PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

Facultad de Ciencias Biológicas

Programa Doctorado en Ciencias Biológicas

Mención Genética Molecular y Microbiología

DOCTORAL THESIS:

DEVELOPMENT OF RECOMBINANT BCG VACCINES TARGETING ANDES ORTHOHANTAVIRUS AND RESPIRATORY SYNCYTIAL VIRUS: EVALUATION ON MURINE AND BOVINE MODELS.

By

FABIÁN ESTEBAN DÍAZ ACEVEDO

Santiago de Chile,

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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 - Molecular Genetics and Microbiology

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FACULTAD DE CIENCIAS BIOLÓGICAS
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ABBREVIATIONS

AECs: Airway epithelial cells

ANDV: Andes orthohantavirus

APCs: Antigen presenting cells

BALF: Bronchoalveolar fluid

BCG: *Mycobacterium bovis* Bacillus Calmette-Guérin

bRSV: Bovine Respiratory Syncytial Virus

BSL-2: Biosafety level 2

BVDV: Bovine viral diarrhea virus

CCT: Comparative cervical test

CFU: Colony-forming units

conA: Concanavalin A

CTL: Cytotoxic lymphocyte

CTV: Cell trace violet

DCs: Dendritic cells

ELISA: Enzyme-linked immunosorbent assay

FDA: Food and Drug Administration

FI-RSV: Formalin-inactivated Respiratory Syncytial Virus

GMP: Good manufacturing practices

HCPS: Hantavirus Cardiopulmonary Syndrome

hRSV: human Respiratory Syncytial Virus

IFN: Interferon

IL: Interleukin

IgA/G: Immunoglobulin A/G

ISP: Instituto de Salud Pública

LMICs: Low- and middle-income countries.

LRTIs: Low respiratory tract infection

MINSAL: Ministerio de Salud de Chile

nAbs: Neutralizing antibodies

NKs: Natural killer cells

NOD: Nucleotide-binding oligomerization domain

NTs: Neutralizing titers

PFUs: Plaque-forming units

PPD-A/B: Purified protein derivative from *Mycobacterium avium/bovis*

PRRs: Pattern recognition receptors

rBCG: Recombinant *Mycobacterium bovis* BCG

RLRs: Retinoic acid-inducible gene I-like receptors

SARS-CoV2: Severe Acute Respiratory Syndrome-Coronavirus 2

SH: Syrian Hamster

TB: Tuberculosis

$TCID_{50}$: Fifty-percent tissue culture infective dose

Th1: T helper 1

Th17: T helper 17

Th2: T helper 2

TLRs: Toll-like receptors

Treg: Regulatory T cells

VED: Vaccine-enhanced disease

WT: Wild Type

ABSTRACT

Respiratory diseases are among the top five causes of mortality and significant causes of morbidity and economic losses, affecting all age groups worldwide. Several infectious agents, mainly bacteria and viruses, are responsible for lower respiratory tract infections, including bronchiolitis and pneumonia episodes. In Chile and worldwide, the human respiratory syncytial virus is the leading etiological agent of low respiratory tract infections and childhood pneumonia, causing millions of hospitalization episodes, with children under five years at risk of severe disease. Additionally, the hantavirus cardiopulmonary syndrome, a zoonotic disease caused by Andes orthohantavirus infection, is a highly lethal cause of endemic respiratory disease in Chile and the Americas, mainly affecting people of working age. To date, no efficient preventive measures are available to prevent neither hRSV nor ANDV-caused respiratory disease. The immune responses elicited in the host upon infection by either hRSV or ANDV are pointed out as significant causes of lung damage that lead to life-threatening conditions, such as bronchiolitis and pulmonary edema, respectively. These immunopathological responses are characterized by dysregulated Th1/Th2 responses leading to an excessive inflammatory milieu in the lung that prevents an efficient antiviral immunity. It has been postulated that Th1 responses with cytotoxic activity and neutralizing antibodies are needed to avoid severe hRSV disease. In this line, a recombinant Bacillus Calmette-Guérin vaccine expressing the nucleoprotein of the hRSV (rBCG-N-hRSV) has shown to be protective against severe disease in mice, conferring efficient Th1-mediated protection against hRSV challenge. This vaccine has shown to be safe in human adult volunteers, generating Th1 immune responses to hRSV, however, it has not been evaluated in neonates, which is the intended age group for vaccination. In this study, we employed a homologous bovine neonate model of bovine RSV infection to test

this candidate vaccine for safety, immunogenicity, and efficacy to prevent severe RSV disease. Vaccination of newborn calves and with rBCG-N-hRSV was safe and well-tolerated, with neither systemic adverse effects nor evidence of vaccine-enhanced disease following bRSV challenge, suggesting that the vaccine is safe for use in neonates. Despite that the vaccinated calves had maternally derived antibodies, rBCG-N-hRSV administration increased virus-specific IgA and virus-neutralization activity in nasal fluid and increased the proliferation of virus- and BCG-specific $CD4^+$ and $CD8^+$ T cells in peripheral blood mononuclear cells and lymph nodes at 7dpi. Furthermore, rBCG-N-hRSV vaccinated calves developed a mild clinical disease as compared to unvaccinated control calves, although neither pathology nor viral burden were significantly reduced in the lungs. These results suggest that the rBCG-N-hRSV vaccine is safe in neonatal calves and induces protective humoral and cellular immunity against RSV and could be considered for infant immunization to prevent severe RSV disease. As a second project, we developed a new recombinant BCG vaccine expressing the ANDV Nucleoprotein. The hypothesis was that its administration would be safe in a preclinical BALB/c model and generate a Th1 cellular and humoral response, being suitable to be tested in other preclinical models. Administration of a 10^8 colony forming unit dose of the new rBCG-N-ANDV vaccine to mice was well tolerated, without adverse systemic effects. Vaccinated animals developed a localized reaction at the inoculation site, similar to those described after wild type BCG and rBCG-N-hRSV vaccination in mice. Further, the vaccine led to N-ANDV specific $CD4^+$ and $CD8^+$ T cell subset associated to a strong IFN-γ secretion in splenocyte cultures, suggestive of a Th1-biased response. An increase in N-ANDV specific IgG serum levels was observed in mice vaccinated with the recombinant formulation after two doses in comparison to pre-immune levels. Further, the new rBCG-N-ANDV vaccine candidate to prevent hantavirus

cardiopulmonary syndrome efficiently induces cellular and humoral immune responses in a preclinical model, supporting efficacy testing assays. Overall, our evaluation of recombinant BCG vaccines suggest that these are safe and immunogenic, and promising vaccine candidates to promote Th1 immunity against respiratory viruses, warranting further evaluation in proper preclinical and clinical stages.

