flu, hADV and probably hRSV [13,15,16••,17].

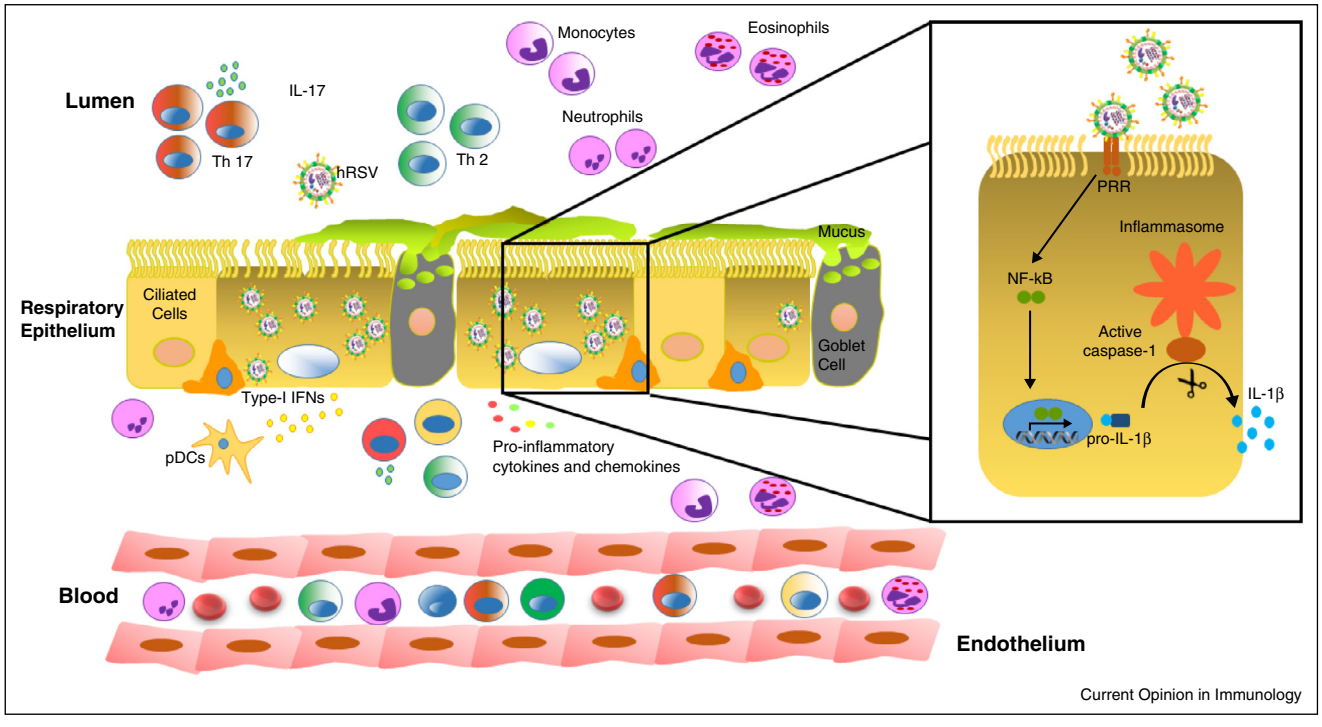
In this article, the major aspects of inflammation processes elicited by hRSV are reviewed, including early stage of infection, acute phase of the pulmonary disease and the late response that promotes the viral clearance, considering also the involvement of the central nervous system.

Acute damage and early immune response against hRSV in the airways

Histopathological studies, performed in lungs from hRSV-infected patients show that the pseudostratified epithelium of bronchiolar airways and alveolar regions are highly compromised due to infection [6•,18]. The main target cells of hRSV infection are ciliated epithelial cells and viral infection promotes loss of the specialized functions of these cells, reduced cilia motility, altered epithelial apoptosis and disruption of cell morphology [5•,19•,20,21]. Furthermore, experiments performed in primary human airway basal cells from multiple donors, show that hRSV can infect basal cells [19•].

After infections, virus particles can be recognized by pattern recognition receptors (PRRs) expressed on AECs [22]. As for hRSV, recognition is mediated mainly by toll like receptors (TLRs) through the interaction between the F protein and TLR4 [23], as well as, viral dsRNA detection by TLR3 [22]. hRSV recognition by AECs leads to the secretion of a broad spectrum of cytokines and chemokines [22]. These molecules promote the recruitment of cells to the peribronchiolar space, including eosinophils, neutrophils and monocytes, as well as the onset of an anti-viral response (Figure 1) [22,24]. A study, comparing nasal-derived and bronchial-derived epithelia from the same individuals, showed a similar pro-inflammatory chemokine response (CXCL10, CCL5, and CXCL11) *in vitro* upon hRSV infection [5•]. Further, patients undergoing hRSV-induced acute bronchiolitis, showed an increase in cytokine and chemokines levels in nasopharyngeal aspirates, but no in peripheral blood. These data suggest that these cytokines are secreted at the target tissue by recruited inflammatory cells [25••].

Figure 1



Immune response elicited by hRSV infection in the airways. Once in the respiratory track, hRSV is recognized by PRRs expressed on epithelial cells, leading to the secretion of cytokines and chemokines. These molecules promote inflammation and the recruitment of innate immune cells into the lungs, such as eosinophils, neutrophils and monocytes. The innate immune response is amplified by IL-1β, which is synthetized by airway epithelial cells and macrophages as an immature form. IL-1β maturation takes place in inflammasome where caspase-1 cleaves pro-IL-β. Further, both hRSV infected epithelial cells and plasmacytoid dendritic cells (pDCs) secrete type-I IFNs, regulating and activating the response of the innate immune system. Thereby, innate immune cell recruitment and mucus production together with an excessive Th2 and Th17 generate an inflammatory environment leading to damage in the respiratory epithelium and the obstruction of distal bronchiolar airways.

The initial response to hRSV infection in the lungs is characterized by the production of interferons (IFNs), which regulate both innate and adaptive immune responses (Figure 1) [22,26]. Type I IFNs engage the IFN-α/β receptor (IFNAR), which is ubiquitously expressed in all cells [27]. As a result, the expression of IFN-stimulated genes is induced, which in turn initiates an antiviral state that extends to non-infected cells [28,29]. Importantly, it was shown in hRSV-inoculated healthy donor blood samples that virus-induced IFN-α production is primarily mediated by plasmacytoid dendritic cells (pDCs) [29]. Also, production of IFN-α is significantly lower in infants as compared to adults, which may contribute to the increased susceptibility of infants and young children to suffering severe hRSV disease [29]. Furthermore, a reduction in type I IFNs was associated to an increase of hRSV loads in lungs [26,30]. In addition, the production of pro-inflammatory cytokines was abrogated in the lungs and bronchoalveolar fluid of IFNAR1$^{-/-}$ mice upon hRSV infection [26]. Also, viral loads were higher in IFNAR1$^{-/-}$ mice as compared to wild type mice [26]. Control of viral infection in AECs is also by the activity of type III IFNs [31••] upon binding to the receptor expressed on epithelial cells at mucosal surfaces [27,32]. Recently, a positive correlation was shown for the clinical score index and the levels of IFN-λ1 in nasopharyngeal washes from patients with hRSV bronchiolitis [31••]. Specifically, IFN-λ1 seems to be associated with an increase in respiratory rate during hRSV infection [31••].

Another important molecule of the innate response against hRSV infection is IL-1β, a cytokine crucial for amplifying the pro-inflammatory response [33]. Macrophages and AECs synthetize IL-1β as an immature pro-IL-1β form, which is transformed into an active form by caspase-1, which in turn is primed by the inflammasome (Figure 1) [34]. It has been recently reported in murine macrophages that the TLR2/MyD88/NF-kB pathway is essential for the expression of pro-IL-1β and inflammasome components during hRSV infection [33]. It would seem, the formation of viral ion channels in membranes of infected human epithelial cells is a signal for the recognition of viral infections and used as host mechanism to induce inflammasome activation [34].

Inflammatory response and viral clearance in lungs

Although the cellular immune response against hRSV plays an important role in virus clearance, it has also been associated to the pathology induced by the infection [35]. hRSV infection is characterized by a predominant differentiation of naïve Th cells into Th2 and Th17 subsets, as well as the production of detrimental Th1 cells [36,37]. This unbalanced immune response promotes a severe inflammation and a diminished secretion of IFN-γ, which suggests that Th2 cells participate in lung damage [36,38]. Viral clearance is mediated by hRSV-specific CD4$^+$ T cells that stimulate both B cell antibody production and a cytotoxic CD8$^+$ T cell response [37]. In addition, studies using animal models suggest that a CD8$^+$ T cell response is able to clear infected cells and resolve hRSV infection without a humoral response [37]. Immunization with FI-hRSV vaccine, which failed to confer an appropriate immunity against hRSV and induced an exacerbated Th2 response supports the notion that Th2-skewed responses against hRSV is detrimental for the host [35].

The Th17 cell response is also associated to hRSV induced pathology in lungs. Th17 cells produce IL-17A to IL-17F, IL-21 and IL-22 [10]. IL-17 promotes the differentiation to Th17 subset and is produced by various cells, such as γδ-T-cells [39,40]. Studies with infant patients with hRSV infection have shown that the concentration of IL-17 was elevated in tracheal aspirates (TAs) as compared to control samples [39,41•]. It was also found that the number of T cells expressing CD161 (surface marker for Th17 phenotype) was elevated in TA samples of hRSV infected patients, as compared to those in autologous peripheral blood, suggesting that an important influx of Th17 cells takes place in the lumen of the lower respiratory tract [41•]. The importance of Th17 cells has been shown in several animal models, in which, an up-regulation of IL-17 could be detected in lungs and peribronchial lymph nodes (LNs) of hRSV infected mice [36]. Elevated level of IL-17 induces mucus production during hRSV infection, which could inhibited by IL-17 blockade with neutralizing antibodies [42]. Consistently with this notion, no mucus secretion could be detected in IL-17$^{-/-}$ mice upon hRSV infection [42]. Furthermore, IL-17 inhibits CD8$^+$ T-cell effector functions, thus reducing viral clearance from hRSV-infected lungs [36,41].

Because it is known that IL-27 plays an important role in the Th phenotype, recent studies have described that IL-27 signaling as an important pathway to control the severity of hRSV infection by suppressing Th17 cells and by promoting the proper T cell response against the virus in the lung [36]. However, this response appears to be dampened by IL-17 induced by hRSV [36]. In fact, by using an IL-27r-KO mouse model infected with hRSV, it was observed an increase in Th17 cell infiltration in lungs with a worse pathology outcome, as compared with WT mice [36]. Further, blockade of IL-17 results in a significant increase in the transcripts of IL-27 subunits p28 and EBI-3 in both, lungs and peribronchial LNs, of hRSV-infected mice. These data suggest that IL-27 not only regulates the cytokines expression during hRSV infection through Th17 cells differentiation, but it also can be regulated by the high levels of IL-17 induced by hRSV [36].

Thus, a complex interplay of T cell subtypes and cytokines induced by the virus are involved in the generation of the lung immunopathology upon hRSV infection.

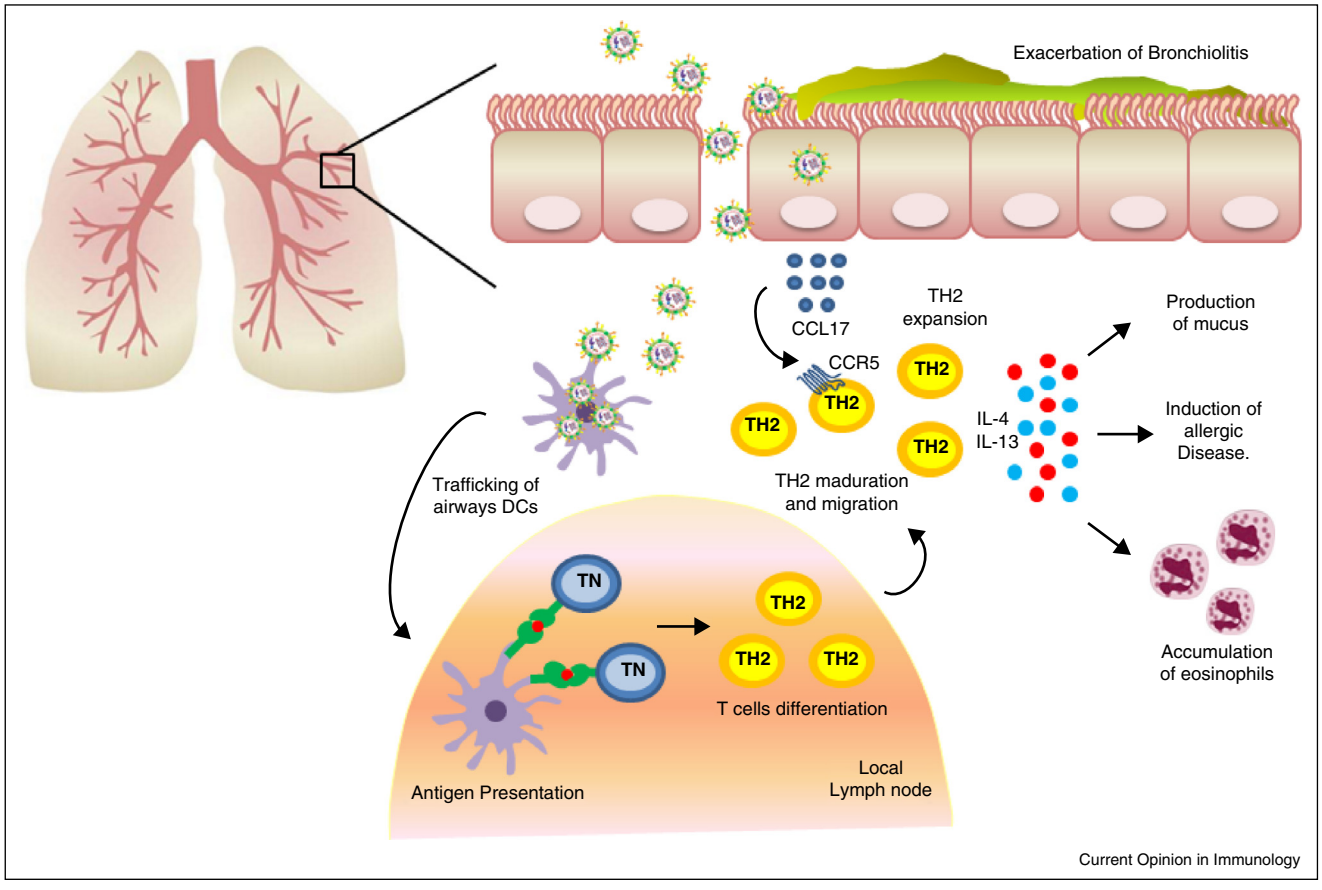
Delayed lung damage induced by hRSV infection

hRSV infection causes an excessive inflammation in small bronchiolar airways of infected infants, increasing the propensity to developing more frequent and severe acute bronchiolitis later in life [6]. Clinical studies have demonstrated the importance of secreted cytokines and chemokines in the airways, in response to hRSV infection, in relation to the severity of bronchiolitis associated with the virus [43]. Specifically, studies have underscored the importance of TARC/CCL17 in the severity of disease caused by infection with hRSV [44]. CCL17 is a chemokine involved in the recruitment of Th2 cells to sites of inflammation during an allergic response, as these cells express CCR4 (the receptor for CCL17) (Figure 2) [44]. An infection model was used to evaluate the expression of CCL17 during hRSV challenge. Monick *et al*. showed that acute hRSV infection of BALB/c mice increases the expression of CCL17 in the lung, further stimulating the ability to generate a Th2 immune environment in hRSV-infected mice [44]. In addition, recent studies in mice have suggested that IL-4 and IL-13 may also contribute to hRSV-induced pulmonary injury [45•]. During primary hRSV infection, production of IL-4/IL-13 by Th2 cells in the lung triggers airway hyperreactivity, chemoattraction of eosinophils and mucus production that could be one of the possible mechanisms involved in the pathogenesis of asthma and wheezing (Figure 2) [46,47]. Therefore, these molecules that are shown to induce a Th2 immune response in lungs of hRSV-infected animals could also induce lung pathology and enhanced disease in patients infected with hRSV.

Inflammatory response in the CNS caused by hRSV

Although it is considered that hRSV infection is confined to the respiratory tract, recent reports have shown the ability of hRSV to spread from the airways to various

Figure 2

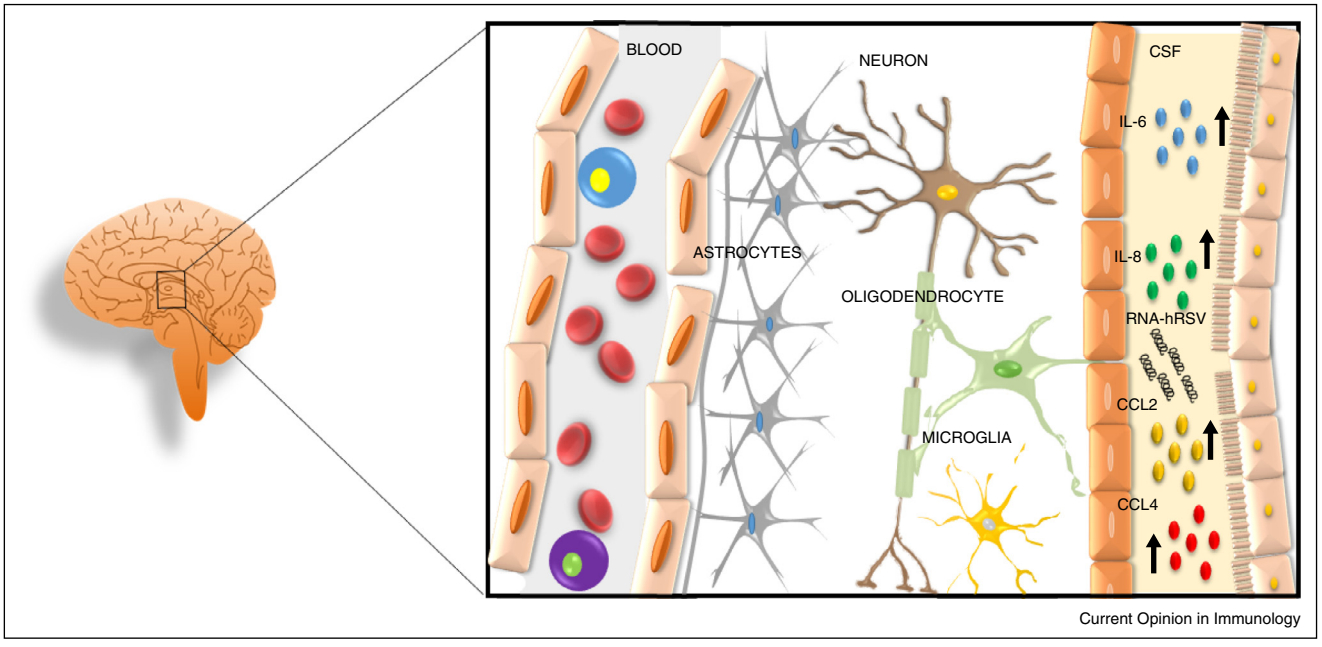


Late effects in the respiratory epithelium after hRSV infection. The main target of hRSV is the respiratory epithelium, which in response to the infection produces TARC/CCL17. CCL17 is a chemokine that leads chemotaxis through their interaction with the chemokine receptor CCR5 expressed on Th2 cells to the infected lung. Once Th2 cells are attracted at the site of hRSV infection in the airways, it secretes cytokines including IL-4 and IL-13, which cause harmful effects, such as the recruitment of eosinophils, hyper-responsiveness and mucus production in the airways. As a result, an exacerbated immune-inflammatory response is induced and contributes to the development of bronchiolitis and possibly asthma in some cases. These data suggest a positive feedback loop between the production of CCL17, Th2 cytokine production and hRSV infection.

tissues in the host producing diverse clinical manifestations. Mainly, these manifestations are cardiopathy, hepatitis and encephalitis [16••]. Moreover, several clinical studies have reported the presence of hRSV RNA in peripheral blood by RT-PCR in a large percentage of newborns [10]. Consistent with these observations, it had been described that hRSV could generate neurological complications in infants, such as central apnea, seizures, encephalopathy and ataxia, among others [11]. Such complications are associated with increased levels of pro-inflammatory cytokines as well as detection of genetic material and anti-hRSV antibodies in the CSF (Figure 3) [11]. A CSF analysis obtained from hRSV infected children with neurological complications suggests that the encephalopathy can be induced by a cytokine storm-like disease [48••]. Patients recruited in this study showed high levels of NO_x in the CNS and were found to be positive for hRSV [48••]. In addition, increased levels of IL-6, IL-8, MCP-1/CCL2 and MIP-1β/CCL4 in the CSF were detected in all patients (Figure 3). Also, IFN-γ and TNF-α were found to be altered in the CSF in of hRSV-infected patients [48••]. Likewise, it is thought that CCL2 and CCL4 contribute to the CNS damage through the recruitment of macrophages, as it has been described for bacterial meningitis [48••]. Studies in animal models for hRSV infection were carried out to determine whether the virus can impair the function of the host CNS. These studies showed that intranasal hRSV infection in BALB/c mice generated the spread of the virus to the CNS and the detection of hRSV nucleoprotein mRNA expression in brain tissues such as hippocampus, ventromedial hypothalamic nucleus and brainstem of hRSV-infected mice. The presence of hRSV in various areas of the infected host CNS may contribute to short-term and long-term learning cognitive impairment related with the failure of the long term potentiation process at the hypocampus [49••].

Despite the evidences of the pathological effects of hRSV in the CNS of infected individuals, the mechanism of the viral entry into the brain remains unknown [50]. Studies in BALB/c mice have shown that blockade of integrin α4 with a neutralizing antibody (anti CD49d) prevents entry of hRSV into the CNS and these results suggests that immune cells carrying the virus would require to cross the blood–brain barrier (BBB) [49••]. Mice treated with anti-CD49d showed no changes in viral loads in the lungs as compared to control mice. On the contrary, viral loads in the CNS were significantly reduced in mice treated with antiCD49d as compared to control hRSV-infected mice [49••]. These findings suggest that hRSV is probably to use a Trojan horse-like mechanism to invade the CNS [49••]. In support of this notion, CD49d blockade prevents, the cognitive impairment caused by hRSV infection in mice [49••], consistently with an impairment in the performance in cognitive test due to hRSV invasion to the CNS by going across the BBB on infected inflammatory cells [49••].

Figure 3



Schematic representation of human CNS inflammation due to hRSV infection. Studies of extrapulmonary manifestations of hRSV infection, principally neurological alterations, have shown elevated levels of cytokines and chemokines in the CSF of patients undergoing a hRSV infection. Furthermore, hRSV-RNA has been detected in the CSF of the patients with encephalopathy due to hRSV infection.

