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

DOCTORAL THESIS:

CONTRIBUTION OF FC GAMMA RECEPTORS TO THE PATHOLOGY INDUCED BY HUMAN METAPNEUMOVIRUS IN A MURINE MODEL

By

ORLANDO ANDRÉS ACEVEDO ACEVEDO

December, 2020

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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

By

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FACULTAD DE CIENCIAS BIOLÓGICAS
PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

ACTA DEFENSA PRIVADA DE LA TESIS DOCTORAL TITULADA

"Estudio de la contribución de los receptores Fc gamma RIIb y Fc gamma RIII en el desarrollo de la respuesta inmune causada por el Metapneumovirus humano"

Presentada por el Candidato a Doctor en Ciencias Biológicas
Mención Genética Molecular y Microbiología

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El Comité Evaluador, constituido por los profesores abajo firmantes, ha evaluado como APROBADO (APROBADO, APROBADO CON MODIFICACIONES, REPROBADO) el trabajo realizado, el manuscrito sometido y la defensa oral.

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ACKNOWLEDGMENTS

I would like to thank to my family in supporting my decision of being part of this PhD program which has been a key element in my scientific formation. I would like to thank them for always bring me his support in every decision that I have made. I would like to thank to my mother Rosa, my grandmother Gladys and my brother Javier who were fundamental in this process. Finally, I would like to my girlfriend for her support, love and company.

I would like to thank to my old friends for their continuous support and to my new friends I have made during this formative process. I would like to thank to Jorge Soto, Angello Retamal, Camila Covian and Mariana Rios for its experimental and intellectual support during the development of this PhD thesis. I also appreciate the support of Fabian Diaz, Gisela Canedo, Farides Saavedra and Gaspar Diaz during the writing of this thesis. I would like to thank members of the Dr. Bueno's laboratory who always make the space to attend my questions and guide me. I think that every one of the members of both laboratories even not mentioned as it is an enormous teamwork are excellent professionals and friends.

I would also like to thank to my thesis director, Dr. Alexis Kalergis Parra, who allowed me to work in his laboratory and always bring his support and trust on my abilities during this formative stage. Also, I will like to thank Dr. Susan Bueno for all the opportunities given during my years as a Ph.D. student, and for being always available to resolve my questions and difficulties that were encountered in the course of this thesis.

I would like to thank CONICYT (grant number 21160962) for the funding given during the development of my PhD thesis that allowed me to develop as a professional. Finally, I would like to thank to the Millennium Institute of Immunology and Immunotherapy for the funding and the support granted during this doctoral program.

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ABBREVIATIONS

AECs: Airway cells epithelial

AMs: Alveolar macrophages

BAL: Bronchoalveolar Lavage

hMPV: Human Metapneumovirus

hRSV: Human Respiratory Syncytial Virus

ICs: Immune-Complexes

IgG: Immunoglobulin G

ssRNA: Single-strand RNA

FcγR: Fc gamma Receptor

FcγRIIb: Fc gamma Receptor IIb

FcγRIII: Fc gamma Receptor III

IgG: Immunoglobulin G

IFN: Interferon

IL: Interleukin

ITAM: Immunoreceptor Tyrosine Based Activation Motif

ITIM: Immunoreceptor Tyrosine Based Inhibition Motif

DPI: Days post infection

DCs: Dendritic cells

IMs: Interstitial macrophages

BALF: Bronchoalveolar lavage fluid

TLR: Toll-like receptor

MHC: Major Histocompatibility Complex

MPO: Myeloperoxidase

NETs: Neutrophil Extracellular traps

pDCs: plasmacytoid DCs

ROS: Reactive Oxygen Species

TSLP: Thymic Stromal Lymphopoietin

T_h1: T helper cells 1

T_h2: T helper cells 2

ABSTRACT

The human Metapneumovirus (hMPV) is a major cause of severe lower respiratory tract infections and hospitalization in children younger than five years-old. Clinical evidence indicates that such severe cases of hMPV infection are accompanied with a massive infiltration of neutrophils orchestrated by the coordinated action of macrophages and dendritic cells (DCs). However, the mechanisms that regulate this inflammatory response remains poorly understood. Evidences from other studies highlight the role of receptors for the Fc portion of IgG (FcγRs) in modulating the function and recruitment of DCs as well as macrophages and neutrophils during inflammation. As part of the doctoral thesis, we investigated the role of the canonically inhibitory IgG receptor FcγRIIb and activating FcγRIII in the neutrophil response triggered by hMPV infection. Furthermore, we also determined if the lack of such receptors regulates the infiltration of macrophages and DCs that, in addition to neutrophils, orchestrate the innate response against hMPV. Using purified neutrophils we also examine the putative role of hMPV as well as IgG-opsonized hMPV in the form of hMPV immune-complexes (hMPV-ICs) in regulating the expression of both receptors, which may in turn modulate neutrophil function on *in vivo* settings. With a similar strategy we also tested whether hMPV alone as well as hMPV-ICs regulate neutrophil apoptosis, the production of reactive oxygen species (ROS) as well as the release of neutrophil extracellular traps (NETs), which together may contribute to increased airway inflammation after hMPV infection.

The results of this study indicate that hMPV infection produce lung damage, evidenced as an increased lung histopathology score in WT mice as compared to uninfected controls which was not observed in FcγRIIb$^{-/-}$ and FcγRIIb$^{-/-}$ mice, indicating that both receptors contribute to the inflammation triggered by hMPV in lungs. We showed that hMPV infection increases the

average number of viable neutrophils in the airways and lungs of WT, but not FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice, suggesting that both receptors contribute to the recruitment of neutrophils after hMPV infection. Furthermore, a deficiency of either FcγRIIb or FcγRIII attenuates the increase of interstitial macrophages (IMs) in the lungs and airways of WT mice following hMPV infection, which together with neutrophils may contribute to airway obstruction. In contrast, a deficiency of either FcγRIIb or FcγRIII prevents the reduction in the levels of viable Alveolar Macrophages (AMs) that was observed after infection of WT mice and which accordingly with previous studies may promote the infiltration of neutrophils into the lung and airways following infection.

On the other hand, we also showed that IgG opsonized hMPV as well as hMPV alone prevents constitutive apoptosis of isolated murine neutrophils whereas triggering the production of ROS and NETs which together can contribute to lung damage during infection.

Taken together, our results suggested that blocking the interaction between hMPV-ICs and specific FcγRs could be used as novel therapeutic strategy to prevent the lung inflammatory response against hMPV. In this context, the usage of Fc-engineered monoclonal Abs against hMPV surface proteins, which can engage FcγRs other than FcγRIIb and FcγRIII, could be used as novel prophylactic or therapeutic treatment to ameliorate the lung inflammatory response triggered by hMPV infection .

RESUMEN

El Metapneumovirus humano (MPVh) es una de las principales causas de infecciones graves de las vías respiratorias inferiores y de hospitalización en niños menores de cinco años. La evidencia clínica indica que estos casos graves de infección por MPVh se acompañan de una infiltración masiva de neutrófilos orquestada por la acción coordinada de macrófagos y células dendríticas (CDs). Sin embargo, los mecanismos que regulan esta respuesta inflamatoria siguen siendo poco conocidos. Las evidencias de otros estudios destacan el papel de los receptores de la porción Fc de la inmunoglobulina G (FcγRs) en la modulación de la función y el reclutamiento de las CDs, así como de los macrófagos y neutrófilos durante la inflamación. Como parte de la tesis doctoral, investigamos el papel del receptor de IgG murino canónicamente inhibidor FcγRIIb y canónicamente activador FcγRIII en la respuesta de neutrófilos desencadenada por la infección por MPVh. Además, también determinamos si la falta de tales receptores regula la infiltración de macrófagos y CDs que, además de los neutrófilos, orquestan la respuesta innata frente al MPVh. Utilizando neutrófilos purificados, también examinamos el papel putativo del MPVh y del MPVh opsonizado con IgG en la forma de inmuno-complejos de MPVh (MPVh-ICs) en la regulación de la expresión de ambos receptores, que a su vez pueden modular la función de los neutrófilos en un contexto *in vivo*. Con una estrategia similar, también determinamos si estos estímulos regulan la apoptosis de los neutrófilos, la producción de especies reactivas de oxígeno (EROS), así como la liberación de trampas extracelulares de neutrófilos (TENs), que en conjunto pueden contribuir a una mayor inflamación de las vías respiratorias después de la infección por MPVh.

Los resultados de este estudio indican que la infección por MPVh produce daño pulmonar, evidenciado como un aumento de la puntuación de histopatología pulmonar en ratones WT en

comparación con controles no infectados, que no se observó en ratones FcγRIIb$^{-/-}$ y ratones FcγRIII$^{-/-}$, lo que sugiere que ambos receptores contribuyen a la inflamación desencadenada por MPVh en los pulmones. Además, la infección por MPVh aumenta el número medio de neutrófilos viables en las vías respiratorias y pulmones de ratones WT, pero no en ratones FcγRIIb $^{-/-}$ y FcγRIII$^{-/-}$, lo que sugiere que ambos receptores contribuyen al reclutamiento de neutrófilos y después de la infección por MPVh. Además, una deficiencia de ya sea FcγRIIb o FcγRIII atenúa el incremento de Macrófagos intersticiales (MIs) en el pulmón y lavado broncoalveolar de ratones WT luego de la infección con MPVh los cuales en conjunto con los neutrófilos podría contribuir a la obstrucción de las vías respiratorias durante la infección. En contraste, una deficiencia de ya sea FcγRIIb o FcγRIII evita la reducción en los niveles de Macrófagos Alveolares (MAs) viables que se observa después de la infección de ratones WT y que de acuerdo con estudios previos podrían contribuir al reclutamiento de neutrófilos en las vías aéreas y el tejido pulmonar.

Por otro lado, también demostramos que el MPVh opsonizado con IgG y el MPVh por si solo previenen la apoptosis constitutiva de neutrófilos purificados los que a su vez desencadenan la producción de EROS y TENs que en conjunto pueden contribuir al daño pulmonar producida por la infección de MPVh.

En conclusión, nuestros resultados sugieren que el bloqueo de la interacción entre FcγRs específicos y MPVh opsonizado con IgG podría usarse como una nueva estrategia terapéutica para prevenir la respuesta inflamatoria pulmonar contra este virus. En este contexto, el uso de anticuerpos monoclonales con porciones Fc modificadas y que reconozcan proteínas de superficie de MPVh, que pueden activar FcγRs distintos de FcγRIIb y FcγRIII, podría usarse como tratamientos profilácticos o terapéuticos novedosos para disminuir la inflamación pulmonar causada por la infección con MPVh.

1. GENERAL INTRODUCTION

1.1. General characteristics of hMPV

HMPV is respiratory virus discovered and isolated in 2001 by the group of Van den Hoogen in Netherlands (van den Hoogen et al., 2001). Although the virus was recently isolated, hMPV-specific antibodies have been detected in every tested individual older than 8 years in serum samples collected since 1958 (van den Hoogen et al., 2001). Thus, hMPV may not be a "new virus", since it has been circulating for more than 50 years.

HMPV belongs to the Paramyxoviridae family, subfamily Pneumovirinae, genus Metapneumovirus (Amarasinghe et al., 2019). There are 2 genotypes, A and B, which are divided in several subgroups according to phylogenetic analyses of the surface glycoprotein F, which is involved in attachment and fusion (Oong et al., 2018). Infection with one genotype only confers partial cross-protective immunity whereas it is described that the circulation of hMPV strains varies annually (Skiadopoulos et al., 2004). These epidemiology and immunological characteristics make difficult the development of an efficient and universal prophylactic or therapeutic strategy against hMPV infection (Kumar and Srivastava, 2018).

1.2 Epidemiology

The presence of hMPV has been reported in 4% to 16% of patients with acute respiratory infections, has a worldwide distribution and affects all age groups (Williams et al., 2004;Chow et al., 2016). Outbreaks occur mainly in winter and spring and the infection is associated with a significant number of hospitalizations and outpatient visits in children under 5 years of age, especially during the first 12 months of life (Edwards et al., 2013). Clinical manifestations of hMPV infection varies from mild upper respiratory tract illness to severe lower respiratory tract

disease including bronchiolitis and pneumonia, often accompanied by high fever and myalgia (van den Hoogen et al., 2003). In Chile, hMPV represents an emerging respiratory pathogen that was firstly described in 2003 from nasopharyngeal aspirates of children under 2 years of age that become hospitalized due to acute respiratory infection (Luchsinger F et al., 2005). According to the available data in the website of Instituto de Salud Publica (ISP), hMPV showed increased number of diagnosed cases from around 900 positive individuals in 2015 to 1600 cases in 2019 (Figure 1A). Of note, during the spring season of this latter year the percentage of hMPV cases overcomes hRSV positivity, which was the main etiological agent of respiratory disease during winter (epidemiological week 30) (Figure 1B). Percentage of hMPV-positive cases peaks on epidemiological week 46 and represents the 34.6% of total respiratory viruses detected, overcoming the percentages of positivity registered in this week for ParaInfluenza (21%), Adenovirus (17.3%), Influenza B (14.9%), Influenza A (6.2%) and hRSV (6.2%). This data indicates that hMPV was the main etiological agent which accounts for respiratory viral infection during spring season on 2019 (Figure 1C).

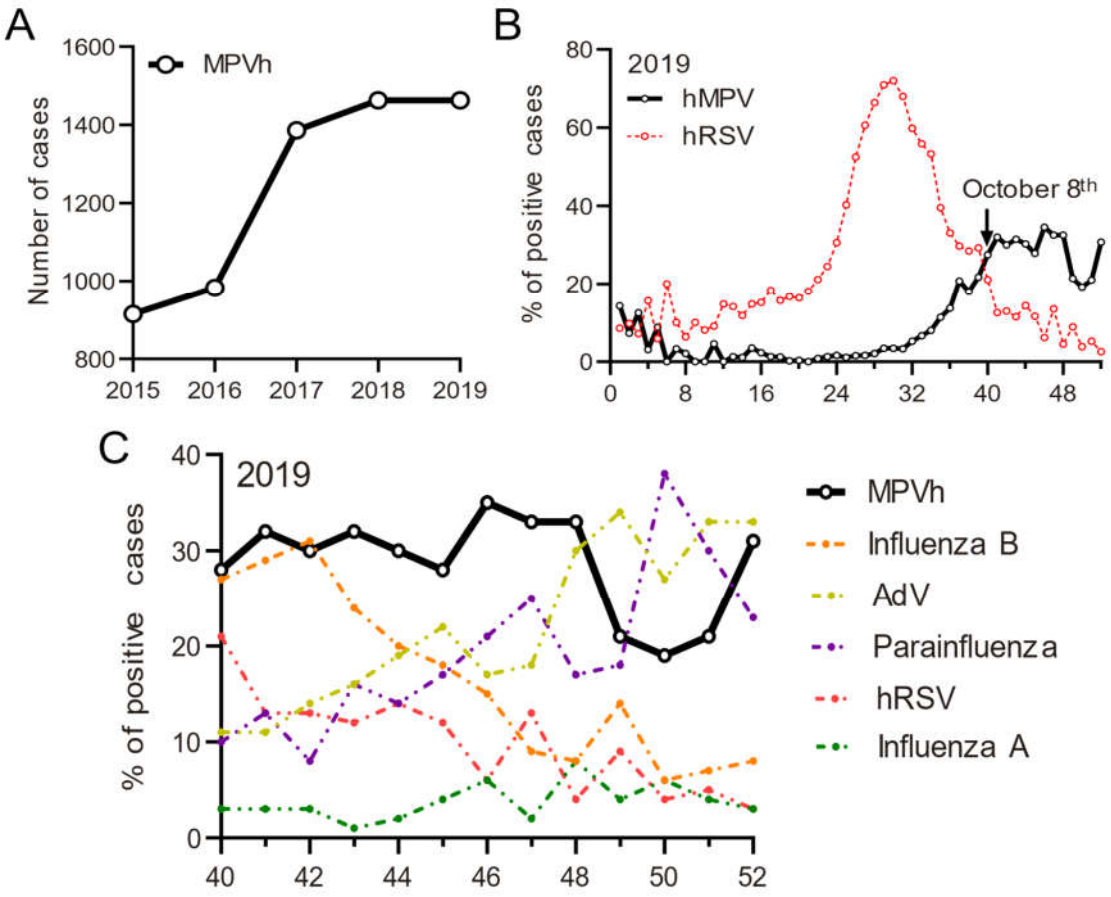

Figure 1. Epidemiologic data for hMPV infection in Chile. Data were acquired from Public Health Institute of Chile (ISP). (**A**) Cases numbers of hMPV infection increased from 2015 to 2019. (**B**) Percentage of positive cases for hRSV and hMPV during 2019. (**C**) Percentage of positive cases for the different respiratory circulating on spring viruses that were included in the weekly reports of respiratory infections in Chile by ISP.

1.3 Genome organization and viral proteins of hMPV

HMPV is a non-segmented negative single-strand RNA enveloped virus which display a pleiomorphic structure with diameters ranging from 150 to 600 nm (Figure 2) (Ogonczyk Makowska et al., 2020). The 13.3-kb genome of hMPV has eight genes that encode nine proteins due to the presence of two overlapping open reading frames, each of them encoding for an individual protein (M2-1 and M2-2) which serve as a co-factors during replication (Ren et al., 2012;Cai et al., 2015). On the lipid surface there are three viral transmembrane proteins: the glycoprotein (G) that *in vivo* promotes neutrophil infiltration and inhibits the production of alpha interferon (IFN-α) (Cheemarla and Guerrero-Plata, 2017); the fusion (F) protein that mediates both attachment and membrane fusion to epithelial cells (Cox et al., 2015); and the small hydrophobic (SH) protein that acts as a viroporin which increases membrane permeability to promote infection (Masante et al., 2014). Beneath viral lipid envelope it is localized the matrix M protein, which determines virion morphology by directing viral assembly and budding (Sabo et al., 2011). As part of the nucleocapsid, there are 3 proteins including nucleoprotein (N) that protects the viral genome from host nucleases (Renner et al., 2016), the phosphoprotein (P) that assist other proteins in viral replication (Derdowski et al., 2008), and the large (L) RNA dependent RNA polymerase that allows the replication of the viral genome (Pan et al., 2020).