RESUMEN

Las enfermedades respiratorias constituyen una de las cinco causas más importantes de mortalidad, morbilidad y pérdidas económicas mundialmente, afectando a personas de todas edades. Varios agentes infecciosos, principalmente bacterias y virus, son causantes de infecciones del tracto respiratorio bajo incluyendo episodios de bronquiolitis y neumonía. En Chile y a nivel mundial, el virus respiratorio sincicial humano (VRSh) es el agente principal de infecciones del tracto respiratorio bajo y neumonía en infantes, causando millones de hospitalizaciones al año, siendo los niños menores a cinco años quienes tienen mayor riesgo de enfermar gravemente. Por otra parte, el síndrome cardiopulmonar por hantavirus, una enfermedad zoonótica causada por Andes orthohantavirus, es una enfermedad respiratoria altamente letal en Chile y el continente americano, afectando principalmente personas en edad laboral. Actualmente no se cuenta con medidas preventivas eficaces para prevenir las enfermedades respiratorias causadas por VRSh y ANDV. La respuesta inmune causada por VRSh y ANDV en el hospedador es considerada una causa importante de daño pulmonar, que lleva a condiciones de riesgo vital, como bronquiolitis y edema pulmonar, respectivamente. Las respuestas inmunopatológicas asociadas se caracterizan por respuestas inmunes Th1/Th2 desbalanceadas que llevan a inflamación excesiva en el pulmón, y que no permiten la generación de una inmunidad antiviral efectiva. Se ha postulado que una inmunidad Th1 en conjunto con células citotóxicas y anticuerpos neutralizantes son necesarios para prevenir infecciones graves por VRSh. Al respecto, una vacuna Bacillus Calmette-Guérin recombinante que expresa la nucleoproteína de VRSh (rBCG-N-hRSV) ha mostrado ser protectora frente a VRSh en ratones, generando inmunidad Th1 virus-específica. Esta vacuna ha demostrado ser segura e inmunogénica en voluntarios adultos, sin embargo, no se ha evaluado en neonatos; la población

objetivo de esta vacuna. En esta tesis, usamos un modelo homólogo de infección con VRS bovino en terneros neonatos para evaluar la seguridad, inmunogenicidad y eficacia de esta vacuna candidata. La vacunación de terneros neonatos con rBCG-N-hRSV fue segura y bien tolerada, sin efectos adversos sistémicos ni evidencia de enfermedad aumentada por la vacunación luego del desafío con VRSb, sugiriendo que la vacuna es segura para uso en neonatos. A pesar de que los terneros tenían anticuerpos maternos circulantes, la administración de la vacuna generó niveles de IgA virus-específica y actividad neutralizante en muestras de fluido nasal, e incrementó la proliferación de células T $CD4^+$ y $CD8^+$ virus y BCG-específicos en células mononucleares de sangre periféricas y ganglios linfáticos a los 7 días post infección. Además, los terneros vacunados con rBCG-N-hRSV generaron una enfermedad clínica atenuada en comparación a terneros no vacunados, a pesar de que no hubo reducción significativa del daño pulmonar microscópico ni de la carga viral en pulmones. Estos resultados sugieren que la vacuna rBCG-N-hRSV es segura en terneros neonatos y confiere inmunidad protectora frente a VRS, pudiendo ser considerada para inmunización de infantes para prevenir la enfermedad severa por RSVh. En un segundo proyecto, generamos una nueva BCG recombinante expresando la nucleoproteína de ANDV, con la hipótesis de que cuya administración en un modelo preclínico murino BALB/c sería segura, y además capaz de generar inmunidad celular y humoral contra N-ANDV, siendo adecuada de evaluarse en otros modelos preclínicos. La administración de 10^8 unidades formadores de colonia de rBCG-N-ANDV fue bien tolerada por ratones, sin causar efectos sistémicos adversos. Los animales vacunados generaron una reacción focalizada en el sitio de inoculación, similar a aquella observada en ratones inmunizados con BCG wild type u otras BCG recombinantes. La vacunación generó poblaciones de células T $CD4^+$ y $CD8^+$ N-ANDV-específicas asociadas a

una potente secreción de IFN-γ por parte de esplenocitos cultivados, sugiriendo una respuesta inmune tipo Th1. Un aumento de IgG específica para N-ANDV fue observada en suero ratones inmunizados con la formulación recombinante luego de dos dosis, en comparación con los niveles pre-inmunes. Además, la vacuna candidata diseñada para prevenir SCPH es capaz de inducir respuestas celulares y humorales en un modelo preclínico, y es por lo tanto adecuada de evaluarse en eficacia en otro modelo preclínico. En conjunto, nuestra evaluación de vacunas BCG recombinantes sugieren que éstas son seguras, bien toleradas e inmunogénicas, siendo candidatas promisorias para generar inmunidad Th1 contra virus respiratorios, garantizando futuras evaluaciones en etapas preclínicas y clínicas adecuadas.