Furthermore, hRSV infects primary neurons *in vitro* and also neuronal processes that innervate the lungs by a manner dependent on the hRSV G protein and the G protein CX3C motif, which is thought to interact with the CX3CR1 expressed on the neuron surface [51]. In addition, the cell-surface receptor nucleolin for hRSV infection is expressed by the nervous tissue. Taken together, these observations support a potential neurotropic capacity for hRSV [52].

Currently, based on the recent evidence about CNS involvement of hRSV, it should be important to evaluate whether the cognitive impairment induced by hRSV infection in animals studies could be extrapolated to infants that had suffered severe bronchiolitis due to this virus, which could reveal an up to now unknown clinical outcome of the respiratory disease caused by this pathogen.

Concluding remarks

Currently, our knowledge about the mechanisms involved respiratory tract illness caused by hRSV remains incomplete. Both, bronchiolitis and pneumonia are due to an excessive inflammation of the airways that induces lung damage and changes in the AEC morphology of bronchioles and alveolus affecting the gas exchange. While these pathologic changes are commonly induced by Flu, hADV, among others, they differ with the hRSV-caused immunopathology [7–9]. The host immune response elicited by hRSV is characterized by a predominant Th2 differentiation, which is not appropriate for an effective viral clearance and induces lung injury and disease. Besides the severe lung damage induced by hRSV, inflammation caused by this virus also affects other tissues, as has it been described for others respiratory viruses, such as hADV [8,10]. Thus, extrapulmonary manifestations have been associated with hRSV bronchiolitis, especially neurological abnormalities [10]. As for the case of Flu, specifically for the H5N1 strain, existing data support the notion that it is neurotropic and that entry to the CNS is through the peripheral nervous system [53]. However, further research would we required to elucidate the mechanisms behind the neurological symptoms caused by hRSV infection and the mechanism of CNS entry.

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Virulence 5:7, 740–751; October 1, 2014; © 2014 Landes Bioscience

Modulation of host adaptive immunity by hRSV proteins

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Keywords: respiratory syncytial virus, virulence proteins, innate immune response, adaptive immune response

Globally, the human respiratory syncytial virus (hRSV) is the major cause of lower respiratory tract infections (LRTIs) in infants and children younger than 2 years old. Furthermore, the number of hospitalizations due to LRTIs has shown a sustained increase every year due to the lack of effective vaccines against hRSV. Thus, this virus remains as a major public health and economic burden worldwide. The lung pathology developed in hRSV-infected humans is characterized by an exacerbated inflammatory and Th2 immune response. In order to rationally design new vaccines and therapies against this virus, several studies have focused in elucidating the interactions between hRSV virulence factors and the host immune system. Here, we discuss the main features of hRSV biology, the processes involved in virus recognition by the immune system and the most relevant mechanisms used by this pathogen to avoid the antiviral host response.

Introduction

HRSV is the leading cause of lower respiratory tract infections (LRTIs) in infants and young children worldwide.[1] Epidemiological data show that more than 70% of children under 1 y old and 100% of children at age 2 have been infected by hRSV.[2-4] HRSV can also infect the elderly and immunocompromised individuals, however the most severe disease manifestations occur in infants younger than 6 mo.[5,6]

HRSV pathology includes a broad spectrum of disease manifestations, ranging from milder upper respiratory tract infection to severe bronchiolitis, alveolitis, and pneumonia.[7,8] Generally, hRSV infections are not lethal and the virus is eliminated from the airways during disease resolution. Nevertheless, global epidemiological studies have estimated that hRSV causes over 34 million LRTIs and more than 200 000 deaths every year.[9] HRSV spread occurs through contact with large-particle aerosol droplets or direct contact with infected patients. The infection begins in the nasopharynx of the host and progress with the spreading of the virus to the lower respiratory tract.[10-12] At these sites the main target of hRSV are epithelial cells present in the airways, but it also may infect lung resident myeloid cells, as evidenced by the detection of infected mononuclear cells in circulation.[13,14]

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Submitted: 03/16/2014; Revised: 07/05/2014; Accepted: 07/31/2014; Published Online: 08/05/2014; http://dx.doi.org/10.4161/viru.32225

Molecular Composition of hRSV

HRSV is an enveloped virus classified in the order Mononegavirales, the *Paramyxoviridae* family, and the *Pneumovirus* genus.[15] HRSV contains a non-segmented, negative-sensed and single-stranded RNA genome of 15.2 kb in length. The hRSV genome has 10 genes in the order 3′-NS1-NS2-N-P-M-SH-F-G-M2-L-5′, which are transcribed into 10 different monocistronic mRNAs. The hRSV genome encodes nine structural proteins and two non-structural proteins. The structural proteins include three envelope glycoproteins (F, G, and SH), the nucleocapsid proteins (N, P, and L), the nucleocapsid-associated proteins (M2–1 and M2–2), the matrix protein (M), and the non-structural proteins NS1 and NS2.[16] Different from other viral mRNAs, the M2 mRNA is translated into two different proteins, namely M2–1 and M2–2, through a process of ribosomal termination-dependent re-initiation mechanism (**Fig. 1**).[17] The M2 gene products, M2–1 and M2–2, are pivotal regulatory proteins that modulate the replication cycle of hRSV. More specifically, M2–1 integrates the ribonucleoprotein complex that mediates transcription of viral mRNAs, whereas the M2–2 protein regulates the switch from transcription to replication.[18,19]

Once hRSV reaches the host target cell, the infection cycle begins with the attachment and entry process, which is mediated by the G and F glycoproteins, respectively.[16] It has been reported that G and F glycoproteins can interact with the cell surface receptors CX3CR1 and TLR4,[20,21] respectively, as well as glycosaminoglycan (GAGs)[22] and C-type lectins[23] to promote viral infection. Further, nucleolin was recently described as another the functional host receptor that interacts with the hRSV F glycoprotein.[24] After fusion of the viral envelope and the cell plasma membrane, the viral nucleocapsid is released into the cytosol of infected cells where transcription of viral mRNAs and replication of the viral genome are initiated.[1,25] HRSV replication requires the synthesis of a complementary, polycistronics ssRNA (+) antigenome, which is used as a template for the synthesis of

new full-length ssRNA(-) genomes.[26] Both, genomes and antigenomes are independently wrapped by the N protein, forming stable nucleocapsids.[27]

HRSV virions are pleomorphic, spherical structures with a diameter ranging from 100–350 nm to 100–100 nm, which are assembled in cholesterol-enriched domains at the host cell membrane.[28] Recent studies suggest that the majority of hRSV strains form long filamentous projections at sites of virus assembly and budding.[29] Furthermore, assembly and maturation of hRSV filaments on the surface of infected cells depend on the proper destination of M protein to cholesterol rich domains of the plasma membrane.[30] Therefore, during genome packaging and virus assembly the hRSV particle acquires a lipid envelope of host origin.[31] Because glycoproteins located at the viral envelope participate in host cell recognition, attachment and infection, this structure is pivotal for the infectivity of hRSV.[32]

Modulation of Host Cell Biology by hRSV Replication

Upon infection, hRSV modulates several biological processes of the infected cells to enhance their replication. Studies performed in A549 and primary human epithelial cells (PHBE cells) have shown that hRSV infection induces the production of TGFβ1 and the decrease of the p53, which results in G1/S and a G2/M cell-cycle arrest and a subsequent enhancement of hRSV replication.[33] HRSV infection also promotes the formation of host cytoplasmic stress granules (SG) in epithelial cells.[34] Although the formation of SG has been associated with increased viral replication,[34] these structures are also recognized by the cytoplasmic RLR receptor MDA5. This protein is activated by viral dsRNA or by 5′-triphosphorylated un-capped viral RNA,[35]which activates the type I interferon (IFN-α/β) response.[36] Recently, it was described that MDA5 specifically recognizes dsRNA replication intermediates in viral infected cells.[34,37,38] SGs increase in size during the course of infection and contain the N, P, M2–1, L, and M proteins.[37] In addition, hRSV induces changes in the expression of neurotrophic factors and receptors, which are involved in airway inflammation and hyperreactivity. Recent reports have shown that hRSV induces the upregulation of the nerve growth factor (NGF) and their receptor tropomyosin-related kinase A (TrkA), with concomitant downregulation of the low-affinity pan-neurotrophin $p75^{NTR}$ receptor.[39] The NGF–TrkA axis prevents apoptosis by increasing the expression of anti-apoptotic Bcl-2 family members, whereas $p75^{NTR}$ signaling promotes apoptosis via JNK. These mechanisms keep the infected cells alive and promote viral replication.[39] The same study suggested that upregulation of the NGF–TrKA axis induced by hRSV infection in human bronchial epithelial cell takes place through silencing of miR-221 expression and also induces the downregulation of other 24 miRNAs.[39] MiRNAs are small ssRNA molecules that modulate the gene expression at the post-transcriptional level.[40] The role of these miRNA in the modulation of both innate and adaptive immune responses has been broadly studied.[41] Indeed, miRNAs participate in the maintenance of the airway epithelial barrier and in the modulation of the antiviral defense of epithelial cells.[40] Infection of A549 cell line with hRSV increases the production of miRNA let-7f expression, probably due to the signaling triggered by the G glycoprotein.[8]

Let-7 miRNAs regulate several key host genes induced during hRSV infection that also controls virus replication.[42] Furthermore, let-7f regulates cell-cycle genes (CCND1, DYRK2, and ELF4),[42] the gene encoding the CCL7 chemokine and the gene encoding the suppressor of cytokine signaling 3 (SOCS3).[17,42] Also, the regulation of ELF4 by let-7f modulates the expression of IL-8, which plays an important role in the pathogenesis of hRSV. Also, it has been described that G hRSV glycoprotein can modulate the expression of IL-8 thought let-7f miRNA.[43,44]

HRSV Recognition by PRRs Receptors

Innate immunity is the first line of defense against virus infection, before induction of the adaptive immune response.[45] It is well established that innate immunity is critical to restrain virus spreading and infection, resulting in diminished disease burden. After hRSV infection, the virus infects epithelial cells, alveolar macrophages and dendritic cells, which trigger an innate antiviral response through pattern recognition receptors (PRRs), including toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-biding oligomerization domain (NOD)-like receptors (NLRs). All these proteins recognize pathogen-associated molecular patterns (PAMPs) from the virus or damage-associated molecular patterns (DAMPs) derived from host cells after virus infection.[43,46]

TLRs play an important role in the recognition of hRSV. The TLR-2 and TLR-6 form the cell-surface heterodimer TLR4/TLR6 on immune cells. After activation by hRSV, this complex triggers a signaling cascade in leukocytes that activates innate immunity by promoting the production of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), CCL2, and CCL5.[47] Also, TLR-4 associates with CD14 and the complex recognizes LPS from gram-negative bacteria and the hRSV-F glycoprotein. Activation of TLR4/CD14 complex requires binding of MD2, leading to NFκB activation. The final result of this activation pathway is the secretion of IL-8, IL-10, and IL-6, and also the upregulation of TLR4 on epithelial cells.[48]

HRSV infection is also sensed by the intracellular receptor TLR-3. This receptor localizes on the surface of endosomes and recognizes double-stranded RNA (dsRNA) produced during viral replication.[45] The induction of TLR3 activates the innate immune response through the TRIF-mediated pathway that promotes the production of CCL-5, IFN-α, and IFN-β.[49] TLR7 is also involved in the recognition of hRSV, which binds to the viral ssRNA genome in endosomes during the fusion process. When TLR7 is activated after hRSV infection it signals via the MyD88 pathway.[50]

Similar to MDA5, RIG-I is a receptor belonging to the RLRs family and is activated by viral dsRNA or 5′-triphosphorylated un-capped viral RNA in the cytoplasm.[51] Specifically, RIG-I recognizes ssRNA viral genomes bearing 5′-triphosphates, whereas

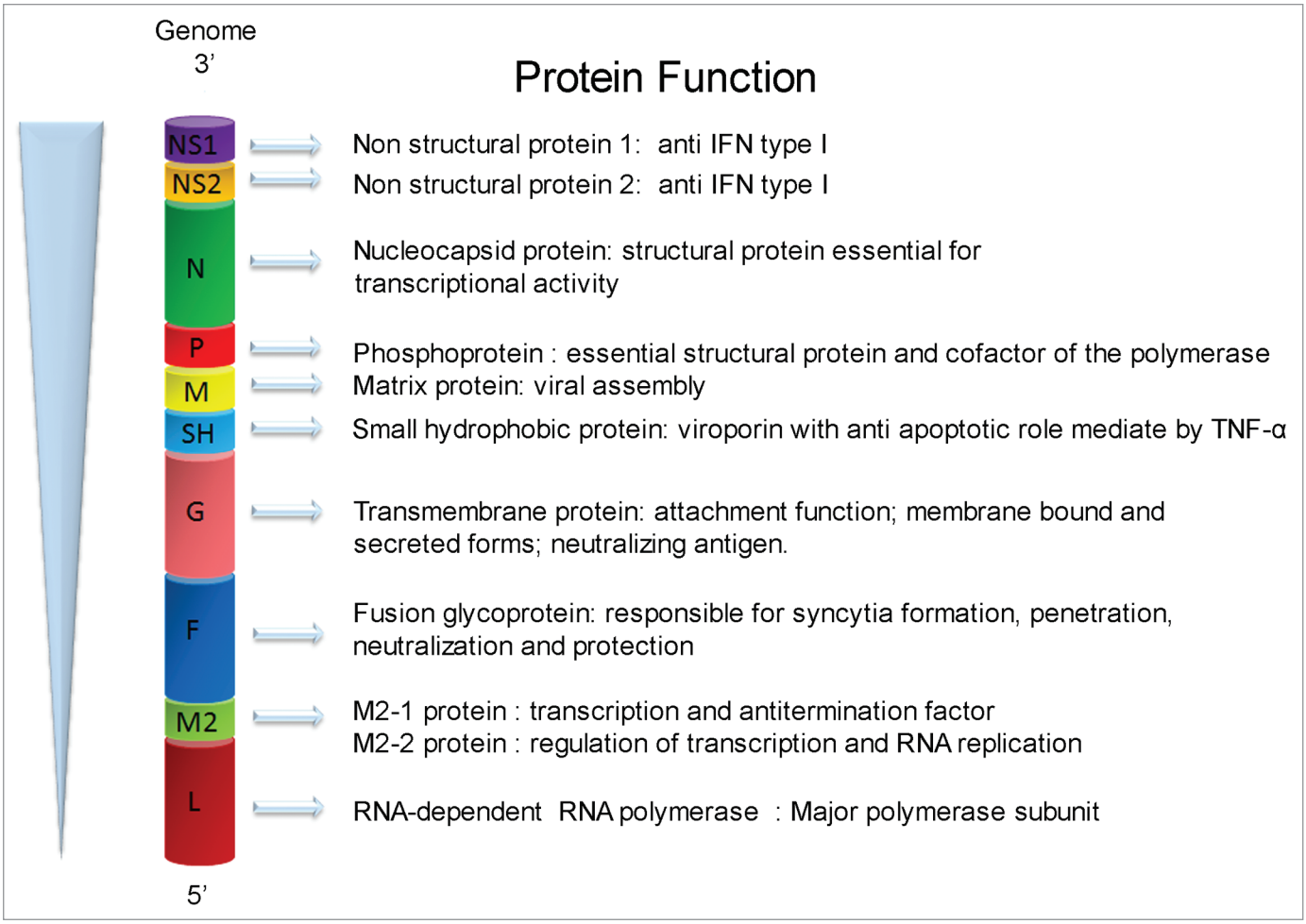

Figure 1. Schematic representation of the hRSV genome indicating the known function for each encoded protein. The figure shows the order of the 10 genes of hRSV in its genome and the known function of the 11 encoded proteins.

MDA5 recognizes long dsRNA molecules.[52] The induction of either RIG-I or MDA5 leads to the activation of downstream NFκB and IRF3 pathways by interacting with the mitochondrial antiviral-signaling protein (MAVS; IFN-β promoter stimulator 1 [IPS-1]). RIG-1 detects hRSV during replication and subsequently activates IFN regulatory factors 3 and 7 (IRF-3 and IRF-7), which are transcription factors for IFN-α and IFN-β.[45] Finally, the nucleotide-binding oligomerization domain 2 (NOD2) detects the ssRNA genome and triggers innate immune activation by binding with the adaptor MAVS. NOD2 is required for IRF3 activation and IFN-β production upon hRSV infection in vitro.[45]

Airway Immune Response against hRSV

The epithelial cells from the airways (tracheal, bronchial, and bronchiolar cells) actively contribute to initiate the immune response after hRSV infection, through the secretion of immunomodulatory molecules with innate antimicrobial activity, as well as secretion of cytokines and chemokines upon infection to recruit immune cells.[53] HRSV infection induces the secretion of surfactant proteins A and D (SP-A and SP-D). Both SP-A and SP-D are polypeptides of the collectin family that bind pathogens and play an important role in host defense and regulation of inflammatory processes in the lung.[54] SPs can act as opsonins, but also stimulate macrophages (Mφ) activation, increasing chemotaxis, phagocytosis and modulating cytokine secretion.[55,56] In vitro infections of human peripheral blood monocytes and HEp-2 cells with hRSV show that SP-A interacts with G and F glycoproteins and favors the binding and uptake of the virus.[57,58] Also, SP-A modulates the activity of Mφ, including activation of the NFκB signaling pathway and the upregulation of cytokine synthesis, mediated by TLR4 complex.[59] SP-A and SP-D bind to CD14,[60] whereas SP-A also binds to TLR2, but does not activate downstream NFκB signaling.[61] Surfactant protein D also binds the hRSV G protein and inhibits hRSV infection in vitro and in vivo.[58]

Recent reports have also shown that infection of airway epithelial cells by hRSV induces the secretion of thymic stromal lymphopoietin (TSLP), an epithelium derived cytokine that plays an important role in the development of allergic asthma

Table 1. HRSV interaction with the host innate and adaptive immune responses

hRSV Proteins	Innate immune response	Adaptive immune response	References
NS1-NS2	Inhibits the induction of IFN-α/β	Suppresses DCs maturation	69–78
	Role in suppressing apoptosis and facilitating virus growth	Suppresses proliferation and activation of two of the protective cell populations ($CD103^+$ $CD8^+$ T cells and Th17 cells)	
SH	Important role of this protein in the inhibition of apoptosis mediated by TNF-α		84, 85
	Required for the trigger of signal 2 of NLRP3		
G	Inhibits of NK cells infiltration and proinflammatory cytokine secretion	Reduces the Th1 cytokines and promote the Th2 response in pulmonary $CD3^+$ T cells	89–100
	Decreases the expression of MIP-1a, MIP-1b, MIP-2, and MCP-1	Induces an exacerbated Th2 type cytokine expression	
F	Induces the secretion of proinflammatory cytokines such as IL-6, IL-1β, and IL-8 mediated by the NFκB pathway.	Activates specific T $CD8^+$ cells	103-105

The table summarizes the pieces of evidence supporting the role of NS1, NS2, SH, G, and F proteins in the modulation of both innate and adaptive immune responses.

via activation of RIG-1 antiviral pathway.[62] Additional evidence supports the notion that TSLP induces myeloid dendritic cells (mDCs) to express the OX40 ligand (OX40L), which is a member of the TNF superfamily that has been involved in the B cell–T cell interaction, the DC–T cell interaction, and the initiation of Th2 cell responses through OX40L expressed on these DCs.[63-65] DCs are professional antigen presenting cells (APCs) with superior capacity to activate antigen-inexperienced T cells, thus being an APC subset linking the innate and adaptive immunity.[66] To achieve their function, DCs that are infected by pathogens or that have up-taken antigens at mucosal tissues of the body need to undergo a phenotype change process known as maturation, which has been demonstrated to be a critical response to establish $CD8^+$ T cell memory to infections.[67] Along these lines, previous studies have shown that TSLP promotes mDCs maturation, evidenced by the upregulation of major histocompatibility molecules (MHC) class I and II and costimulatory molecules (CD40, CD80, CD83, and CD86).[43,64] In addition, other reports have shown that TSLP can act either in an autocrine or paracrine manner on epithelial cells. Thus, airways cells contribute to the TSLP response and drive the production of the Th2 chemokine CCL17, allowing epithelial cells to induce a Th2-biased response due to hRSV infection.[62] Another study performed in primary rat airway epithelial cells (PRAECs) shows that hRSV induced the production of both TSLP mRNA and protein at 18 h post-infection.[64] In this work, it was shown that hRSV-treated PRAECs induce the maturation of mDCs, which have enhanced levels of OX40L and CCL17 mRNAs.[64] It has been also described that the presence of TSLP in mixed lymphocyte reactions increases the expression of MHC-II and CD86 and promotes enhanced T-cell proliferation.[64] CCL17 have a key role in the Th2 response, because it binds to their chemokine receptor CCR4, which is expressed in almost 100% on Th2 cells that produce IL-4, IL-5, and IL-10.[64,68] This chemokine also participates in the recruitment of Th2 cells and eosinophils into the lungs.[68]

Role of hRSV Proteins in Immune System Evasion

During host-virus co-evolution, several strategies has been developed by viruses to interfere with critical functions of the immune system, including antigen presentation, T-cell activation, and the development of the host humoral response.[69] Among the viral components modulating the host immunity, several hRSV proteins have been attributed with the capacity to modulate the function of either innate or adaptive immune cells (summarized in **Table 1**). In the next sections, we review the virulence determinants used by hRSV to negatively modulate the antiviral immunity, from the early innate responses to the highly specific T-cell responses required to clear the infection.