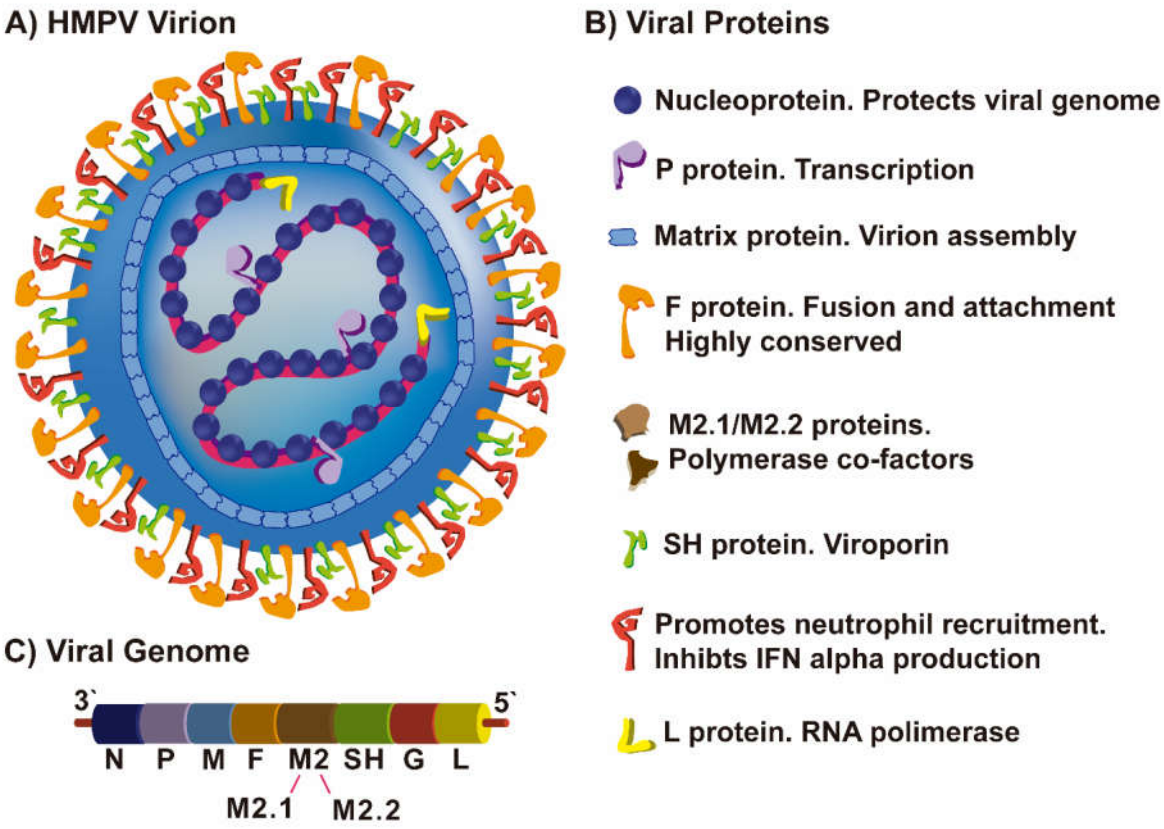

Figure 2. HMPV structure and function of viral proteins during infection. (**A**) The structure of hMPV virion with its internal and external proteins is depicted. (**B**) The roles of viral proteins during infection is described in the right of hMPV virion. Inside of virion it is present the nucleoprotein that protects viral genome from host nucleases. Associated to the nucleocapsid P protein also associates with other proteins during hMPV replication. Matrix protein supports viral assembly and budding together with maintaining virion morphology. On the surface, F protein can mediate both fusion and attachment to target cells. SH proteins act as a viroporin that increase membrane permeability which in turns may promote hMPV infection. G protein promotes neutrophil recruitment whereas inhibits the production of IFN-α. The L protein is an RNA dependent RNA polymerase that replicates the viral genome inside of host cell cytoplasm. (**C**) HMPV genomic organization is depicted below hMPV virion. The genes codifying for M2.1 and M2.2 participate in the replicative viral cycle but were absent in the mature virion.

1.4 Innate immune response against hMPV

1.4.1 Cellular Innate immunity

Cells of the respiratory tract that primarily sense hMPV in the airways include Airway Epithelial cells (AECs), Alveolar Macrophages (AMs) and DCs (Andrade et al., 2020). Through different entry pathways which involve endocytosis, the presence of lipid rafts together with the action of pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) these different cells can recognize hMPV and initiate infective cycle and innate immune responses (Figure 3) (Bao et al., 2008;Goutagny et al., 2010;Cox et al., 2015;Gillespie et al., 2016;Chen et al., 2019). The recognition of hMPV pathogen associated molecular patterns (PAMPs) by AECs triggers the release of pro-inflammatory cytokines such as TSLP that in turn promotes the recruitment of neutrophils, DCs and macrophages that differentially regulates the immunopathology triggered by hMPV (Kolli et al., 2014;Lay et al., 2015;Cheemarla et al., 2017a). The role of these distinct myeloid sub-sets to hMPV immune response and pathology is further detailed below.

1.4.1.1 Alveolar Macrophages

Alveolar macrophages (AMs) represent a first line of defense against airway invading pathogens due to their strategic location in direct contact with the alveolar space (Hu and Christman, 2019). During hMPV infection it was shown that depletion of AMs prior to infection leads to a more severe pathology in the mice model (Kolli et al., 2014). Remarkably, AMs-depleted mice showed decreased levels of neutrophils after hMPV infection as early as 1 dpi (Kolli et al., 2014), suggesting that depletion of AMs may alleviate hMPV pathology by preventing an excessive neutrophil recruitment to the airways (Kolli et al., 2014). The same study showed that hMPV infection triggers cell death on *in vitro* cultured AMs, leading to the

release of pro-inflammatory mediators that can contribute to lung inflammation. However it is not clear what are the drivers of this process and if this event of cell death may contribute to augmented hMPV pathology *in vivo* (Kolli et al., 2014).

1.4.1.2 Interstitial Macrophages

Interstitial Macrophages (IMs) constitute another class of lung macrophages which in contrast to AMs are localized in the interface between the alveolar epithelium and vascular endothelium (Schneberger et al., 2011). Although there is no evidence about the specific role of IMs during hMPV infection several reports indicate that IMs perform an important anti-inflammatory function during lung inflammation in mice (Bedoret et al., 2009;Kawano et al., 2016;Sabatel et al., 2017). These studies showed that lung IMs constitutively produce IL-10, which is further increased in response to inflammatory stimuli such as lipopolysaccharide (LPS), or house dust mite extracts (HDM) (Bedoret et al., 2009;Kawano et al., 2016;Sabatel et al., 2017). The action of IL-10 inhibits the pro-inflammatory responses mediated by DCs and neutrophils by decreasing the levels of pro-inflammatory cytokines such as GM-CSF and TNF-α (Kawano et al., 2016). These results suggest that IMs, in contrast to AMs, may contribute to the resolution of the inflammation following hMPV infection.

1.4.1.3 Dendritic cells

Dendritic cells (DCs) play a crucial function in the recognition of invading pathogens. Their strategic location at the entry site of microbes makes them a key target to initial viral invasion (Peebles and Graham, 2001). In the mice model two subset of DCs can be recognized, including plasmacytoid DCs (pDCs) and $CD11b^{+}$ DCs (Webb et al., 2005). In the context of hMPV infection, it was shown that pDCs are early recruited to the lung tissue and following *in vitro* incubation with hMPV secrete several chemokines that promote the recruitment of neutrophils

(Guerrero-Plata et al., 2009), suggesting that *in vivo* pDCs subset may also promote the infiltration of neutrophils into the airways during infection (Guerrero-Plata et al., 2009).

In contrast to suggested role of pDCs in promoting neutrophil infiltration during hMPV infection (Guerrero-Plata et al., 2009) the immune response mediated by $CD11b^+$ DCs is mainly related to their ability to prime and activate antiviral T-cell responses (Mueller, 2017). In the context of hMPV infection it was described that hMPV infected $CD11b^+$ DCs become unable to prime $CD4^+$ T cells leading to inadequate immune responses that in turn may promote increased lung inflammation and the impairment of memory responses (Cespedes et al., 2013). However, incubation of DCs with IgG-opsonized hMPV can subvert this detrimental effects (Cespedes et al., 2013). Therefore, targeting hMPV to specific FcγRs on this particular DC sub-set can be a useful approach to increase the immunogenicity of DCs during hMPV infection to promote appropriate T helper responses. In this line, no reports about the contribution of specific FcγRs on modulating the recruitment of DC sub-sets during hMPV infection is actually available.

1.4.1.4 Neutrophils

Although neutrophils are central players in the inflammatory response against hMPV and represent the major myeloid population recruited to site of infection (Cheemarla et al., 2017a), their specific role in tissue damage and the obstruction of the alveolar space during hMPV infection remains still limited. Evidence from clinical samples indicate that neutrophil recruitment is correlated with airway obstruction as well as lung tissue damage. Thus, suggesting that neutrophils recruited to the lungs may contribute to lung injury (Cavallaro et al., 2017). However, depletion of neutrophils prior to hMPV infection in the mice model leads to a more severe immunopathology, indicating that a fine balance of neutrophil recruitment is

necessary for an optimal and controlled inflammatory response (Cheemarla et al., 2017a). Evidences from other lung inflammatory contexts including infection by hRSV, influenza and *Streptococcus pneumoniae*, indicate that activated neutrophils contribute to tissue damage and obstruction (Beiter et al., 2006;Souza et al., 2018;Zhu et al., 2018) by producing reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) (Beiter et al., 2006;Souza et al., 2018;Zhu et al., 2018). However, it remains unknow whether such neutrophil responses are developed during the onset of hMPV infection. In this context, it was recently shown that crosslinking of specific FcγRs with monoclonal antibodies leads to the production of ROS and NETs by human neutrophils (Alemán et al., 2016), opening the possibility that engagement of such receptors by IgG opsonized hMPV can also trigger ROS production and NETosis during hMPV infection.

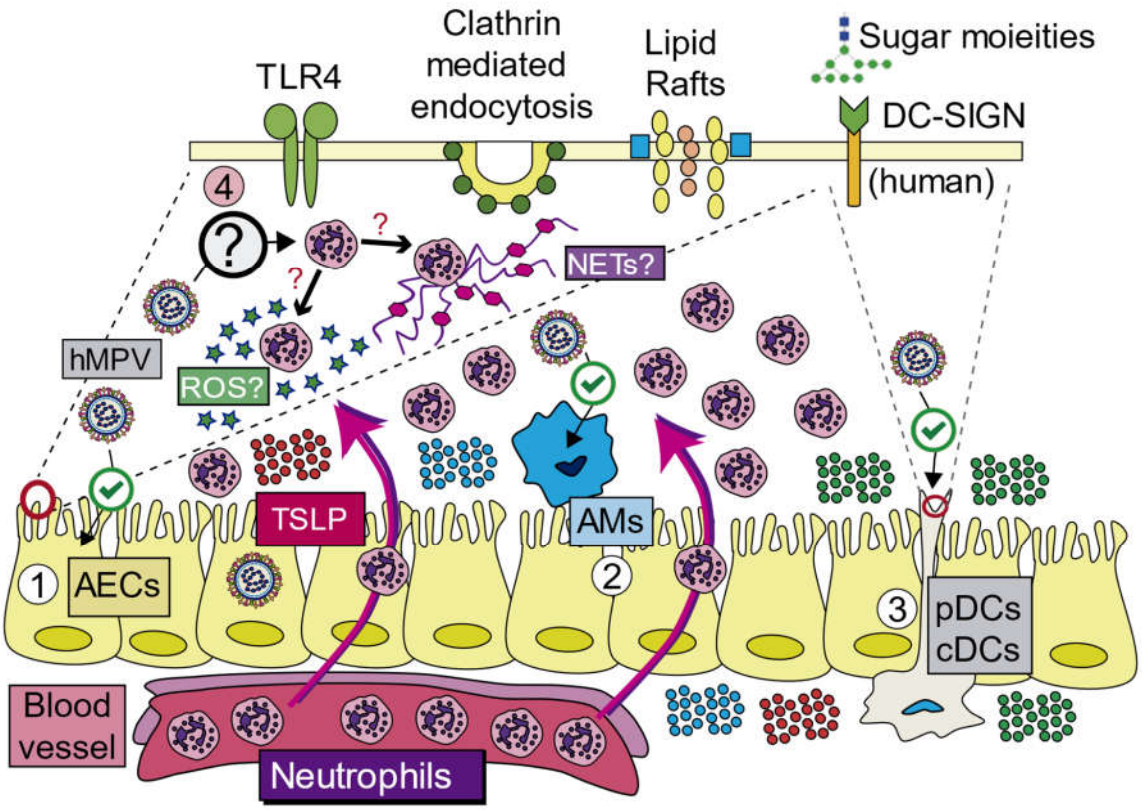

Figure 3. Innate immune response against hMPV infection. **(1)** HMPV infection occurs mainly in AECs which in turns secrete pro-inflammatory cytokines such as TSLP, which contribute to neutrophil recruitment. Other cells that are susceptible to infection are **(2)** Alveolar Macrophages (AMs), **(3)** plasmacytoid dendritic cells (pDCs) and conventional DCs (cDCs, $CD11b^{+}$ DCs) which also secrete pro-inflammatory cytokines which contribute to neutrophil recruitment. **(4)** A Direct interaction of neutrophils with hMPV is not characterized and can eventually lead to the generation of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs).

1.5 Adaptive immunity

1.5.1 Cellular Adaptive immunity

The primary response elicited by the innate immune system is then followed by the adaptive response mediated by T cells which can assist innate cells such as macrophages in the elimination of cells infected by intracellular pathogens (Jankovic and Feng, 2015). The main cytokine that support the elimination of such cells is IFN-γ which is related to the development of a T_h1 biased immune which is appropriate against viral infection (Graham et al., 1994;Fischer et al., 1997;Snell et al., 2016). However, several viruses including hMPV can shift this T helper response towards a T_h2 profile which lead to airway obstruction and pulmonary disfunction (Alvarez et al., 2004;Soto et al., 2018). Although the exact mechanisms that account for this T_h2 biased immune response against hMPV is not currently known, it was demonstrated that the infection of DCs with hMPV alone impairs their ability to induce Th1 biased $CD4^+$ T cells (Cespedes et al., 2013). However, this ability is retained by DCs incubated with hMPV previously opsonized with IgG containing serum (Cespedes et al., 2013). Thus, suggesting that targeting specific FcγRs on DCs by hMPV-ICs can increase the immunogenicity of DCs that in subsequently activate naïve T cells contributing to viral clearance and reduced lung inflammation (Cespedes et al., 2013).

1.5.2 Humoral Adaptive immunity

Humoral adaptive immunity in another arm of the immune system that can fight against the infection with several pathogens, including bacteria and viruses (Newton et al., 2016). In this context, evidences from the mice model showed that hMPV infection triggers the production of IgG antibodies which are able to neutralize the infection of epithelial cells *in vitro* but were not enough to promote viral clearance and the resolution of the inflammation *in vivo* (Alvarez

et al., 2004). This apparently paradoxical results can be explained due to the dual function of IgG antibodies in the recognition of pathogens through its variable region but at the same time to their ability to activate or inhibit cellular functions that modulate the inflammatory response through the interaction with specific FcγRs (Ben Mkaddem et al., 2019) which are present in both humans and mice cells and are further described in the following sections.

1.6 Receptors for the Fc portion of IgG antibodies (FcγRs).

Receptors for the Fc portion of IgG antibodies represents a bridge between the innate and adaptive immunity. Engagement of FcγRs by antigen-IgG immune-complexes (ICs) lead to the inhibition or activation of innate and adaptive immune cells that in turn promote or inhibit inflammatory responses (Ben Mkaddem et al., 2019). Several process are regulated by the engagement of FcγRs including cell survival, migration to the sites of infection as well as the development of antimicrobial responses against invading pathogens (Fossati et al., 2002;Xiong et al., 2006;Amezcua Vesely et al., 2012;Alemán et al., 2016). A general description of human and mouse FcγRs, their role inflammatory process and its possible contribution during hMPV infection is discussed below.

1.7 Classification and general features of human and mouse FcγRs

Accordingly with their different properties, several classes of FcγRs can be identified in mice and humans. Based on their affinity to IgG, FcγRs can be divided into low o high affinity receptors (Figure 4). Among them, it is highlighted the role of low affinity FcγRs which upon engagement by IgG opsonized targets lead to the inhibition or activation of cellular immune responses. In mice and humans a single inhibitory Fc gamma receptor, namely FcγRIIb is actually conserved (Figure 4) (Brauweiler and Cambier, 2003). This receptor has a single Imuno-Tyrosin-Inhibiting motif (ITIM) involved in several cell inhibitory pathways (Stopforth et al., 2016).

Similarly, only a single low affinity activating FcγR is conserved between mouse and humans corresponding to FcγRIII and FcγRIIIa respectively (Mechetina et al., 2002). Both receptors are characterized by the presence of two Immuno-Tyrosin-activating motif (ITAM) involved in different cell activation pathways (Giorgini et al., 2008). In the case of humans, two additional activating FcγRs are recognized and correspond to FcγRIIa and FcγRIIc both of which present a single ITAM (Anania et al., 2019). The functional properties of low affinity inhibitory FcγRIIb and activating FcγRs due to the presence of ITIM and ITAM motifs is discussed below.

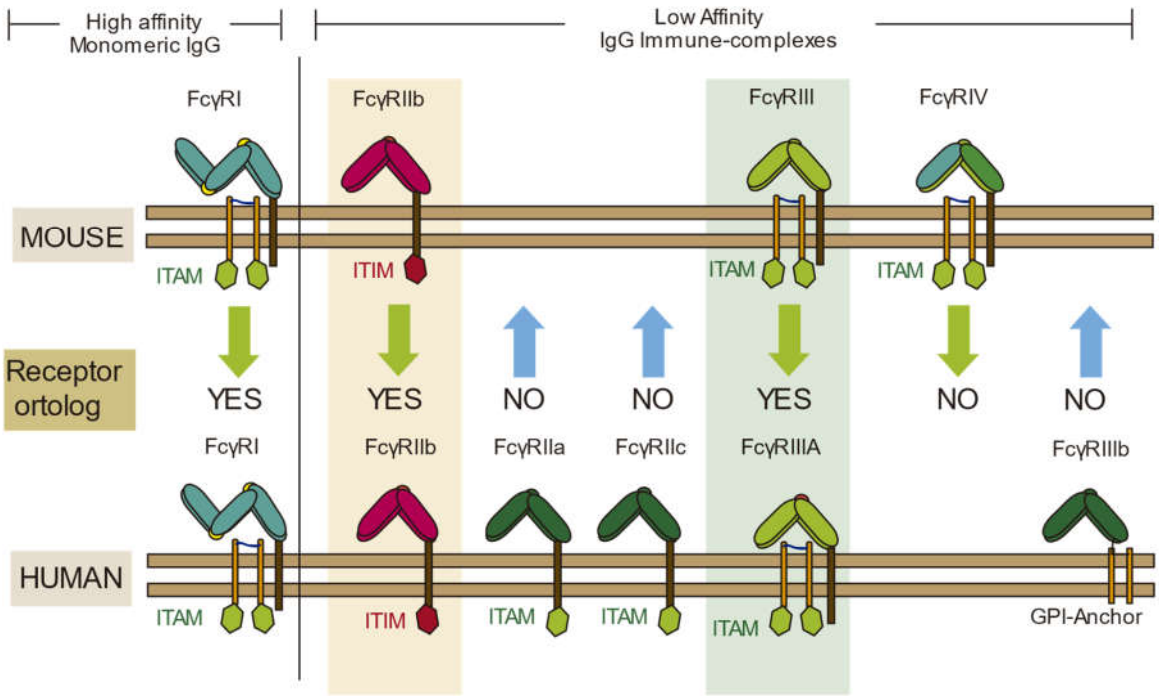

Figure 4. Surface expression of Fc gamma receptors in mice and humans and their respective orthologues. Murine and human Fc gamma receptors can be classified as High affinity receptors which recognize monomeric IgG as it is the case of activating Fc gamma receptor I which is present in both mouse and human phagocytic cells and possess a cytoplasmic ITAM motif. Low affinity FcγRs recognize ICs composed by IgG and specific antigens such as cells, virus or bacteria. Murine and human FcγRIIb is the only inhibitory FcγR which counteracts the action of activating FcγRs by signaling through an ITIM cytoplasmic motif . Mice express the activating FcγRIII which is orthologue to human FcγRIIIA and the activating FcγRIV which is orthologue to IgE receptor FcεR (not shown). Activating FcγRIIa, FcγRIIc, FcγRIIIa and FcγRIIIb are present in humans but only FcγRIIIa have a murine ortholog.