1. INTRODUCTION

1.1 THE GLOBAL BURDEN OF ACUTE RESPIRATORY VIRAL INFECTIONS

Acute respiratory diseases lead charts as the most frequent illnesses worldwide, affecting all age groups. The respiratory tract and the lungs are the internal structures most exposed to insults, infections, and injury due to the continuous exposure to particles, chemicals, microorganisms, and pathogens (FIRS, 2012; van Doorn & Yu, 2020). Due to the delicate homeostasis that the respiratory system needs to guarantee gas exchange and oxygen tissue perfusion, any compromise of its normal function can significantly impact health (Haddad & Sharma, 2019; Brinkman & Sharma, 2020). Thus, respiratory diseases are major threats to human health, one of the top five causes of mortality worldwide, and a significant cause of morbidity and economic losses (FIRS, 2012).

While most acute respiratory disease episodes are self-limiting and are restricted to the upper respiratory tract, a minor percentage might develop or progress to lower respiratory tract infections (LRTIs), including bronchiolitis and pneumonia episodes. Children and elderly people are at higher risk of suffering from respiratory problems, especially in low or middle-income countries (LMICs) (FIRS, 2012). In this line, childhood pneumonia is the leading cause of mortality in individuals under five years old, responsible for 15% of all deaths in that age group in 2017 (FIRS, 2012; van Doorn & Yu, 2020). The human Respiratory Syncytial Virus (hRSV) is the leading causal agent of LRTI and childhood pneumonia, with young infants in LMICs at risk of severe disease, and more than 30 million LRTI episodes in children worldwide. Despite decades of research, no FDA-licensed vaccine is available to prevent hRSV illness in any age group (Soto et al., 2020). Licensing vaccines to protect infants from severe hRSV

disease and to reduce morbidity in children is considered a global public health priority, and therefore vaccines should be affordable, and meet safety and efficacy standards (Neuzil, 2016; WHO, 2017).

Besides, several emerging pathogens have threatened human health worldwide during the last decades with significant incidence or lethality, including Middle East respiratory syndrome-coronavirus, severe acute respiratory syndrome-coronavirus, severe acute respiratory syndrome-coronavirus 2 (SARS-CoV2), pandemic influenza viruses, hantaviruses, and *Legionella* spp (Zumla et al., 2014; Herwaldt & Marra, 2018; Zhou et al., 2020; Saavedra et al., 2021). For most of these pathogens, no FDA-licensed vaccines are available, apart from new vaccines targeting SARS-CoV2 currently licensed for emergency use in several countries to contain the coronavirus disease 2019 (COVID19) pandemic (Kyriakidis et al., 2021).

Although field respiratory virus infections stimulate the immune system to develop defenses against future infections from a pathogen, those naturally induced responses often are unable to provide complete protection (Broadbent et al., 2015). The development of safe, effective vaccines that prepare the immune system to mount protective responses against pathogens without inducing immunopathogenic effects is therefore a key component of prevention of LRTIs (Broadbent et al., 2015). Vaccination is also critical to prevent or control disease outbreaks and potential epidemic - and pandemic – episodes (WHO, 2017), as exemplified by the 2009 swine flu and COVID19 pandemic vaccines.

1.1.1 Epidemiology of Acute Respiratory Infections in Chile

In Chile, acute LRTIs, are responsible for one-third of total hospitalizations in children ten years old or younger, being hRSV the leading cause (Zepeda et al., 2016). After analyzing the total positive confirmed cases of respiratory viruses, national surveillance programs for respiratory viruses in Chile during 2015-2020 reveals a predominance of hRSV, followed by Influenza A virus (IAV) infections, every year, except for 2020, in which the prevalence of respiratory viruses was heavily affected by the COVID19 pandemic (ISP, 2020). Human RSV infections account for around half of total positive cases during the 2015-2020 period, with the highest case incidence reported in the cold seasons (Figure 1A, B). Other relevant viral agents include IBV, human Metapneumovirus (hMPV), human Parainfluenza virus (hPIV), and Adenoviruses (Figure 1A, B) (ISP, 2020).

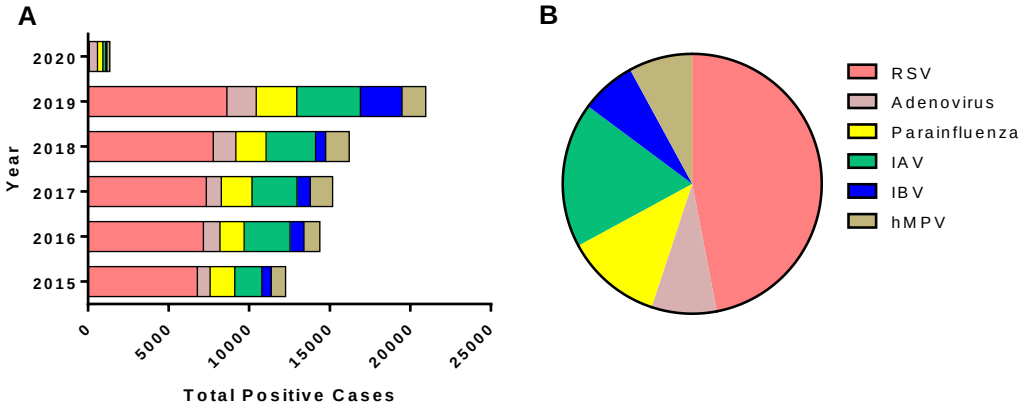

Figure 1. National Surveillance of circulating respiratory viruses in Chile, 2015-2020. A. Total positive cases of hRSV, Adenovirus, Parainfluenza, Influenza A and B virus and hMPV per year on the 2015-2020 period. B. Accumulated positive cases of each virus during the 2015-2020 period (ISP, 2020).

Besides the previously mentioned prevalent circulating respiratory viruses, another endemic viral agent of public health importance in Chile is Andes orthohantavirus (ANDV), the causative agent of the Hantavirus Cardiopulmonary Syndrome (HCPS) in Chile, and Argentina. The HCPS is a severe acute respiratory disease of seasonal presentation, first described in Chile in 1996, with a high case fatality rate that has historically oscillated between 30-40% (Figure 2) (MINSAL, 2020). Since 1996, more than 1000 cases of HCPS have been reported in Chile, with adults of productive age being the most affected group (MINSAL, 2020). In Chile, and due to its impact on public health and the need to prevent outbreaks, HCPS is listed in the group of communicable diseases whose immediate notification to health agencies is mandatory. To date, neither licensed vaccines nor specific treatments are available to prevent HCPS, and treatment of patients relies on supportive care (Saavedra et al., 2021). The global emergence of ANDV and hantaviruses in general evidences a need for further interventions to efficiently prevent their impact (Krüger et al, 2011; Saavedra et al., 2021).