The Nonstructural NS1 and NS2 Proteins

NS1 and NS2 are two small proteins (139 and 124 amino acids respectively), which are not included as structural elements in the viral particle.[70] NS1 and NS2 are encoded by the first two genes of the hRSV genome and their mRNAs are the most abundant during the infective cycle.[70] Accordingly, NS1 has been shown to be the most abundantly produced protein in hRSV-infected cells, suggesting that these proteins are involved in the modulation of the innate immune response during early stages of the virus replication cycle.[71] In agreement with this notion, both proteins modulate the host immune responses by impairing the induction/signaling of interferons, DC maturation and T lymphocyte activation.[72-74] Also, NS1 and NS2 have been associated with the inhibition of apoptosis, thus prolonging the life of the infected cell and increasing viral yields.[75,76]

Inhibition of the Type I Interferon Response by NS Proteins

The first evidence supporting that NS proteins inhibit the type I interferon pathway showed that virus lacking both NS1 and NS2 failed at preventing the activation of the interferon regulatory factor 3 (IRF-3) and its nuclear translocation.[72,76,77] Further studies demonstrated that NS1 inhibits the phosphorylation of IRF-3, thereby interrupting the binding of this protein to the interferon gene promoter. On the other hand, NS2 causes the degradation of STAT2, an important signaling component of the JAK/STAT cascade that is triggered by interferon receptors. Additionally, NS2 interacts with the RIG-I, preventing the activation of IRF-3 and the interferon stimulated genes involved in the innate antiviral response.[76-78] Moreover, NS1 and NS2 activate the phosphoinositide 3-kinase (PI3K) pathway, which promotes survival of infected epithelial cells and mediates virus maturation and budding.[79-81] In agreement with this notion, suppression of NS1 and/or NS2 expression by either small interfering RNAs (siRNAs) or by viral gene deletion suppressed the activation of the PI3K pathway, which resulted in accelerated apoptosis of hRSV-infected cells and a reduction in virus yield.[78] By activating the PI3K pathway, NS1 and NS2 increased the survival time of the infected cells and increased the yield of viral progeny.[82]

Effects of NS Proteins in the Maturation of Dendritic Cells

It has been demonstrated that hRSV mutants lacking either NS1 or NS1/NS2 have an increased capacity to induce DC maturation as compared with wild-type viruses, as evidenced by an increased expression of maturation markers and secretion of proinflammatory cytokines, both of which are known changes associated with DCs maturation.[73] However, NS1 appears to exert most of the modulatory effect over DCs maturation, as evidenced by a non-significant modulation of NS2 knockout hRSV. The upregulation described was inhibited by pretreatment with a blocking antibody against the type I IFN receptor, suggesting that suppression of DCs maturation by NS1/NS2 is associated with antagonism of the type I IFN pathway by these proteins.[73] Furthermore, suppression of DCs maturation negatively affected antigen presentation and T cell activation, suggesting that reduced immune responses against hRSV are at least in part due to the effects of NS proteins over DCs maturation.[73]

Negative Modulation of T-Cell Responses by NS Proteins

The hRSV NS proteins can modulate the activation and proliferation of T cells. As described by Munir and coworkers, deletion of NS1, but not NS2, produced an increased activation and proliferation of $CD8^+$ T cells expressing the tissue homing integrin CD103. Because this integrin leads $CD8^+$ T-cell recruitment into the respiratory tract mucosa, which favors its cytolytic activity in infected airways, it is thought that NS1 negatively modulates cytotoxicity in vivo.[74] Also, NS1 knockout mutants display increased activation and proliferation of Th17 cells within the lungs, which have anti-viral effects and also indirectly attract neutrophils; and decreased activation of IL-4-producing $CD4^+$ T cells and reduced proliferation of total $CD4^+$ T cells.[74] Except for total $CD4^+$ T-cell proliferation, none of the T-cell effects appeared to be due to increased type I IFN signaling. Data from a previous study show that in infected DCs, deletion of the NS1 and NS2 genes strongly upregulated the expression of cytokines and other molecules involved in DCs maturation.[73] This was partly IFN-I-independent, and thus might account for the T-cell effects. Taken together, these reports demonstrate that the NS1 protein suppresses proliferation and activation of two protective T-cell populations ($CD103^+$ $CD8^+$ T cells and Th17 cells), and promotes proliferation and activation of deleterious Th2 cells that may enhance the pulmonary immunopathology.[74]

The Small Hydrophobic (SH) Glycoprotein

The SH glycoprotein gene encodes 64 or 65 amino acids depending of the hRSV serotype (A or B),[83-85] which is highly conserved among all hRSV A subtypes.[84] In cells infected with hRSV strain A2, the SH glycoprotein can adopt several forms, such as SH 0, SH g, and SH p,[84,85] while in cells infected with B1 strain similar glycosylated and non-glycosylated forms are found.[85] Depending of the glycosylation pattern of the SH glycoprotein, three forms have been described, which are: a 7.5 kDa non-glycosylated form (SH0), a 13–15 kDa N-linked glycosylated form (SHg), and a polylactosaminoglycan-modified form of the protein (SHp), which varies between 21 and 30 kDa.[84] The SH glycoprotein is found anchored at the cellular membrane by the N-terminus and the C-terminal amino acids are extracellular.[84] Several reports indicate that SH glycoprotein can form pentamers and when expressed in *Escherichia coli*, it changes the membrane permeability of the bacteria, allowing the entry of low-molecular-weight compounds.[86-88] Structural modeling analyses have demonstrated that this glycoprotein is an ion channel-forming viroporin.[84,85,88,89] Viroporins belong to a group of small/highly hydrophobic virus proteins that can oligomerize forming pores in the cell membrane, causing varying effects in the physiology of infected cells.[86]

Currently, it is know that SH glycoprotein is not important for the viral replication but hRSV lacking SH glycoprotein was attenuated in mouse and chimpanzee models, which indicates that SH glycoprotein is important for hRSV pathogenesis.[82-84,88] Mutant viruses generated by reverse genetics, in which the SH gene has been deleted, suggest that it is dispensable for virus growth, virus entry into host cells or syncytium formation, but may be necessary for the evasion of the host immune system.[84,85] To evaluate the participation of the SH glycoprotein as a virulence factor, experiments has been performed comparing the hRSV SH protein with another member of the *Paramixoviridae* family, Parainfluenza virus 5 (PIV5), because hRSVΔSH shows a phenotype similar to rPIV5ΔSH: a normal growth in vitro but attenuated growth in vivo.[85] Also, studies of rPIV5ΔSH have shown the role of this protein in the inhibition of apoptosis mediated by TNF-α.[85] In the absence of SH glycoprotein during

PIV5 infection, there is an increase of production of TNF-α and activation of NFκB, due to the translocation of the p65 subunit of NFκB into the nucleus of PIV5ΔSH-infected L929 cells.[85] A similarly ability to inhibit the activation of NFκB by TNF-α in the L929 cells has been observed for hRSVΔSH, independent of the strain used.[85] Other characteristic of hRSVΔSH infection is the high cytophatic effect and the high rate of the apoptosis produced in infected cells, compared with the wild-type hRSV.[85] This observation suggests that hRSV SH glycoprotein plays an important role in the inhibition of apoptosis during the infection, to favor the viral replication.[85]

The hRSV infection induces the secretion of IL-1β in the respiratory tract in mouse and humans and its secretion is relevant for the anti-viral immune response to clear viruses.[90] Recently, the activation of the nucleotide binding oligomerization domain like receptor (NLR) inflammasome, principally NOD-like receptor family, pyrin domain containing 3 (NLRP3) has been described following infection with hRSV, resulting in the pro-IL1β cleavage and secretion of the processed cytokine.[90] Given that the triggering of NLRP3 inflammasome requires the permeability of cellular membrane, the participation of the SH glycoprotein was evaluated.[87] In this study it was observed that a mutant strain of hRSV lacking SH glycoprotein fails at triggering inflammasome activation.[87] This result suggests that SH glycoprotein is required for the triggering signal 2 (**Fig. 1**), due to the formation of a pore or channel on the plasma membrane.[87] More studies are required to understand the role of SH glycoprotein as a hRSV virulence factor.

The Attachment (G) Glycoprotein

The G hRSV glycoprotein contains 298 amino acid residues and besides of its role in the attachment process, it seems to have additional functions, unrelated with attachment proteins from other *Paramyxoviridae* family members.[91] The G glycoprotein has a trans-membrane domain near the N-terminus and the major part of the molecule, including the C-terminus, is external. However, the G glycoprotein exists also in a secreted form lacking this trans-membrane domain.[91] The G glycoprotein has a central conserved cysteine region that contains a CX3C chemokine motif at amino acid positions 182–186.[92] This CX3C motif interacts with the CX3CR1 receptor, whose ligand is CX3CL1, also known as fractalkine. Therefore, the G glycoprotein establishes a competitive inhibition with CX3CL1 for the binding to CX3CR1, facilitating infection and impairing CX3CL1-mediated responses.[92]

CX3CL1 has several functions related to leukocyte biology, including adhesion, chemoattraction and immunomodulation of T cells.[93] The membrane-anchored form of CX3CL1 promotes cell adhesion with CX3CR1 expressed primarily on cytotoxic cells, e.g., T cells, natural killer NK cells, and monocytes/macrophages, and the soluble form acts as a chemoattractant for $CX3CR1^+$ cells.[94] Studies of the interaction between CX3CL1-CX3CR1, using either blocking antibodies for CX3CL1/CX3CR1 or knockout mice for CX3CR1, have shown a high inhibition of leukocyte migration and chemotaxis after hRSV infection.[20,93-95] Also, it has been suggested that G glycoprotein is important for the development of enhanced pulmonary disease in the vaccination model of formalin-inactivated hRSV,[95] and also increases the expression of the pro-inflammatory tachykinin sustance P during hRSV infection.[96] Indeed, antibodies that block hRSV G glycoprotein CX3C–CX3CR1 interaction prevent many of the immunomodulatory effects associated with RSV G glycoprotein,[97] supporting the idea that the interaction of CX3 mimetic domain in the G glycoprotein with CX3CR1 receptor have an important role in the hRSV infection and disease pathogenesis.[98]

Modulatory Effects on the Host Innate Immune Response by the G Glycoprotein

HRSV G glycoprotein has the ability to modify the immune response at different levels, affecting the function of chemokines, cytokines and leukocytes. Competition with the CX3C chemokine is one of the most described effects of G glycoprotein attributed to the central conserved region, which contains a CX3C motif. Also the G glycoprotein presents a structural homology with the fourth subdomain of the tumor necrosis factor receptor (TNFr).[89] TNF-α and TNF-β are important cytokines of the inflammatory response, and the structural homology with the TNFr of the G glycoprotein suggest that this protein could bind to TNF-α and TNF-β, affecting the antiviral response against hRSV.[89]

Experimental approaches using mutant hRSV virus lacking the G gene showed a increases in the recruitment of natural killer cells into the lungs, as well as increases in the production of IFN-γ and TNF-α, supporting the involvement of the G glycoprotein in the inhibition of NK cell infiltration and proinflammatory cytokine secretion.[99] The G glycoprotein has been associated with the induction of a Th2 response and the increased recruitment of eosinophils into the lungs after hRSV infection. The secreted form of G glycoprotein increases the IL-5 levels, producing a more severe immunopathology due the activation and migration of eosinophils.[100] Other studies have also shown that G glycoprotein decreases the expression of macrophage inflammatory protein (MIP-1a), MIP-1b and MIP-2 and monocyte chemoattractant protein (MCP-1), which have attracting function over NK cells into the lungs.[98]

Modulation of the Host Adaptive Immune Response by the G Glycoprotein

Several pieces of evidence support the notion that hRSV G glycoprotein has important immune modulatory effects. For instance, it has been shown that during hRSV infection the G glycoprotein promotes a Th2 immune response in pulmonary $CD3^+$ T cells (high expression of IL-4 and IL-5) by negatively modulating Th1 cytokines, including IFN-γ and IL-2. It is possible that this phenotype is due to alterations in DCs

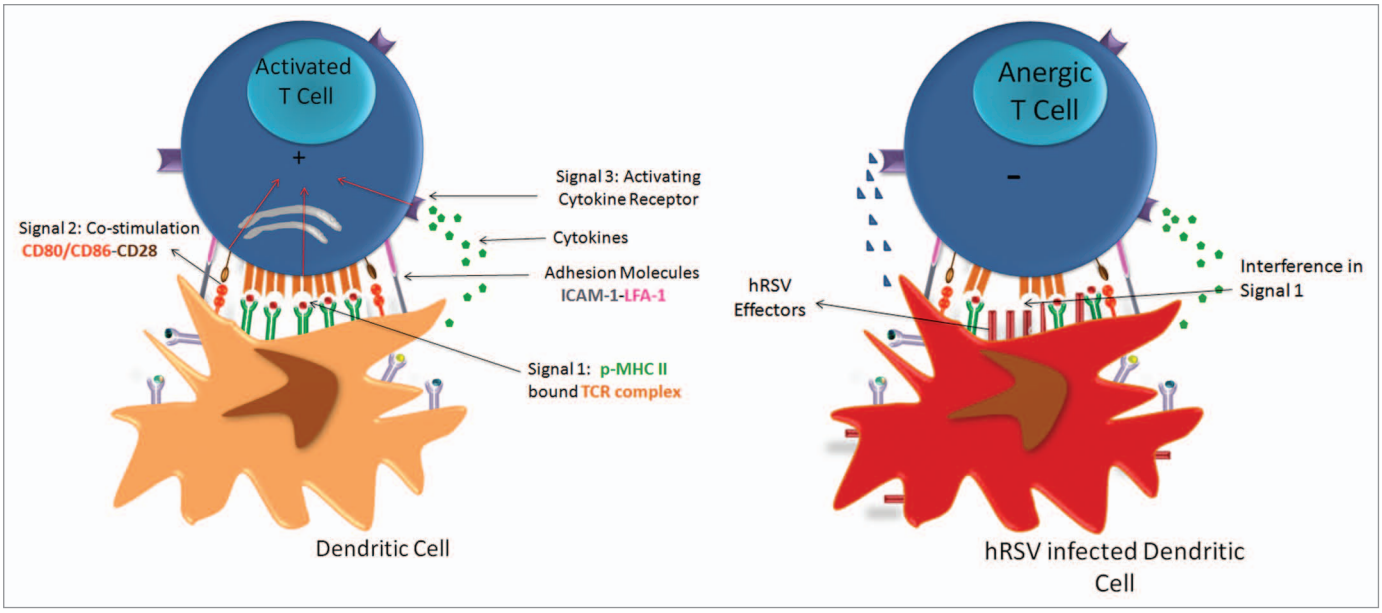

Figure 2. HRSV infection blockade of IS assembly between infected DCs and T cells as a novel RSV virulence mechanism. A novel mechanism to avoid the host immune response elicited by hRSV is the impairment of immunological synapse (IS) assembly between hRSV-infected DCs and naïve T cells that impairs the activation of T cells. During IS assembly DCs provide three different signals to promote the T-cell activation: Signal 1 (antigenic presentation in p-MHC), signal 2 (Co-stimulation) and signal 3 (cytokines) represented in the left part of cartoon. In hRSV infected DCs, as shown in the right part of the cartoon, hRSV effectors are expressed in the surface membrane interfering with the molecular interaction between the T-cell receptor (TCR) and antigenic pMHCs impairing the T-cell activation. This phenomenon is evidenced by a reduction in the Golgi apparatus polarization toward the DC and decreases in IL-2 secretion by T cells.

recruitment/activation or to effects in signaling add pathways important in T-cell activation, such as substance P.[99] Moreover, recent studies have suggested a possible mechanism by which the G glycoprotein may be interfering with the cytotoxic T-cell response (which is essential for viral clearance and the control of virus replication) by antagonizing the activities of the chemokine CX3CL1 over the CX3CR1$^+$ cells, characterized by a Th1 response.[94] Several studies performed in the murine model suggest that hRSV suppresses the effector activity of CD8$^+$ T cells and the development of pulmonary CD8$^+$ T-cell memory,[101] which can be recovered by exogenous IL-2 treatment.[102] These findings are consistent with hRSV G glycoprotein-associated reduction of Th1-type cytokine responses.[97] Because CX3CR1 plays an important role as a chemotactic and adhesion receptor for CX3CL1$^+$ cells, it is though that the hRSV G glycoprotein through its CX3C motif may be involved in the negative regulation of T-cell function observed in vivo. Indeed, the G glycoprotein differentially affects the trafficking of CD8$^+$CX3CR1$^+$ T cells into the lungs and the mediastinal lymph nodes (MLN) of hRSV infected mice. Furthermore, additional evidence suggest that the hRSV G protein may affect the antiviral response through the modulation of perforin and granzyme B expression in cytotoxic CX3CR1$^+$ cells[94] and also expression of the G glycoprotein during hRSV infection induces an exacerbated Th2 type cytokine expression.[98]

The Fusion (F) Glycoprotein

The F hRSV glycoprotein is a type I integral membrane protein of 574 amino acid similar to the fusion proteins of other *Paramyxoviridae* family members, which participates in both the fusion of the viral envelope with the host cell membrane during viral entry and the formation of syncytia.[103] The F glycoprotein is highly conserved among hRSV genogroups, displaying amino acid sequence identities of 90% or higher between serogroups A and B.[104] The F glycoprotein is synthesized as an inactive F0 precursor; three F0 monomers assemble into a trimer, which is further modified and activated in the Golgi apparatus by the host furin-like protease.[82,83] This protease cleaves at amino acid positions 109 and 136, therefore forming three polypeptides. Finally, the N-terminal and C-terminal polypeptides (named F2 and F1 subunits) are linked by two disulfide bonds.[83] During the replication of hRSV, the F mRNA is produced in the cytosol and the functional F protein exists as a trimer located in the virion membrane as a metastable pre-fusion form, which upon binding to its relevant ligand; the nucleolin, undergoes a refolding process into a post-fusion conformation.[105] This conformational change promotes the fusion of the virus and cell membranes, allowing virus entry and the initiation of the hRSV replication cycle (reviewed in[103]). Because of this, the F glycoprotein is essential for the infective cycle, as evidenced by studies showing complete loss of virus

infectivity both in vivo and in vitro in hRSV strains lacking the F glycoprotein (RSVΔF).[106]

As the major protein mediating infection of target cells, the F protein has been attributed as a major target for the pattern recognition receptors (PRRs) of the innate immune system. Indeed, through binding to the TLR4/CD14 complex expressed on the surface of monocytes, the F protein triggers the NFκB pathway and the secretion of pro-inflammatory cytokines, including IL-6, IL-1β, and IL-8 in vitro.[107] Because these cytokines act as chemoattractants, they promote the recruitment of neutrophils and macrophage cells into injured tissues.[108] Furthermore, the TLR4 pathway appears to be essential for the efficient elimination of hRSV from the infected lungs, as evidenced by studies showing severe impairment of viral clearance in TLR4 null mice. Although the mechanism underlying increase susceptibility to hRSV in TLR4 null mice is not fully understood, studies with these mice show an overall impairment of the innate antiviral immunity, including IL-12 expression and diminished numbers of NK cells and $CD14^+$cells in hRSV-infected lungs. Also, the infiltrating NK cells have a significantly diminished cytotoxicity.[108]

Besides being a major hRSV signature for the innate immune system, the hRSV fusion protein has been recognized as a major $CD8^+$ T-cell antigen, as evidenced by the identification of several F-derived antigenic peptides both in humans and mice.[83,109,110]

CTL responses against F protein in mice

In agreement with this notion, it has been shown that approximately 4.8% of the pulmonary $CD8^+$ T cells activating the adaptive antiviral response during the infection peak (day 8) are indeed $F_{85–93}$-specific.[83] Nevertheless, the F-specific T-cell repertory generated during the experimental infection with hRSV display a substantially reduced cytotoxic capacity when compared with the F repertory induced through vaccination with a recombinant influenza or adenovirus vaccine,[111] suggesting that hRSV negatively modulates the expansion and generation of $CD8^+$-specific T cells. Furthermore, F-specific $CD8^+$ T cells isolated from hRSV infected mice exhibited lower IFN-γ synthesis and impaired cytolytic activity ex vivo.[83,112] Although the mechanisms accounting for inefficient cytolytic activity of $CD8^+$ T cells are not fully understood, it has been shown that the heterologous expression of the hRSV F protein in epithelial cells reduced their sensitivity to $CD8^+$ cytotoxicity in vitro.

Recently, studies reported the interaction of F glycoprotein with the intracellular adhesion molecule (ICAM)-1 expressed on the cell surface, suggesting a possible role of this molecule in the viral fusion. Indeed, hRSV infection increases expression of ICAM-1 on epithelial cells and the ICAM-1 cross-linking of human epithelial cells induced the expression of IL-1β, which is also induced by hRSV infection, suggesting the participation of ICAM-1 in this process.[113] The high conservation of the F protein and its pivotal role in the infection of target cells and the modulation of the host immune response, make the pre-fusion form of the F glycoprotein an ideal target for neutralizing antibodies and the development of antiviral therapies.[104]

The Nucleoprotein (N) as a Novel Modulator of T-Cell Activation

The hRSV N protein is a non-glycosylated, 43 kDa protein with essential roles in virus transcription and replication, acting as a scaffold for the assembly of the hRSV ribonucleoprotein complex. Although its major role in infected cells has been attributed to the protection of viral RNA species, including the wrapping of genomes and polycistronic antigenomes,[114,115] a recent work of our group has proposed that N protein could be expressed in the surface of infected cells at early stages of the viral replication cycle (Céspedes et al., manuscript accepted). Although the mechanism of surface N protein destination is still elusive, it may occur through interactions with the M and P proteins.[116] M protein has a highly positive face able to interact directly with the inner leaflet of the plasma membrane,[117] and the P protein serving as chaperone of N protein that prevents its interaction with RNA.[118] Furthermore, surface expression of N was shown to interfere with the assembly of the T-cell activating immunological synapse (IS) when expressed in the surface of infected APCs, mainly by interfering with the molecular interaction between the T-cell receptor (TCR) and antigenic pMHCs. This observation was in agreement with our previous work showing impairment of IS assembly between hRSV-infected DCs and naïve T cells, which was characterized as a contact-dependent mechanism[119] as shows the **Figure 2**.

Because the priming of T cells largely depends on the assembly of the IS with DCs (and in a lesser extent with other APCs), these observations suggest that the major mechanism explaining the broad impairment of $CD4^+$ and $CD8^+$ T-cell activation by hRSV is due to expression of hRSV proteins in APCs (**Fig. 2**).

Concluding Remarks

HRSV has been recognized as the major viral agent causing severe acute respiratory infections in children. However, and despite considerable efforts made to understand the molecular mechanisms explaining the immunopathology and the evasion of the host-immune response, there is still no appropriate vaccine for hRSV prophylaxis. It is known that hRSV pathology is due to an excessive inflammation of the respiratory tract, characterized initially as a Th2, allergic-like immune response. Because the hRSV-elicited immune response is non-optimal for virus clearance (due to broad impairment with T-cell functions), infected individuals can eliminate the virus at the expense of an exaggerated inflammatory response in the lungs. Furthermore, the aberrant immune response observed in the airways of hRSV-infected animals have been associated with several viral proteins, each of them with unique complementary functions in the modulation of the innate antiviral response of the host, thus facilitating viral propagation. Indeed, hRSV use multiple mechanisms to avoid the host immune response including interference with type I IFN responses (mediated by hRSV NS1 and NS2 proteins) and the antagonism of CX3CL1 chemokine mediated by the mimetic

domain of the hRSV G glycoprotein. The pieces of evidence suggesting immune regulatory roles for hRSV F and SH glycoproteins is not conclusive yet. However, our knowledge is still insufficient to understand the complete picture of hRSV infection necessary to design an effective and safe vaccine available for the population most affected by this pathogen.