1.7.1 Activating ITAM signaling

Engagement of activating low affinity FcγRs by IgG-ICs leads to the activation of different cell responses which rely on the recruitment of different kinases that phosphorylate the ITAM motif present on activating FcγRs (Mkaddem et al., 2017). This process in turn activates numerous downstream effector proteins that promote several effectors functions including the initiation of phagocytosis, ROS production and the release of pro-inflammatory cytokines (Huber et al., 2001;Fossati et al., 2002).

1.7.2 ITAM Inhibitory (ITAMi) signaling

Although the canonical function of activating FcγRs is to trigger cell activation, it was recently described that ITAM bearing FcγRs can also induce inhibitory signaling through its ITAM motif after low avidity interaction with IgG (Pinheiro da Silva et al., 2008). ITAM inhibitory (ITAMi) signaling leads to weak ITAM phosphorylation and the recruitment of the tyrosine phosphatase Src homology 2 (SH2) domain-containing phosphatase-1 (SHP-1) (Ben Mkaddem et al., 2014). *In vitro* studies indicate that ITAMi signaling decreased the release of neutrophil chemoattractants by macrophages, whereas in neutrophils this inhibitory pathway decreases ROS production (Ben Mkaddem et al., 2014). However, it remains unknown whether ITAMi signalling can also control other cell functions including cell apoptosis which is modulated by inhibitory FcγRIIb as discussed below.

1.7.3 Inhibitory ITIM signaling by FcγRIIb

Functions of activating FcγRs is counteracted by the action of inhibitor FcγRIIb. When both activating and inhibitor FcγRs become co-aggregated after the engagement by IgG-ICs different associated kinases phosphorylate the ITIM motif leading to the recruitment of several tyrosine phosphatases which de-phosphorylates the ITAM motif in activating FcγRs, thus inhibiting

several cellular processes including the release of pro-inflammatory cytokines as well as FcγR-mediated phagocytosis (Ooi et al., 2014). Of note, FcγRIIb associated ITIM signaling inhibits cell responses by inducing cell death on several cell types including B cells (Amezcua Vesely et al., 2012), neuronal cells (Kam et al., 2013) and also $CD8^+$ T cells (Morris et al., 2020). However it remains unknown whether this inhibitory signaling pathway can also lead to cell death of other immune cells including macrophages and neutrophils.

1.8 Role of FcγRs in inflammation

Many of the studies about the role of human FcγRs have focused in FcγRIIb as well as FcγRIIIa since both are the unique low-affinity IgG receptors which are shared between humans and mice (Kerntke et al., 2020). The role of these receptors during infection as well as other inflammatory conditions are further described in the following sections.

1.8.1 Contribution of FcγRIIb to inflammation

FcγRIIB was the unique canonically inhibitory FcγR present in both humans and mice (Hargreaves et al., 2015;Roghanian et al., 2018). In humans it was shown that a polymorphism in FCGR2B leading to loss of function was associated with protection against malaria, but increased susceptibility to systemic lupus erythematosus (Willcocks et al., 2010). On the other hand, the presence of this receptor was detected at low levels on resting human AMs but is subsequently up-regulated after the interaction of these cells with pro-inflammatory stimulus (Bruggeman et al., 2019). Thus, suggesting that this receptor could be one important factor that contribute to maintain airway homeostasis. Expression of this receptor is also detected in circulating pDCs, cDCs as well as neutrophils (Su et al., 2007). Thus suggesting that a diverse repertoire of cells can be regulated through the action of this receptor. In the mice model presence of FcγRIIb contributes to decreased lung inflammation in a murine model of allergic

rhinitis (Watanabe et al., 2004) whereas the the lack of this receptor results into exacerbated allergic airway inflammation in a murine model of asthma (Dharajiya et al., 2010b). In the context of viral infection it was described that the lack of FcγRIIb in mice leads to increased airway neutrophilia and eosinophilia following hRSV infection and lower levels of viral loads which can be related to decreased viral replication at expenses increased airway inflammation (Gómez et al., 2016). Together, these results highlight the inhibitory role of FcγRIIb on inflammation opening the possibility that this receptor may also play an anti-inflammatory response during infection by hMPV.

1.8.2 Role of FcγRIII in inflammation

The actual evidences about the role of FcγRIII on human disease has been focused in the ability of this receptor to modulate the efficacy of mAb therapy against inflammatory diseases such as rheumatoid arthritis (Ruyssen-Witrand et al., 2012) and Crohn's disease (Louis et al., 2004). In the case of lung-related disease it is showed that specific allelic variants of FcγRIII increase the risk of developing chronic disease after sensitization with allergens such as beryllium (Liu et al., 2019). Despite such findings, the information about the role of FcγRIIIa in lung disease during infection remains unexplored. Studies of FcγRIII$^{-/-}$ mice have highlighted the role of this receptor in the pro-inflammatory response in several organs including the skin and the lungs. In the skin, the lack of this receptor ameliorates the symptoms associated to atopic dermatitis characterized by the accumulation of macrophages, mast cells, and eosinophils (Abboud et al., 2009). In the gut, it was shown that inhibitory ITAM signalling through FcγRIII on macrophages impairs their ability to kill *E. coli* and allows its spread with the result of sepsis and decreased mice survival in the absence of IgG antibodies (Pinheiro da Silva et al., 2007). Thus suggesting that FcγRIII can also mediate inhibitory innate responses in the absence of IgG

antibodies (Pinheiro da Silva et al., 2007). In the lungs, it is described that this receptor is required for the initiation of T_h2 responses which in turn may promote airway inflammation (Bandukwala et al., 2007). During viral infection it was shown that deficiency of FcγRIII contributes to decreased airway neutrophilia during hRSV infection but lower viral loads as opposite to the results obtained from FcγRIIb deficient mice in the same study (Gómez et al., 2016). Together, these findings highlight the prominent pro-inflammatory role of activating FcγRIII which may also contribute to airway inflammatory response triggered by hMPV infection in lungs.

1.10 Role of FcγRs in triggering activation of neutrophils.

Neutrophils are the most prominent leukocytes in the blood. Neutrophils migrate from the circulation to sites of infection in where they represent the first line of defense against invading pathogens (Mocsai, 2013). Several antimicrobial mechanisms are used by neutrophils. Among them, neutrophils use the of release neutrophil extracellular traps (NETs) to kill and entrap microbes (Brinkmann et al., 2004). NETs are composed of DNA and neutrophil-granule proteins including myeloperoxidase (MPO) (Parker et al., 2012). NETosis, the cell death pathway that leads to the release of NETs, involves activation in most cases of nicotinamide adenine dinucleotide phosphate oxidase (NOX), which produces reactive oxygen species (ROS) (Stoiber et al., 2015). Neutrophil produced ROS allow the release of MPO from neutrophil granules (Figure 5) (Almyroudis et al., 2013). Translocation of MPO into the nucleus triggers chromatin breakdown, which after ROS-triggered membrane break get released as spread structures constituted by DNA, MPO and other proteases. Several stimuli, including TLR4 signalling by hRSV is able to induce NETosis of human resting neutrophils (Pruchniak and Demkow, 2019). Furthermore, engagement of FcγRIIa as well as FcγRIIIb by mAbs can trigger

NETosis on resting human neutrophils (Chen et al., 2012;Alemán et al., 2016). In the case of murine neutrophils it was shown that co-engagement of FcγRIII and FcγRIV by immobilized ICs leads to ROS production (Jakus et al., 2008). However it remains unknown whether this activation pathway leads to the release of NETs in the case of murine neutrophils. It also remains unknown whether hMPV alone as well as IgG-ICs formed between hMPV and IgG antibodies can lead to NETosis of murine neutrophils. Thus, in the present work we evaluated whether such hMPV-ICs as well as hMPV alone modulates the expression of these receptors together with promoting ROS and NETs production.

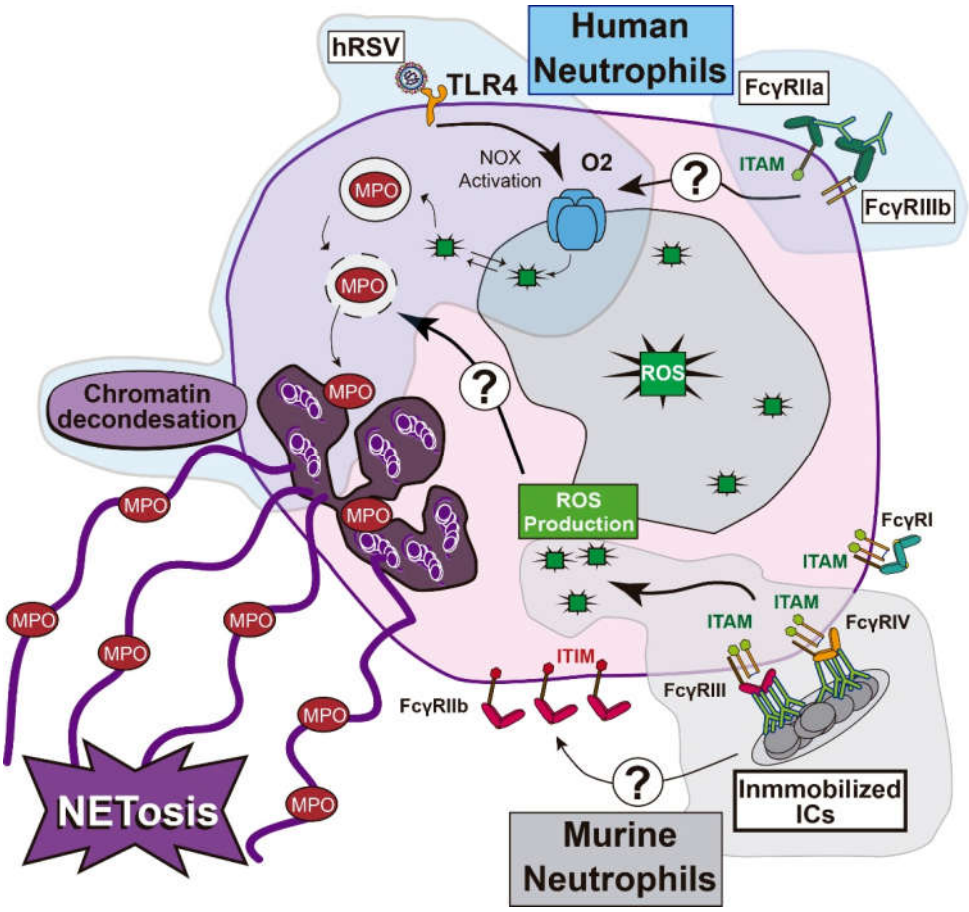

Figure 5. Viral infection and FcγRs engagement can regulate ROS and NET release by murine and human neutrophils. Human neutrophils can be activated by several stimuli such as hRSV and monoclonal and mAbs against FcγRIIIb which in turns is recognized by FcγRIIa through it Fc region. TLR4 triggering by hRSV can induce the production of ROS which subsequently leads to the release of NETs. Resting human neutrophils can trigger ROS and NETs production after hRSV challenge. In murine neutrophils immobilized ICs can trigger ROS production due to co-engagement of FcγRIII and FcγRIV.

2. HYPOTHESIS STATEMENT

2.1 Hypothesis 1

Inhibitory Fc gamma receptor IIb (FcγRIIb) and activating Fc gamma RIII play opposing roles in hMPV pathogenesis by respectively inhibiting and promoting the inflammatory response mediated by neutrophils, macrophages and DCs.

2.2 Hypothesis 2

The interaction of murine neutrophils with hMPV as well as IgG opsonized hMPV triggers neutrophil activation, which results into reduced constitutive apoptosis, increased ROS production and the release of NETs.

3. AIMS

3.1 General Aims

1. To evaluate the contribution of FcγRs (FcγRIIb and FcγRIII) in the lung inflammatory response caused by hMPV infection in murine models

2. To evaluate whether hMPV and hMPV-ICs triggers the activation of murine resting neutrophils.

3.2 Specific Aims for the Hypothesis 1

1. To evaluate *in vivo* whether the absence of FcγRIIb or FcγRIII modulate the parameters of the disease induced by hMPV infection in terms of weight loss, viral load, neutrophil infiltration and lung immunopathology .

2. To evaluate *in vivo* whether the absence of FcγRIIb or FcγRIII modulate the recruitment and survival of other innate cells including macrophages as well as DCs.

3.3 Specific Aims for the Hypothesis 2

To evaluate *in vitro* whether IgG opsonized hMPV as well as hMPV alone can prevent constitutive apoptosis of isolated murine neutrophils whereas promoting the production of ROS and NETs.

4. CHAPTER 1. FC GAMMA RECEPTORS IIB AND III CONTRIBUTE TO LUNG INFLAMMATION DURING THE ONSET OF HMPV-INDUCED DISEASE.

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Keywords: hMPV, Fc gamma receptors, neutrophils, inflammatory response, NETs, ROS production

4.1 ABSTRACT

Human Metapneumovirus (hMPV) is a major cause of severe lower respiratory tract infections and hospitalization in children younger than five years-old. Clinical evidence indicates that severe cases of hMPV infection are accompanied with a massive infiltration of neutrophils into the airways, which is orchestrated by the cooperative action of macrophages and dendritic cells (DCs). However, the host factors that modulate this immune response *in vivo* remains still limited. In this work, we investigated the role of inhibitory IgG receptor FcγRIIb and activating FcγRIII in modulating the recruitment and survival of these innate cell subsets together with their role in the immunopathology induced by hMPV infection *in vivo* . Results from FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice infected with hMPV indicate that both receptors promote the infiltration of neutrophils and Interstitial Macrophages (IMs) into the lungs and airways. Thus, suggesting that both receptors promote the recruitment of both innate subsets after hMPV infection. Interestingly, we also observed that IgG opsonized hMPV as well as free hMPV can prevent constitutive apoptosis of isolated neutrophils, whereas triggering the production of ROS and NETs both of which neutrophil activation pathways can contribute to lung inflammation and airway obstruction. Thus highlighting the contribution of FcγRs to the immunopathology caused by hMPV infection on *in vivo* and *in vitro* settings.

4.2 INTRODUCTION

Human metapneumovirus (HMPV) is one of the leading causes of acute lower respiratory tract infection (ALRTI) in children, the elderly as well as immunocompromised people (Manoha et al., 2007;Schildgen et al., 2007). HMPV was discovered in 2001 but had been circulating worldwide for at least 65 years ago (van den Hoogen et al., 2001). Symptoms related to hMPV disease are like those of other respiratory viral illnesses, extending from cough, rhinorrhea, and fever to more serious ones such as pneumonia and bronchiolitis, hence contributing to increased rates of hospitalization worldwide (Lopez et al., 2006;Ji et al., 2009;Ditt et al., 2011). Despite its prominent prevalence on humans the pathophysiology of hMPV infection is largely unknown and only few reports have described the role of innate immunity in controlling or promoting the inflammatory response against hMPV (Schildgen et al., 2007). Evidences from the mice model highlight the role of neutrophils as well as other myeloid sub-sets, including macrophages and DCs to the pathology induced by hMPV (Kolli et al., 2014;Cheemarla et al., 2017a). However the current information about the factors that modulate the function and recruitment of such cells is still limited.

Evidences from other inflammatory conditions indicate that receptors for the Fc portion of IgG antibodies can act as a key regulators of innate immunity by either activating or inhibiting the recruitment and cellular functions of neutrophils, macrophages and DCs (Kalergis and Ravetch, 2002;Santegoets et al., 2014). In humans and mice, there are two shared FcγRs, namely FcγRIIb and FcγRIII, which accordingly to several reports play opposing role during airway inflammation by allergens (Watanabe et al., 2004;Bandukwala et al., 2007;Dharajiya et al., 2010a) as well as respiratory viruses, including hRSV (Gómez et al., 2016). However it remains

unknown what is the contribution of these receptors to the inflammatory response in the lungs and the airways that is triggered by hMPV infection.

In order to address this question we used multi-color flow cytometry to determine the levels of relevant myeloid cells including neutrophils, and particular sub-sets of macrophages and DCs that were present in WT, FcγRIIb$^{-/-}$ FcγRIII$^{-/-}$ mice prior and after hMPV infection. Using isolated neutrophils we further characterize the role of hMPV alone as well as IgG opsonized hMPV in modulating the expression of both receptors that in *in vivo* settings contribute to lung inflammation. Using the same approach we also investigate the role of hMPV alone as well as IgG opsonized hMPV in the modulation of neutrophil apoptosis as well as the release of ROS and NETs which together may contribute to increased lung tissue damage and airway obstruction.

The results of this study indicate that hMPV infection increased the lung histopathology score in WT mice ($p=0.0008$) but not in FcγRIIb$^{-/-}$ ($p>0.999$) and FcγRIIb$^{-/-}$ mice ($p=0.8711$), suggesting that both receptors contribute to the inflammation triggered by hMPV in lungs. Furthermore, the increased levels of airway and lung neutrophils and IMs observed in WT mice after hMPV infection which were attenuated in FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice, suggest that both receptors contribute to the accumulation of these cells after infection.

On the other hand, we also showed that hMPV-ICs as well as free hMPV can prevent constitutive apoptosis of neutrophils whereas triggering the production of ROS and NETs by murine resting neutrophils. Thus suggesting that circulating hMPV-ICs as well as free hMPV can trigger the production of ROS and NETs which in turn can contribute to the lung damage in the mice model.

Taken together, our results suggested that the usage of Fc-engineered monoclonal Abs against hMPV surface proteins which can engage FcγRs other than FcγRIIb and FcγRIII human orthologs, could be used as novel prophylactic or therapeutic treatment against hMPV infection.

4.3 MATERIALS AND METHODS

4.3.1 Mouse infection and ethical considerations

6 to 8 weeks old C57BL/6 male WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice were initially obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at the pathogen-free animal facility at the Pontificia Universidad Católica de Chile (Santiago, Chile). For infection, mice were intraperitoneally anesthetized with a mixture of ketamine and xylazine (80 mg/kg and 4 mg/kg, respectively) and intranasally instilled with ~1 x 10^6 PFU of hMPV A, strain CZ0107 in a final volume of 70 µL. Lung and Bronchoalveolar Lavage fluid (BALF) samples were obtained at 3 d.p.i. All animal experiments were repeated two independent times using at least two independent mice on each individual replicate of the corresponding experimental condition.