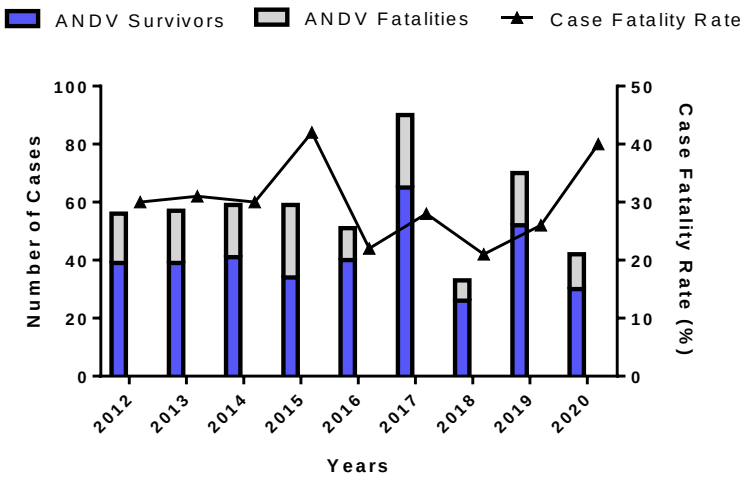

Figure 2. Confirmed cases, fatalities, and Case Fatality rates for HCPS in Chile, 2012-2020. (MINSAL, 2020).

1.2 THE HUMAN RESPIRATORY SYNCYTIAL VIRUS

1.2.1 Generalities and Epidemiology of hRSV

The human RSV (family Paramixoviridae, genus Pneumovirus), recently named human orthopneumovirus (Afonso et al., 2016), is a human pathogen that causes a major burden in public health, both in developing and in industrialized countries (Kuhdari et al., 2018; Nair et al., 2010; Simoes, 2003). hRSV is the leading cause of acute respiratory infection in newborns and severe LTRI in children, causing an estimated of 33.8 million LRTIs in children less than 5 years old each year and 3.4 million hospital admission (Nair et al., 2010). Importantly, this virus is a major cause of mortality in young children in developing countries. In 2015, it was estimated that 59,600 hospitalized infants younger than 5 years old have died from hRSV-related LTRD worldwide (Shi et al., 2017; Scheltema et al., 2018). hRSV-induced disease presentations range from cold-like symptoms to severe respiratory disease, with fever, dyspnea, cough, wheezing, bronchiolitis, and pneumonia (Uhari et al., 1995). Furthermore, severe hRSV infection is a predisposing factor for otitis media (Uhari et al., 1995) and has been associated with the development of asthma and recurrent wheezing (Silvestri et al., 2004; Sigurs et al., 2010). More recently, extrapulmonary symptoms that include central nervous system pathology and neurological signs have also been linked to hRSV (Bohmwald et al., 2018; Peña et al., 2020). Multiple hRSV genotypes categorized into two subtypes co-circulate in human populations with differential prevalence, facilitating repeated epidemics (Pangesti et al., 2018). By 2 years of age, all children have been infected at least once with hRSV and recurrent hRSV infections occur throughout life in adults (Glezen et al., 1986; Hall et al., 1991), which is one of the most important features of this virus. Relevant risk factors associated with developing severe

LRTI upon hRSV infection include prematurity, low birth weight, being male, having siblings, maternal history of smoking, history of atopy, deficient breastfeeding, and crowding (Shi et al., 2015). Despite its widespread impact, no licensed vaccines are currently available to prevent hRSV disease (Soto et al., 2020).

1.2.2 Structural and genome organization of hRSV

The hRSV is an enveloped, negative-sense, and single-stranded (-ss) RNA virus (Collins et al., 2013). The hRSV genome contains 10 genes (3′-NS1-NS2-N-P-M-SH-F-G-M2-L-5′) that encode for 11 proteins: the non-structural proteins 1 (NS1) and 2 (NS2) the nucleoprotein (N); the phosphoprotein (P); the matrix protein (M); the small hydrophobic protein (SH); the glycoprotein (G); the fusion protein (F), and the RNA-dependent RNA polymerase (RdRP) (L) (Figure 3). Additionally, the M2 gene contains two ORFs, one encoding for M2-1 and the other encoding for M2-2 (Collins et al., 2013). The hRSV attaches and merges with the cell membrane using the G and F proteins, respectively. The SH acts as a viroporin, modifying the host cell permeability (Fuentes et al., 2007). The M protein interacts with the viral envelope, playing essential roles in replication, virion budding and assembly. The N protein wraps the viral genome and forms a ribonucleoprotein complex with P and L proteins, required for viral replication and transcription (Ouizougun-Oubari et al., 2015). As discussed in the following sections, N is an important virulence factor that impairs the proper development of adaptive immunity. The NS1 and NS2 are small proteins that participate in the assembly of the viral envelope and are important virulence factors that impair the IFN-α/β signaling, which is crucial to prevent the activation of the host innate immune response (Hastie et al., 2012; Collins et al.,

2013). Through different mechanisms involving its different proteins, hRSV evades the host immunity to ease respiratory tract infection and virion spread.