Considering the accumulated knowledge of hRSV proteins that act as negative modulators of T-cell physiology, we propose that during host-virus co-evolution the hRSV has selected/evolved virulence determinants with complementary functions at inhibiting T-cell activation and effector functions. Indeed, hRSV proteins impair several critical steps occurring during the development of T-cell responses; from naïve T-cell priming (by hRSV N protein), the acquisition of proper Th1 anti-viral $CD4^+$ responses (by hRSV NS1 protein), the proper recruitment of CTLs and helper T cells within infected lungs (by hRSV G glycoprotein) and the execution of antigen-specific $CD8^+$ cytotoxicity (by hRSV F glycoprotein). Accordingly, new, rational vaccine design strategies should consider the development of T-cell responses with the capacity to circumvent the mechanisms imposed by hRSV to restraint the host adaptive immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Millennium Institute on Immunology and Immunotherapy from Chile (P09/016-F for AMK and SB), La Région Pays de la Loire through the "Chaire d'excellence program" for AMK and Grant "Nouvelles Equipes-nouvelles thématiques"(to AMK and SMB), the ECOS France-Chile grant, FONDECYT no 1070352, FONDECYT no 1050979, FONDECYT no 1040349, FONDECYT no 1100926, FONDECYT no 1110397, FONDECYT no 1131012, FONDECYT no 1140010, FONDECYT no 3140455 and the Biomedical Research Consortium CTU06. JAE and KB are CONICYT-Chile fellows.

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Rev. Med. Virol.
Published online in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.1813

Reviews in Medical Virology

REVIEW

Central nervous system alterations caused by infection with the human respiratory syncytial virus

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SUMMARY

Worldwide, the human respiratory syncytial virus (hRSV) is the leading cause of infant hospitalization because of acute respiratory tract infections, including severe bronchiolitis and pneumonia. Despite intense research, to date there is neither vaccine nor treatment available to control hRSV disease burden globally. After infection, an incubation period of 3–5 days is usually followed by symptoms, such as cough and low-grade fever. However, hRSV infection can also produce a larger variety of symptoms, some of which relate to the individual's age at infection. Indeed, infants can display severe symptoms, such as dyspnea and chest wall retractions. Upon examination, crackles and wheezes are also common features that suggest infection by hRSV. Additionally, infection in infants younger than 1 year is associated with several non-specific symptoms, such as failure to thrive, periodic breathing or apnea, and feeding difficulties that usually require hospitalization. Recently, neurological symptoms have also been associated with hRSV respiratory infection and include seizures, central apnea, lethargy, feeding or swallowing difficulties, abnormalities in muscle tone, strabismus, abnormalities in the CSF, and encephalopathy. Here, we discuss recent findings linking the neurological, extrapulmonary effects of hRSV with infection and functional impairment of the CNS. Copyright © 2014 John Wiley & Sons, Ltd.

Received: 7 August 2014; Revised: 31 August 2014; Accepted: 2 September 2014

INTRODUCTION

Acute respiratory tract infections (ARTI) are the major cause of childhood mortality/morbidity worldwide [1]. Among pathogens causing ARTI, the human respiratory syncytial virus (hRSV) is the leading cause of severe lower respiratory tract disease in infants and young children [2–5]. Respiratory tract infections caused by hRSV produce a wide range of clinical manifestation including cough, rhinorrhea, congestion, low-grade fever, decreased appetite, otitis, and respiratory distress, the latter ultimately associated with bronchiolitis and pneumonia [6]. The severity of the disease caused by hRSV depends on the presence of host risk factors, such as preterm birth (before 35 weeks of gestation) [7], congenital heart disease [7,8], and immunosuppression [9].

hRSV is highly contagious, infecting approximately 70% of infants before the age of 1 year and nearly 100% of children by the age of 2 years. A 2010 report estimated that approximately 33.8 million new cases of hRSV–lower ARTI occurred

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Abbreviations used
ADH, antidiuretic hormone; ARTI, acute respiratory tract infections; BBB, blood–brain barrier; hRSV, human respiratory syncytial virus; LTP, long-term potentiation; MB, marble burying; MWM, Morris water maze; SH, small hydrophobic protein.

worldwide in children under the age of 5 years, accounting for 22% of the total number of cases of ARTI [10]. At least 3.4 million of the infected individuals needed hospitalization [10]. Based on these statistics, hRSV is considered a pathogen leading to a health problem that is extremely costly for individuals, governments, and health care systems globally, and the total annual medical cost in the USA for the hRSV infection is estimated in $394M US [11]. Thus, it is likely that a safe and effective vaccine against this virus will display a very favorable cost/benefit ratio.

Because infectious viral particles can be efficiently delivered from infected individuals to susceptible new ones, either through inhalation of aerosolized droplets or via direct contact of these droplets with the ocular mucosa, hRSV spreads rapidly throughout the population [12,13]. Indeed, hRSV epidemics are recurrent, in part, because individual reinfections are extremely frequent during an outbreak. Epidemiological studies indicate that up to 36% of individuals can suffer at least one reinfection episode during the same season [11,14,15]. Compelling evidence suggests that recurrent epidemics are favored by the fact that, following disease resolution, individuals that recovered from infection develop somewhat limited or hampered cellular and humoral immunological memory responses [4,16–18].

Since first identified in 1957, global efforts against hRSV have focused both on addressing and understanding the mechanisms involved in pulmonary pathology, as well as on the design of vaccines and therapies against this virus [19–21]. Recently, several reports have shown the ability of hRSV to spread from the airways to various tissues in the host, including the heart, liver, and brain, consequently producing diverse clinical manifestations, such as cardiopathy, hepatitis, and encephalitis [6,22–24]. Here, we review extrapulmonary manifestations developed during hRSV infection focusing on recently described functional alterations of the CNS.

COMPOSITION, INFECTIOUS CYCLE, AND PATHOGENESIS OF HRSV

hRSV is a *Paramyxoviridae* virus belonging to the *Pneumovirinae* subfamily and more specifically to the *Pneumovirus* genus [3]. Other members of the *Paramyxoviridae* family include measles virus, parainfluenza viruses, Hendra virus, Nipah virus, and human metapneumovirus, some of which have also been shown to alter CNS function [25–30]. The hRSV virion is enveloped and encapsidates a single-stranded, negative-sensed non-segmented RNA genome 15.2 kb long. Because hRSV replicates in the cytoplasm of infected cells, a viral RNA-dependent RNA polymerase is needed to synthesize antigenome single-stranded RNA (ssRNA; +) for producing genomic ssRNA (−) [31]. The hRSV genome encodes 11 proteins, eight of which are present in the virion and therefore are considered structural [2,5,32,33]. The disulfide-bonded fusion glycoprotein F, the large attachment glycoprotein G, and the small hydrophobic protein (SH) are located at the virion surface, with the two first being the major antigenic determinants that induce anti-hRSV antibody responses [2,5,32]. Current reports suggest that the G protein mediates viral attachment by binding to negatively charged cell surface carbohydrates, such as glycosaminoglycans [34]. Indeed, virus attachment can be inhibited by heparin, which is not expressed in epithelial cells [34]. While glycoprotein G is not essential for infection of target cells *in vitro*, as demonstrated in deletant viruses for this gene, this protein seems to play more important roles *in vivo* as an antibody decoy for immune evasion [35–37]. In contrast, the fusion protein F is key for cell infection both *in vitro* and *in vivo* [38]. Given this pivotal role for infection, the F protein has been the main target of blocking drugs intended to reduce infection, such as neutralizing antibodies and antiviral drugs [39,40]. Although several cell surface molecules had been proposed to be receptors for F, only nucleolin was recently shown to meet all the criteria for such a role in hRSV entry. On the other hand, the SH, which forms a pentameric ionic channel in target cell membranes, was recently shown to be involved in inflammasome activation [41], and whether it is involved in the entry or exit of hRSV, as suggested for viroporins from other viruses, remains yet somewhat unclear [42]. Beneath the envelope lies the matrix protein (M) [2,32] and further below the M2 protein, the nucleoprotein (N), the phosphoprotein (P), and the large RNA-dependent RNA polymerase (L), the latter three in the nucleoprotein complex are also known as the nucleocapsid. Finally, NS1 and NS2 are non-structural proteins that are found only in infected cells but not in virions [2] (Figure 1).

hRSV infection can occur through inhalation of infectious droplets or through their direct contact with the ocular mucosa [13,43]. The principal

DOI: 10.1002/rmv

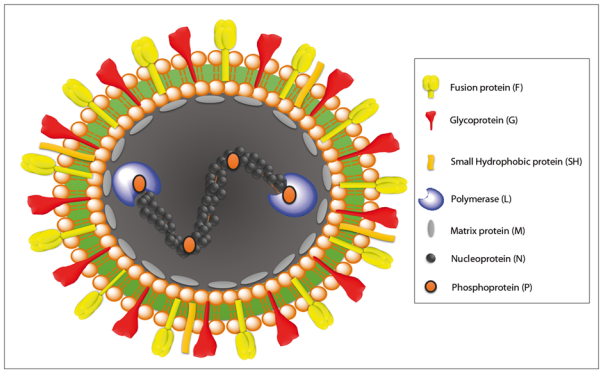

Figure 1. hRSV structure. Schematic representation of the hRSV virion depicting its surface proteins: attachment glycoprotein G, fusion glycoprotein F, and small hydrophobic protein SH. Beneath the envelope reside the matrix protein M, the nucleoprotein N, the phosphoprotein P, and the RNA-dependent RNA polymerase. The hRSV genome is single-stranded, negative sense RNA (RNA (−))

cellular targets of this virus seem to be epithelial cells in the respiratory tract, where the pathogen can replicate to produce thousands of new infectious viral particles per cell [44]. Also, it has been described that hRSV can infect other cell types, such as structural cells of the airway and immune cells [45–47].

The first step in the replication cycle of hRSV starts with the virus attaching to the host cell surface by means of the G protein. Then, the viral envelope fuses with the plasma membrane of the host cell through the F protein. Consequently, the viral nucleocapsid is released into the cellular cytoplasm, where transcription and replication of the viral genome take place [2,5,32]. Transcription of viral genes is initiated mainly at inclusion bodies. At this phase, several studies have provided evidence for the involvement of host proteins, which display modulating capacity over the viral polymerase complex. For instance, actin promotes viral polymerase activity by working as a transcription factor [48], and the actin-modulatory protein profilin regulates the transcription of hRSV genes by participating as a transcriptional cofactor associated with actin [48].

The viral ssRNA(−) genome functions as a template to generate messenger RNA used for the translation of viral proteins. Alternatively, this ssRNA(−) serves as a template for the synthesis of ssRNA(+) antigenome, which will then be used to synthesize the ssRNA(−) genome again [2,5,32]. hRSV transcription develops rapidly after virus entry; indeed, it has been described that viral antigens can be detected as soon as 9 h postinfection, and new virus particles can be released within 11 to 13 h postinfection, in cell culture media.

Recent reports have defined several molecular events taking place during the process of hRSV replication inside the host cell. For example, during hRSV genome replication and protein translation in the cytoplasm, the M protein is translocated to the nucleus [49], where it binds to host RNA and likely blocks host-gene transcription [50,51]. Later during infection, the M protein is retrotranslocated into the cytoplasm [52], as it would be required for promoting an efficient production of large amounts of virus particles [52]. Furthermore, it is thought that virion assembly is regulated by the M matrix protein, which brings together viral surface proteins and the encapsidated genome at the cellular membrane for packaging [53]. Finally, it has been proposed that virus budding from the surface of infected cells occurs through the apical recycling endosome system by an uncommon mechanism for enveloped RNA viruses, which involves the participation of the host Rab11 family interacting protein 2 [54]. Although available data relative to the infection, replication, assembly, and budding allow significant understanding of the hRSV infectious cycle, further research is needed to elucidate the detailed molecular mechanisms behind these processes. Unveiling these molecular elements will allow effective drugs to be identified that target specific steps of the replication cycle.

To complete infection, hRSV requires F and G glycoproteins that are only necessary for increasing the infection [43]. Recent evidence attributes an important role for the G protein in the pathogenesis of the virus by showing that this protein is essential to promote an efficient infection of sensitive cells in lungs [55]. Indeed, treatment with G protein-specific monoclonal antibodies caused a decrease in viral infection by interfering with the pathogenic properties of both the soluble and membrane-bound forms of the G protein [56].

hRSV infection leads to a significant inflammatory response in the airways, which is thought to establish a hyperreactive immunological environment that fails to efficiently clear the virus [4,57]. Following virus entry to the respiratory tract, hRSV reaches alveoli and infects airway epithelial cells. hRSV-derived pathogen-associated molecular patterns, such as double-stranded RNA, engage TLR3

DOI: 10.1002/rmv

leading to nuclear factor KB pathway activation and the secretion of IFNα/β and IL-8 (CXCL-8) [31]. At the same time, engagement of surface TLR4 by the F protein induces the secretion of several cytokines by epithelial cells, such as IL-1β, IL-6, IL-8, and TNF-α [4]. In the absence of infection, lymphocytes and phagocytes, including granulocytes and monocytes, circulate in the blood in the alveolus periphery [4]. However, following hRSV infection, activation of the innate immune system can lead to a massive infiltration of inflammatory cells into the lung parenchyma and alveoli [31]. The host response to the viral replication involves alveolar infiltration of neutrophils, eosinophils, natural killer cells, myeloid dendritic cells (DCs), plasmacytoid DCs, macrophages, B lymphocytes, and $CD4^+$ and $CD8^+$ T lymphocytes [4,58]. Despite the fact that $CD8^+$ T lymphocyte infiltration can be detected, their cytotoxicity function is significantly diminished [59,60]. Furthermore, increased expression of TLR3 and TLR4 on the surface of epithelial cells is observed after hRSV infection [4,58]. In addition, lung pathology of hRSV infection is associated with a strong T helper 2 (Th2)-polarized T cell immunity against the virus [61] characterized by the production of pro-inflammatory cytokines, such as IL-4, IL-5, and IL-13 [62]. These cytokines increase the recruitment of inflammatory cells, such as eosinophils, neutrophils, and monocytes, and impair the activity of cytotoxic $CD8^+$ T cells [60]. Several studies have suggested that hRSV can modulate the polarization of the host adaptive immune response to promote a detrimental Th2-biased T cell responses. Simultaneously, the virus blocks the priming, expansion, and function of Th1 and cytotoxic T cells needed for viral clearance [3,4,17,63–68].

CLINICAL PATHOLOGY CAUSED HRSV INFECTION

Recently, clinical reports have described that hRSV infection in humans can lead to respiratory symptoms, as well as pathological manifestations in other tissues. The first data were registered by the Children's Hospital in Cleveland in 2004 (OH, USA) [69], when authors described the symptoms in infants with hRSV admitted in the pediatric intensive care unit. hRSV extra-pulmonary effects were particularly important and involved cardiovascular, endocrine, and nervous system abnormalities [22].

During the initial states of disease, hRSV infection shares several clinical signs with other viral infections. The most common symptoms induced by hRSV include cough, fever, and rhinitis [70,71]. In children under 1 year old, the infection is associated with less specific symptoms, such as failure to thrive, periodic breathing or apnea, and feeding difficulties. [71]. X-ray examination commonly shows hyperinflation, peribronchial thickening, and, in some cases, scattered interstitial infiltrates. Further, areas of atelectasis are usually present in the right middle and upper lobes of the patient lungs [70,71]. The acute stage of illness lasts for about 5–7 days, but symptoms, such a cough, may be extended for several weeks [70]. Younger patients that have been hospitalized and required mechanic ventilation show a significantly increased probability to develop asthma later during childhood and even in adulthood [71]. Meanwhile, recent data suggest that asthma can also predispose to severe symptoms after hRSV infection [72,73] In the elderly, upper or lower respiratory tract infection with hRSV may promote exacerbations of asthma and/or chronic obstructive pulmonary diseases [71]. Diagnosis of hRSV infection is accomplished by tests such as immunofluorescence, EIA, or cell cultures of nasopharyngeal samples [39]. Although these tests have shown good sensitivity and specificity, faster and less costly diagnostic tools are required for detecting hRSV, as soon as possible after exposure and infection [45].

Studies in human patients have revealed that the predominant immune response triggered by hRSV is an exacerbated Th2 cellular response, with limited capacity to clear virus-infected cells and reduce cytotoxic T lymphocyte activity [71,74–76]. Depending on the nature and intensity of the immune response, several features of hRSV-induced illness can differentially affect patients. For instance, immunocompetent individuals with hRSV usually show increased airway obstruction, with alveolar sparing, as compared with immunocompromised patients. In these latter individuals, disease manifests with higher frequency as progressive pneumonia, with cellular and fluid infiltration at alveoli, but with reduced wheezing [71,77,78]. Furthermore, studies have correlated viral loads in blood with disease severity, which suggests that hRSV itself could play an important role in pathogenesis, although this notion remains controversial [79,80].

DOI: 10.1002/rmv

NEUROLOGICAL MANIFESTATIONS OF HRSV INFECTION

Epidemiological studies show that approximately 1.2–1.8% of patients admitted with severe bronchiolitis resulting from hRSV infection can manifest some kind of CNS disorder [6,81], such as seizures, central apnea, lethargy, feeding or swallowing difficulties, abnormalities of tone, strabismus, abnormalities in the CSF, and encephalopathy [22,81–83]. Among the neurological alterations described, the most frequent clinical symptomatology in the course of hRSV infection is central apnea and seizures [84]. However, the percentage of children suffering from neurological complications can reach up to 40% in infants receiving intensive care as a result of severe hRSV infection [6]. Neurological complications are more frequently observed in children younger than 2 years of age [6]. Further, the detection of viral RNA and specific antibodies for hRSV in CSF of infected patients supports the notion of an association between neurological alterations and hRSV infection or the immune response induced by this pathogen. Furthermore, recent studies show that patients with neurological symptomatology associated to hRSV usually have altered cytokine profiles, as compared with patients infected by hRSV but do not develop such symptoms [85,86]. These data are consistent with a role of cytokines and inflammation in the neurological complications caused by hRSV in some groups of infected children (Table 1) [87,88].

Because central apnea is the most common symptom associated with hRSV neurological alteration, which can reach up to 21% of hospitalized children [84], it is important to understand the pathogenesis behind this process. A possible explanation involves the laryngeal chemoreflex in sleeping infants that can be significantly prolonged in infants with hRSV bronchiolitis, as compared with those infected with hRSV but do not develop bronchiolitis [89]. Although the pathophysiologic mechanisms responsible for this symptom remain unclear, it has been proposed that hRSV infection can cause disruptions in the neural control pathways by reducing non-adrenergic, non-cholinergic inhibitory responses [90,91]. Further, hRSV causes abnormal cholinergic responses in an animal

Table 1. Extrapulmonary manifestations associated with hRSV infection

Extrapulmonary Manifestations	Clinical signs	Laboratory observations	References
Neurological	Apnea Status epilecticus Seizures Encephalopathy Strabismus	hRSV RNA, antibodies, and elevated proinflammatory cytokines in CSF	[6,22,65,69,82–86,93]
Cardiovascular	Heart block Ventricular tachycardia Ventricular fibrillation Myocarditis	hRSV RNA in a patient with myocarditis and correlation between viral load and sinoatrial blocks	[23,105–107]
Hepatic	Hepatitis	hRSV detected by culture of liver from an immunocompromised patient and elevated transaminase levels	[22,24]
Endocrine	Hyponatremia	Patients with hyponatremia and hRSV bronchiolitis show elevated ADH levels	[22,109]

DOI: 10.1002/rmv

model, which suggests that hRSV can directly alter specific CNS responses [92], which can contribute to the inflammation of this tissue. However, the observed alterations in the cytokine profiles shown by patients suffering from neurological symptoms suggest an involvement of inflammation in the CNS and thus alterations caused by hRSV infection [85,86]. However, no significant correlation was found between nasopharyngeal IL-6 levels and the occurrence of apnea [89]; it is possible that IL-6 was produced systemically or at the CSF could contribute to this pathological condition. Furthermore, it is likely that, in addition to IL-6, other cytokines or pro-inflammatory molecules could influence the generation of apnea.

A direct role for hRSV at inducing encephalopathy is supported by the detection of hRSV antibodies, as well as virus nucleic acid in the CSF of infants with neurological abnormalities after hRSV infection [6,82]. Furthermore, radiographic changes in the brains of infected infants have been observed, as a result of hRSV-induced encephalitis [82]. Data from several studies support the notion that hRSV infection leads to an inflammatory status in the CNS, which is likely because of the secretion of pro-inflammatory cytokines [85,86] (Table 1).

In addition, two types of seizures constitute other important neurological manifestations of hRSV infection: generalized tonic–clonic and partial seizures with altered consciousness and focal motor features or eye deviation (Table 1) [69,82]. Although the molecular or cellular mechanisms responsible for the induction of seizures after hRSV exposure remain largely unknown, it is likely that inflammation of the CNS region might be the cause.

Furthermore, although the ultimate pathophysiologic basis for hRSV-caused encephalopathy remains unknown [22,82,93], it is likely that this virus promotes the production of pro-inflammatory cytokines and free radicals in the CSF, such as IL-6 and nitric oxide, which could contribute to disease [81,86]. Consistent with this notion is the clinical observation made in an 11-month-old infant showing seizures after hRSV bronchiolitis [86] and high IL-6 levels in the CSF [85,86]. Additionally, elevated protein levels, increased cell numbers, and low glucose levels have been reported in CSF of patients infected with hRSV [22]. Interestingly, these findings have even been observed in patients with no apparent neurological alteration, suggesting that in some cases, hRSV can produce subclinical CNS alterations [69].

The mechanisms for the induction of seizures by hRSV infection have not been yet elucidated, but it is likely that hyponatremia and the production of cytokines and free radicals could contribute to this symptom [81,82]. As for the case of the reported hRSV-infection-induced strabismus, in the form of esotropia [82], it could result from a direct infection of the cranial nerves by hRSV. Consistent with this notion is the observation that this virus is one of the pathogens that most frequently invades the middle ear and causes otitis [94].

It is important to mention that hRSV is not the only respiratory virus that can affect the proper function of the CNS. Examples of respiratory viruses causing neurological abnormities include influenza and human metapneumovirus [28,84]. However the prevalence of hRSV is much higher and so is the potential for causing neurological and cognitive damage inside a population of infected individuals [95].

POSSIBLE MECHANISMS BEHIND THE NEUROLOGICAL ALTERATIONS CAUSED BY HRSV

It is possible that the ability of hRSV to spread from the respiratory tract to other tissues, including CNS, relies on the infection of PBMCs infiltrating the airways during the acute phase of infection. Consistent with this notion, hRSV has been detected in PBMCs both in animal models of disease and in patients naturally acquiring infection [96–98]. Detection of hRSV RNA by real-time PCR in blood or PBMCs of neonates and children with hRSV lower respiratory tract infection has been reported [96–98]. Furthermore, recent studies show that the presence of hRSV RNA in PBMCs correlates with disease severity in mice [98]. *In vitro*, hRSV infects DCs and B cells [3,99,100].