All animal procedures were conducted according to ethical standards and the local animal protection law, number 20.800. All experimental protocols followed the standards of the Sanitary Code of Terrestrial Animals from the World Organization for Animal Health (OIE, 24ª Edition, 2015) and were approved by the Scientific Ethical Committee for Animal and Environment Care of the Pontificia Universidad Católica de Chile Protocol Nº 161026006. All procedures were performed under the guidance of an experienced veterinarian. A clinical score was used for monitoring mouse wellness in terms of several parameters such as weight loss, pain signs, and respiratory changes.

4.3.2 Preparation of hMPV viral stocks

LLC-MK2 cells (American Type Culture Collection, CCL-7^{TM}) were used to propagate hMPV serogroup A, strain CZ0107 (clinical isolate obtained from the Laboratorio de Infectología y Virología of the Hospital Clínico, Pontificia Universidad Católica de Chile). Briefly, cell monolayers were grown in T75 flasks with Opti-MEM supplemented with 5% FBS for until

70–80% confluence. Flasks containing 5 mL of infection medium (Opti-MEM 5% FBS medium, supplemented with $CaCl_2$ [100 µg/mL] and trypsin [5 µg/ml]) were inoculated with 1 × 10^5 plaque formation units (PFU) of hMPV and incubated at 37°C. Following viral adsorption (2 hours), supernatants were replaced with fresh medium and incubated for 72 h until the visible cytopathic effect was evidenced. For harvesting, cells were scraped, and the flask content was pooled and centrifuged at 300 g for 10 min to remove cell debris. Supernatants of non-infected cells monolayers were used as non-infectious control (mock). Viral titers of supernatants were determined by immunocytochemistry in 96-well plates containing a confluent monolayer of LLC-MK2 cells, as previously described (Cespedes et al., 2013).

4.3.3 Processing of BALF and lung samples

To collect BALF we inject and recover 1 mL of sterile PBS through the trachea to the lungs using a sterile syringe (twice). The suspension of cells was then centrifuged at 300 g at 4° C for 5 minutes. Afterwards, the supernatant was stored at -80°C for further analyses. The cells were treated with ACK (Ammonium-Chloride-Potassium) lysis buffer consisting of NH_4Cl 150 mM, $KHCO_3$ 10 mM, and Na_2EDTA 0.1 mM (pH 7.2) to lyse red blood cells at RT for 5 minutes. For interstitial pulmonary cell studies, lung tissue was cut into small pieces with scissors and placed in a collagenase PBS (1 mg/mL) solution and incubated in a shaker (150 rpm) at 37˚C for 1 h. Then, tissues were incubated in PEB buffer (1X PBS, EDTA 2 mM, 0.5% BSA, pH 7.2) to avoid forming cell clumps that interfere with flow cytometry and to stop the collagenase reaction by reagent dilution. Tissue samples were homogenized using a 70 µm cell strainer, then rinsed with ACK lysing Buffer to remove red blood cells at RT for 5 minutes. Later, samples were centrifuged at 300 g for 5 min, and pellets were washed with 1X PBS.

4.3.4 Flow cytometry analyses

Flow cytometry staining was performed to assess the levels of different myeloid subsets in lungs and BALF following hMPV infection. For this purpose recovered cells from the BALF and lungs were stained with viability stain Zombie Violet (Biolegend® Cat: 423113) to discriminate between dead and live cells, then washed and stained with anti–**CD45**-BV510 (BD Biosciences® ,Clone 30-F11), anti–**CD11b**-PE (BD Biosciences® ,Clone M1/70), anti-**CD11c**-PE-Cy7 (BD Biosciences® Clone HL3), anti-**I-A/I-E** (Biolegend® ,Clone M5/114.15.2) anti-**CD64**-Alexa Fluor 647 (BD Biosciences® ,Clone X54-517.1), anti-**Siglec-F** (BD Biosciences® ,Clone E50-2440) anti–**Ly6G**-PerCP-Cy5.5 (BD Biosciences® Clone 1A8). Data was acquired on a BD Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJowV10 software.

4.3.5 Determination of viral load by RT-qPCR

Viral RNA loads in lungs was determined by the quantification of hMPV's nucleoprotein (N) expression in lung tissue. Total RNA was obtained from the lung section by using TriZol Reagent (Invitrogen® Cat: 15596026), according to the manufacturer's instructions, and reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (BioRad® Cat: 1708891). Then, hMPV N protein and β-actin cDNA were detected by real-time PCR by using Brilliant qPCR Master Mix (Agilent, San Diego, California USA) on a StepOne thermal cycler (Applied Biosystem®). Primers used for hMPV-N were 5'-ACA GCA GAT TCT AAG AAA CTC AGG-3' (Forward) and 5'-TCT TTG TCT ATC TCT TCC ACC C-3' (Reverse), with an amplicon length of 153 bp. Primers for detection of mouse β-actin primers were 5'-AGG CAT CCT GAC CCT GAA GTA C-3' (Forward) and 5'-TCT TCA TGA GGT AGT CTG TCA G-3' (Reverse), with an amplicon length of 384 bp. Amplification program was the same for N and β-actin and consists in 40 cycles of the following steps: Denaturation at 95ºC for 5 s, primer

annealing and extension at 60ºC for 30s. To obtain melting curves of generated PCR products samples were subjected to 1 cycle of the following conditions: 95ºC for 15s, 60ºC 60s, 95ºC 15s. To calculate viral loads we used standard curves generated by serial dilutions of known DNA copies corresponding to each gene of interest. Viral load was expressed as copies of N gene for each 5000 copies of β-actin.

4.3.6 Histological analyzes

For histopathology analyses, the major bronchi of the left lung were clamped using a 10 cm hemostatic forceps. Following BALF extraction of the right lung, the left lung was fixed with 4% paraformaldehyde (PFA) and then paraffin-embedded using a Leica ASP300S automatic tissue processor (Leica Microsystems, Wetzlar, Germany). Then, 5 µm-thick tissue sections were obtained using a Microm HM 325 Rotary Microtome (Thermo Scientific). Tissue sections were mounted in silanized Glass Microscope Slides, deparaffinized, then stained for analysis using hematoxylin & eosin (H&E). Evaluation of histopathological score was summarized in Supplementary Table 1 accordingly to a previous characterized criteria (Stack et al., 2000).

4.3.7 Isolation of murine bone marrow-derived neutrophils

Bone marrow-derived neutrophils (BMDNs) were isolated from the femurs of C57BL/6 WT mice by means of Magnetic Assisted Cell Sorting (MACS, Miltenyi), according to the manufacturer's instructions. Cell viability was assessed after purification with trypan blue exclusion. Cell purity was assessed by flow cytometry by gating on singlets, $CD11c^{-}$ /$CD11b^{+}$/$Ly6G^{+}$/ population. Cell purity in all experiments was over 90%.

4.3.8 Opsonization of hMPV

Virus stock stored at -80 °C was thawed at 37°C, then an aliquot of 300 µL of the virus stock ($1x10^7$ PFUs / mL) was incubated with 1 µL of anti-F Alexa Fluor 700 conjugated antibody (Novus Biologicals, clone 5E5 Cat Nº NB110-37245) (stock 1000 µg / mL) for 30 min at 37°C. Therefore, the final concentration of anti-F for IgG opsonization was 3,3 µg / mL. After that, the opsonized virus was kept at 4°C on ice until used in the experiments to measure the expression of FcγRIIb and FcγRIII together with ROS and NETs production.

4.3.9 Measurement of FcγRIIb and FcγRIII expression in BMDNs after challenge with free and opsonized hMPV by flow cytometry.

To determine the expression of FcγRIIb and FcγRIII in BMDNS after free or opsonized hMPV challenge, $2x10^5$ isolated cells were either left untreated or challenged with 100 µL of free or opsonized hMPV (MOI=5) for 3 h then separately stained with PE-conjugated goat anti-mouse anti FcγRIIb or PE-conjugated goat anti-mouse anti FcγRIII for 20 mins. This approach allowed to compare the expression pattern of the two different receptors in the same color channel. Final concentration of antibodies used to for staining was 1 µg / mL. 3 independent pools of cells ($2x10^5$ cells) were used for each experimental group in 1 independent experiment.

4.3.10 Measurement of reactive oxygen species (ROS)

For the detection of ROS we used cell permeant ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen,D-399). Lyophilized H_2DCFDA was dissolved in DMSO to obtain a 1 mM stock solution that was diluted before use. WT BMDNs ($2x10^5$ cells / 100 µL of HBSS) in 1.5 mL microcentrifuge tubes were carefully mixed with 100 µL of 2 µM H_2DCFDA staining solution diluted in 1X HBSS without calcium and

magnesium (Cat Nº 14185052, Gibco) in the dark for 30 min in a 5% CO_2 incubation chamber at 37 °C. This loading step allows the dye to be internalized accordingly with manufacturer`s instructions. Remaining dye in cell supernatant was removed by centrifugation at 300g for 5 mins at 4ºC. Cells were then washed with 200 µL of HBSS 1X and incubated with 100 µL of the following treatments: Optimem-I 5% FBS (which is the media for hMPV propagation), hMPV (MOI of 5), IgG-opsonized hMPV, 0,05% H_2O_2 diluted in Optimem-I 5% FBS (which serves as positive control due to its reaction with H_2DCFDA). Treatment of cells after 5 or 10 mins was stopped by adding 100 µL of 4% PFA followed by the incubation of cells on ice. After 15 mins of incubation with PFA the cells were washed with PBS and analyzed by flow cytometry. Percentage of $FITC^+$ positive cells was used as a readout of ROS production.

4.3.11 Measurement of NETosis, apoptosis and necrosis by flow cytometry using Sytox green and Annexin.

Measurements of NETosis, apoptosis and necrosis were evaluated in BMDNs following incubation with IgG opsonized and free hMPV. Cells were then stained with Annexin V / Sytox green and PE conjugated anti-MPO and analyzed by flow cytometry. BMDNs ($2x10^5$ cells / 100 µL) in 1.5 mL microcentrifuge tubes were carefully mixed with 100 µL of opsonized or free hMPV (MOI = 5) or left untreated for 3h. Afterwards, cells were centrifuged at 300xg for 5 mins at 4º C and the supernatant was carefully removed. Cells were resuspended in 100 µL of Annexin V Binding Buffer (cat # 422201) then 5 µL of stock Annexin solution (Cat Nº 640919, Biolegend) was added together with a mixture containing Sytox™ green (final concentration 1 µM) and 1 : 300 dilution of PE conjugated anti-MPO (Invitrogen® Cat: PA5-16672). Since the anti-MPO antibody was originally unconjugated to PE, we used an antibody conjugation commercial kit to conjugate this antibody (Lightning-Link® R-PE Antibody

Labeling Kit, Cat Nº: 703-0030) following manufactures instructions. For staining, cells were incubated for 15 mins at room temperature with the mixture, then centrifuged at 300g x 15 mins. Cells were resuspended in PBS containing 4950 counting beads (Cat Nº C36950 Molecular Probes Invitrogen) in a volume 300 µL and analyzed by flow cytometry. NET^+ cells were defined as: SYTOX $green^+$/ Annexin V^- /MPO^+ ; apoptotic as: SYTOX $green^-$/ Annexin V^+ whereas necrotic as: SYTOX $green^+$/ Annexin V^+ cells.

4.3.12 Visualization of NETs by immunofluorescence

Isolated BMDNs were seeded in a 24-well plate containing in each well a sterile autoclaved 10 mm glass coverslip. Cells were seeded at a density of $2x10^5$ cells per well in a total volume of 200 µL. The cells were left to adhere to glass by incubation for 1 h at 37 ºC, 5% CO_2 in a culture incubator. Afterwards, the cells were incubated with 200 µL of either opsonized or free hMPV at a MOI of 5. As a positive control, cells were incubated with 5 µM of PMA to induce NET formation, as previously reported (Gray et al., 2013). Cells were incubated with the different treatments by 3 hours, then washed with PBS and fixed overnight with PFA 4% at 4°C. During the next day, the wells were blocked with PBS 3% BSA, then stained with 1 µM Sytox™ Green (Invitrogen® Cat: S7020) and a polyclonal anti-MPO antibody (Invitrogen® Cat: PA5-16672) diluted 1:20 for 30 mins at RT. Finally, the cells were incubated with 100 µL of 1:500 dilution of a secondary goat anti-mouse Alexa Fluor 555 (Invitrogen® Cat: A28180, final concentration 2 µg/mL). Microscopy assays were examined under the Bright Light and Fluorescence Microscope Olympus, model BX51.

4.3.13 Statistical analyzes.

All statistical analyses were performed using GraphPad Prism software version 8.00 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis data are expressed as mean ± SEM.

Comparison between two groups was conducted by Student t-test to individually compare between mock and hMPV treatments in WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice. Additionally, two-way ANOVA followed by post Tukey T test was used to analyze all the possible comparisons between WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice subjected to either mock or hMPV challenge. *p* values less than 0.05 were considered statistically significant.

4.4 RESULTS

4.4.1 A deficiency of either FcγRIIb or FcγRIII decreases the clinical and histopathological hallmarks of hMPV infection without modulating viral loads.

To assess the role of both FcγRIIb and FcγRIII receptors in the clinical disease and inflammation caused by hMPV infection, WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice were intranasally infected with 1x10^{6} PFUs of hMPV. Weight loss was registered for three days and lungs and BAL suspensions were recovered for flow cytometry analyses, quantification of viral loads together with the assessment of lung histopathology. A significant decrease of body weight was observed in WT hMPV-infected mice at 2 d.p.i. as compared to their respective mock-treated control group accordingly with two-way ANOVA the post-Tukey test (Figure 6A). In the case of FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice, body weight remains unaltered for the whole evaluation period in hMPV infected mice as compared to the corresponding mock treated group. Together, these results indicate that both FcγRIIb and FcγRIII promotes the clinical outcome elicited by hMPV infection in terms of body weight loss, suggesting that both receptors contribute to hMPV pathogenesis *in vivo*.

Another parameter of disease progression due to hMPV infection quantified was viral loads in the lungs of WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ at day 3 p.i. We did not find any differences between viral loads from WT and any of the KO infected mice (Figure 6B), suggesting that clinical differences related to weight loss due to hMPV infection may not be related to the ability of hMPV to replicate in lung tissue.

Three days after hMPV challenge, lung tissue was obtained and analyzed for histopathology. Representative images showed that WT hMPV infected mice had significant inflammatory cell

infiltration in alveolar space when compared to mock treated group (Figure 6C). In contrast, FcɣRIIb$^{-/-}$ and FcɣRIII$^{-/-}$ mice showed reduced cellular infiltration in airways following hMPV challenge as observed in the respective Mock control. Data quantification from lung samples was performed in three independent mice from where we calculate the score of at least four representative images (Figure 6D). In the case of WT mice we observed a significant increase in the histopathological score (Figure 6D) based on a previous characterized criteria (Supplementary table 1). In the case of WT mice we observed that following hMPV infection 4 from 6 images representative of 3 independent mice showed a reduced area of alveolar space together with perivascular (PVI) and peribronchial inflammation (PBI) foci (Score 3 accordingly with histopathology score, Supplementary Figure 2). In contrast, FcγRIIb$^{-/-}$ hMPV-infected showed a histopathology score that reaches a maximal score of 2 in only 2 from 6 infected mice. Such score value of 2 indicates the presence of PVI and PBI foci without a discernible reduction in the area of alveolar spaces (Supplementary Figure 2). Furthermore, FcγRIII$^{-/-}$ mice also showed a maximal clinical score of 2 in 4 from 6 images evaluated that were representative from at least 3 independent infected mice.

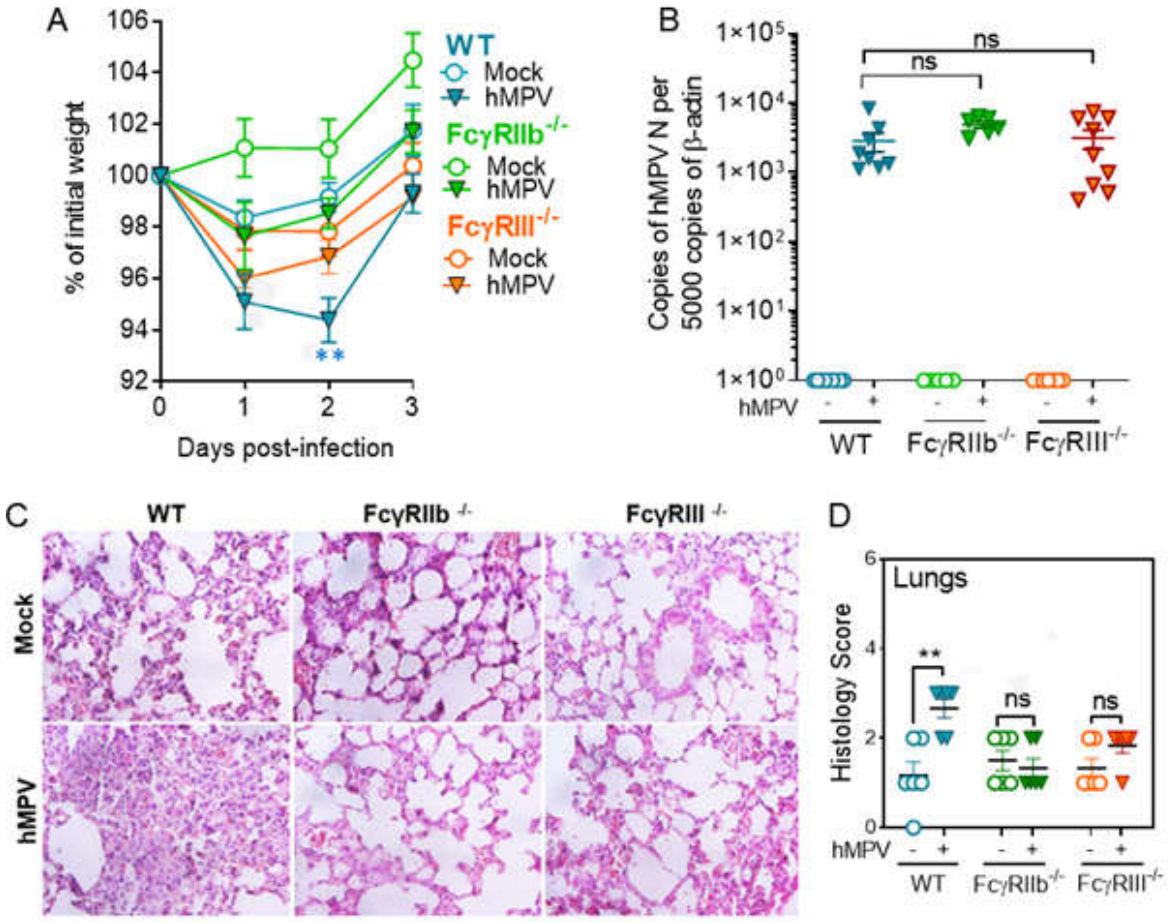


Figure 6. A deficiency of either FcγRIIb or FcγRIII decreases the clinical and histopathological hallmarks of hMPV infection without modulating viral loads. (**A**) Weight loss was measured daily for 3 days as a parameter of hMPV disease progression. (**B**) Levels of hMPV viral load were quantified in the lungs by RT-qPCR. (**C**). Mock and hMPV infected lung samples were stained for H&E original magnification 40X. (**D**) Histopathology analyses of lung sections from each experimental group was performed at day 3 post-infection. Significant differences were determined by two-way ANOVA then post-Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). The error bars represent the Standard Error of Mean (SEM).