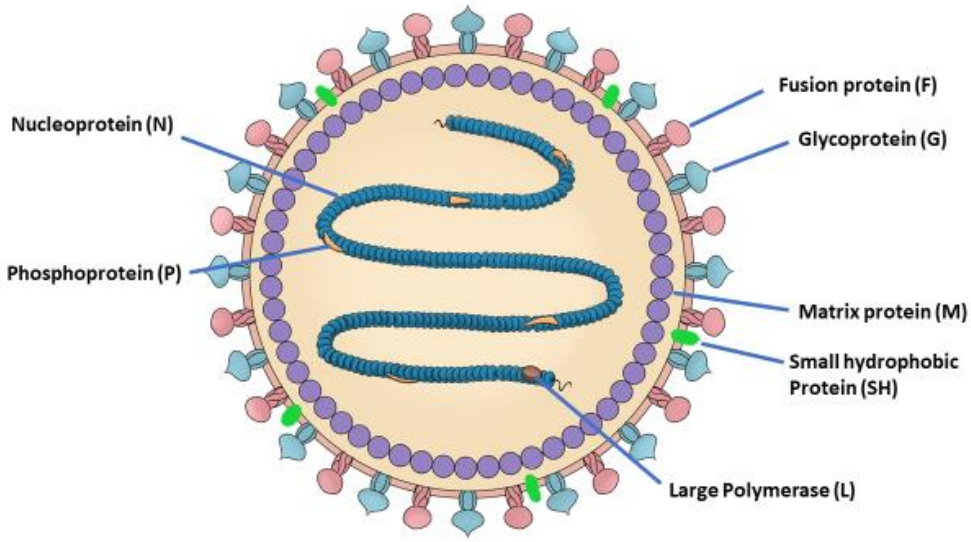

Figure 3. Schematization of the hRSV virion. The fusion protein (F), Glycoprotein (G), and Small Hydrophobic protein (SH) are transmembrane proteins. The internal proteins are: Matrix protein (M) and the nucleocapsid proteins, which include the Nucleoprotein (N), the Phosphoprotein (P), and the Large polymerase protein (L). Original Figure, adapted from Canedo-Marroquín et al., 2017.

1.2.3 Interplay between hRSV and the human host; early recognition and innate response

The primary target cells of hRSV are airway epithelial cells (AECs) (Zhang et al., 2002). Replication starts in the upper respiratory tract epithelium, and then in the bronchial and alveolar epithelium. *In vitro* studies indicate that hRSV predominantly replicates on apical ciliated cells of the stratified airway epithelium, leading to superficial damage and sloughing of the airway lining (Zhang et al., 2002). However, hRSV also infects immune cells, including macrophages, dendritic cells (DCs) (de Graaff et al., 2005; González et al., 2008), natural killer cells (NKs) (Van Erp et al., 2019), B cells, and T cells (Raiden et al., 2017), leading to different outcomes that impact of the viral-host interplay in a complex way. Notably, the lung pathology

experienced in severe hRSV is primarily due to an exacerbated immune response with infiltration of neutrophils and other immune cells, other than by direct damage from viral replication (Canedo-Marroquín et al., 2017; Griffiths et al., 2017).

Innate recognition of hRSV by epithelial and sentinel cells starts from engagement with pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors, driving an antiviral pro-inflammatory cytokine and chemokine response. Ligation of TLR4/CD14 increases expression of TLR4 and activates NF-kB, which is also activated upon recognition of dsRNA by TLR3 and RLR, and ssRNA by NOD2, TLR7, TLR8 (Kim & Lee, 2014). Lack of TLR4 in mice leads to persistent hRSV infection (Kurt-Jones et al., 2000). A type I IFN response driven by IRF3 and 7 activates the type I IFN receptor to activate interferon-stimulated genes and impair viral replication. However, NS1 and NS2 restrict the IFN response in AECs and antigen-presenting cells (APCs) through different mechanisms, easing a productive infection (Lo et al., 2005; Hastie et al., 2012). These proteins also restrict cell apoptosis at early infection times (Bitko et al., 2007). Additionally, BALB/c mice infected with recombinant hRSV lacking the NS genes have shown increased frequency of cytotoxic T cells (CTLs) in comparison to mice infected with WT hRSV (Kotelkin et al., 2006), suggesting that NS proteins also impair the CTL response. *In vitro* studies have also indicated that the hRSV N protein impairs the formation of the immunological synapse between APCs and T cells (González et al., 2008; Céspedes et al., 2014). The nucleoprotein expression in the DC surface impedes the clustering of peripheral MHC molecules, which is required for an efficient synapse. Thus, this virulence mechanism ultimately impacts T cell memory responses (Céspedes et al., 2014). Further, *in vitro* studies indicate that the G protein is another important virulence factor, that

impairs the AEC innate response to hRSV by blocking the CX3CL1-CX3CR1 interaction, which diminishes the I and III and TNF response from the host cell (Chirkova et al., 2013, 2015). Further, hRSV activates the NLRP3 inflammasome through the SH protein leading to IL-1β secretion (Triantafilou et al., 2013). As concluded from these studies, innate recognition and the modulation of immune responses impairs an efficient antiviral immunity, and are closely related to the pathogenesis of severe hRSV disease (Canedo-Marroquín et al., 2017), as discussed in the following sections.

1.2.4 hRSV-associated pathology and the host immune response

Airway obstruction associated with bronchiolitis and pneumonia is a severe consequence of hRSV infection and the immune response in the infant host. hRSV causes direct pathology *in vivo*, causing cell sloughing and airway obstruction through a mechanism involving the NS-2 (Liesman et al., 2014). However, the immunopathologic response elicited by the host is the main factor leading to lung damage and disease severity. Following natural hRSV infection, $CD4^+$ T cells promote an aberrant response where dysregulation of Th1 and Th2 responses are linked to immunopathology. Th17 responses have also been linked to excessive inflammation and neutrophil infiltration in infants and animal models (Stoppelenburg et al., 2013, 2014¸ McGill et al., 2019), however, evidence is still inconclusive.