The observations that hRSV or the immune response triggered by the virus can reach and alter CNS function and modify the CSF composition [85] have promoted searches for the mechanisms behind these phenomena. Studies in animal models, such as BALB/c mice and Sprague Dawley rats, show the presence of hRSV nucleic acid and proteins in brain tissues of animals challenged intranasally with the virus [101]. Although the neuroinvasion mechanism remains unknown, the observation that blockade of integrin α_4 with a neutralizing anti-CD49d antibody prevents entry of hRSV into the CNS suggests that cells carrying

DOI: 10.1002/rmv

the virus would need to cross the blood–brain barrier (BBB). CD49d blockade prevents leukocyte extravasation through the BBB, and mice treated with the neutralizing antibody showed no hRSV in the CNS but normal viral loads in the lungs [101]. One of the implications of hRSV accessing the CNS was an alteration in the cognitive function of mice and rats several weeks after infection with hRSV [101]. Consistently, several weeks after infection with hRSV, we evaluated possible behavioral and learning alterations in mice and rats through the marble burying (MB) and Morris water maze (MWM) tests [101]. The MB test measures the capacity of mice to dig and hide marbles, which is related with the hippocampal function [102]. On other hand, the MWM test is a challenging task for rodents. It allows the acquisition and spatial localization of relevant visual cues that are subsequently processed, consolidated, retained, and then retrieved to successfully navigate and thereby locate a hidden platform to escape from water [103]. This model is useful for the study of neurodegenerative and neuropsychiatric illnesses where cognition is impaired and is directly associated to the hippocampus, which is an essential structure for spatial learning [103,104]. Both evaluations showed a significant reduction in their capacity to optimally perform behavioral and learning tests, as compared with control animals. Furthermore, electrophysiological assays suggested that the impaired performance in learning was because of a failure to efficiently induce the long-term potentiation responses in the stratum radiatum in the hippocampus area. These data support the previously proposed idea that hRSV might possess neurotropic properties, including the ability to infect CNS tissues in a disseminated pattern and the capacity to disrupt cognitive functions by impairing the synaptic plasticity of the infected brain tissue (Figure 2) [101]. Consistent with this notion, hRSV has been shown to infect primary neuronal cells *in vitro*, as well as processes innervating the lungs, a process that would be mediated by the glycoprotein G [55].

The contribution of the damaging inflammatory response triggered by hRSV to the cognitive impairment caused by the infection by this virus was underscored by the observation that vaccine-induced protective T cell immunity prevented virus

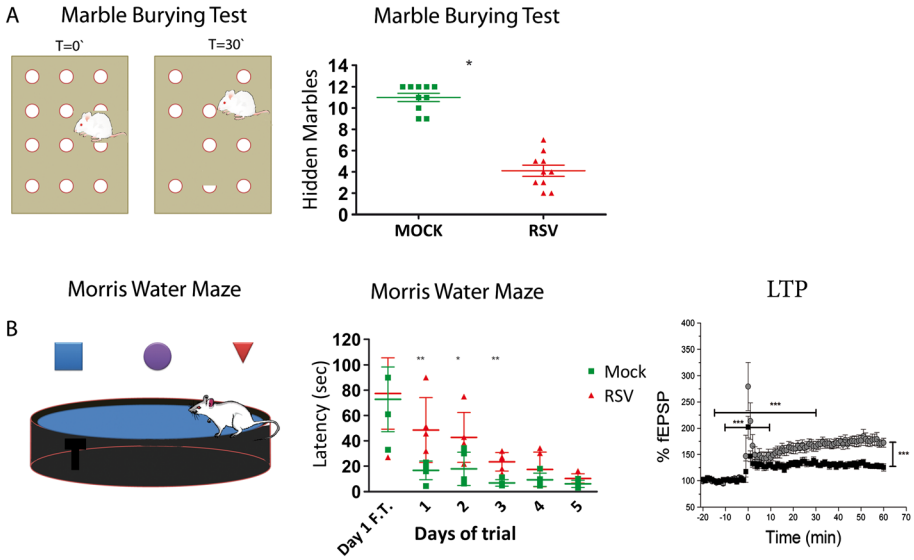

Figure 2. Behavioral and learning impairment caused by hRSV infection. (A) The MB tests measure the capacity of mice to dig and hide marbles and evaluate hippocampal function. Rodents that were infected 30 days before with hRSV showed a significantly reduced marble-hiding activity as compared with controls. The graph shows the quantification of the results obtained in MB tests in rodents [101]. (B) The MWM test measures learning. The rodent spatially localizes relevant visual cues that it will subsequently process, consolidate, retain, and then retrieve in order to successfully navigate in subsequent challenges to locate a hidden platform under the water. hRSV-infected rats showed significant differences compared with control animals 30 days postinfection. The graph shows the latency time obtained as results of MWM tests in rats [101]. (C) Electrophysiological assays, such as long-term potentiation (LTP) in the brain of rats submitted to the MWM test. This type of synaptic plasticity is generally considered the closest neural model for the cellular mechanism responsible for learning and memory storage. These assays showed that hRSV-infected animals displayed a significant impairment in LTP response. This is consistent with increased latency shown by hRSV-infected rats in MWM tests [101]

DOI: 10.1002/rmv

spread to the CNS, as well as the neurological alterations caused by infection [101]. Thus, it is likely that, when presented to a naïve immune system, hRSV induces neuroinflammation that would persist even after virus clearance from the CNS. A possible explanation is that hRSV may enter in the CNS associated with leucocytes or as free virus particles causing an elevated secretion of proinflammatory cytokines that affect normal neuronal function (Figure 3).

CLINICAL PATHOLOGY CAUSED BY HRSV IN OTHER TISSUES

In addition to damaging the CNS, hRSV can cause alterations in other tissues in children hospitalized because of severe bronchiolitis [23]. Other extrapulmonary symptoms shown more frequently by children admitted to the intensive care unit include cardiovascular failure with hypotension and inotrope requirement, which is associated with myocardial damage, cardiac arrhythmias, and pericardial tamponade [23]. It has also been shown that infection with hRSV can cause fatal interstitial myocarditis [105] and second-degree heart block during disease [106]. Recently, hRSV nucleic acid was detected by PCR in the myocardium of a patient with myocarditis (Table 1) [107].

Further, a recent study that included 69 patients (34 hRSV positive and 35 hRSV negative controls) showed the occurrence of sinoatrial blocks in 76.5% of hRSV positive patients [23]. Sinoatrial blocks were significantly more frequent in patients with high viral loads (≥100 000 copies per ml) than in those with lower loads (Table 1) [23].

Liver alterations have also been associated with hRSV infection, as demonstrated by elevated transaminase levels in children suffering from hRSV bronchiolitis. Severe hepatitis with elevated alanine aminotransferase levels, reaching up to 3000 IU/l, has been described and associated with coagulopathy [108]. Direct liver invasion was shown in a hRSV-infected immunocompetent infant and was documented after successfully isolating the virus from a biopsy [24]. Adipose hepatic infiltration was described in a fatal case of Reye's syndrome associated with hRSV infection [22], as well as the development of hepatitis in the course of an infection by hRSV (Table 1) [24].

Finally, it is thought that hRSV causes alterations to the endocrine system leading to hyponatremia [22,109], which was manifested by 33% of infants requiring intensive care with hRSV infection; 11% had serum sodium levels below 130 mM/l. Patients with hyponatremia and hRSV bronchiolitis show

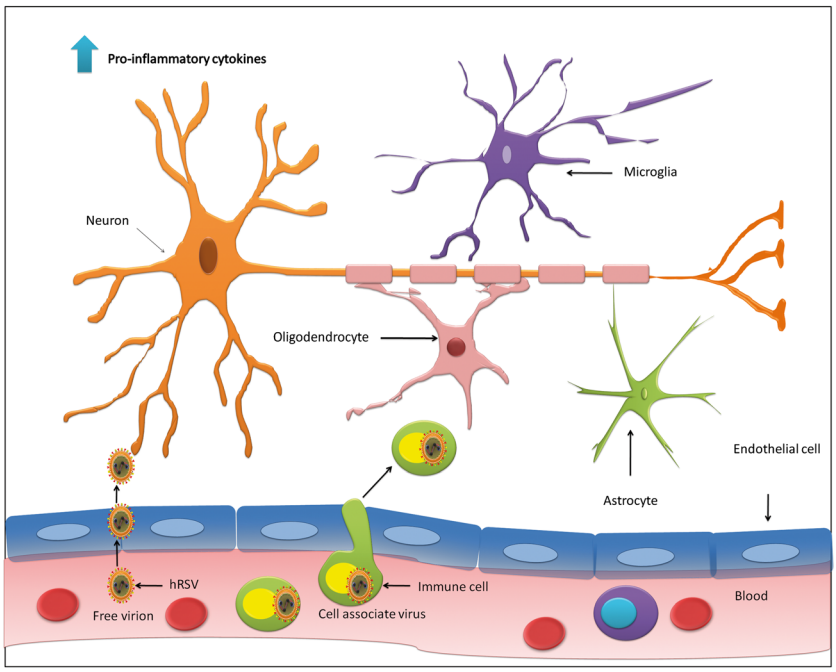

Figure 3. Model for hRSV entry into the CNS and consequences at this site. The figure proposes two possible entry mechanisms for hRSV into the CNS. The arrow points to an increase in the levels of proinflammatory cytokines that could explain the cognitive impairment in mice

DOI: 10.1002/rmv

elevated antidiuretic hormone (ADH) levels [109]. Other studies have revealed that ADH levels are significantly higher in patients with bronchiolitis, as compared with patients with apnea or upper respiratory tract infections with hRSV [109]. Further, increased ADH levels have been associated with higher arterial partial pressure of CO_2 and hyperinflation visible on chest X-ray (Table 1).

PREVENTION, PROPHYLAXIS AND TREATMENT OF HRSV-CAUSED DISEASE

Vaccine trials aimed to generate protection against hRSV were initially carried out in the mid-1960s by means of a formalin-inactivated hRSV formulation (FI-RSV) [17]. However, children immunized with this vaccine experienced exacerbated pulmonary disease and required hospitalization upon subsequent hRSV infection, while non-vaccinated control children showed significantly milder symptoms [19,94,110]. Since those trials, many vaccination strategies have been evaluated, such as attenuated [111] or inactivated viruses [112], recombinant proteins with or without adjuvants [113], modified viruses [114], peptides, and polypeptides [115]. However, very few of these strategies have been evaluated in clinical trials [110]. More than 50 years after the unfortunate results obtained with the FI-RSV, an effective and safe vaccine against this virus is still not available.

One strategy that has shown strong and protective capacity against hRSV in preclinical studies is a recombinant bacillus Calmette–Guérin formulation expressing the virus nucleoprotein (hRSV N-rBCG) [68]. This vaccine candidate has demonstrated the capacity to promote hRSV clearance from the lungs of infected animals [68,101,113], as well as to block virus entry into CNS and the development of behavior anomalies following a lower respiratory tract infection [101]. Adoptive transfer experiments suggest that protection conferred by this vaccine was mediated by T cell immunity [113].

For the last 20 years, important efforts have been invested into the identification of safe, effective, and well-tolerated drugs for hRSV prophylaxis or treatment. Currently, hRSV prophylaxis is recommended for high-risk individuals, such as preterm infants and infants with congenital heart disease, chronic lung disease, neuromuscular diseases, cystic fibrosis, and congenital and acquired immunodeficiency [20,116]. For these patients, a humanized neutralizing monoclonal antibody directed to the hRSV F protein is recommended [116,117]. This antibody, known as palivizumab (Synagis®), has been shown to reduce hospitalization rates by up to 50% in these risk groups [20,116]. However, the elevated costs of this treatment impair its widespread use.

CONCLUDING REMARKS

The association between acute encephalopathy and hRSV infection has been recently shown in several studies [6,22,69,83,93,118]. Despite the increasing number of reports suggesting an association between neuropathologic symptoms and hRSV-induced severe bronchiolitis, the mechanisms involved in these pathological processes remain largely unknown. hRSV-caused neurologic manifestations include esotropia, preceding convulsive seizures, and impaired consciousness. Regarding other extrapulmonary manifestations, some patients with hRSV-induced encephalopathy presented hyponatremia, apnea, or cardiopulmonary arrest without convulsions. These patients also show altered cytokine profiles, with elevated levels of IL-6, IL-8, and chemokines, such as chemokine ligand 2 (CCL2) and CCL4. However, a correlative but not causative association has been established in these children, and the mechanisms underlying these manifestations remain to be defined [87]. Experimental data support the notion that hRSV reaches brain tissues after an intranasal inoculation and that this causes cognitive deficits in laboratory animals. Learning impairment and CNS damage caused by hRSV likely result from deficient inductions of long-term potentiation in the hippocampus. These findings contribute to understanding how hRSV causes pathogenesis in the CNS and provide novel insights into the mechanism by which hRSV spreads from the airways into CNS, causing functional damage to the brain.

CONFLICT OF INTEREST

The authors have no competing interest.

ACKNOWLEDGEMENTS

This work was supported by funding from the Millennium Institute on Immunology and Immunotherapy from Chile (P09/016-F for AMK and SB), La Région Pays de la Loire through the "Chaire d'excellence program" for AMK and Grant "Nouvelles Equipes-nouvelles thématiques" (to AMK and SMB), Institut national de santé et de la

recherche médicale contrats à durée déterminée grant, the Évaluation Orientation de la Coopération Scientifique France–Chile grant, Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) no 1070352, FONDECYT no 1050979, FONDECYT no 1040349, FONDECYT no 1100926, FONDECYT no 1110397, FONDECYT no 1110604, FONDECYT no 1140011 and Biomedical Research Consortium CTU06. KB and JAE are Comisión Nacional de Investigación Científica y Tecnológica—Chile fellows.

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DOI: 10.1002/rmv

Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

Induction of protective effector immunity to prevent pathogenesis caused by the respiratory syncytial virus. Implications on therapy and vaccine design

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doi:10.1111/imm.12313
Received 22 March 2014; revised 29 April 2014; accepted 01 May 2014.
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Summary

Human respiratory syncytial virus (hRSV) is the leading cause of respiratory illness in infants and young children around the globe. This pathogen, which was discovered in 1956, continues to cause a huge number of hospitalizations due to respiratory disease and it is considered a health and economic burden worldwide, especially in developing countries. The immune response elicited by hRSV infection leads to lung and systemic inflammation, which results in lung damage but is not efficient at preventing viral replication. Indeed, natural hRSV infection induces a poor immune memory that allows recurrent infections. Here, we review the most recent knowledge about the lifecycle of hRSV, the immune response elicited by this virus and the subsequent pathology induced in response to infection in the airways. Novel findings about the alterations that this virus causes in the central nervous system and potential therapies and vaccines designed to treat or prevent hRSV infection are discussed.

Keywords: hRSV; lung; T helper type 1/type 2 cells; vaccination; viral.

Introduction

In 1956 Morris and co-workers isolated a cytopathogenic agent from a colony of chimpanzees at the Walter Reed Army Institute of Research, which presented a respiratory illness characterized by coughing, sneezing and mucopurulent nasal discharge.[1,2] The infected animals showed inflammatory damage in the upper respiratory tract and this condition was rapidly spread to other members of the colony, suggesting the presence of a highly infectious pathogen.[1] Because the major sign of disease in the affected monkeys was coryza – or nasal inflammation – the pathogen was termed 'chimpanzee coryza agent'. One year later, Chanock and Finberg[3] reported the isolation of a similar agent from two throat swab samples of infants with severe respiratory illness. These viruses were identical to the 'chimpanzee coryza agent' reported by Morris, suggesting that this pathogen could infect both chimpanzees and humans.[3] The unusual cytopathic effect caused by the virus on HEp-2 cells, characterized by the syncytia formation and giant cells in cultures, led to its current denomination as human respiratory syncytial virus (hRSV).[1]

Human RSV is now the most important cause of acute lower respiratory tract infections (ALRTI) that include acute bronchitis, bronchiolitis, pneumonia and tracheitis in infants and young children worldwide.[4] Data from a recent meta-analysis showed that this pathogen causes up to 33·8 million ALRTI in children under 5 years of age each year, of which around 3·4 million of cases need hospital admission worldwide.[5] Further, hRSV infection causes the deaths of 66 000–199 000 children every year

in developing countries.[5] For these reasons, hRSV is considered a global health burden.

The success of hRSV as a respiratory pathogen is probably explained by its extremely contagious capacity. Estimates suggest that approximately 70% of infants under 1 year of age are infected with this virus, while 100% of 2-year-old children have been infected at least once with hRSV.[6,7] Infections in children and adults are recurrent during life and protective immunity against the pathogen is inefficient, despite the production of antibodies after infection.[6,8] The inefficient immune response against hRSV is partly due to virulence factors, such as the NS1 and NS2 proteins that interfere with the immune response against this pathogen.[8]

The severity of hRSV infection is associated with the pre-existence of several risk factors, the most important being age and sex.[9] Regarding age, the groups that present severe complications are babies, infants and the elderly.[9] In fact, 10–28% of hospitalized infants infected with hRSV are < 6 weeks old, 49–70% below 6 months and 66–100% under 1-year-old.[10] The severity of the disease in the elderly has been associated with additional pathological conditions like cardiopulmonary and immunosuppressive diseases.[11] Moreover, it has been reported that males are most susceptible to suffer severe ALRTI than females.[10] Indeed, male infants are 1·5 times more likely to require hospital admission due to hRSV infection than females.[12] Other conditions such as prematurity and congenital diseases have been implicated in the risk for severe hRSV infection.[9] Among the most important risk factors are chronic lung disease, cystic fibrosis and congenital heart problems; all these conditions contribute to severe ALRTI and patients need intensive care and mechanical ventilation.[9] Further, it has been reported that malnutrition is an important risk factor in developing countries and both smoke exposure and maternal smoking increase the severity of ALRTI due to hRSV infection.[9]

Despite more than 50 years of intensive research on hRSV pathogenesis, antiviral drugs and treatment against the virus are very limited and no vaccine is currently available to induce long-term protection against hRSV. The study and design of new approaches of prophylactic drugs and vaccines against hRSV is imperative to control the annual outbreaks of the virus and to decrease the high rate of infant hospitalization. To accomplish these aims it would be necessary to understand the virus life cycle and the pathology it causes. Here, we review and describe the most recent findings associated with hRSV infection, pathology and virulence. Also, we discuss strategies developed recently to prevent and treat hRSV infection.

Human RSV virology

Classification

Human respiratory syncytial virus belongs to the *Mononegavirales* order in the *Paramyxoviridae* family, and *Pneumovirinae* subfamily, genus Pneumovirus.[13] The *Paramyxoviridae* family also includes other viruses such as metapneumovirus, and parainfluenza, mumps, measles, Nipah and Hendra viruses.[13] Human RSV has two antigenic subgroups, A and B, that shows divergence in sequencing analysis.[14] Other members of this genus are bovine RSV, ovine RSV and pneumonia virus of mice.

Human RSV characteristics

Human RSV is an enveloped non-segmented negative sense single-stranded RNA virus. The viral particle consists of a helical nucleocapsid covered by a lipid membrane derived from the infected host cell.[15,16] Although hRSV is a spherical particle of 100–350 nm diameter, the virus can also take the form of long filaments. Indeed, a recent study suggests that this can be the most predominant morphology of the virus.[16,17] The hRSV genome is 15·2 kb in length comprising 10 genes encoding 11 proteins, as there are two overlapping open reading frames, each of them encoding for an individual protein (M2-1 and M2-2).[16] The lipid envelope contains three viral transmembrane glycoproteins: the attachment G protein, the fusion F protein and the small hydrophobic SH protein. Underneath the envelope is the matrix M protein, which is a non-glycosylated protein involved in the assembly of the viral particle.[18] As part of the nucleocapsid there are four proteins: nucleoprotein N, the phosphoprotein P, the transcription factor M2-1 and the polymerase L.[19] Human RSV expresses two non-structural proteins, named NS1 and NS2,which inhibit the production of type I interferon activity by the host cell.[16]

Human RSV infective cycle

The transmission of hRSV requires direct contact of secretions from infected individuals.[20–23] Small droplets containing hRSV can enter the host through the nose, eyes and upper respiratory tract, which deliver the virus to epithelial cells.[8,15,24] Although the main targets of hRSV infection are the airway epithelial cells, this virus can also infect other cell types, such as structural cells of the airway and immune cells.[25,26] Human RSV infection in host cells begins with the attachment and entry of the virus through the activity of the G and F glycoproteins, respectively. The RNA of the virus enters the cells upon the fusion of the viral envelope with the cell plasma membrane.[25] Once inside the host cell, the transcription

of viral genes and viral genome replication are initiated, two processes essential for the infective cycle. While *in vitro* studies have shown that mRNA and proteins from the virus are detected inside the cell 4–6 hr after infection, expression peaks at 20 hr after infection.[25] The transcription leading to mRNA synthesis and the replication of genomes for new viral particles are separate processes, which are modulated by the activity of the M2-2 protein.[25]

The production and delivery of viral particles start after 12 hr after infection and persist up to 48 hr after viral entry.[13,27] Cells infected with hRSV show cytoplasmic inclusion bodies that contain viral RNA and proteins, including N, P, M2-1 and L.[27] It has been suggested that inclusion bodies support the RNA synthesis and recent studies showed that these structures can also sequester cellular signalling components to impair the cellular response to infection.[27] The structural components of hRSV are mobilized to the plasma membrane for the assembly and budding of viral particles.[18] The minimum molecular requirement for viral particle assembly are the F, M, N and P proteins, in addition to the genome and anti-genome.[27] The budding of hRSV takes place at the apical membrane in polarized cells. The F protein goes to the apical membrane through the secretory pathway from the endoplasmic reticulum and Golgi, where it is associated with the lipid raft.[18] The rest of the hRSV structural proteins and the RNA genome also traffic to the apical membrane from the cytoplasm and from viral inclusion bodies.[28] The matrix protein is localized in the nucleus in early stages after infection, but is mostly cytoplasmic in the late phases of infection.[28]

Immune response elicited by hRSV

Innate immune response to hRSV

Once in the airways, hRSV is recognized by pattern recognition receptors (PRRs) expressed on epithelial and immune cells that induce the secretion of innate cytokines and chemokines. These molecules promote inflammation and the recruitment of eosinophils, neutrophils and monocytes into the lungs, as well as the onset of an anti-viral response. To date, there are three types of PRRs identified, which include toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), all involved in eliciting the immune response against hRSV.[29] Several TLRs are activated by hRSV, including TLR2, TLR3, TLR4 and TLR7.[25,30–33] As detailed in Fig. 1, TLR2 and TLR4 are expressed in the cell surface and recognize hRSV when

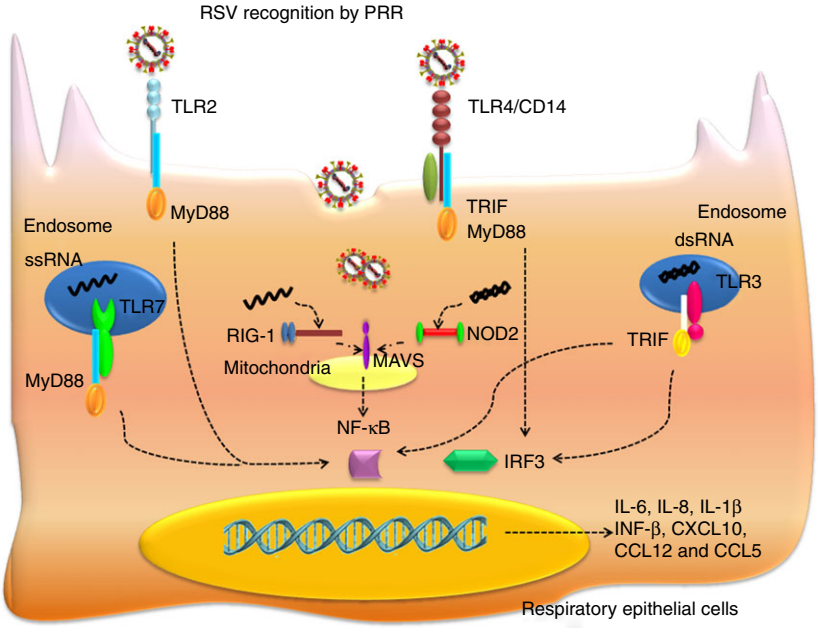

Figure 1. Human respiratory syncytial virus (hRSV) recognition by airways epithelial cells: upon infection, different hRSV components activate pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are crucial in the hRSV recognition and the triggering of innate immune response against this pathogen; TLR2 and TLR4, expressed in the cell surface, and TLR3 and TLR7, present in cytoplasmatic endosomes, are activated in response to hRSV and promote the secretion of pro-inflammatory cytokines, such as interleukin-8 (IL-8), IL-1β and IL-6 through the nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3) pathway. Also retinoic acid-inducible gene (RIG-1) and nucleotide-binding oligomerization domain (NOD2), which belong to RIG-I-like receptors (RLRs) and NOD-1-like receptors (NLRs), respectively, participate in the recognition of viral RNA associated with mitochondrial anti-viral signalling (MAVS) in the mitochondrial membrane in the cytoplasm leading to activation of NF-κB and IRF3.

associated with the co-receptors TLR6 and CD14, respectively.[34] TLR4 interacts with hRSV F protein, leading to nuclear factor-κB (NF-κB) activation and promotes the secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8 by epithelial cells. TLR3 is an intracellular receptor that recognizes dsRNA generated during the viral replication. In response to hRSV, TLR3 activates NF-κB and interferon regulatory factor 3 (IRF3) through the adaptor protein TRIF, with the subsequent secretion of interferon-β (IFN-β), CXCL10, CCL12 and CCL5. TLR7 is expressed in the endosomal membrane and recognizes ssRNA. Entry of hRSV into the cytosol is detected by TLR7, which regulates the secretion of IL-12 and IL-23 through signalling via MyD88.[29]

In addition, RIG-1 is a cytosolic RLR (that belongs to the RNA helicase family) that detects intracellular viral RNAs.[29] Upon hRSV infection, RIG-1 is activated by the 5′ triphosphate structure of viral RNA, which activates the NF-κB and IRF3 pathways using the mitochondrial anti-viral signalling (MAVS) adaptor localized in the mitochondrial membrane, inducing the expression of IFN-β, IP-10 and CCL5 in the airway epithelium.[29] Furthermore, NOD2 is an NLR that belongs to the large cytosolic receptor family. NOD2 is activated by hRSV ssRNA and then is translocated to the mitochondria, where it interacts with MAVS to induce activation of both IRF3 and NF-κB.[29] Recognition of RSV though PRR is schematized in Fig. 1.