4.4.2 A deficiency of FcγRIIb or FcγRIII prevents recruitment of neutrophils to the airways of hMPV-infected mice.

Neutrophil recruitment during hMPV-induced pathology is associated with an increased severity of pulmonary illness in the murine model (Lay et al., 2015;Cheemarla et al., 2017b). Therefore, we sought to determine the recruitment of neutrophils in lung and BALF tissue of WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ hMPV-infected mice. To determine the number of infiltrating neutrophils we performed flow cytometry analysis of cell preparations from BALF and lung tissue. The gating strategy for neutrophil discrimination was as follows: Total cells/Singlets/Live cells/CD45^{+}/CD11b^{+}/CD11c^{-}/Ly6G^{+}/SiglecF^{-} (Supplementary Figure 1).

In the lung tissue, we observed a 2.1-fold significative increase in the absolute number of viable neutrophils of WT hMPV-infected mice as compared to the corresponding mock treated group (Figure 7A). In contrast, average number of neutrophils in FcγRIIb$^{-/-}$ decreased 0.7-fold after hMPV infection as compared to the Mock treated group. In FcγRIII$^{-/-}$ mice we observed a 1.2-fold increase in neutrophil number in hMPV infected mice as compared to mock treated groups. Two-way ANOVA indicates that differences were statistically significant only in WT mice.

Neutrophils counts in BALF of WT mice showed 2.1-fold increase after hMPV infection (Figure 7B). This difference was statistically significant accordingly to two-way ANOVA analyses. Interestingly, in both FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ the average number of BAL neutrophils does not change after hMPV infection accordingly to two-way ANOVA and post-Tukey test. Together these findings suggest that weight loss observed in WT mice after hMPV infection might be related to the infiltration of neutrophils in lung and airways. As the results obtained in both KO mice were similar, we further investigate the infiltration of other immune cells that

contribute to inflammation and may explain the mechanism underlying the reduced lung inflammation observed in mice lacking FcγRIIb or FcγRIII.

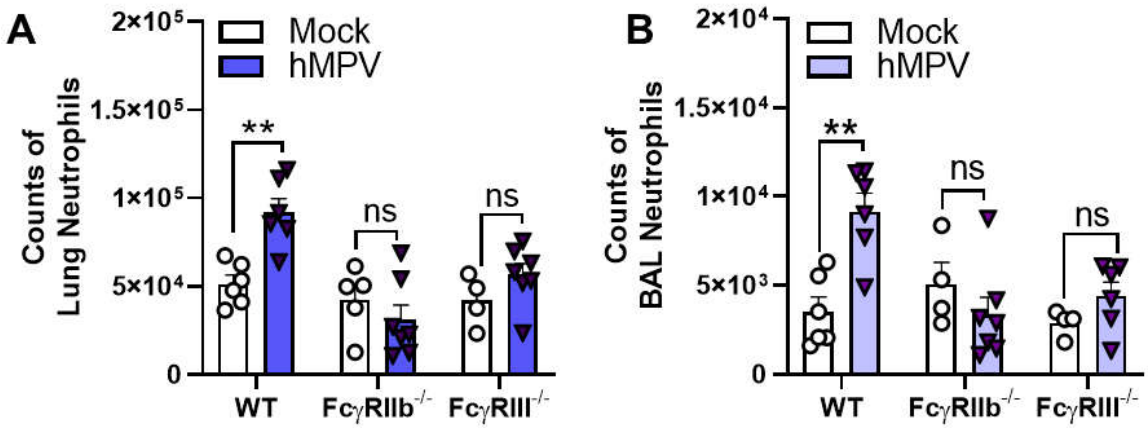


Figure 7. FcγRIIb or FcγRIII deficiency prevents recruitment of neutrophils to the airways of hMPV infected mice. The levels of lung (**A**) or BAL (**B**) neutrophils after challenge with Mock or hMPV infection was measured at day 3 p.i in WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice. Significant differences were determined by two-way ANOVA then post-Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). The error bars represent the Standard Error of Mean (SEM).

4.4.3 The number of IMs and AMs in lungs and BALF after hMPV infection is modulated in mice lacking either FcγRIIb or FcγRIII.

Since in this study we used constitutive *knockout* (KO) mice which lack the expression of FcγRIIb$^{-/-}$ and/or FcγRIII$^{-/-}$ in the entire subset of myeloid cells that are recruited to the lungs and BALF during hMPV infection, we analyze by cytometry other myeloid sub-set rather than neutrophils that can be modulated by hMPV-infection in the absence of either FcγRIIb and/or FcγRIII . The panel for cell identification was based in a previous publication (Misharin et al., 2013) which allows us to discriminate between myeloid cells that differentially regulates hMPV pathogenesis and lung inflammatory responses such as AMs, IMs, pDCs and CD11b^{+} DCs (Supplementary Figure 1). Remarkably some relevant significant differences were found related to macrophage levels (Figure 8). First, we found that the levels lungs and BAL IMs showed a significant increase following hMPV infection in WT mice (Figure 8A) but not in FcγRIIb$^{-/-}$ mice and to a lesser extent in FcγRIII$^{-/-}$ mice accordingly with two-way ANOVA then post Tukey test (Figure 8B). Together, our results suggest that deficiency of either FcγRIIb or FcγRIII down-regulates the recruitment of IMs into the lung parenchyma and the alveolar space *in vivo*.

On the other hand, we found that the levels of AMs decreased significantly in the lungs (Figure 8C) and airways (Figure 8D) of WT mice after hMPV infection but not in any of the KO mice analyzed in this study. In addition, both KO mice showed decreased levels of lung and BAL AMs after mock treatment when compared to WT mice (Figure 8D), thus suggesting that deficiency of both receptors may regulate the number of lung and airway AMs in basal conditions.

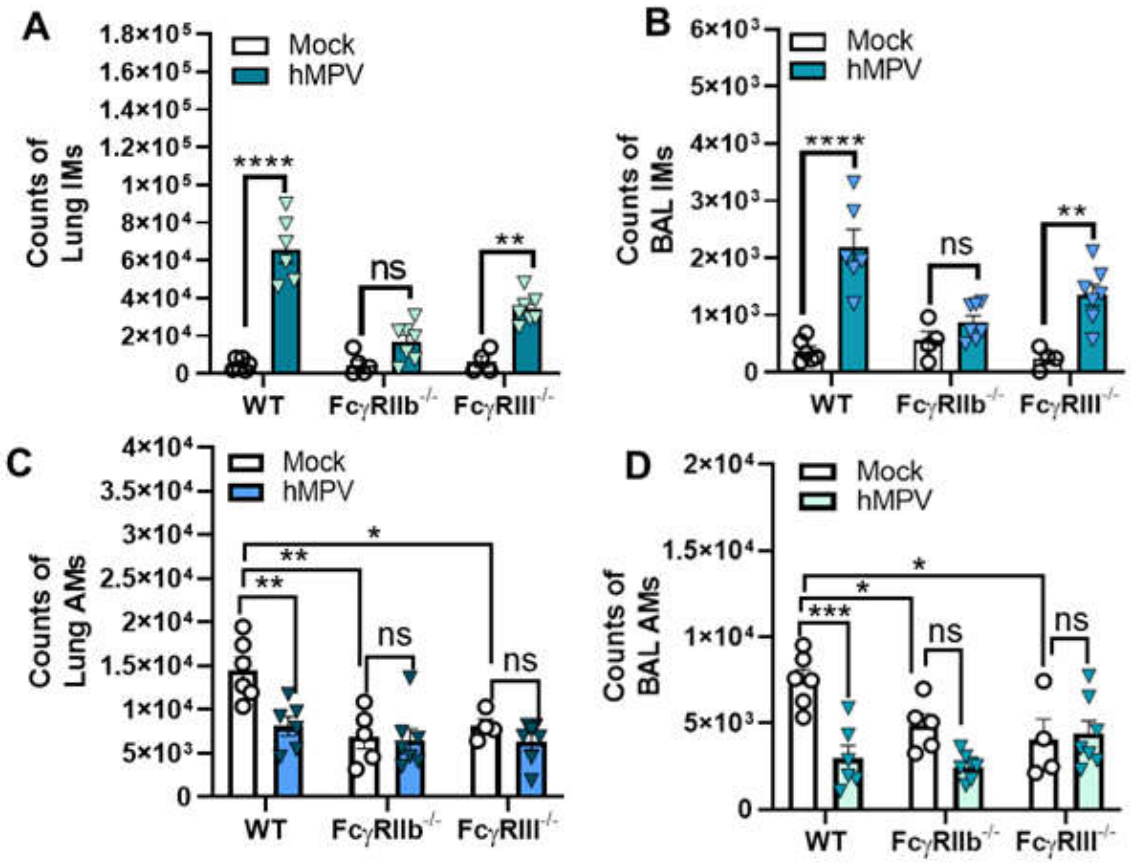


Figure 8. The number of IMs and AMs in lungs and BALF after hMPV infection is modulated in mice lacking either FcγRIIb or FcγRIII. The recruitment of different macrophage subsets was evaluated on day 3 post-infection with $1x10^6$ PFUs of hMPV in BALF and lung tissue of WT, FcγRIIb and FcγRIII deficient mice. Recovered cells were stained and analyzed by flow cytometry. Significant differences were analyzed by two-way ANOVA then post-Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). The error bars represent the Standard Error of Mean (SEM).

4.4.4 FcγRIIb or FcγRIII deficiency fails to modulate the levels of pDCs and CD11b$^+$ DCs in lung parenchyma and the airways during hMPV infection.

Previous studies have shown that pDCs as well as CD11b$^+$ DCs accumulates in the airways as well as lung tissue after hMPV infection (Guerrero-Plata et al., 2009) . However, it is remains unknown what are the factors that can contribute to the recruitment of such cells following hMPV infection. Since there, we evaluated if the basal and post-infection levels of these cells are modulated by deficiency of either FcγRIIb or FcγRIII. As shown in Figure 9, we observed that a deficiency of either FcγRIIb or FcγRIII does not lead to any significant differences between any of experimental groups in pDCs levels in lungs (Fig. 9A) as well as in BAL (Figure 9B). We also found that deficiency of either FcγRIIb or FcγRIII does not impact the levels of CD11b$^+$ DCs in either lungs (Fig. 9C) or BAL (Fig. 9D). Thus suggesting that deficiency of these receptors does not contribute to the recruitment of pDCs as well as CD11b$^+$ DCs during infection. Furthermore, supporting a major role of neutrophils as well as AMs and IMs in the innate inflammatory response caused by hMPV infection *in vivo*.

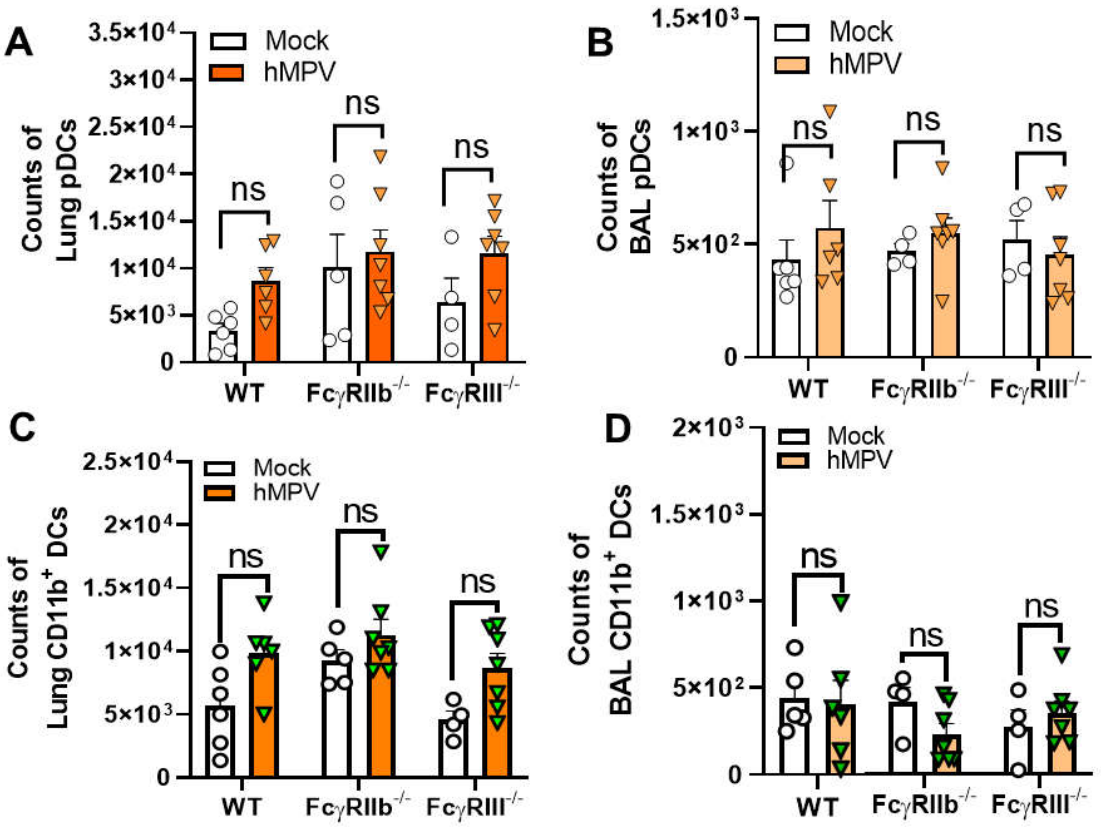


Figure 9. The numbers of pDCs and CD11b+ DCs in lungs and BALF after hMPV infection are not modulated in mice lacking either FcγRIIb and/or FcγRIII. The recruitment of pDCs and CD11b^{+} DCs was evaluated ay 3 post-infection as described above. Recovered cells were stained and analyzed by flow cytometry. Significant differences were analyzed by two-way ANOVA then post-Tukey test. The error bars represent the Standard Error of Mean (SEM).

4.4.5 Incubation of BMDNs with opsonized and free hMPV leads to the upregulation of FcγRIIb and FcγRIII

Since we observed that both FcγRIIb and FcγRIII contribute to the *in vivo* recruitment of neutrophils into the BAL and lungs of hMPV-infected mice we further investigated the possibility that ICs formed between hMPV and antibodies raised against hMPV F protein can modulate the expression of both FcγRIIb and FcγRIII on resting neutrophils. We focused our analysis in F protein since it has been proposed that this protein can mediate both attachment and fusion thus represents a key regulator of the hMPV replicative cycle (Cox and Williams, 2013). Furthermore, we tested whether hMPV alone can also modulate the expression of both receptors, since it is described that inflammatory stimulus can modulate the expression of such receptors on humans neutrophils (Wang and Jönsson, 2019). However, it has not been described if the interaction of hMPV with murine neutrophils can modulate the expression of FcγRIIb or FcγRIII.

In order to compare the relative expression of both receptors in neutrophils after the challenge with hMPV alone as well as hMPV-ICs we stained different pools of BMDNs with PE conjugated antibodies that recognize either FcγRIIb or FcγRIII. We found that both FcγRIIb and FcγRIII were up-regulated when murine neutrophils were incubated with free hMPV (Figure 10). We observed a 16-fold increase in GMFI of FcγRIIb on murine BMDNs incubated with hMPV alone that was even more pronounced (35-fold) in neutrophils incubated with hMPV-ICs, Statistically significant differences between FcγRIIb GMFI between neutrophils incubated with hMPV alone and hMPV ICs (2.2-fold) were also found (Figure 10). Therefore, suggesting that hMPV-ICs are a more powerful inducer of the inhibitory FcγRIIb on resting neutrophils. On the other hand we observed a 24-fold increase in the GMFI of FcγRIII in resting neutrophils incubated with free hMPV when compared to untreated cells. No significant

differences were observed in the GMFI of FcγRIII when we compared between free hMPV and hMPV-ICs challenged BMDNs. Therefore suggesting that hMPV alone as well as hMPV-ICs induces the expression of FcγRIII at the same extent on resting neutrophils. Of interest, we observed a 2.1-fold difference in the GMFI of FcγRIII in comparison to FcγRIIb in BMDNs incubated with hMPV, thus suggesting that hMPV is a stronger inducer of FcγRIII than FcγRIIb on resting neutrophils. Altogether our results suggest that hMPV as well as hMPV-ICs modulate the expression of both FcγRIIb and FcγRIII on resting neutrophils.

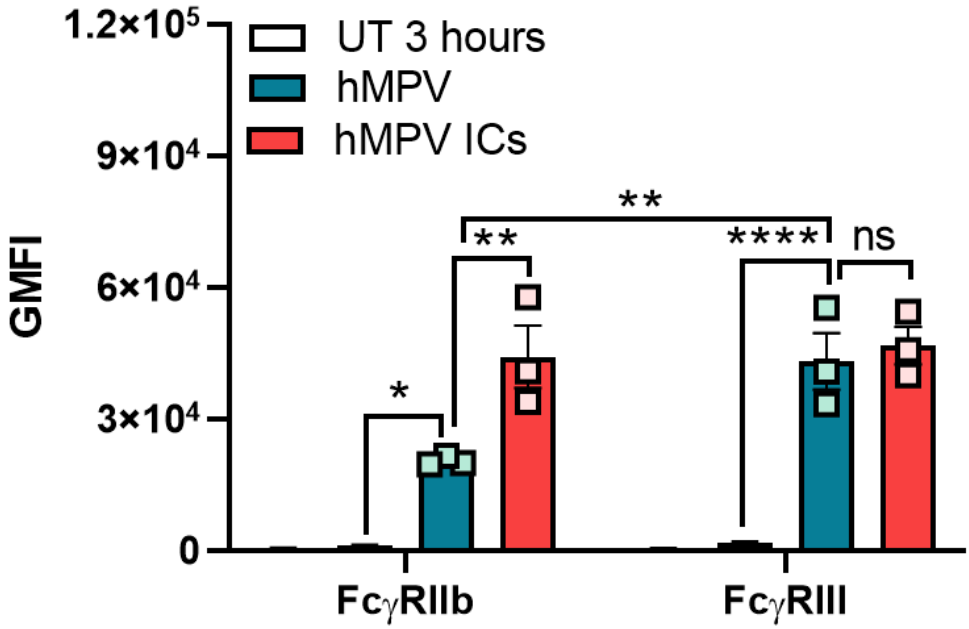


Figure 10. Membrane expression of FcγRIIb and FcγRIII is up-regulated following incubation of BMDNs with opsonized hMPV. The expression of FcγRIIb and FcγRIIb was analyzed by flow cytometry in BMDNs that were left untreated or challenged with either free or opsonized hMPV for 3 hours. . Significant differences were analyzed by two-way ANOVA then post-Tukey test . The results in the figure represents 1 independent experiment with three samples on each experimental group.