Engagement of PRRs by hRSV triggers the secretion of pro-inflammatory cytokines, including IL-6, IL8, CXCL8, CCL3, CCL5, TNF and TSLP (Bohmwald et al., 2019). The early cytokine secretion is associated with massive infiltration of polymorphonuclear cells, along with the development of bronchiolitis and pneumonia. The secretion of IL-33 by AECs triggers IL-13, IL4 and IL-5, which are associated to a Th2 phenotype, by ILC2s, and promote eosinophil

recruitment, which is associated to ventilation requirement. These cytokines have also been associated with asthma exacerbation in infants (Griffiths et al., 2017). Massive airway inflammation is typically seen in severe cases, with up to 70-90% of neutrophil component (McNamara et al., 2003). Interleukin-8 secretion participates in the neutrophil recruitment and promotes release of neutrophil extracellular traps (Brinkmann et al., 2004; Vázquez et al., 2019), generating cellular debris and the free DNA that causes airway obstruction, along with the production of mucus. *In vivo* studies analyzing bronchoalveolar fluid (BALF) have shown increased transcripts and protein levels of IL-6, TNF-α, CXCL8, CCL3, CCL4, CCL2, and CCL5 in ventilated infants when compared to control groups (McNamara et al., 2004, 2005). Studies from infants with severe hRSV LRTI have shown that cytokines associated to a Th2 phenotype, including IL2, IL-4, IL-6, IL-9, IL-10, IL-13 are elevated in upper and lower respiratory tract samples (Bermejo-Martin et al., 2006; Bertrand et al., 2015). This unbalanced, Th2 skewed response, in the absence of a strong IFN response, is unable to mediate an effective antiviral immunity, and is otherwise associated with the severe disease and tissue damage, which has also been observed in mice models related to tissue damage (Bohmwald et al., 2019; Vázquez et al., 2019).

1.2.5. Adaptive responses to hRSV infection and correlates of protection

Adaptive responses and T cell immunity are considered essential to resolve an acute infection and to establish virus-specific immune memory (Openshaw et al., 2017). As stated before, hRSV natural infection generates a short-lived, waning immunity, and reinfections are common. In this line, *in vitro* studies indicate that both activation of neonatal DCs and the generation of a DC-T cell synapse are impaired during hRSV infection, leading to poor antiviral T cell

responses and immune memory (González et al., 2008; Lau-Kilby et al., 2020). CD4+ and CD8+ T cells are important for viral clearance, and play a role in mediating pathologic responses during hRSV infection, according to *in vivo* studies in mice and cotton rats (Christiaansen et al., 2014; Chiu & Openshaw, 2015). Following natural hRSV infection, CD4+ T cells promote an aberrant response where dysregulation of T helper responses is linked to immunopathology. Further, natural infection triggers weak CD8+ T cell responses. These CD8+ T cells have a critical role in hRSV clearance and in conferring protection from reinfection, as suggested by studies in animal models (Graham et al., 1991). In infants and animal models, higher frequency and number of CD8+ T cells have been observed after 7-10 dpi, concomitant with viral clearance and disease recovery (Taylor et al., 1995; Gaddum et al., 1996; Mcinnes et al., 1999; West et al., 1999; Heidema et al., 2007). In the bovine model of RSV infection, $CD8^+$ T cell depletion leads to more severe disease and prolonged viral shedding (Mcinnes et al., 1999). Less $CD8^+$ T cell infiltration has been observed in the lungs of deceased infants (Welliver et al., 2007). Th1 immunity developed in human adults have been linked to protective memory responses, and Th1 biased cellular immune response generated by some vaccines have shown to be protective against hRSV in mice, suggesting that Th1 biased T cell responses and IFN-γ driven responses might be suitable for an efficient antiviral immunity after hRSV infection, avoiding a harmful Th2 response (Bueno et al., 2008; Cautivo et al., 2010; Lambert et al., 2015; Openshaw et al., 2017).

Neutralizing antibodies also have an important role for protection against severe RSV infection, as exemplified by the anti-F neutralizing monoclonal antibody palivizumab, which reduces disease severity (Connor, 1998). However, natural RSV infection in infants generates a weak, transient primary IgG and IgA response that wanes within less than four months (McIntosh et

al., 1978, 1979; Sande et al., 2014). Moreover, the generation of antibody responses might be affected by circulating maternal antibodies (Murphy et al., 1986; Shinoff et al., 2008), which are not fully protective, but can reduce disease (Kimman et al.,1987; Kimman & Westenbrink, 1990). Importantly, mucosal RSV-specific IgA has been correlated to protection in both adults (Mills et al., 1971; Habibi et al., 2015) and infants (Tsutsumi et al., 1995), but serum NTs are considered only an indirect correlate of protection in adult volunteers (Openshaw et al., 2017). Nevertheless, serum antibodies might be more relevant for long-term protection since IgA and mucosal NTs wane. The development of a strong CTL and broad neutralizing antibody response is sought to develop vaccines to prevent hRSV infection in infants (Soto et al., 2020). Importantly, mechanistic studies in mice suggest that neutralizing antibodies are essential to dampen potential pathological roles of CTLs in RSV disease (Schmidt et al., 2020), highlighting the relevance of concomitant humoral and cellular response.

1.2.6. hRSV Vaccine development

Developing vaccines to tackle hRSV infection has been challenging, and no effective vaccines have been licensed to prevent severe RSV infection. During the 60's, a failed vaccine trial using a formalin-inactivated hRSV (FI-RSV) led to a vaccine enhanced disease (VED), in which seronegative infants exhibited increased disease severity upon subsequent natural hRSV infection compared to the controls (Kapikian et al., 1969; Kim et al., 1969; Acosta et al., 2016). After several studies on individuals and animal models, results suggest that the failure to elicit protective immunity was related to suboptimal antibody and memory $CD8^+$ T cells along with a strong inflammatory $CD4^+$ T cell activation (Olson et al., 2008; Delgado et al., 2009; Knudson