Among the pro-inflammatory cytokines described below, IL-8 is a key molecule produced by epithelial cells and macrophages during the early response to hRSV and works as a chemoattractant in the recruitment of neutrophils, which infiltrate the site of infection.[35] Another important molecule of the innate response against hRSV infection is IL-1β, a pro-inflammatory cytokine involved in the antiviral response. First, hRSV stimulates PRR to induce the expression of pro-IL-1β (IL-1β precursor) and inflammasome components, trigged by TLR2/MyD88 that activates the NF-κB pathway.[33,35] Second, the assembly of the inflammasome complex takes place and caspase-1 cleaves pro-IL-1β into IL-1β in response to the production of reactive oxygen species, cellular potassium efflux, or cathepsin leakage into the cytosol after lysosomal disintegration.[34,36]

The NF-κB pathway is important for the activation of an innate response against hRSV, not only for the cytokine response, but also for the formation of tight junctions between nasal epithelial cells.[37] Infection with hRSV induces the up-regulation of genes encoding structural components of tight junctions, including claudin-2, -4, -7, -9, -14, -19, occludin, ZO-2, cingulin and MAG-1, mediated by the protein kinase Cδ signalling.[37] This phenomenon seems to be beneficial for the replication of the virus, because inhibition of NF-κB and protein kinase Cδ activation leads to an impairment of viral replication and formation of virus filaments.[37] In addition, the induction of tight junctions could increase the cell polarity necessary for viral budding.[13]

Adaptive immune response against hRSV

Human RSV infection has been associated with an inefficient adaptive immune response, characterized by an excessive T helper type 2 (Th2) and a deficient antiviral Th1 response.[15,36,38] The Th1 responses usually involve the production of IFN-γ, IL-2 and tumour necrosis factor-α, whereas IL-4, IL-5, IL-10 and IL-13 secretion characterize Th2 responses. Further, a Th17 response has been associated with hRSV pathogenesis because it contributes to the development of asthma in infected children.[15,39] Studies using an *in vitro* model comprising both human airway epithelial cells (A549 cells) and human immune cells (peripheral blood mononuclear cells) have shown that hRSV infection induces the production of IFN-γ,IL-4 and IL-17, suggesting that the three subsets (Th1, Th2 and Th17) can be activated upon viral infection.[40] Assays performed with peripheral blood mononuclear cells co-cultured with hRSV-infected A549 cells have also shown a Th2 and Th17 differentiation and the suppression of the generation of regulatory T cells.[8,41] Indeed, as shown in Fig. 2, epithelial cells infected with hRSV expressed MHC-II and the co-stimulatory molecules CD80 and CD86, suggesting that these cells can directly activate naive $CD4^+$ T cells during hRSV infection, promoting a Th2 and Th17 differentiation and suppressing the differentiation of regulatory T cells.[8,25,36,42,43] Studies from hRSV infection in mice demonstrated a Th1 response with production of IFN-γ, IL-2 and IgG2a followed by the production of cytotoxic T lymphocytes.[13] Also, studies using murine models have shown that the vaccination with different hRSV proteins and peptides followed by hRSV challenge allows the modulation of T-cell responses and disease severity. The immunization with recombinant vaccinia viruses expressing F protein induced a Th1 $CD4^+$ T-cell response and a strong cytotoxic lymphocyte response, leading to a secondary hRSV disease with polymorphonuclear cell efflux. Immunizing mice with hRSV G protein promoted a Th2 $CD4^+$ T-cell response and eosinophilic infiltration in lungs after subsequent infection with hRSV. In humans, production of both Th1 or Th2 cytokines has been detected in blood, nasopharyngeal aspirates and bronchoalveolar lavage taken from infants with hRSV disease.

Antibody responses also play an important role in hRSV infection, preventing the occurrence of re-infection by neutralizing or opsonizing extracellular viral particles. However, hRSV fails to induce a long-lasting antibody response. G and F glycoproteins are the major antigens of hRSV-specific neutralizing antibodies. IgA and IgG are secreted during hRSV infection and confer protection in

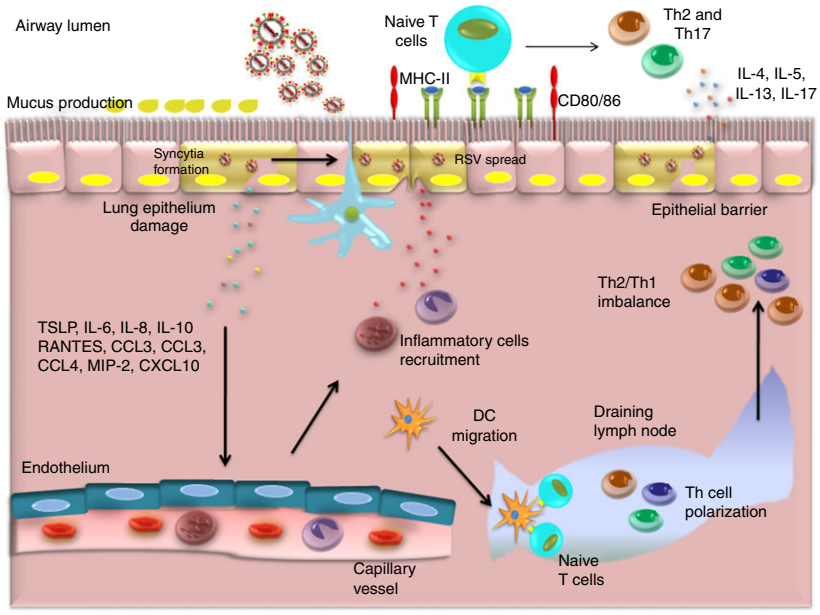

Figure 2. Inflammatory environment after human respiratory syncytial virus (hRSV) infection. Human RSV infection has been associated with an inefficient immunity characterized by a T helper type 2 (Th2) and Th17 polarized response and a weak Th1 response. Human RSV enters lung epithelial cells and induces the secretion of cytokines and chemokines such as interleukin-6 (IL-6), IL-8, RANTES, CCL3, MIP-2, CXCL10 to promote the recruitment of neutrophils infiltrating the infection site. Also, resident dendritic cells (DCs) migrate to draining lymph nodes to activate T-cell responses against hRSV characterized by an exacerbated Th2 and Th17 response. Indeed, epithelial cells expressed MHC-II, the co-stimulatory molecules CD80 and CD86 that can activate naive T cells to Th2 and Th17 polarization after hRSV infection. The pro-inflammatory cytokines and effector cells in the environment cause damage in the epithelium, which causes the characteristic pathology of hRSV.

the upper and lower respiratory tract.[44] In humans, IgA and IgG titres decreased quickly after acute hRSV infection, especially in young children.[45] The declining of antibody titres is thought to contribute to re-infection with hRSV and is also correlated with an increased susceptibility to hRSV infection in the elderly. Young children have an immature immune system and combined with the presence of maternal antibodies develop poor antibody responses against hRSV.[45] Indeed, neutralizing hRSV-specific antibodies are detected only in 50–75% of children younger than 6 months of age. Hence, hRSV infection induces a deficient antibody response that fails to produce long-term protection against the pathogen and results in re-infections throughout life.[45] The stimulation of primary antibody responses against hRSV occurs mostly in the lymph nodes draining the respiratory tract. In those tissues, virus-specific extrafollicular and marginal zone B cells found viral components and hRSV antigens, to initiate the engagement of their surface immunoglobulin B-cell receptor. Simultaneously, naive $CD4^+$ T cells interact with dendritic cells (DCs) that have migrated from the airways to lymph nodes and become activated through the assembly of an immunological synapse. In this step the presence of co-stimulatory molecules (e.g. inducible co-stimulatory molecule) and the secretion of inflammatory cytokines (e.g. IL-6) is critical for differentiation of hRSV-specific T follicular helper cells. Hence, T follicular helper cells contribute to the differentiation of extrafollicular B cells into antibody-secreting plasma cells by promoting germinal center formation and affinity maturation.

Epithelial cells also participate in the adaptive immune response elicited by hRSV infection through the secretion of thymic stromal lymphopoietin, a cytokine that promotes the activation of T cells.[46] A recent study that used primary rat airway epithelial cells infected with hRSV and co-cultivated with DCs, showed that these latter cells displayed increased expression of MHC-II and CD86 on their surface.[47,48] Blockade of thymic stromal lymphopoietin in this system decreased significantly the expression of both maturation markers.[47] It has also been described how DCs infected with hRSV up-regulate the expression of molecules that promote Th2 polarization as represented in Fig. 2,[36,49] such as thymus- and activation-regulation chemokine and OX40 ligand.[47] These data suggest that epithelial cells infected with hRSV contribute to the nature of T-cell differentiation through the modulation of DCs.

Pathogenesis in the respiratory tract

The respiratory disease caused by hRSV begins with viral replication in the nasopharynx.[50] The spread from the upper respiratory tract to the lower respiratory tract takes

place possibly through the direct spread along the respiratory epithelium and/or the aspiration of nasopharyngeal secretions.[13] Spreading from cell to cell is also common for hRSV by means of the induction of cell fusion and syncytia formation (Fig. 2). Another mechanism proposed to explain the spread of hRSV in lungs is the infection of macrophages that migrate to the lower respiratory tract. Evidence supporting this mechanism consists of the detection of infected alveolar macrophages *in vivo* and the infection of monocyte-derived macrophages *in vitro*.[51]

During the first days of hRSV infection, patients show mild compromise of the upper respiratory tract, presenting signs such as cough and low-grade fever. The signs of disease in the lower respiratory tract include tachypnoea, wheezing, dyspnoea and retractions of the chest wall.[50,52] During hRSV bronchiolitis, the ciliated epithelial cells are destroyed and in severe cases an extensive bronchiolar epithelial necrosis is observed. Severe cases of hRSV infection included peribronchiolar mononuclear cell infiltrates accompanied by submucosal oedema and bronchorrhoea. This phenomenon leads to bronchiolar obstruction with irregular atelectasis and areas of compensatory emphysema. Also, pneumonitis can occur when the alveoli become filled with fluid. In cases of milder bronchiolitis, the infection affects mostly lower airways, with peribronchiolar and interstitial inflammation.

Damage to central nervous system as a novel effect of RSV infection

In addition to the multiple deleterious effects of hRSV in the airways, during the last decade several reports have provided evidence for an association between hRSV infection and alterations in other tissues, such as the heart, liver and brain. Infection by this virus has been shown to produce different clinical manifestations, such as cardiopathy, hepatitis and encephalitis.[53,54] Infection of the central nervous system (CNS) by hRSV has been supported by the presence of viral RNA in human cerebrospinal fluid,[53] which correlates with neurological symptoms including seizures, central apnoea, lethargy, feeding or swallowing difficulties, abnormalities of muscle tone, strabismus, abnormalities of the cerebrospinal fluid and encephalopathy.[54] Our group evaluated whether the CNS of mice and rats challenged with hRSV can be reached by this virus after intranasal infection.[55] The presence of hRSV was corroborated in brain tissues using immunofluorescence and real-time PCR assays, which showed hRSV proteins and nucleic acids in several zones of the brain, supporting the notion that hRSV infection reaches the CNS.[55] Entrance of hRSV to the CNS was dependent on the blood–brain barrier, because the blockade of CD49d by a monoclonal antibody that targets integrin α_4 and impairs leucocyte extravasation through the blood–brain barrier decreased viral loads in the brain but not in the lungs.[55] As a result of hRSV infection, impairment in cognition was revealed in rodents submitted to water-maze as a spatial learning test and to marble burying as a behavioural test.[55] These alterations were correlated with electrophysiological studies that showed an impairment in the induction of long-term potentiation in stratum radiatum at the hippocampus area.[55] Together, these observations support the previously described notion that hRSV has the ability to infect CNS tissues in a disseminated pattern and that this virus is capable of disrupting cognitive functions by altering the synaptic plasticity of the infected brain tissue.[55]

Drug design to treat RSV infection

Human RSV is considered an important health burden affecting mainly children and the elderly. Unfortunately, currently available treatments for infections by this pathogen are limited and it is not possible to use them broadly because of their high cost. However, there are many efforts invested in the design of new drugs to control the symptoms and unwanted effects caused by hRSV infection. The knowledge of the life cycle of hRSV and the pathology induced in the infected host is essential for the design of drugs with curative or prophylactic purposes. Along these lines, the most relevant processes in the life cycle of hRSV are replication, transcription and fusion, which are potential targets for antiviral drugs.[56] Table 1 summarizes the antiviral drugs designed up to date against hRSV infection.

Ribavirin is an antiviral drug that interferes with the replication of DNA and RNA viruses. This drug was the first antiviral drug approved for the treatment of hRSV infection in humans.[57] Even though ribavirin is effective against hRSV when tested *in vitro* and in animals models, the clinical use of this molecule is currently very limited because of poor efficiency and difficult administration (nasal by aerosol), in addition to a potential elevated risk of tissue toxicity.[56]

Another therapeutic strategy has focused on the inhibition of hRSV replication by using drugs, such as RSV604. RSV604 is a benzodiazepine that affects the replication and promotes the positive selection of hRSV variants with mutations in the gene encoding the N protein. A phase 1 trial has been completed for RSV604 and a phase II trial is currently in progress, showing positive results as an antiviral drug for hRSV.[58] Another promising antiviral drug is a derivative of the antibiotic geldanamycin, named 17AAG and 17DMAG, used commonly against cancer.[59] These compounds inhibit the heat-shock protein hsp 90, which plays an important role in the replication of hRSV and is also efficient against other respiratory viruses; however, to date no clinical trials aim to use this drug for hRSV treatment are in progress.[59]

Table 1. Therapeutic approaches to treat respiratory syncytial virus (RSV) infection (Source Clinical trials.gov)

Name	Mechanism	Remarks
Ribavirin	Inhibition of RSV replication	High toxicity Difficult administration FDA approved
RSV604	Inhibition of RSV replication	Safety, Tolerability and Pharmacokinetic Study in Healthy Subjects complete Phase I, complete; Phase II, in progress
17-DMAG	Inhibition of the heat-shock protein hsp 90	Impairs the RSV replication Antitumoral drug No clinical trial
HR121 and HR212	Fusion inhibitor peptides	IC_{50} of 4·13 and 0·95 μM, respectively No clinical trial
JNJ2408068 and BMS-433771	Chemical compounds that prevent the fusion process	Phase I/II, discontinued
RFI-641	Blocks viral F protein-mediated fusion and cell syncytium formation	Anti-RSV agent with potent *in vitro* and *in vivo* activity Phase I/II, discontinued
ALN-RSV01	Small interfering RNA specific against NS1	Phase 2b Study of ALN-RSV01 in Lung Transplant Patients Infected with Respiratory Syncytial Virus complete

Another class of antiviral drugs are inhibitors of the fusion process. These molecules are synthetic compounds that block the fusion of the virus with the host cells, avoiding the entry of hRSV.[56] Fusion inhibitors that target hRSV have been designed to bind the conserved region of the F protein. For instance, the peptide T-118 blocks the fusion activity of the F hRSV protein and it has been shown to be effective as an antiviral drug to prevent hRSV infection.[56] There are other peptides similar to T-118, namely HR121 and HR212, which differ in effectiveness. Although the peptides described above have shown high anti-hRSV activity in *in vitro* assays, none of them has been reported in clinical trials, probably because of the lack of oral availability, high cost of production and relatively low half-life in the circulation.[60] A similar pharmacological approach consisted of the peptide RhoA, which inhibits the syncytia formation that is characteristic of hRSV infection. RhoA is a small GTPase that is involved in the fusion process and the inhibitor of this protein has been tested in HEp-2 cells and mice, with promising results.[56,61]

Besides peptides that inhibit hRSV fusion, there are several other chemical compounds that impair the fusion process. The benzimidazole JNJ2408068 has shown a high antiviral activity, 100 000 times higher than ribavirin and acts by preventing virus fusion and syncytia formation.[62] Similarly, another synthetic compound is the antiviral BMS-433771,[63,64] a benzotriazole derivative that interacts with the F protein and alters the conformation of this protein. RFI-641, a biphenyl triazine, is another drug that has shown the most potent anti-hRSV activity *in vitro* and *in vivo*.[65] BMS-433771 and RFI-641 have been evaluated in clinical trials, but all of these trials were discontinued, mainly because of the disadvantageous pharmaceutical properties of the compounds.[60]

Nanotechnology has brought new options for hRSV treatment and prophylaxis, using the anti-microbial activity of metals, such as silver and gold.[66] Although due to their toxicity, the clinical use of these metals in humans seems unfeasible, the development of silver or gold nanoparticles combined with polyvinylpyrrolidone have been shown to efficiently inhibit hRSV replication, showing low toxicity in cell lines. Further, gold nanoparticles fused with inhibitor peptides displayed a high inhibitory capacity against hRSV.[66] Human RSV F protein nanoparticle vaccines have recently initiated clinical and preclinical studies to evaluate safety.[67]

Another interesting therapeutic approach is the use of interference RNA that targets different steps during the hRSV infective cycle. The small interfering RNA (siRNA) strategy was initially used to target the expression of NS2[68] and the P[69] proteins, the latter showing an efficient capacity to protect mice against hRSV infection. This approach was also used to target the F gene, showing inhibition of hRSV infection.[70] Nanotechnology has also been applied in combination with the siRNA approach to target the NS1 gene, resulting in the increase of IFN-β production by DCs and stimulated the Th1 differentiation of $CD4^+$ cells.[71] Such a strategy protected mice against RSV infection, because treated mice showed decreased viral loads in lungs and reduced inflammation in this tissue. A new siRNA specific against NS1(ALN-RSV01) showed high antiviral activity that impaired nucleocapsid expression.[72] Studies in mice reported that administration of this molecule reduces RSV titres in the lungs.[73] This antiviral drug has also been evaluated in human clinical

trials, demonstrating their safety and tolerance in healthy adults.[72] In addition, the effectiveness of ALN-RSV01 against hRSV infection was evaluated in humans, with a 44% reduction of hRSV infection without adverse effects[74] and the phase IIb clinical trial has concluded. Further, this drug has been tested in lung transplant patients, where it has demonstrated safety and effectiveness.[74]

Another strategy to combat the disease caused by hRSV is to target the harmful immune response elicited by hRSV infection. The exacerbated Th2 response associated with the hRSV bronchiolitis is characterized by high production of IL-4. Along these lines, a study generated an antisense oligomer to promote local silencing of *il4* gene expression, which was delivered intranasally.[75] This approach was evaluated in neonatal murine models, showing a reduction of Th2 response and decreasing the airway damage caused by hRSV.[75]

To improve the specificity of siRNA technology as an antiviral approach for hRSV, the use of phosphorodiamidatemorpholino oligomers (PMOs) has been proposed. PMOs consist of oligomers in which the nucleobases are covalently attached to the morpholine ring, replacing the deoxyribose sugar while the phosphodiester bond is replaced by the phosphorodiamidate linkage.[76] This strategy gives specificity, stability and target delivery.[77] The function of PMOs is blocking the interaction of proteins with the target RNA. This method has been applied against the L gene of hRSV to impair infection in cell lines and in animal models.[76]