4.4.6 Incubation of BMDNs with hMPV-ICs and free hMPV trigger ROS production *in vitro*.

Our results showed that lung damage due to hMPV infection *in vivo* is reduced by either FcγRIIb or FcγRIII deficiency. One possibility is that part of this tissue injury arises from neutrophil ROS produced due to the interaction of hMPV-ICs with FcγRs expressed on the surface of neutrophils. In this context, a previous report indicate that immobilized ICs formed by OVA and anti-OVA-IgGs can trigger the release of ROS by resting murine neutrophils due to the co-engagement of FcγRIII and FcγRIV (Jakus et al., 2008). Therefore, we tested whether hMPV-ICs as well as hMPV alone can trigger production of ROS by resting neutrophils. Accordingly, we observed that following 5 mins of incubation both free hMPV and hMPV-ICs increased the percentage of ROS producing BMDNs (Figure 11). Incubation of BMDNs with hMPV produces a 2.3-fold increase in the percentage of $FITC^+$ (ROS^+) cells in comparison with untreated control after 5 mins. Compared to hMPV alone, hMPV-ICs treated neutrophils showed a 1.5-fold increase in the percentage of ROS^+ neutrophils after 5 mins of treatment (Figure 11). However such increased levels reach the basal values as observed in untreated cells after 10 mins of treatment. On the contrary control cells that were incubated with H_2O_2 which reacts with the dye used in the assay and serves as a positive control, showed no differences in the percentage of ROS^+ cells between 5 and 10 mins of treatment. Therefore suggesting that hMPV alone as well as hMPV-ICs trough interaction with FcγRs can trigger only a transient increase in ROS production by resting neutrophils.

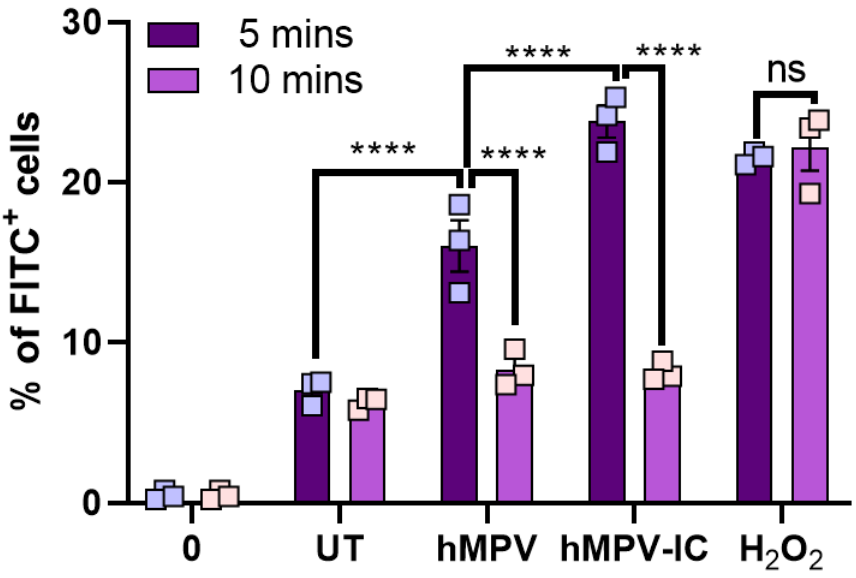


Figure 11. Effect of free and IgG opsonized hMPV on the stimulation of reactive oxygen species production by BMDNs. Purified neutrophils were incubated in HBSS medium with or without hMPV, hMPV-IC for 5 and 10 min at 37ºC 5% CO_2. hMPV-IC was prepared using by mixing 1 µL of a commercial mAb against hMPV F protein with a 300 µL aliquot of hMPV stock that was incubated for 30 mins at 37ºC. Data are reported as means ± SEM for n = 3 different pools of cells. Significant differences were analyzed by two-way ANOVA then post-Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). The error bars represent the Standard Error of Mean (SEM).

4.4.7 Opsonization of hMPV with an anti-F protein mAb triggers NETosis of murine BMDNs and can prevent constitutive apoptosis of isolated BMDNs.

We investigated whether neutrophil interaction with hMPV has any effects on cell survival as hMPV infection *in vivo* is characterized by a significant increase in neutrophil levels in both the airways and lung tissue. Therefore, a possible explanation of such result is a reduced neutrophil death which has been characterized for other viruses such as HCMV (Pocock et al., 2017). Further, to investigate if hMPV fusion protein and possibly Fc gamma receptors contribute in such interaction we determined if opsonized hMPV with a commercial mAb against hMPV F protein modulates neutrophil survival in terms of NETosis, apoptosis and necrosis (Figure 12). In this assay we use the apoptosis marker Annexin V, which binds to externalized phosphatidylserine on the membrane of apoptotic cells (Park et al., 2016). In addition, we use the Sytox green dye which binds to extracellular DNA under non-permeabilizing conditions (Gupta et al., 2018). Depending on the staining pattern of cells we can distinguish between, NET forming, apoptotic and necrotic cells (Figure 12). Remarkably, we observed that the number of necrotic neutrophils remains unchanged despite of the treatment with hMPV or hMPV-ICs (Figure 12A) . On the other hand, we observed that apoptosis of neutrophils occurs to a significant extent when the cells were left untreated for 3 h in comparison with freshly isolated cells (Figure 12B). Of note, such increase in neutrophil apoptosis was prevented when the cells were incubated with free or opsonized hMPV. NET forming neutrophils (Annexin V^- $Sytox^+$ MPO^+) were increased by the incubation of BMDNs with free hMPV and hMPV-IC. Remarkably, a statistically significant decreased NETosis was observed when the cells were incubated with hMPV-ICs in comparison to hMPV alone (Figure 12C).

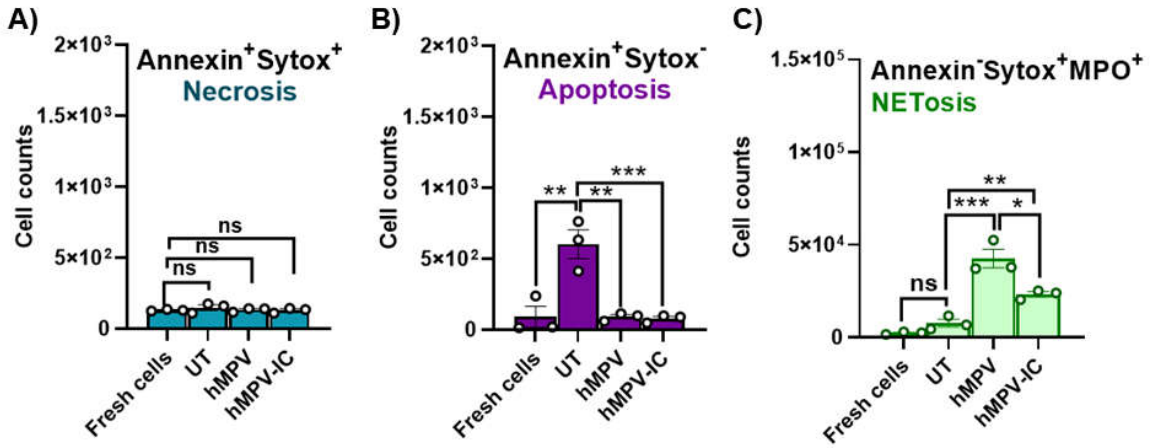


Figure 12. The interaction of BMDNs with hMPV alone as well as hMPV-ICs prevent constitutive apoptosis of isolated murine neutrophils and promote the formation of neutrophil extracellular traps. Flow cytometry analysis of neutrophil NETosis, apoptosis and necrosis in cells challenged with free and opsonized hMPV (MOI 5, $n = 3$). (**A**) Necrotic cells were identified as Annexin V^+ $Sytox^+$ as the express the apoptotic marker Annexin V and were positive for extracellular DNA. (**B**) Apoptotic cells were defined as Annexin V^+/ $Sytox^-$ cells which express the apoptosis marker Annexin V but lack the signal for extracellular DNA. (**C**) NET forming cells were considered as Annexin V^- $Sytox^+$, as lack the apoptotic marker Annexin V whereas were positive for extracellular DNA. Significant differences were analyzed by two-way ANOVA then post-Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$). The error bars represent the Standard Error of Mean (SEM).

4.4.8 Opsonization of hMPV with an anti-F protein mAb triggers different patterns of NETosis in murine BMDNs

Since our previous results suggest that the interaction of hMPV alone as well as hMPV-ICs can modulate the release of NETs by resting BMDNs (Figure 12C), we then validate our results by means of immunofluorescence of BMDNs challenged with hMPV alone as well as hMPV-ICs (Figure 13A). With this approach we can visualize the released neutrophil DNA (observed as a decondensed green signal) co-localized with other relevant markers such as MPO (in red) which serves to identify NETs (Rada, 2019). This kind of visualization also allow us to evaluate morphological changes in the appereance of nuclei which can appear as condensed or de-condensed, together with NET^{+} cells that appear with a diffuse or spread pattern of NETosis (Figure 13B). Interestingly, BMDNs incubated with hMPV showed increased proportion of a diffuse pattern of NETosis when compared to untreated cells (Figure 13C) which is not as evident in hMPV-IC and PMA treated neutrophils. On the contrary, we observed that the spread pattern of NETosis was reduced in neutrophils incubated with hMPV alone whereas it is increased on neutrophils incubated with hMPV-ICs in comparison to hMPV alone (Figure 13D). The criteria used to discriminate between the distinct staining patterns is described in Supplementary Figure 3.

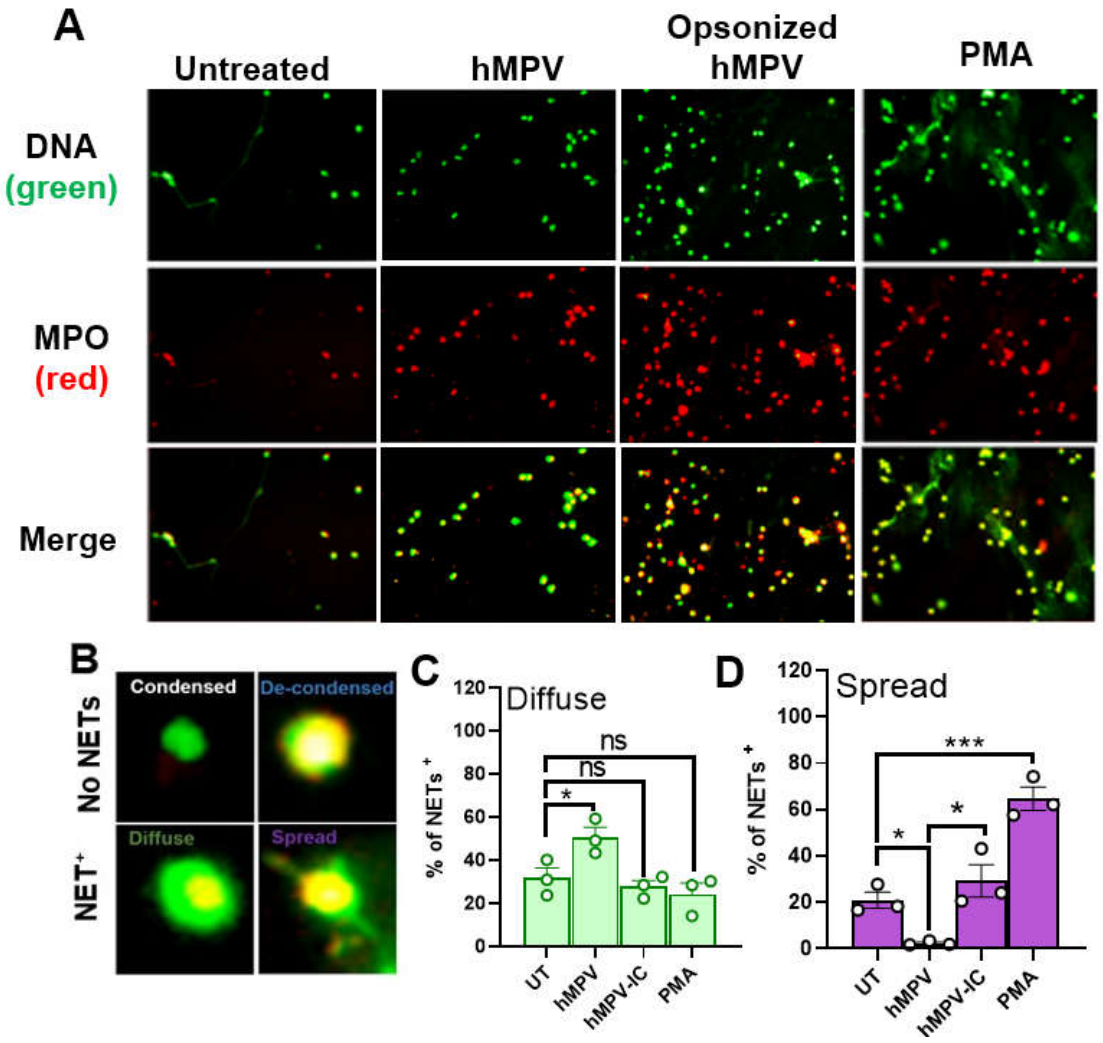


Figure 13. The interaction of WT murine neutrophils with hMPV-ICs promotes the release of NETs by resting murine neutrophils. (**A**) BMDNs were either left untreated or incubated with free or opsonized hMPV for 3h then stained for immunofluorescence detection of NETs. In green SYTOX DNA stain, Red: Myeloperoxidase. Yellow: the merge of green and red channels. Opsonized hMPV was generated by incubating an aliquot of hMPV with a mAb that recognize hMPV F protein. As a positive control BMDNs were incubated with PMA for 3h. (**B**) Four nuclear morphologies were identified as described by Hakkim el al., 2011 namely: condensed, de-condensed nuclei as well as diffuse and spread NETs. (**C**) Quantification of diffuse NETosis pattern in the different treatments accordingly to the morphologies described in B. (**D**) Quantification of spread NETosis pattern in the different treatments accordingly to the morphologies described in B. The results represent 1 independent experiment in which 3 independent fields were analyzed for each condition. Significant differences were analyzed by two-way ANOVA then post-Tukey test. *P<0.05; **P<0.005; ***P<0.0005

4.5 DISCUSSION

HMPV infections represent a major healthcare problem due to the ability of this virus to impair the function of innate and adaptive immune system (Bao et al., 2008;Kolli et al., 2011;Cespedes et al., 2013;Lay et al., 2015). Also, other studies indicate that the presence of neutralizing antibodies is not enough to promote viral clearance and the resolution of the inflammation triggered by hMPV infection (Alvarez et al., 2004;Falsey et al., 2010). From a general point of view, IgG opsonization of pathogens will also trigger effector function through the activation of FcγRs on macrophages, DCs as well as neutrophils. The engagement of these receptors can trigger the inhibition or activation of such cells which in turn may inhibit or promote tissue damage (Michon et al., 1998;Selvaraj et al., 2004;Santegoets et al., 2014). In this work we investigated whether constitutive deficiency of inhibitory IgG receptor FcγRIIb or activating FcγRIII may also play such opposing roles in terms of the clinical outcome produced by hMPV infection which is characterized by reduced corporal weight after infection. Interestingly, we found that deficiency of these receptors produces the same effect on weight loss following hMPV infection (Figure 6A). Of note, no differences in viral loads were observed between hMPV-infected WT, FcγRIIb and FcγRIII mice (Figure 6B). Thus, in agreement with other reports indicating that viral load is not necessarily related to increased hMPV pathology in the mice model, but on the contrary to an exacerbated immune response (Cheemarla et al., 2017a). In this context, the increased weight loss observed in hMPV-infected WT mice correlates with increased immunopathology after infection which was not observed in neither FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ hMPV-infected mice (Figure 6C). Thus, supporting the idea that as stronger is lung inflammation stronger it is also the clinical outcome in terms of weight loss after infection.

Notably, we also found that a deficiency of FcγRIIb prevents the increase of lung (Figure 7A) and airway (Figure 7B) neutrophils observed in WT mice. Thus suggesting that FcγRIIb is responsible for up-regulating the influx of neutrophils from the circulation to the lungs as well as the alveolar space. Furthermore, deficiency of FcγRIII also prevents recruitment of neutrophils into the airways and the lung tissue. Thus, suggesting the mechanism by which these two different receptors control the inflammatory response against hMPV may converge mechanistically.

In order to examine in detail what are other myeloid subsets whose recruitment was altered upon hMPV infection as a result of deficiency of either FcγRIIb or FcγRIII we further examine the contribution of these receptors in regulating the levels of macrophages as well as DC subsets which in cooperation with neutrophils may promote lung inflammation. One of these subset correspond to IMs which accordingly with our results showed a significant increase after hMPV infection in the lungs of WT mice but not in FcγRIIb$^{-/-}$ mice and to a lesser extent in FcγRIII$^{-/-}$ mice. Thus, suggesting a more prominent role of FcγRIIb rather than FcγRIII in promoting the recruitment of IMs after hMPV infection (Figure 8A).

On the other hand, we observed that WT mice showed decreased levels of viable lung (Figure 8A) and airway AMs (Figure 8B) after hMPV infection which was not observed in FcγRIIb$^{-/-}$ as well as FcγRIII$^{-/-}$ mice. Since the levels of viable AMs were reduced in WT mice after hMPV infection and that cell death may account for this reduction, we then evaluate if the levels of death AMs were increased after hMPV infection of WT mice. Interestingly, we determined that in these mice the levels of death AMs were not increased after hMPV infection in comparison to the mock treated group (Supplementary Figure 4). Thus, suggesting that cell death may not be the mechanism that accounts for the reduction of AMs after hMPV infection

of WT mice. In this scenario, another possibility is that inhibitory ITIM signalling trough FcγRIIb and inhibitory ITAMi signalling through FcγRIII can down-regulate the expression of SiglecF on AMs and this cell population was then identified as IMs accordingly with the described phenotype for this cell sub-set (Misharin et al., 2013). In agreement with this hypothesis, we observed that the reduction of viable AMs was not observed in mice lacking either FcγRIIb or FcγRIII. However, further studies are required to determine if a particular subset of IMs found after hMPV infection is derived from AMs in which Siglec-F expression was down-regulated after infection through such inhibitory signalling pathway. Of importance, we also observed that basal levels of AMs in lungs were statistically decreased in FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice when compared to WT mice and the same tendency was observed in the BAL fluid. Thus, suggesting the existence of a mechanisms that relies in the presence FcγRIIb and FcγRIII to regulate the basal levels of AMs in murine airways and lungs.