et al., 2015). The deleterious effects of FI-RSV vaccination were probably compounded with the naïve infant immune system to create a pathogenic immune response with a Th2-biased phenotype and deleterious T cell responses (Openshaw et al., 2017). Nowadays, promising vaccine candidates are being evaluated in clinical trials, including live-attenuated, recombinant vector-based, and subunit vaccines. The WHO lists two priority approaches to develop a high quality, safe and efficacious vaccine: (i) maternal immunization during pregnancy to prevent severe hRSV through passive antibody transfer, and (ii) direct infant immunization (WHO, 2017). Elderly people are also a critical target population and several vaccines have been designed to be applied in this population (Openshaw et al., 2017; Biagi et al., 2020). Infant immunization seeks to activate the host immune response directly; however, the immune system of very young infants might not be equipped to mount a robust and long-lived adaptive immune response (Openshaw et al., 2017; Blanco et al., 2018; Mazur et al., 2018). Besides, infant vaccination must overcome the possibility of interference from maternally-derived antibodies (MDA). On the other hand, maternal immunization is a promising strategy for protecting infants shortly after birth (Openshaw et al., 2017; Blanco et al., 2018; Mazur et al., 2018). Maternal immunization is being actively pursued in clinical trials, as discussed below. However, serum levels of MDA wane within a few months and might not be protective against hRSV (Yamazaki et al., 1994; Stensballe et al., 2009; Jans et al., 2017). Most of the current vaccine candidates in clinical trial seek to generate systemic IgG-mediated antibody responses, and therefore, might not be best for controlling viral transmission in the population (Openshaw et al., 2017). Multiple approaches will likely be required to reduce the global burden of infection, and candidate vaccines should be tested in proper animal models to elucidate their efficacy to prevent severe RSV disease in high-risk groups (Altamirano-Lagos et al., 2019; Biagi et al., 2020).

1.2.7. Animal models to study hRSV and the neonatal calf model

The hRSV is a human pathogen with a high specificity, and no animal reservoir recapitulating the disease is found in nature (Collins & Graham, 2008). Implementing diverse animal models for studying hRSV is much needed to develop immunotherapies and vaccines against hRSV (Hurwitz, 2011). Since no animal model reflects all aspects of this viral infection and disease, several models have been used to study hRSV (Taylor, 2017; Altamirano-Lagos et al., 2019). Rodent species, including the lab mice and the cotton rat have been relevant for carrying out mechanistic studies and testing candidate vaccines and immunotherapeutic drugs. However, these animals are only semi-permissive to hRSV infection, and do not adequately recapitulate LRTI and clinical signs found in humans (Taylor, 2017; Altamirano-Lagos et al., 2019). Cotton rats have been successfully employed to investigate VED (Prince et al., 1986; Sawada & Nakayama, 2016; Widjaja et al., 2016) and the effect of maternal immunity on the offspring (Prince et al., 1983; Blanco et al,. 2015). The study of pneumoviruses in their natural hosts has gained increasing attention lately due to the fully permissive replication of a virus in its primary host (Taylor, 2017; Altamirano-Lagos et al., 2019). Among them, bovine RSV (bRSV) infection in calves has been instrumental in studying pathogenesis and preventive interventions in infants. After bRSV infection, calves recapitulate hRSV disease seen in infants, with clinical signs such as fever, rhinorrhea, cough, serous or mucopurulent nasal discharge, abnormal pulmonary auscultation, tachypnea, and hypoxia (Sacco et al., 2012, 2014; Taylor, 2013). Disease severity is age-dependent, with calves being more prone to severe disease at 1-6 months of age (Hägglund & Valarcher, 2016). As in infants, Th2 biased immunity and neutrophil infiltration have been related to severe RSV disease (Taylor et al., 1989; Sacco et al., 2012; Blodörn et al., 2015; Hägglund et al., 2017). Moreover, calves also could present VED after vaccination, and

are therefore a suitable model to investigate potential vaccine-induced pathogenesis (Kimman et al., 1989; Schreiber et al., 2000; Kalina et al., 2004; Acosta et al., 2016; Altamirano-Lagos et al., 2019). Vaccine development against bovine and human RSV faces similar challenges, such as the need to vaccinate very young individuals in the presence of MDAs, and to overcome a Th2 neonatal bias (Chase et al., 2008; Ellis, 2017; Guerra-Maupome, et al., 2019). Successful strategies to overcome those major hurdles include mucosal vaccination and triggering cell-mediated immune mechanisms, i.e., by adjuvanted parenteral vaccines (Chase et al., 2008; Cortese, 2009). The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is a well-recognized immunogenic vaccine or adjuvant, being a potent stimulator of Th1 immunity in adults and newborns, triggering antigen-specific $CD4^+$ and $CD8^+$ T cells (Ravn et al., 1997; Marchant et al., 1999; Hanekom, 2005). Efficient cell-mediated immunity and IFN-γ secretion have been observed after s.c. BCG vaccination of calves as early as 8 h after birth (Buddle et al., 2003), and one-week-old BCG-vaccinated calves show cellular and IFN-γ responses to PPD-B comparable to adult animals (Nonnecke et al., 2005). Thus, BCG-based vaccines might be suitable vector to boost the immune system of young individuals towards a Th1 phenotype.

1.3. ANDES ORTHOHANTAVIRUS AND THE HANTAVIRUS CARDIOPULMONARY SYNDROME.

1.3.1. Generalities and Epidemiology of HCPS and ANDV

The HCPS is a endemic zoonotic disease in the Americas whose etiological agents are hantaviruses (Hantaviridae family, Bunyavirales order) of the New World (Jonsson et al., 2010; Saavedra et al., 2021). ANDV and Sin Nombre orthohantavirus (SNV) cause most of the cases in South and North America, respectively, but more than 20 pathogenic species have been described in the continent (Jonsson et al., 2010; Jiang et al., 2017). HCPS is characterized by an acute sere respiratory disease with case fatality rates of up to 20–40% (Alonso et al., 2019; Avšič-Županc et al,. 2019; Vial et al., 2019). After an incubation period of 10-40 days, and a short prodromal phase of flu-like signs, a significant proportion of cases develops acute respiratory insufficiency, with severe dyspnea and cough caused by a massive cell infiltration and vascular leakage in the alveolar niche (Vial et al., 2019). Cardiac insufficiency, hypotension and other systemic complications are present in severe cases (Vial et al., 2019). Hantaviruses are naturally hosted by rodents, showing a strong degree of host-virus specificity (Schountz & Prescott, 2014). Transmission to humans occurs after inhalation of contaminated droplets from rodent excreta, and risk factors of HCPS in South America include being male of working age, performing outdoor activities and living in rural areas (Jonsson et al., 2010; Vial et al., 2019). Despite that humans are usually dead-end hosts for these viruses (Krüger et al., 2011), person-to-person transmission leading to outbreaks has been described for ANDV (Wells et al., 1997; Martinez et al., 2005, Martinez-Valdebenito et al., 2014). In Chile, and due to its impact on public health and the need to prevent outbreaks, HCPS is listed in the group of communicable