Novel strategies to induce protective RSV immunity

Passive immunization

There are limited options of prophylaxis and currently no vaccines are available to prevent hRSV infection (Table 2). Current clinical approaches to control hRSV infection comprise passive immunization with neutralizing antibodies against F and G proteins, which has been successful at decreasing the symptoms of hRSV infection. Further, these strategies can reduce the severe detrimental effects caused by hRSV infection in patients with risk factors, who can develop serious illness.[78,79] A humanized monoclonal antibody that prevents hRSV fusion to the host cells by the neutralizing F protein (palivizumab or Synagis®; MedImmune, Gaithersburg, MD), is the most efficient and used antibody to prevent severe cases of hRSV disease.[80,81] Motavizumab is another humanized antibody that binds the fusion protein after attachment to the host cell, but before starting the transcription of the viral genome.[82,83] Neither monoclonal antibody completely prevents viral entry to the host cell but they decrease the viral replication and prevent hRSV infection. Despite the effectiveness of palivizumab in the treatment of hRSV infection, the use of this drug is seriously limited due to high costs and is restricted to patients with high risk of severe bronchiolitis associated with congenital diseases and preterm birth.[84]

Table 2. Prevention of respiratory syncytial virus (RSV), passive immunization and new vaccines. Source Clinical trials.gov

Name	Mechanism	Remarks
Palivizumab (Synagis®)	Humanized monoclonal antibody that prevents RSV fusion to the host cells (passive immunization)	The most efficient and used antibody to cure or treat severe cases of RSV disease FDA approved
Motavizumab Medi-524	Humanized antibody that binds to fusion protein after attachment to the host cell (passive immunization)	Safety, Tolerability and Immunogenicity of MEDI-524 After Dosing for a Second Season complete Phase 1-III, complete
DNA vaccines	RSV G protein construct	Induces neutralizing antibodies and balances the pulmonary T helper type 1 (Th1)/Th2 cytokines
NDV-F	Newcastle disease virus (NDV) vector plus F protein	Induces a high interferon-β response
rBCG-RSV	Bacillus Calmette–Guérin (BCG attenuated *Mycobacterium bovis*) modified to express RSV N and M2 RSV proteins	Induces a Th1 immune response specific against RSV
RSV Fusion protein particle vaccine	Trimers Form F protein Nanoparticles (Nanovax)	Safety study complete

Novel vaccine approaches for hRSV

The development of an efficient vaccine against hRSV requires that the formulation promotes protective and efficient immunity against the virus, without adverse effects. Human RSV proteins are immunogenic and are good candidates to design vaccines, but it is important to consider that some hRSV proteins or peptides can negatively modulate the host immune response.[85] Further, an efficient vaccine candidate has to prevent the Th2 immune response and needs to promote viral clearance before the development of the disease.[85]

Vectors comprising hRSV genes or parts of the genome of this virus have been used as DNA vaccines, in combination with adjuvants that promote Th1 immunity. An example of this approach is an hRSV-G construct that induces neutralizing antibodies to balance the production of pulmonary Th1/Th2 cytokines during hRSV infection.[86] The gene coding for the F protein has also been used as a DNA vaccine, an example of this approach is the insert of the F gene into the Newcastle disease virus vector (NDV-F). Immunization with this vaccine induces high levels of IFN-β.[87]

Another approach of the DNA vaccine was a strategy designed as an immunization methodology including a mucosal adjuvant,[88] consisting of two F gene fragments, DRF-412 and DRF-412P, which were cloned into the phCMV1 vaccine vector. Immunization with this recombinant formulation induced neutralizing antibody responses (IgG, IgG1, IgG2a and IgG2b) and a mix of Th1/Th2 cytokine responses in mice.[88]

Attenuated bacterial vectors expressing hRSV proteins are another interesting strategy to induce protection against hRSV and induce Th1 immunity. Recently, a recombinant bacillus Calmette–Guérin bacteria (BCG-attenuated *Mycobacterium bovis*) modified to express N and M2-1 proteins from hRSV (rBCG-RSV) was shown to induce protective hRSV immunity in animal models.[55,77,89,90] This vaccine was able to induce a Th1 immune response against hRSV, characterized by the presence of T cells secreting IFN-γ and a significant decrease of lung damage and inflammation after infection.[89,90] Further, the immunization with rBCG-RSV prevented viral replication in the lungs of infected animals.[55,89,90] One important feature shown by this vaccine was the ability to prevent the CNS alterations caused by hRSV.[55] The BCG-based vaccine prevented the cognitive and behavioural impairment observed in hRSV-infected mice and rats.[55] These data suggest that rBCG-RSV vaccination induces a specific T-cell response that protects against hRSV infection and prevents the spread of the virus to the CNS. BCG vaccination has been used worldwide as a vaccine against tuberculosis in newborns, hence the safety of this vaccine candidate might lead to an efficient and reachable vaccine against hRSV.

Using bacteria as a delivery system of plasmid-expressing viral antigens is also an efficient strategy that allows activation of the natural immune response. This system activates the innate immunity of the host through TLRs and redirects the immune response to the efficient clearance of the pathogen. This is the case of an attenuated *Salmonella typhimurium* strain SL7207 containing a plasmid encoding the F hRSV protein. This live attenuated vaccine was administered orally to mice and induced an efficient humoral and cellular response, as well as mucosal immunity.[91] Attenuated viruses have also been used as vaccines, which consist of the replacement of structural genes with hRSV genes. This method was applied with the Venezuelan equine encephalitis virus and immunization with this prototype vaccine confers protection against RSV and induces a balanced Th1/Th2 immune response.[92]

The use of subunit vaccines has also been evaluated to prevent hRSV infection. Human RSV F was the most accepted subunit vaccine because this is a conserved protein in the paramyxoviridae family. The rF255 is a region of F protein that has been cloned into a vector containing the gene encoding ctxA2 B, which encodes the cholera toxin and induces a Th1 response in mice.[56] Further, another strategy of subunit vaccine is a multivalent recombinant protein that mixes the F, M2 and G proteins cloned in a bacterial pET32a(+) vector, named rFM2G. Immunization with this prototype vaccine promotes an increase of IgG titres, reducing illness in infected mice.[93]

Recent studies of the structure of hRSV proteins have allowed a new candidate vaccine to be designed based on the different conformations adopted by the F protein. The hRSV F protein displays conformational changes during hRSV cell attachment, forming two states; a metastable pre-fusion and a stable post-fusion form.[94] The conformation and reactivity of the different variants of the F protein neutralizing antibodies were analysed and these studies suggest that in the metastable pre-fusion form of F protein there are more relevant exposed epitopes than in the stable post-fusion form. Supporting this notion, it has been described that the pre-fusion epitopes induce a strong anti-F neutralizing humoral response.[94] An example of this strategy is the vaccine designed based on a recombinant prefusion-like form of the F protein bound to bacterium-like particles derived from the food-grade bacterium *Lactococcus lactis*.[94]

Recently, the idea of maternal immunization to prevent hRSV infection in young infants has become the focus of research efforts leading to an hRSV vaccine. This strategy aims to increase the serum-neutralizing antibody levels against hRSV during the second or third trimester of pregnancy to transfer these antibodies through the placenta to the fetus.[95] In addition, it is expected that passive immunization continues during the breastfeeding period, protecting the infant from early and recurrent

infections. Maternal antibodies can confer effective hRSV protection in young infants.[96,97]However, the major concern of this strategy is whether the maternal antibody transfer is enough to induce protective immunity without the need for infant vaccination.[95] The first clinical trials in pregnant women showed reduced antibody responses possibly due to maternal immunosuppression in the pregnancy third trimester. These data suggest that maternal immunization could be more effective in the second trimester of pregnancy.[95] Vaccines used to immunize pregnant women must be safe for the mother and the fetus, as has been shown by the influenza vaccine experience, which opens the possibility of maternal immunization for hRSV using attenuated viral or bacterial vectors.[98]

Concluding remarks

Since the isolation of hRSV more than 50 years ago, several groups have tried to explain the mechanism implicated in the respiratory disease caused by this pathogen. They established the consensus that hRSV induces a detrimental inflammation in the airways, characterized by an exacerbated Th2 response, the result of which is not efficient for viral clearance, promoting destruction of ciliated epithelial cells and peribronchiolar cell infiltrates. In addition to the airway damage caused by hRSV infection, our group has also described that hRSV infection leads to alterations in cognition and behaviour associated with the presence of mRNA and viral protein in the brain tissue of infected animals. There are several strategies in course to develop new prophylactic drugs and vaccines based on inhibition of different processes of the viral life cycle, such as the fusion and replication. An efficient vaccine candidate has to promote the differentiation of T cells in an appropriate antiviral response to elicit the viral clearance. Until now, our knowledge was insufficient to understand the complete picture of hRSV infection but progress is promising an effective and safe vaccine available for the population most affected by this pathogen.

Acknowledgements

This work was supported by grants FONDECYT no 107 0352, FONDECYT no 1050979, FONDECYT no 1040349, FONDECYT no 1100926, FONDECYT no 1110397, FON DECYT no 1100971, FONDECYT no 1110604, FONDEC YT no 1130996, CONICYT Proyecto de Inserción de Capital HumanoAvanzado en la Academia no 791100015 and Millennium Institute on Immunology and Immunotherapy (P09-016-F), Grant from La Région Pays De La Loire through the 'Chaird'excellence program', Grant 'NouvellesEquipes-nouvellesthématiques'from the La Région Pays De La Loire, INSERM CDD grant.

Disclosures

The authors declare no financial or commercial conflict of interest.

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Journal of Thermal Biology 71 (2018) 17–23

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Is there an effect of environmental temperature on the response to an antigen and the metabolic rate in pups of the rodent *Octodon degus*?

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ARTICLE INFO

Keywords:
Ecoimmunology
Lipopolysaccharide
Precocial
Thermoregulation
Energy expenditure

ABSTRACT

Environmental temperature is a variable that influences all aspects of organisms, from physiological, e.g. immune function, and morphological traits to behavior. Recent studies have reported that environmental temperature modulates organisms' thermoregulatory capacity and immune response, suggesting that trade-offs must be made between thermoregulation and immune function. Despite this, studies that evaluate this trade-off in developing endotherms are scarce. The aim of this study was to evaluate the effects of environmental temperature experienced during development on the response to an antigen and its energetic costs in the precocial rodent *Octodon degus*. To accomplish this, we acclimated pups from birth to weaning at temperatures of 15 °C and 30 °C. At weaning, animals were inoculated with lipopolysaccharide (LPS) and cytokine interleukin-1β levels, sickness behavior, changes in body temperature and basal metabolic rate, and body mass were measured. Our results showed that environmental temperature influences cytokine levels, body temperature, and some aspects of sickness behavior. Specifically, acclimatization at 30 °C has a suppressive effect on the response to LPS, possibly due to a control to avoid overproduction of interleukin-1β. Body mass and basal metabolic rate were not affected by environmental temperature experienced during development, but inoculation with LPS affected both variables. Our results suggest that ambient temperature may be a key factor that affects the response to an antigen in pups of *O. degus;* however, no evidence of a trade-off between thermoregulation and immune function was found here.

1. Introduction

Ecoimmunological research focusing on trade-offs between the immune system and other fitness-related traits has received considerably more attention in recent decades (Bonneaud et al., 2003; Deerenberg et al., 1997; Cichon et al., 2002; Ilmonen et al., 2000; Nordling, 1998; Soler et al., 2003). These studies have shown that immune function is a costly trait in terms of energy and nutrients (Schmid-Hempel, 2011), and mounting an immune response negatively affects various components of fitness, including breeding effort (Bonneaud et al., 2003; Deerenberg et al., 1997; Ilmonen et al., 2000; Nordling, 1998), nestling growth rates (Soler et al., 2003) and thermoregulation (Cichon et al., 2002). Accordingly, it is assumed that in stressful conditions, typically accompanied by an increase in energy demand, the immune response is suppressed, thereby liberating resources that are reallocated to other costly traits such as thermoregulation (Ilmonen et al., 2003; Sheldon and Verhulst, 1996).

Thermoregulation is an important energetically expensive biological function (Konarzewski and Diamond, 1994). Studies have reported that animals acclimated at temperatures under the lower limit of the thermoneutral zone have higher metabolic rates than those acclimated at temperatures within the thermoneutral zone (Dawson et al., 1983; Hinsley et al., 1993; Maldonado et al., 2009; Repasky, 1991; Swanson, 1993; Wiersma et al., 2007; Withers and Williams, 1990). Hence, in endotherms, thermoregulation could be a major constraint to energy needed for immune function, and, thus, both functions could be compromised due to competition for common resources (Cichon et al., 2002). Therefore, it is expected that immune functionality is suppressed during the winter season or during conditions of low environmental temperatures (Cichon et al., 2002; Dabbert et al., 1997; Svensson et al.,

Abbreviations: mb, Body mass; Tb, Body temperature; LPS, Lipopolysaccharide; IL-1β, Interleukin-1β; BMR, Basal metabolic rate

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http://dx.doi.org/10.1016/j.jtherbio.2017.10.005
Received 19 July 2017; Received in revised form 30 August 2017; Accepted 20 October 2017
Available online 25 October 2017

1998). While this reasoning is sound, studies evaluating the effects of environmental temperature on immune function have reported contradictory results (Burness et al., 2010; Cichon et al., 2002; Dabbert et al., 1997). For example, a negative effect of low temperatures on immune response was detected in the passerine *Cyanistes caeruleus* (Svensson et al., 1998), but not in the Bobwhite Quail (Dabbert et al., 1997).

In mammals, exposure to low environmental temperatures for periods of approximately 10 days negatively affects the immune response of adult mice (Cichon et al., 2002), while shorter exposures of about 24 h have no effect. This suggests that effects on the immune response depend on an individual's thermal history. In this sense, it would be expected that environmental temperatures experienced during development might impose a strong modulatory effect on the immune function of growing animals. Therefore, acclimation to two contrasting experimental ambient temperatures during development would provide an opportunity to explore potential trade-offs between immune function, thermoregulation, and growth.

To date, ecologists have predominantly focused on the immune system adaptive response as a measure of overall immunocompetence, but little is known about innate immunity (Bonneaud et al., 2003; Lee et al., 2006;Owen-Ashley and Wingfield, 2006). It has been proposed that the acute phase response (APR), belonging to the innate immune response, could be an important mediator of trade-offs between immunity and life-history traits (Bonneaud et al., 2003; Lee et al., 2006;Owen-Ashley and Wingfield, 2006) due to the very high costs involved in activating an APR (Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006). The APR refers to a global immune reaction to infection, tissue injury, trauma, neoplastic growth, or immunological disorders (Gordon and Koy, 1985; Gruys et al., 1999). During this reaction, macrophages and other immune cells synthesize pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFβ; Baumann and Gauldie, 1994), and promote adaptive physiological and behavioral responses. Activation of the hypothalamic–pituitary–adrenal axis (HPA), synthesis of liver enzymes, and changes in core temperature are part of the physiological response. Sickness behaviors include crouching, piloerection, reductions in general activity and exploration, anorexia and increased lethargy to conserve energy (Aubert, 1999; Hart, 1988). These behaviors are required to control infection, collectively conserve heat, and promote fever (Oka, 2001); anorexia under these circumstances acts to reduce iron required for pathogen replication (Hart, 1988).

In order to explore putative trade-offs between traits, we evaluated the effects of environmental temperature experienced during development on the antigen response and basal metabolic rate of *Octodon degus* (Octodontidae; Hystricognathi). *Octodon degus* is a diurnal and precocial rodent (Fulk, 1976, Yáñez, 1976) naturally occurring in north-central Chile (Woods and Boraker, 1975). The social nature and communal breeding of this organisms results in an elevated risk of exposure to pathogens; thus, there are clear advantages to investing in immunity during the first days of life. We used lipopolysaccharide (LPS), the immunogenic component of the cell wall of Gram-negative bacteria, as the experimental antigen. It has been shown that LPS produces an inflammatory response in *O. degus* (Nemzek et al., 2008). Responding to LPS appears to be costly, thus providing an opportunity to study the trade-offs between the immune response and thermoregulatory abilities (Owen-Ashley and Wingfield, 2006).

2. Materials and methods

2.1. Animal capture and experimental setting

The experiments were run on the offspring of 29 females and 25 males of *Octodon degus*. The adults were captured in April and May of 2013 and 2014 in the Rinconada de Maipú (33°23′S, 70°31′W) in central Chile. Parental animals were maintained in laboratory conditions at an environmental temperature of 23 ± 1 °C and a photoperiod of 12 L: 12D. For breeding, females were monitored daily to determine when their vulva opened. Once opened, one female and one male were kept together for two weeks in rat cages (dimensions of cages were 58 × 36 × 30 cm) with a bedding of hardwood chips and water and food (commercial rabbit pellets) provided ad libitum. Once pregnancy had been confirmed, females were moved to individual cages and weighed every day. By the end of the gestation period (approximately three months) the cages were checked daily for the presence of pups. Subsequently, the date on which pup presence in the cage was detected was registered as the date of birth (day 0).

After parturition, each litter was randomly assigned to one of two experimental conditions, with one group being acclimated to 15 °C and the other to 30 °C. The temperature of 30 °C was selected because it is within the thermoneutral zone (Rosenmann, 1977) and therefore does not represent a challenge in terms of thermoregulation for adults of this species. Conversely, 15 °C was selected because it is below the thermoneutral zone and would likely be associated with increases in energy expenditure thus producing changes in thermoregulatory behavior (Nuñez-Villegas et al., 2014). The offspring were acclimated from day 0 until day 28, which is within the weaning period for this species (Reynolds and Wright, 1979). On day 28, pups of both groups were separated from the mother and transferred to a room with an environmental temperature of 23 ± 1 °C and photoperiod of 12 L: 12D. Both groups were inoculated on day 30 after birth. For each acclimation temperature, two pups were selected from each litter, resulting in two groups (15 °C and 30 °C) each with 16 pups and with equal male to female ratios. These two groups were then split again into experimental and control subgroups. One subgroup from each group was inoculated with lipopolysaccharide (LPS purified *Salmonella enterica*, Sigma; 500 μg/kg; Nemzek et al., 2003) while the other subgroup was injected with saline solution as a control (0.9% NaCl). In both cases, a final volume of 200 μl was used for the inoculations, and this was administered intraperitoneally. All inoculations were performed between 18:00 h and 19:00 h.

2.2. Body mass and body temperature

Using a digital scale (± 0.01 g), offspring body mass (mb) was measured before inoculation (0 h) and 24 h (24 h) after the immune challenge. Body temperature (Tb) was recorded in the abdominal area with a VeraTemp non-contact digital laser thermometer (± 0.2 °C) before inoculations (0 h) and 12, 15, 19, and 24 h after the immune challenge. These data allowed us to estimate Tb changes in response to inoculation with LPS.

2.3. Blood sampling and determination of Interleukin-1β (IL-1β) levels

After 24 h of inoculation with LPS or saline solution, blood samples (200 μl) were obtained from the lateral saphenous vein (UBC Animal Care Guidelines) for all pups. The total handling time measured from the initial restraint of an animal to the completion of the blood collection did not exceed 2 min. Blood samples were centrifuged at 15,000 rpm for 6 min, and plasma was separated and stored at −80 °C until subsequent assays were performed.

We used plasma samples to determine the levels of the pro-inflammatory cytokine IL-1β. The levels of IL-1β were measured using enzyme-linked immunosorbent assays (ELISA). Wells of a 96-well microtiter plate were coated overnight with monoclonal capture antibody (Life Technologies). Previous studies have reported cross-reactivity in the mouse ELISA kit (Becker et al., 2007). The plates were then blocked for one hour with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3. Serum samples were diluted in PBS, and placed in the wells in duplicate and incubated for 2 h at room temperature. Monoclonal antibody detection (Life Technologies) was subsequently added, and they were again incubated for a period of 2 h. After that, a solution (1:1 mixture of

H_2O_2 and tetramethylbenzidine) was added to each well and stopped with a solution of H_2SO_4 2 N after 20 min. The absorbance of the plates was measured at 450 nm with a correction wavelength of 540 nm using an automated ELISA plate reader. All washings were done with PBS supplemented with Tween 20 (0.05%). Finally, the absorbance was used as a measure of the concentration of IL-1β, using a standard curve.

2.4. Sickness behavior data collection

The behavior of pups was recorded with a video camera (Handycam HDR CX220) mounted on a tripod in front of each cage. Video recordings were made over a period of 30 min after 15 h post-immune challenge. A record of the following behaviors was maintained: 1) crouching: characterized by a hunched posture with lowered head and hidden feet for a period of 60 s (number of periods in this position), 2) locomotion: movement from end to end of the cage (number of movements), and 3) eye closure: eyes closed for a period of 30 s (number of periods). These three behaviors have been reported during the acute phase response in pups of the species *Cavia porcelus*, another caviomorph rodent (Hennessy et al., 2004). Despite this, there are no previous records of sickness behavior in degus nor the exhibition of crouching behavior, eye closure, or decrease in locomotion as part of the inflammatory response in the young of this species.

2.5. Basal metabolic rate estimations (BMR)

The BMR of animals in the two acclimation groups was estimated by determining the rates of oxygen consumption (VO_2). Rates were measured 24 h before and after challenge with LPS or with the control. Oxygen consumption was measured using a computerized open-flow respirometry system (Sable Systems, Henderson, NV) calibrated with a known mix of oxygen (20%) and nitrogen (80%) that was certified by chromatography (INDURA, Chile). The measurements were performed in darkness in glass chambers, at an environmental temperature corresponding to the thermoneutral zone for this species (i.e., 30 ± 0.5 °C; Ronsenman, 1977). Metabolic chambers received dry air at a rate of 750 ml/min from a flow controller (Sierra Instruments). The air was dried before and after the chamber and monitored every 5 s using an oxygen analyzer 1FC-1B (Sable System). CO_2 was removed before entering the O_2 analyzer, and oxygen consumption was calculated using the Withers equation (1977, p 122): $VO_2 = [FR * 60 * (Fi\ O_2 - FeO_2)] / (1- Fi\ O_2)$, where FR is the flow rate in ml/min, and Fi and Fe are the fractions of O_2 concentration at the entrance and exit of the metabolic chamber, respectively.

2.6. Statistical analysis

The data were tested for normality prior to running any analyses. To meet the assumption of normality, body mass and basal metabolic rates were log-transformed. Additionally, the body temperature data were subjected to an inverse function transformation, and locomotion data were square root transformed. We analyzed the effects of environmental temperature and type of challenge (i.e., saline serum vs. LPS) on the dependent variables (i.e., response to antigen and BMR). The effect of environmental temperature on body mass prior to inoculation (0 h) was evaluated with an analysis of variance (ANOVA). After inoculation (24 h), the effect of environmental temperature and type of challenge on mb was assessed using a two-way analysis of variance (ANOVA). To evaluate specific differences *a posteriori Tukey* tests were used. Using a two-way repeated measures analysis of variance, the Tb of animals in the four treatments was compared over a period of 12 h after each inoculation; here, the effect of environmental temperature and type of challenge were the main factors, and time of inoculation was included as a random factor. An *a posteriori Tukey* test was used to assess specific differences in treatments.