Analysis of DC subsets recruited to the lungs and airways after hMPV infection showed a mild but not significant increase in the levels of pDCs in lungs (Figure 9A) and airways (Figure 9B) which was not statistically significant in mice lacking FcγRIIb or FcγRIII. Furthermore, as we also did not find any differences in the levels of pDCs as well as CD11b^{+} DCs in either lung or BALF tissue (Figure 9C and D), we cannot exclude the possibility that expression of FcγRIIb and FcγRIII on DCs can also modulate other aspects of DCs function rather than recruitment that include the production of pro and anti-inflammatory cytokines together with chemokines that can modulate lung inflammation.

Several lines of evidences highlighted the role of neutrophil antimicrobial mechanisms in promoting tissue damage during inflammatory condition such as infection. Neutrophil activation, through various membrane receptors initiate several defense mechanisms including

the release of ROS and NETs which are induced by many pathogens including virus and bacteria. Recent reports suggest a role of FcγRs in triggering the initiation of such antimicrobial responses. In this work, we found that incubation of isolated neutrophils with hMPV alone as well as IgG opsonized hMPV up-regulates the expression of both FcγRIIb as well as FcγRIII in resting neutrophils (Figure 10). Thus suggesting that both receptors can modulate the function of neutrophils during hMPV infection. Furthermore, incubation of non-adherent WT neutrophils with hMPV alone as well as IgG opsonized hMPV triggers the production of ROS (Figure 11) whereas prevents neutrophil apoptosis and on the contrary promotes the release of NETs (Figure 12) that together may contribute to increased lung damage and airway obstruction during infection as observed in the *in vivo* assays. In order to validate our result regarding NETs production by hMPV alone as well as IgG opsonized hMPV we use an immunofluorescence assay in which adherent neutrophils were incubated with hMPV alone as well as IgG opsonized hMPV using PMA as a positive control of NET formation (Figure 13) . Our results suggest that a diffuse pattern of NETosis predominates in neutrophils incubated with hMPV alone whereas a spread pattern of NETosis is more frequently observed in neutrophils incubated with IgG opsonized hMPV. As previous studies indicate that this diffuse pattern of nuclear staining appears prior to the appearance of spread pattern of NETosis (Hakkim et al., 2011) it can be speculated that IgG opsonization of hMPV increase the rate of NET formation that occurs after the incubation of neutrophils with hMPV alone. However, another possibility is that hMPV impairs the ability of neutrophils to extrude DNA during NETosis a process which is dependent on the disassembly of actin cytoskeleton (Thiam et al., 2020) but which can also be modulated by hMPV in the case of AECs (El Najjar et al., 2016). However, further studies are required to determine if the same principle applies for neutrophil NETosis induced by hMPV.

Overall, we suggest that the findings in the present work highlight the possibilities to develop new therapies against hMPV based in the blockade of the interaction between hMPV-ICs and specific FcγRs to prevent the immunopathology caused by infection. In this context it would be of interest to determine whether Fc-engineered antibodies targeting hMPV to FcγRs different from FcγRIIb and FcγRIII could be used as novel therapeutics to control hMPV disease progression.

4.6 CONFLICT OF INTEREST

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.

4.7 AUTHOR CONTRIBUTIONS

OA, JS, AR and CC are responsible for the writing of this research article. MR, AK and SB reviewed the manuscript. AK is the leading investigator and assisted in the organization and revision of this article. All authors listed approved the version to be published and have made a substantial and intellectual contribution to the work

4.8 FUNDING

This work was supported by Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) Programa Formación de Capital Humano Avanzado-Beca de Doctorado en Chile N° 21160962 and N° 21170620. FONDECYT (N°1150862 and 1190830)

5. GENERAL DISCUSSION

Human Metapneumovirus (hMPV) is a major cause of severe lower respiratory tract infections and hospitalization in children younger than five years. Innate response against this virus is orchestrated by several myeloid subsets including neutrophils, macrophages and DCs. However, the mechanisms that regulate the function and recruitment of these cells remains poorly understood. Recently, several pieces of evidence have been demonstrated that receptors for the Fc portion of IgG antibodies can act as key immune regulators by controlling the activation and inhibition of cellular functions. In this thesis, we studied the role of two different FcγRs; inhibitory FcγRIIb and activating FcγRIII both of which and accordingly with several reports play opposing roles during inflammation by triggering cell inhibition and activation respectively but which accordingly with our results contribute to promote lung inflammation after hMPV infection as discussed below.

5.1 FcγRIIb together with FcγRIII contribute to the pathology of hMPV infection without modulating viral load

In the present study, we have examined the role of FcγRIIb and/or FcγRIII in hMPV-mediated acute disease, as well as immune responses, and found that both FcγRIIb and FcγRIII plays an important role in promoting hMPV-induced inflammatory responses and disease pathogenesis *in vivo*. Interestingly, our results suggest that FcγRIIb and/or FcγRIII deficiency does not alter hMPV load after infection when compared to WT mice. Thus, suggesting that hMPV replication occurs efficiently despite of individual deficiency of such receptors (Figure 6). Our results agree with the idea that hMPV associated immunopathology may not rely on viral replication but on the contrary to the recruitment of inflammatory cells. In agreement with this idea studies in TLR4$^{-/-}$ mice showed that deficiency of this receptor does not cause significant

changes in viral loads after infection (Velayutham et al., 2013). However it leads to a reduced clinical outcome after hMPV infection due to a reduction in the number of inflammatory cells including neutrophils (Velayutham et al., 2013). At this point, and despite of the role of neutrophils in contributing to lung damage in models of acute lung injury (Grommes and Soehnlein, 2011), it has been showed that depletion of neutrophils prior to hMPV infection leads to a more severe hMPV pathology due to an exacerbated recruitment of other inflammatory cells such as γδ T cells without a significant impact in viral loads (Cheemarla et al., 2017a). Thus supporting the idea that hMPV pathology relies in a more pronounced way in the recruitment of inflammatory cells rather than viral replication.

In this context, we found that both FcγRIIb and FcγRIII deficiency prevents the increase of neutrophils in lungs and BAL after hMPV infection observed in WT mice (Figure 7). Thus suggesting that both receptors contribute to an increased neutrophilic response in the lungs and airways due to hMPV infection. This result agrees with the previous characterized pro-inflammatory role of FcγRIII in terms of neutrophil recruitment during hRSV infection in the airways (Gómez et al., 2016) but is in opposite to the inhibitory role of FcγRIIb in the same study(Gómez et al., 2016). In this context one explanation about the opposite results obtained in our work in comparison to the study related to hRSV may rely in our exclusion of dead cells which was not considered in the previous study. In this context, a side by side comparison about the role of FcγRIIb and FcγRIII in the recruitment of viable neutrophils into the lungs and airways after hRSV and hMPV infection is needed.

5.2 A deficiency of FcγRIIb or FcγRIII prevents a reduction of AMs whereas differentially modulating the recruitment of IMs into the lung and airways

We further investigated the role of FcγRIIb in the recruitment of other myeloid sub-set such as IMs and AMs during hMPV infection (Figure 8). Interestingly, we found that recruitment of IMs in lungs and airways was down-regulated after hMPV infection in FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice. Thus, suggesting that both receptors contribute to the recruitment of IMs in the lung and airways. On the other hand we found the levels of AMs decrease in both lungs and BAL of WT mice after hMPV infection and such difference was attenuated in FcγRIIb and FcγRIII deficient mice. In this context, we reasoned that cell death may be involved in the reduction of AMs in WT mice after hMPV infection. However, we determined that in these mice the levels of death AMs were not increased after hMPV infection in comparison to the mock treated group (Supplementary Figure 4). Thus, suggesting that cell death may not be the mechanism that accounts for the reduction of viable AMs after hMPV infection of WT mice. In this scenario, another possibility is that inhibitory ITIM signalling trough FcγRIIb and inhibitory ITAMi signalling through FcγRIII can down-regulate the expression of Siglec-F on AMs and this cell population was then identified as IMs accordingly with the previously described phenotype for this cell sub-set (Misharin et al., 2013). In agreement with this hypothesis, we observed that the reduction of viable AMs was not observed in mice lacking either FcγRIIb or FcγRIII. Thus, suggesting that both receptors contribute to the overall decrease of AMs observed in WT mice and in contrast contribute to the accumulation of SiglecF^{+} IMs after hMPV infection. However, further studies are required to determine if a particular subset of IMs-like cells found after hMPV infection is derived from resident AMs in which Siglec-F expression was down-regulated after infection. Finally, it is also a matter of future investigations how deficiency of either FcγRIIb and FcγRIII leads to decreased levels of AMs under basal conditions in

comparison to WT mice and which suggest a role of both receptors in the generation or maintenance of AMs in the airways and lungs.

5.3 The recruitment of lung and airway pDCs and CD11b$^+$ DCs triggered by hMPV infection is not modulated by a deficiency of FcγRIIb or FcγRIII

In response to hMPV infection, we did not observe any significant difference in the levels of pDCs as well as CD11b$^+$ DCs in lungs or airways of WT mice as well as in any of the KO mice used in this study (Figure 9). This result is unexpected as previous reports indicate that hMPV infection triggers the accumulation of pDCs into the lung tissue of WT mice. However such study differs from our experimental setting as in our work we used C57BL/6 mice in contrast to the BALB/c mice used in the previous study. Since there, but at the same time to the similar contributions of both receptors in the lung immunopathology observed after hMPV infection further studies are required to clarify what are the exact pathways that regulate the inflammatory responses mediated by FcγRIIb and FcγRIII during hMPV infection. In this context, as we did not find any differences in the levels of pDCs as well as CD11b$^+$ DCs in either lung or BALF tissue, we cannot exclude the possibility that expression of FcγRIIb and FcγRIII on DCs can also modulate other aspects of DCs function rather than recruitment that include the production of pro and anti-inflammatory cytokines together with chemokines that can favor the recruitment of inflammatory cells such as neutrophils.

5.4 The expression of FcγRIIb and FcγRIII on resting BMDNs neutrophils is up-regulated following incubation with hMPV alone as well as hMPV-ICs

While the contribution of various Fc-receptors in neutrophil responses *in vitro* and *in vivo* models has been evaluated; it remains unclear whether hMPV infection can modulate the expression of inhibitory and activating FcγRs on murine neutrophils. We found that hMPV

alone as well as hMPV-ICs up-regulates the expression of both FcγRIIb and FcγRIII on resting neutrophils, thus suggesting that both receptors are involved in the activation and / or inhibition of neutrophil function during hMPV infection (Figure 10). Our results represent a novel data regarding the expression of FcγR expression due to hMPV challenge of resting neutrophils to improve our current knowledge about the factors modulating the interaction of neutrophils with this virus.

5.5 The interaction of resting BMDNs with hMPV alone as well as hMPV-ICs promotes ROS production

Activation and recruitment of neutrophils plays a central role in the pathogenesis of several viral diseases. Excessive release of ROS is thought to contribute to inflammation and have been described to be elicited by the interaction of neutrophils with viruses such as hRSV (Muraro et al., 2018). Furthermore, interaction of murine neutrophils with immobilized IgG-ICs has also described as an stimulus that induces ROS production (Jakus et al., 2008). In this context, it remains unknown whether viruses such as hMPV as well as ICs formed between IgG and this virus can trigger ROS production by resting murine neutrophils. Our results suggest that hMPV alone can trigger ROS production by murine neutrophils (Figure 11) and such production can be increased by the interaction of murine FcγRs expressed by neutrophils with hMPV-ICs. However this increase is modest and only detected at 5 mins following stimulation. Thus, suggesting that hMPV-ICs as well as hMPV alone are not inducers of sustained ROS production by murine resting neutrophils.

5.6 The interaction of resting BMDNs with hMPV alone or hMPV-ICs prevents constitutive apoptosis of BMDNs whereas triggering the release of NETs

Since our *in vivo* studies suggest that neutrophil survival is increased following hMPV infection and this process may depends on the interaction with hMPV alone as well as hMPV-ICs with FcγRs we further characterize different class of cell death that can occur in neutrophils including necrosis, apoptosis and NETosis which are differentiated by the expression of several markers including Annexin V and the presence of extracellular DNA (Figure 12). Interestingly, we found that necrosis of neutrophils was not modulated by the interaction of BMDNs with hMPV alone as well as hMPV-ICs. On the contrary, we found that constitutive apoptosis of untreated BMDNs was prevented by the incubation of BMDNs with hMPV alone as well as hMPV-ICs. Thus suggesting that the virus exerts regulatory functions on neutrophils to avoid cell apoptosis which in turn may allow neutrophil activation and lung inflammation which was observed in WT mice after *in vivo* infection. Furthermore, the release of NETs which was evidenced in cells negative for Annexin V stain but positive for extracellular DNA ($Sytox^{+}$) and MPO was also up-regulated by the incubation of BMDNs with hMPV alone as well as IgG-opsonized hMPV. Of note, we also found that IgG opsonization decreased the numbers of NET^{+} cells in comparison to cells treated with hMPV alone. Since we use a neutralizing antibody against the F protein, this results suggest that IgG opsonization with anti-F antibodies triggers a signalling pathway that inhibits the NETosis program initiated by non-neutralized F protein present in non-opsonized hMPV. However, further studies are required to test such hypothesis.

5.7 The interaction of adherent BMDNs with hMPV alone or hMPV-ICs triggers different patterns of NET forming neutrophils

In order to validate our results about the role of hMPV alone and hMPV-ICs in triggering NETosis we perform immunofluorescence microscopy to determine the presence of NETs in BMDNs incubated with hMPV alone as well as hMPV-ICs after 3 h.p.i (Figure 13). Incubation of the cells with hMPV as well as hMPV-ICs induce different morphological change of neutrophils which may vary from a condensed nuclei (NET negative cells) or decondensed nuclei (also NET negative cells) to diffuse (initial) or spread (later) NETs (Figure 13B)(Hakkim et al., 2011). Of interest, we observed that frequency of diffuse NET^+ neutrophils in the hMPV alone treated group is statistically higher from untreated cells . Furthermore, incubation of BMDNs with hMPV-ICs does not impact the average proportion of neutrophils showing a diffuse pattern of NETosis. This observation contrast with the results obtained by flow cytometry in which NETosis is increased significantly by incubation of these cells with hMPV alone as well as hMPV-ICs. One explanation of these different results is the use of non-adherent cells in the flow cytometry assays which contrast to the adherent cells used for immunofluorescence. Along this line, it was shown that certain NETs inducers require neutrophil adhesion to a matrix before NETosis can occur (Erpenbeck et al., 2019). However, it seems that for the specific context of hMPV infection such situation is the opposite as non-adherent neutrophils respond more readily to hMPV to triggering NETosis when compared to adherent cells. Incubation of BMDNs with hMPV alone triggers a significant decrease of spread NET^+ cells which is not observed after the incubation of these cells with hMPV-ICs (Figure 13D). One explanation is that the incubation of hMPV alone delays the formation of spread NETs from diffuse NETs, but this delayed response is alleviated after the interaction of BMDNs

with hMPV-ICs. However, further studies are necessary to determine if diffuse NETs triggered by free hMPV can lead to spread NETs at later time points than the evaluated in this study.

6. CONCLUSIONS

The results from this doctoral thesis, allow us to determine that two different FcγRs which canonically exert opposing function during inflammatory responses contribute to increased lung inflammation after hMPV infection. Also, we characterize different myeloid populations present in lungs during the first 3 d.p.i that include neutrophils, AMs, IMs, $CD11b^{+}$ DCs and pDCs. We showed that neutrophils up-regulate the expression of both receptors following interaction with hMPV alone as well as in the form of IgG-ICs. Thus, indicating a novel role of hMPV alone and hMPV-ICs in regulating the expression of both receptors. Furthermore, the observation that hMPV infection triggers an overall reduction of AMs rather than inducing increased number of dead AMs, suggest that hMPV infection may regulate other aspects of AMs function rather than cell death to modulate inflammatory responses. One possibility in accordance with previous studies is that cooperative ITIM inhibitory signalling through FcγRIIb and inhibitory ITAMi signalling through FcγRIII triggers a down-regulation on the expression of Siglec-F which is expressed by AMs and such change may represent an initial signal that promotes the inflammatory response against hMPV (Figure 14). In this regard a limitation of this study is the lack cell-specific KO mice to elucidate the contribution of FcγR expression on different myeloid subsets including AMs which may be useful to test our hypothesis. Nowadays, the use of these constitutive KO mice brings us a general overview about the pro- or anti-inflammatory role which a specific receptor plays *in vivo*. Moreover, the implementation of *in vitro* assays allows us to the elucidate the unrecognized role of hMPV alone as well as hMPV-ICs in triggering ROS and NETs production by resting murine neutrophils during hMPV infection which could be probably extrapolated to *in vivo* settings. Our results provide new insights into the development of monoclonal mAbs that can target

hMPV to FcγRs other than FcγRIIb or FcγRIII to control exacerbated lung inflammation as a consequence of hMPV infection.

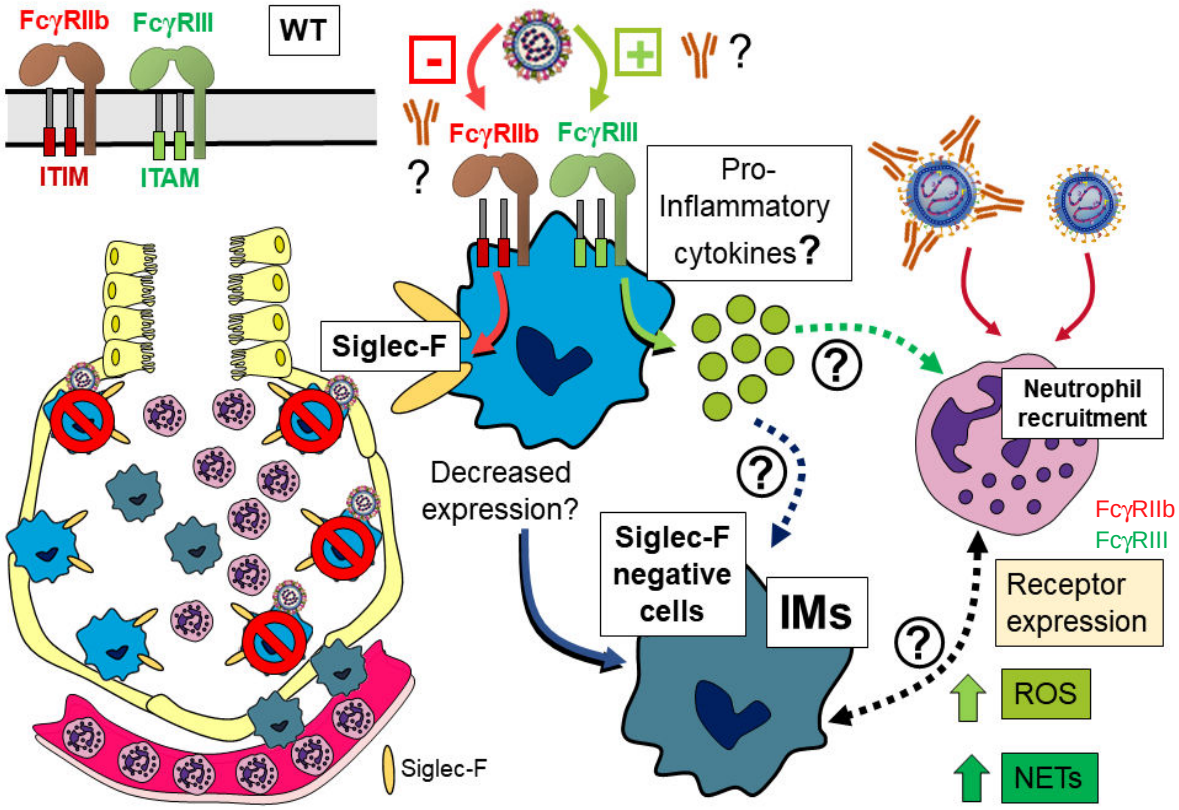
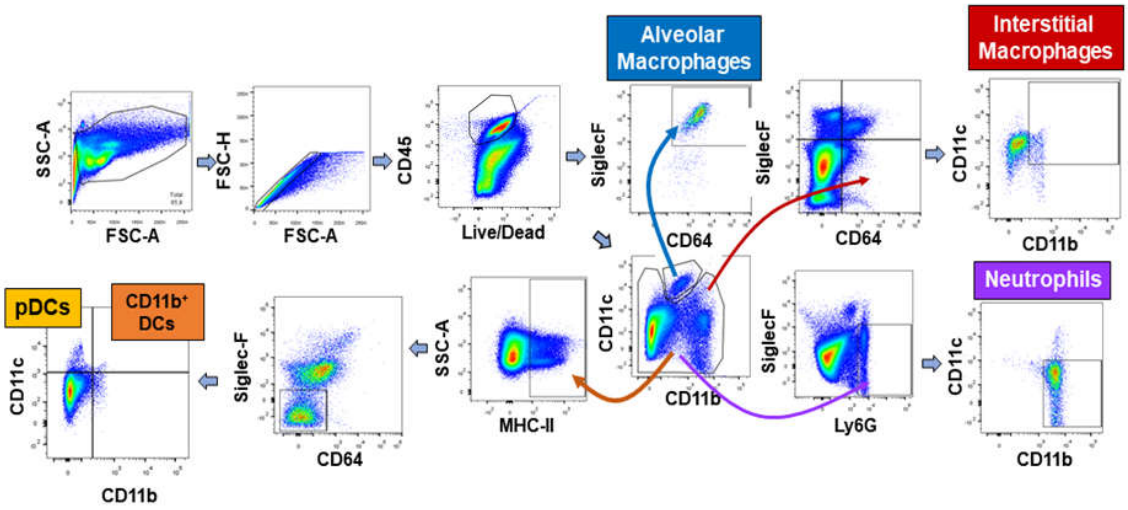


Figure 14: Proposed model for the contribution of FcγRIIb and FcγRIII in triggering lung inflammation and hMPV-ICs as well as hMPV. In the airways of WT mice hMPV infection may downregulate the expression of SiglecF on AMs leading to the generation of cells with a IMs-like phenotype which may contribute to airway obstruction. Through a mechanisms that was not fully understood both receptors FcγRIIb and FcγRIII promote the recruitment of neutrophils after hMPV infection. The interaction of recruited neutrophils with hMPV as well as hMPV-ICs upregulates the expression of FcγRIIb and FcγRIII. Engagement of FcγRs by hMPV-ICs triggers a transient increase in ROS levels followed by the formation of NETs which also may contribute to airway obstruction.

7. SUPPLEMENTARY MATERIAL



Supplementary Figure 1: Gating strategy for identification of myeloid subsets during hMPV infection. Total events were gated then singlets were selected. Using the viability stain Zombie Violet, which stain dead cells, viable cells were gated. Total immune cells ($CD45^+$) cells were selected then subsets expressing either CD11b and/or CD11c were analyzed. $CD11b^-$ /$CD11c^+$ population was further analyzed and identified as $SiglecF^+/CD64^+$ alveolar macrophages (AMs) and $SiglecF^-/CD64^-$ plasmacytoid DCs (pDCs). Double positive $CD11b^+/CD11c^+$ were identified as Interstitial macrophages (IMs) that were $SiglecF^-/CD64^+$ whereas $CD11b^+$ DCs correspond to $SiglecF^-/CD64^-$ population. Neutrophils were identified as CD11b+/CD11c-/$SiglecF^-/Ly6G^+$.

7.1 Histopathological score

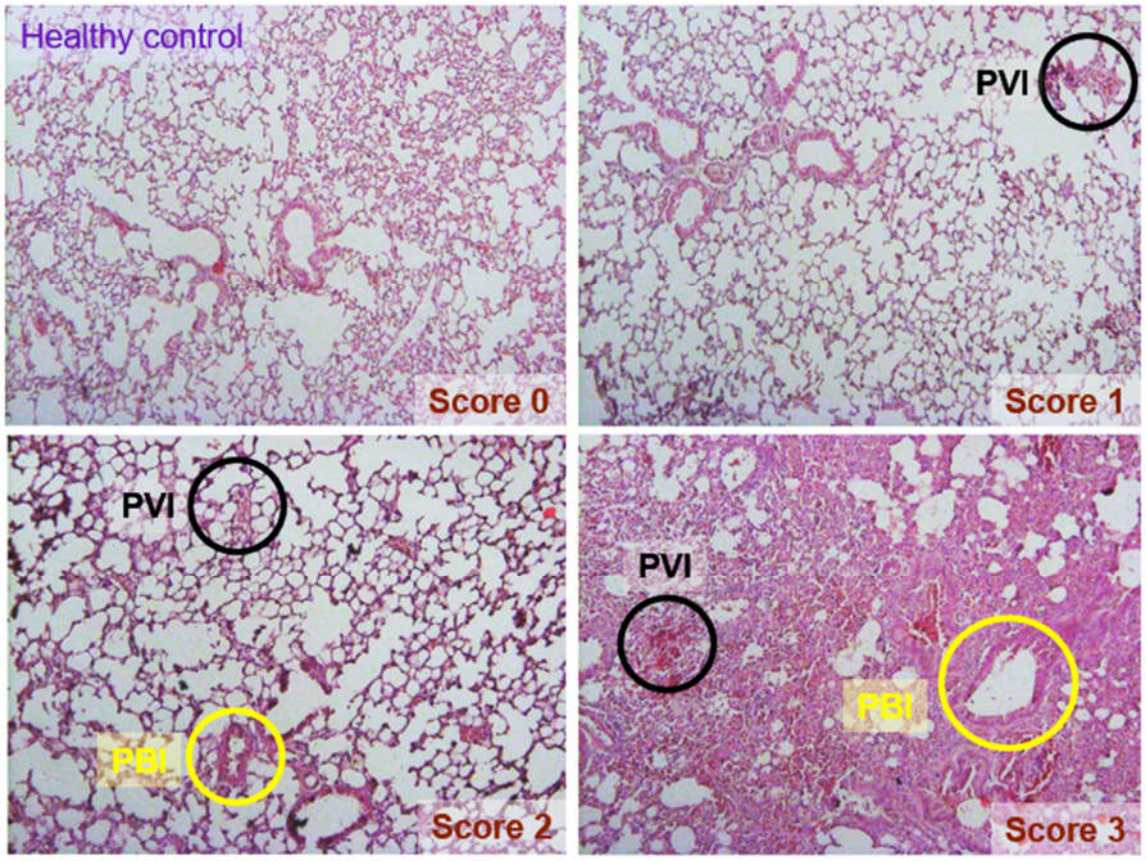
Following the criteria of a published article (Stack et al., 2000) perivascular and peribronchial inflammation was assessed and summarized in Supplementary Table 1.

Representative lung images of the different histopathological scores are depicted in Supplementary Figure 2.

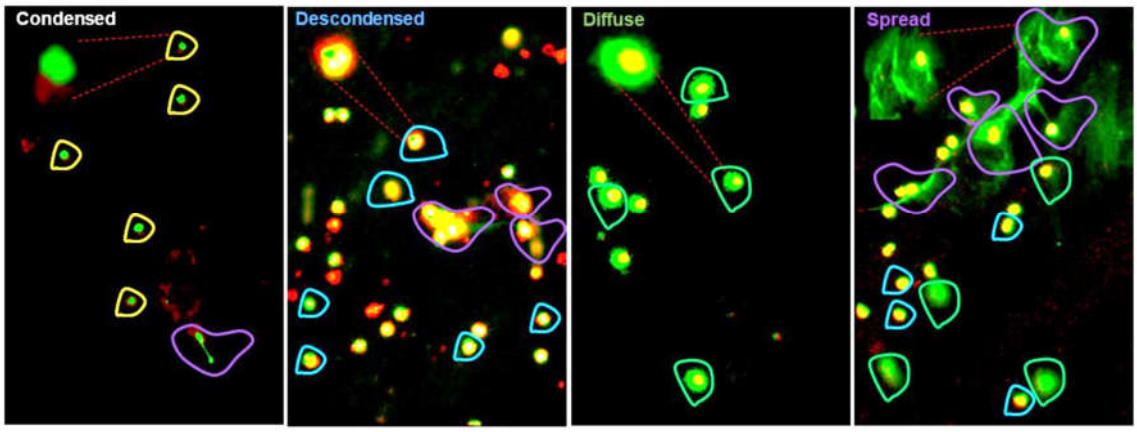
At least 3 independent fields for each lung section representative of three independent mice was counted to calculate the scores.

Parameter	Observation	Score
Normal Lung	Area of alveolar spaces remain similar to the lowest infiltrated WT Mock mice. No foci of perivascular inflammation are found	0
Morphological change I	Area of alveolar spaces remain similar to the lowest infiltrated WT Mock mice. Perivascular or Peribronchial foci of inflammation are found	1
Morphological change II	Area of Alveolar spaces remain similar when compared to the lowest infiltrated WT Mock mice. Perivascular together with Peribronchial foci of infiltration are found in the same field	2
Morphological change III	Area of alveolar spaces is decreased when compared to the lowest infiltrated WT Mock mice. Foci of perivascular infiltration are found	3

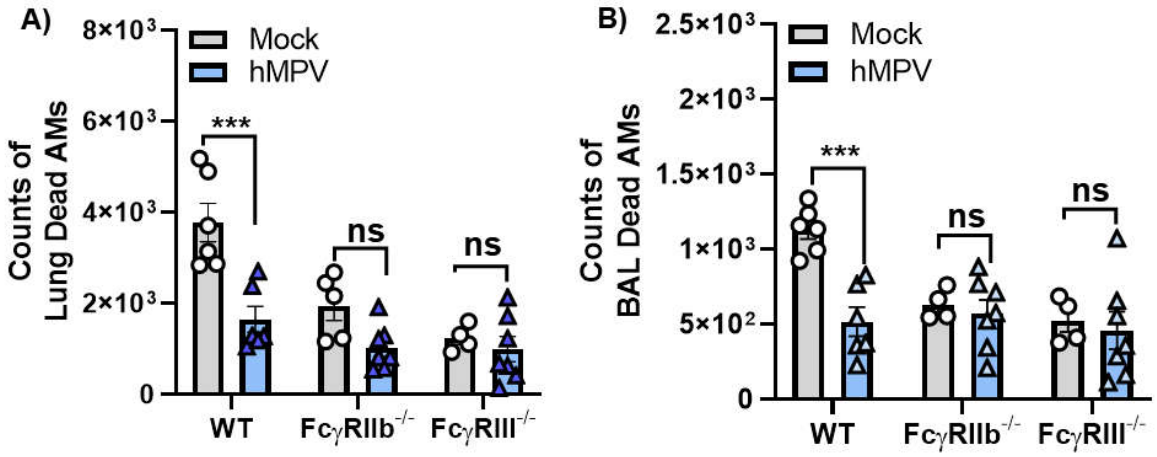
Supplementary Table 1. Pulmonary pathology scores for hMPV intranasal infection in C57BL/6 WT, FcγRIIb$^{-/-}$ and FcγRIII$^{-/-}$ mice



Supplementary Figure 2. Representative images of lung sections with the different associated scores. PVI (black): Perivascular inflammation. PBI (yellow): Peribronchial inflammation



Supplementary Figure 3. Patterns of nuclear DNA together with MPO defines 4 different appearance of neutrophils incubated with hMPV alone as well as hMPV-ICs. (**A**) Condensed nuclei appear as a compact DNA (green) signal that do not co-localize with MPO (red). MPO signal appears outside of DNA signal (red dashed lines). (**B**) De-condensed nuclei appear as red signal surrounding a yellow signal due to co-localization of DNA with MPO. (**C**) Diffuse NETs are distinguished by colocalization of DNA with MPO in the center surrounded by an expanded green signal due to DNA extrusion. (**D**) Spread NETs showed a diffuse yellow channel near to a compacted yellow signal due to the co-localization of DNA with MPO



Supplementary Figure 4. The absolute numbers of dead AMs were decreased after hMPV infection of WT but not FcγRIIb or FcγRIII deficient mice. The number of lung (A) and BAL (B) dead AMs after mock or hMPV infection was determined by flow cytometry. Significant differences were analyzed by two-way ANOVA then post-Tukey T-test. *P<0.05

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9. APPENDIX

7.1 Publications generated in this thesis and PhD training

1. Acevedo, O.A., Diaz, F.E., Beals, T.E., Benavente, F.M., Soto, J.A., Escobar-Vera, J., Gonzalez, P.A., and Kalergis, A.M. (2019). **Contribution of Fc gamma Receptor-Mediated Immunity to the Pathogenesis Caused by the Human Respiratory Syncytial Virus**. Submitted to Frontiers in Cell Infection Microbiology.

2. Canedo-Marroquin, G., Acevedo-Acevedo, O., Rey-Jurado, E., Saavedra, J.M., Lay, M.K., Bueno, S.M., Riedel, C.A., and Kalergis, A.M. (2017). **Modulation of Host Immunity by Human Respiratory Syncytial Virus Virulence Factors: A Synergic Inhibition of Both Innate and Adaptive Immunity**. Submitted to Frontiers in Cell Infection Microbiology

frontiers in Cellular and Infection Microbiology

REVIEW
published: 29 March 2019
doi: 10.3389/fcimb.2019.00075

Contribution of Fcγ Receptor-Mediated Immunity to the Pathogenesis Caused by the Human Respiratory Syncytial Virus

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Specialty section:
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *08 January 2019*
Accepted: *05 March 2019*
Published: *29 March 2019*

Citation:
Acevedo OA, Díaz FE, Beals TE, Benavente FM, Soto JA, Escobar-Vera J, González PA and Kalergis AM (2019) Contribution of Fcγ Receptor-Mediated Immunity to the Pathogenesis Caused by the Human Respiratory Syncytial Virus. Front. Cell. Infect. Microbiol. 9:75. doi: 10.3389/fcimb.2019.00075

The human Respiratory Syncytial Virus (hRSV) is the leading cause of severe acute lower respiratory tract infections (ALRTIs) in humans at all ages and is the main cause of hospitalization due to pneumonia, asthma, and bronchiolitis in infants. hRSV symptoms mainly develop due to an excessive host immune and inflammatory response in the respiratory tissue. hRSV infection during life is frequent and likely because of non-optimal immunological memory is developed against this virus. Vaccine development against this pathogen has been delayed after the detrimental effects produced in children by vaccination with a formalin-inactivated hRSV preparation (FI-hRSV), which caused enhanced disease upon natural viral infection. Since then, several studies have focused on understanding the mechanisms underlying such disease exacerbation. Along these lines, several studies have suggested that antibodies elicited by immunization with FI-hRSV show low neutralizing capacity and promote the formation of immune complexes containing hRSV (hRSV-ICs), which contribute to hRSV pathogenesis through the engagement of Fc gamma receptors (FcγRs) expressed on the surface of immune cells. Furthermore, a role for FcγRs is supported by studies evaluating the contribution of these molecules to hRSV-induced disease. These studies have shown that FcγRs can modulate viral clearance by the host and the inflammatory response triggered by hRSV infection. In addition, ICs can facilitate viral entry into host cells expressing FcγRs, thus extending hRSV infectivity. In this article, we discuss current knowledge relative to the contribution of hRSV-ICs and FcγRs to the pathogenesis caused by hRSV and their putative role in the exacerbation of the disease caused by this virus after FI-hRSV vaccination. A better understanding FcγRs involvement in the immune response against hRSV will contribute to the development of new prophylactic or therapeutic tools to promote virus clearance with limited inflammatory damage to the airways.

Keywords: hRSV, Fc gamma receptors, re-infection, inflammatory response, lung disease, immune complexes, opsonized virus

frontiers in Cellular and Infection Microbiology

REVIEW
published: 16 August 2017
doi: 10.3389/fcimb.2017.00367

Modulation of Host Immunity by Human Respiratory Syncytial Virus Virulence Factors: A Synergic Inhibition of Both Innate and Adaptive Immunity

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Edited by:
Steven Varga,
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Reviewed by:
Michael Teng,
University of South Florida, United States
Nathalie Grandvaux,
Université de Montréal, Canada

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Received: *09 May 2017*
Accepted: *31 July 2017*
Published: *16 August 2017*

Citation:
Canedo-Marroquín G, Acevedo-Acevedo O, Rey-Jurado E, Saavedra JM, Lay MK, Bueno SM, Riedel CA and Kalergis AM (2017) Modulation of Host Immunity by Human Respiratory Syncytial Virus Virulence Factors: A Synergic Inhibition of Both Innate and Adaptive Immunity. Front. Cell. Infect. Microbiol. 7:367. doi: 10.3389/fcimb.2017.00367

The Human Respiratory Syncytial Virus (hRSV) is a major cause of acute lower respiratory tract infections (ARTIs) and high rates of hospitalizations in children and in the elderly worldwide. Symptoms of hRSV infection include bronchiolitis and pneumonia. The lung pathology observed during hRSV infection is due in part to an exacerbated host immune response, characterized by immune cell infiltration to the lungs. HRSV is an enveloped virus, a member of the Pneumoviridae family, with a non-segmented genome and negative polarity-single RNA that contains 10 genes encoding for 11 proteins. These include the Fusion protein (F), the Glycoprotein (G), and the Small Hydrophobic (SH) protein, which are located on the virus surface. In addition, the Nucleoprotein (N), Phosphoprotein (P) large polymerase protein (L) part of the RNA-dependent RNA polymerase complex, the M2-1 protein as a transcription elongation factor, the M2-2 protein as a regulator of viral transcription and (M) protein all of which locate inside the virion. Apart from the structural proteins, the hRSV genome encodes for the non-structural 1 and 2 proteins (NS1 and NS2). HRSV has developed different strategies to evade the host immunity by means of the function of some of these proteins that work as virulence factors to improve the infection in the lung tissue. Also, hRSV NS-1 and NS-2 proteins have been shown to inhibit the activation of the type I interferon response. Furthermore, the hRSV nucleoprotein has been shown to inhibit the immunological synapsis between the dendritic cells and T cells during infection, resulting in an inefficient T cell activation. Here, we discuss the hRSV virulence factors and the host immunological features raised during infection with this virus.

Keywords: hRSV, genes, evasion of host immunity, N protein, immunological synapse