The effect of environmental temperature and the type of challenge on IL-1β level was evaluated using a two-way analysis of variance (ANOVA). An *a posteriori Tukey* test was used to assess specific differences in IL-1β. Sickness behavior was analyzed using parametric and non-parametric analysis when ANOVA assumptions were not met. The effects of environmental temperature and the type of challenge on crouching and locomotion were evaluated using a two-way analysis of variance (ANOVA). Eye closure was evaluated by Aligned Rank Transform (ART; Wobbrock et al., 2011). This approach allows for factorial analysis of nonparametric data, including the interaction between factors. Here, a transformation is first performed that aligns the data of each effect; then the data are ranked. Then, the aligned and ranked data were analyzed using a factorial analysis of variance. An *a posteriori Tukey* test was again used to assess specific differences in sickness behavior.

The effect of environmental temperature on BMR prior to inoculation (0 h) was evaluated using an analysis of covariance with body mass at 0 h as the covariate. To evaluate the effect of type of challenge and environmental temperature on BMR, a two-way analysis of covariance was performed (ANCOVA) with body mass as the covariate. All statistical analyses were performed using Statistica for Windows program 7. The results are presented with the mean ± standard error.

3. Results

3.1. Changes in body mass (mb) and basal metabolic rate (BMR)

Initial mb (prior to inoculation, 0 h) was significantly affected by environmental temperature (ANOVA: $F_{1,18} = 7.16$; $p = 0.015$) where individuals reared at 15 °C had the lowest body mass (a *posteriori Tukey* test $p < 0.05$; Table 1). Challenge with LPS resulted in a significant loss of body mass (Table 2) with pups losing 2.7% of their initial weight (*a posteriori Tukey* test $p < 0.05$; Fig. 1). Neither environmental temperature nor the interaction between environmental temperature and type of challenge were found to affect body mass (Table 2).

BMR prior to inoculation (0 h) was not affected by environmental temperature (ANCOVA: $F_{1,30} = 0.004$; $p = 0.95$; Table 1). Significant effects on BMR due to the type of challenge were detected post inoculation (24 h); specifically, BMR increased in LPS challenged pups (*a posteriori* Tukey test, $p < 0.008$; Table 2; Fig. 2). Environmental temperature did not affect BMR (Table 2) nor did the interaction between

Table 1
Body mass (mb) and basal metabolic rate (BMR) measured pre (0 h) and post-inoculation (24 h) in offspring acclimated at 15 °C and 30 °C. Values are expressed as mean ± standard error.

	Environmental temperature			
	30 °C		15 °C	
	LPS (n = 8)	Saline (n = 8)	LPS (n = 8)	Saline (n = 8)
Mb pre-inoculation	76.75 ± 6.01	73.37 ± 6.77	53.79 ± 2.59	52.07 ± 3.24
Mb post-inoculation	72.52 ± 6.1	71.79 ± 5.81	49.73 ± 1.04	57.6 ± 3.26
BMR pre-inoculation	81.56 ± 8.53	80.18 ± 11.4	56.16 ± 5.05	70.19 ± 8.55
BMR postinoculation	100.47 ± 7.76	78.17 ± 7.72	79.83 ± 5.37	69.87 ± 6.23

Table 2
Results of the two-way analysis of variance (ANOVA).

	Challenge			Temperature			Temperature x challenge		
	(df), F, p			(df), F, p			(df), F, p		
Mb	(1,29)	8.49	0.007	(1,29)	3	0.09	(1,29)	2.73	0.11
BMR	(1,27)	12.17	0.002	(1,27)	0.00003	0.1	(1,27)	0.03	0.86
IL−1b	(1,16)	8.71	**0.01**	(1,16)	3	0.1	(1,16)	7.46	0.01
Locomotion	(1,29)	13.76	0.001	(1,29)	3.99	0.06	(1,29)	1.11	0.3
Crouching	(1,29)	10.23	0.003	(1,29)	2.92	0.09	(1,29)	0.26	0.4
Eyes closed	(1,29)	103.67	< **0.0001**	(1,29)	60.4	< **0.0001**	(1,29)	26.78	0.5

Analysis performed to evaluate changes in body mass and BMR, levels of IL-1b and sickness behavior of pups challenged with LPS and a saline solution and acclimated to two different environmental temperatures (30 °C and 15 °C). Significant values of the factorial ANOVA are shown in **bold**. d.f. = degree of freedom; F = F-value; p = p-value.

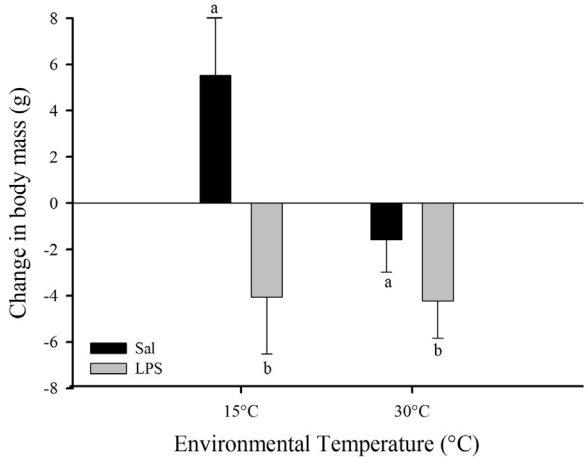

Fig. 1. Change in body mass of *O. degus* pups challenged with LSP or a saline serum and acclimated at one of two environmental temperatures (15 °C and 30 °C). Different letters denote significant differences between treatments.

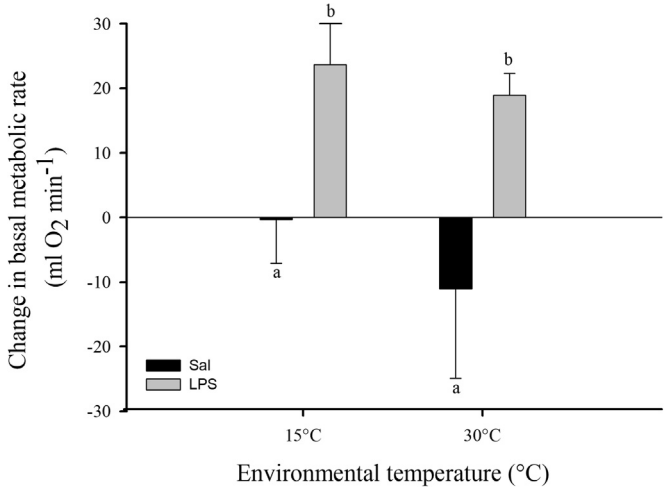

Fig. 2. Change in basal metabolic rate of offspring of *O. degus* challenged with LSP or saline serum and acclimated at one of two environmental temperatures (15 °C and 30 °C). Different letters denote significant differences between treatments.

type of challenge and environmental temperature (Table 2).

3.2. Sickness behavior, body temperature, and IL-1β levels

Comparison of body temperature at 0 h, 12 h, 15 h, 19 h and 24 h post-challenge showed a significant effect of post-inoculation time and of the interaction between post-inoculation time and type of challenge (Table 3). At 19 h post-challenge, the lowest body temperatures were detected in pups treated with LPS (a *posteriori Tukey* test p = 0.001; Fig. 3). There were no significant effects on body temperature due to

Table 3
Results of two-way repeated-measures analysis of variance (ANOVA) on body temperature.

	d.f.	F	p
Between effects			
Temperature	1,18	0.01	0.92
Challenge	1,18	1.9	0.19
Challenge × Temperature	1,18	1.1	0.31
Within effects			
h.p.i	4,72	17.7	< **0.0001**
h.p.i × temperature	4,72	1.8	0.14
h.p.i × challenge	4,72	2.7	**0.03**
h.p.i. × challenge × temperature	4,72	1.4	0.24

Analysis performed to evaluate changes in body temperature (°C) in pups challenged with LPS and saline solution at two different environmental temperatures (30 °C and 15 °C) and among different hours post-inoculation (h.p.i). The significant values of the factorial repeated-measures ANOVA are shown in **bold.** d.f. = degree of freedom; F = F-value; p = p-value.

the interaction between post-inoculation time and environmental temperature (Table 3) or due to the interaction between the three variables (Table 3).

A significant effect of type of challenge and of the interaction between environmental temperature and type of challenge on IL-1β levels was observed (Table 2). Specifically, higher levels of this cytokine were detected in LPS challenged pups acclimated to the lower temperature range (*a posteriori Tukey* test $p < 0.05$; Fig. 4). Environmental temperature did not affect IL-1β levels (Table 2).

Inoculation with LPS produced a significant reduction in locomotion (Table 2; Fig. 5a) although environmental temperature was not significantly affected (Table 2). The interaction between environmental temperature and type of challenge did not significantly affect this behavior (Table 2). While the crouching intervals of LPS challenged pups were observed to increase (Table 2; Fig. 5b), neither acclimation to environmental temperature (Table 2) nor the interaction between type of challenge and environmental temperature (Table 2) had significant effects on crouching behavior. Since eye closure events were not observed in several cases (and therefore are represented as zeros) non-parametric tests were used. The analysis of variance with a two-way ART procedure (see methodology) showed a significant effect of environmental temperature (Table 2) on eye closure; pups reared at 15 °C displayed this behavior more frequently than those reared at 30 °C. Similarly, type of challenge significantly affected the number of times pups performed this behavior (Table 2); specifically, pups treated with LPS closed their eyes more frequently. Finally, there was a significant effect of the interaction between type of challenge and environmental temperature (Table 2); eye closure intervals occurred most frequently in LPS challenged young acclimated to 15 °C (a *posteriori Tukey* test $p < 0.05$; Fig. 5c).

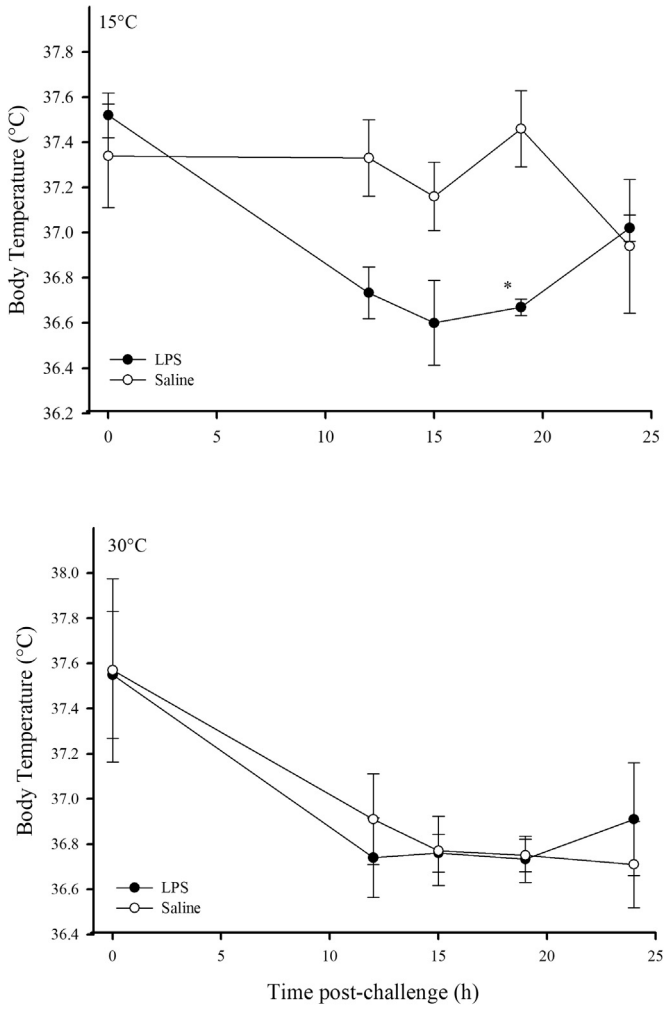

Fig. 3. Body temperature recorded at 0, 12, 15, 19 and 24 h after LPS challenge (closed circles) or saline inoculation (open circles) in pups of *O. degus*, acclimated at two different temperatures: 15 °C (upper panel) and 30 °C (lower panel). The inoculations began at 19:00 (0 h). Asterisk denotes a significant difference.

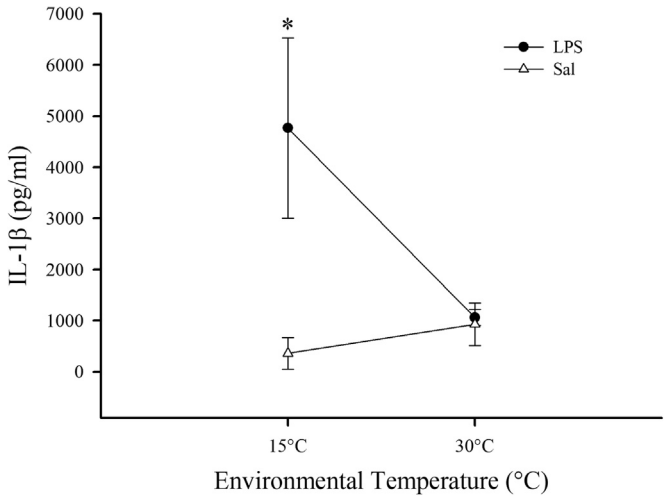

Fig. 4. IL-1β levels of *O. degus* pups challenged with LPS (closed circles) or treated with saline serum (open circles) and acclimated to one of two environmental temperatures (15 °C and 30 °C). Asterisk denotes a significant difference.

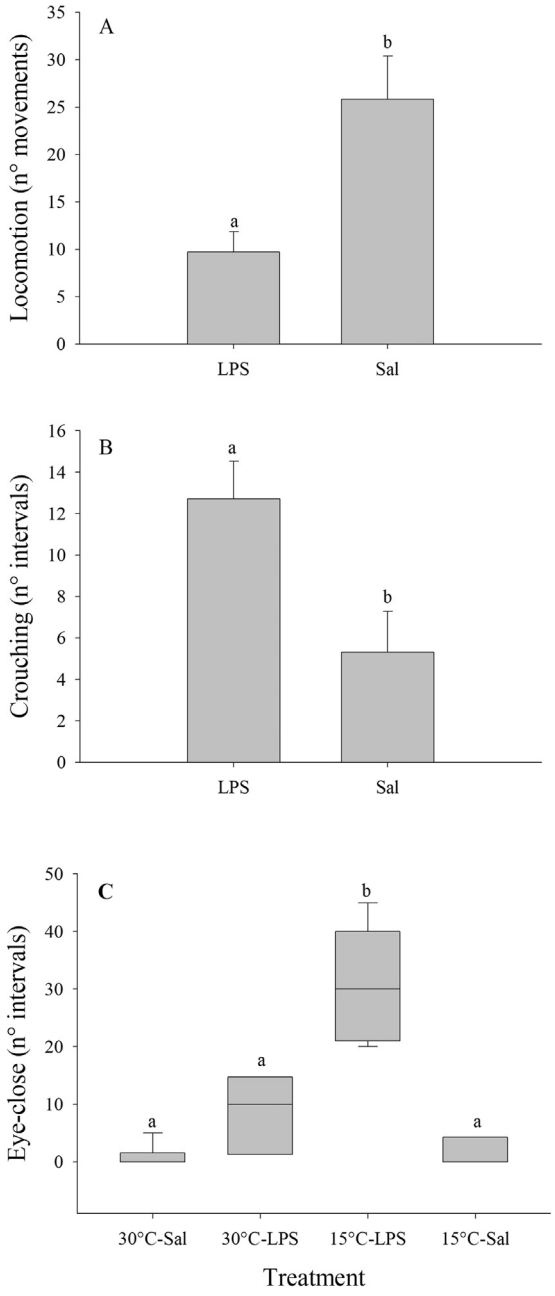

Fig. 5. Sickness behavior. A) locomotion, B) crouching and C) eyes closed. Records were made 15 h after treatment with LPS (black bars) or saline (gray bars) in pups of *O. degus* acclimated to 15 °C or 30 °C. Different letters denote significant differences between treatments.

4. Discussion

In this study, we evaluated the effect of exposure to contrasting environmental temperatures during development (15 °C and 30 °C) on the response to LPS in pups of *Octodon degus*. We hypothesized that there would be a trade-off between the cost of responding to an antigen and the cost of thermoregulation. Thus, we predicted a reduction in the magnitude of the response to an antigen in individuals acclimated to low environmental temperatures. Despite this, we found no evidence of such a reduction.

4.1. Changes in body mass and basal metabolic rate

Environmental temperature was found to affect body mass prior to

the LPS challenge, with greatest reductions in mass observed in pups reared at 15 °C. After LPS injection, we expected pups reared at 30 °C to display greater reductions than pups reared at 15 °C, since the response to LPS is assumed to be energetically costly (Owen-Ashley and Wingfield, 2006), and only pups in good condition or with the greatest body masses should theoretically be able to mount a strong immune response (Burness et al., 2010). However, there was no significant effect on body mass following LPS inoculation of animals acclimated to either environmental temperature; reductions in mass following LPS injections in both treatments were similar (Fig. 1). Several studies have reported a reduction in body mass in adults and young animals challenged with an antigen (Brommer et al., 2004; Burness et al., 2010; Moreno-Rueda, 2011). This reduction may, in part, be attributable to the cost of the antigen response and the associated re-allocation of resources (Ots et al., 2001) or to a reduction in feeding rate as a consequence of sickness behavior (Bonneaud et al., 2003). We cannot rule out either of the two explanations, however, since we do not have data on feeding rates after pups were challenged with the antigen, nor do we have estimates of the energetic cost of the response to LPS.

Relative to the control group, the BMR of the group acclimated to 15 °C and challenged with LPS increased in 28.5% of the pups while the BMR of the group acclimated to 30 °C and challenged with LPS increased in 14.3% of the pups. In this vein, Demas et al. (1997) found that the resting metabolic rate of adult mice increases by 27% in response to keyhole limpet hemocyanin (KLH) inoculation. However, Pilorz et al. (2005) report that the metabolic rate of guinea pig pups does not change in response to the KLH inoculation. The increase in BMR in our study is well within the values previously reported for others rodent individuals (Demas et al., 1997; Schmid-Hempel, 2011), but the increases in BMR for both environmental temperature were not significantly different (Fig. 2). The absence of a significant effect of environmental temperature on the basal metabolic rate of LPS challenged animals may be explained in part by the duration of the period of acclimation. It has been reported that prolonged exposure to cold in adult mice results in suppression of the immune response due to adjustments in thermoregulatory capacity (Cichon et al., 2002). However, it is possible that the opposite might occur in pups where prolonged exposure to certain environmental conditions involves more acclimation and therefore reduced susceptibility to immune challenge (Thaxton, 1978). Thus, the acclimation from birth to weaning may involve the maintenance of a safe energy margin in case of unexpected energy demands (Diamond and Hammond, 1992).

4.2. IL-1β levels and changes in body temperature

Our results reveal that *degus* acclimated to 15 °C and challenged with LPS possessed the highest IL-1β levels in this study (Fig. 4). It has been documented that the overproduction of IL-1β is related to septic shock and death in animals, including humans (Natanson et al., 1989; Rees et al., 1990). Fairchild et al. (2000) report that at temperatures near 37 °C an explosion/suppression of the production of proinflammatory cytokines is observed so that individuals avoid prolonged exposure to the potentially cytotoxic effects of these cytokines. Thus, it is possible that exposure to 30 °C causes a reduction in the production of IL-1β to avoid decontrolled expression of this cytokine and accompanying cell injury, septic shock, and death of the animal (Muñoz et al., 1991; Tracy et al., 1990). Similar results have been observed in chicks of domestic chickens where temperatures around 32 °C produce a reduction in antibody levels (Thaxton, 1978). On the other hand, there are several studies reporting that low temperatures stimulate immune responses in both adult (Kaunisto et al., 2015; Zhang et al., 2015) and young individuals (Henken et al., 1983a; Thaxton et al., 1978), and conversely, high temperatures suppress immune responses. Consequently, Henken et al. (1983) propose that more extreme reduced environmental temperatures (below 15 °C) are necessary to cause a suppression of the immune response in developing organisms as there is a trade-off between maintaining normothermia and mounting an immune response.

Regardless of environmental temperature, the body temperature of pups challenged with LPS decreased while the control group (saline) displayed no changes in this respect throughout the study (Fig. 3). It is well documented that the febrile response (hyperthermia) is part of the APR (Owe-Ashley and Wingfield 2006). Here, pups of *Octodon degus* acclimated at both temperatures (15 °C and 30 °C) showed hypothermia. Hyper or hypothermia has been reported to result as a response to LPS in endotherms (Rudaya et al., 2005). In fact, it has been argued that both hyperthermia as well as hypothermia represent two different strategies to combat systemic inflammation, each having developed as an adaptive response to environmental conditions, specifically habitat productivity (Romanovsky and Szekely, 1998). In this sense, in geographic regions characterized by low habitat productivity (as is the case in central Chile), hypothermia represents an adaptive response against pathogens (Martin et al., 2008).

4.3. Sickness behavior

Our results show evidence of a stereotype behavior in response to a nonpathogenic antigen in *O. degus*. Specifically, inoculation with LPS resulted in an increase in crouching behavior and a decrease in locomotion. Behavioral changes following LPS inoculation, such as those recorded here, have been previously described in studies conducted in other precocial rodents (Hennessy et al., 2004) and birds (Burness et al., 2010). These studies have documented that individuals challenged with LPS showed a decrease in locomotion and an increase in resting (crouching) states (Hennessy et al., 2004). A decrease in activity and increased rest intervals may have an adaptive function if they allow individuals to overcome infection and therefore enhance their probability of survival (Hart, 1988; Wingfield, 2003). Both locomotion and crouch intervals were not affected by the interaction between environmental temperature and type of challenge. The only behavior affected by the interaction between treatments was the duration of eye closure; contrary to our expectations, LPS challenged pups acclimated at 15 °C engaged in this behavior for longer periods of time than pups challenged with LPS and acclimated to 30 °C.

In conclusion, our results suggest that environmental temperature, at least to some degree, affects the response to LPS by developing degus. In this regard, and contrary to our expectations, exposure to low temperatures appears to enhance the production of the cytokine IL-1β. Further, the increase in the levels of this cytokine is not related to an increase in BMR. It is possible that the dietary regime of animals in this study could mask effects of environmental temperature on rates of energy expenditure. For example, in chicks of *Riparia riparia* (Brzęk and Konarzewski, 2007), the costs of immune response have been found to be dependent on environmental factors that include food availability. The fact that food was provided ad libitum in this study could explain the absence of environmental temperature effects on most of the variables analyzed. Future research should consider the possible effect of diet quality and caloric intake on immune function and its possible interaction with other environmental variables.

Acknowledgment

We are grateful to Alfredo Bermudez for his help and assistance with data collection. This study was funded by CONICYT 21110063 and FONDECYT 3160133 to NRO and Millennium Institute of Immunology and Immunotherapy, Pontificia Universidad Católica de Chile. Animals were captured with permits from SAG, Chile (No. 2355/2013). All protocols were approved by the Institutional Animal Care Committee of the Universidad de Chile where the experiments were performed.

Conflict of interest

The authors declare that they have no conflict of interest.

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