diseases whose immediate notification to health agencies is mandatory (MINSAL, 2020). Worldwide, ANDV is categorized as an emerging virus that pose a threat to global health and security, with a potential use as a bioweapon (D'Souza & Patel, 2020). To date, neither licensed vaccines nor specific treatments are available to prevent HCPS, and treatment of patients relies on supportive care (Saavedra et al., 2021). The complex ecoepidemiology of the disease and the lack of preventive measures leads to sustained reports of lethality, mainly in South America, making preventive interventions a priority.

1.3.2. Structural and genome organization of ANDV

Andes orthohantavirus contains a segmented -ssRNA genome. The hantavirus virion is pleomorphic and enveloped, with a size around 70-160 nm (Goldsmith et al., 1995; Parvate et al., 2019). A large (L) viral genomic segment encodes an RNA-dependent RNA polymerase (RdRP), a medium (M) segment encodes a glycoprotein precursor (GPC), and a small (S) segment encodes a nucleoprotein (NP) and a non-structural protein (NS) (Figure 4) (Saavedra et al., 2021). The GPC is processed in the host cell to generate two glycoproteins, Gn and Gc. A tetrameric assembly of the Gn and Gc glycoproteins on the virus surface constitutes the spike complex that mediates cell entry and virus assembly (Tischler et al., 2005). The N protein is a multifunctional protein with the canonical function of protecting the RNA genome generating a panhandle structure with each of its three parts. It directly interacts with the RdRP and is critical to support genome transcription and replication. It also has RNA chaperone activity and acts as DNA nuclease (Reuter & Krüger, 2018). Importantly, N protein is an important virulence factor

in pathogenic hantaviruses and immune evasion through reverting the translational shutoff and early innate responses (Muyangwa et al., 2015; Reuter & Krüger, 2018). The ANDV NS protein also plays a role in impairing innate responses (Vera-Otarola et al., 2020) .

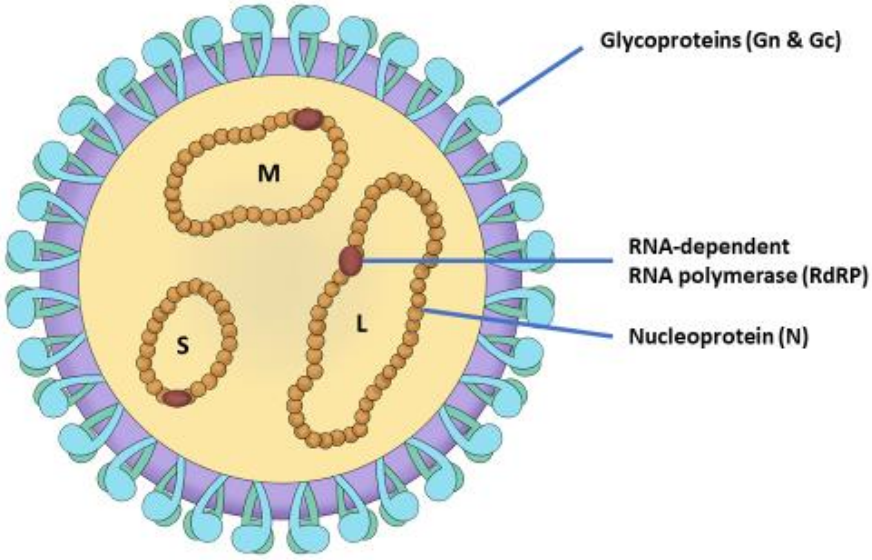

Figure 4. **Schematization of the ANDV virion**. ANDV has a tripartite -ssRNA genome, with the three segments spanning different sizes: A large (L) genome segment that encodes for a RdRP, A medium (M) segment encoding for a GPC that is processed to generate the Gc/Gc glycoproteins, and a small (S) segment that encodes for a nucleoprotein (N) and a small non-structural (NS) protein (Muyangwa et al., 2015) Original Figure, adapted from Swiss Institute of Bioinformatics.

1.3.3. Human host and ANDV interplay: immune mechanisms of pathology

The pathogenesis of HCPS is still not fully understood, but damage leading to AEC dysregulation and vascular leakage seems to be due to both direct viral effects and immunopathological responses (Vaheri et al., 2013). Hantaviruses replicate predominantly in microvascular ECs, macrophages and DCs (Jonsson et al., 2010). Little or no cytopathic effect is seen after viral replication (Temonen et al., 1993; Khaiboullina et al., 2000). Hantavirus replication elicits EC dysfunction in the vascular niche by increasing in the secretion of vascular

endothelial growth factor A that enhances vascular permeability and ultimately leads to pulmonary edema (Gavrilovskaya et al., 2012; Vaheri et al., 2013). Thrombocytopenia, leucocyte activation and release of pro-inflammatory cytokines and chemokines have also been implicated in pathogenesis (Vaheri et al., 2013).

Immune activation seems to be a key mechanism of pathology (Schönrich & Raftery, 2019; Raftery et al., 2020). Upon viral infection, PAMPs are recognized by different host cell receptors and subsequently activate innate immune responses. Scarce information is reported for ANDV, but *in vitro* studies indicate that hantaviruses are recognized by TLR3 activating the synthesis of alpha/beta interferon (IFN-α/β) and ISGs to fight the viral infection. RIG-I and MDA5 activate mitochondrial antiviral signaling protein (MAVS), inducing the expression of type I IFN and pro-inflammatory cytokines at the early stages of infection.However, ANDV presents various ways to inhibit both RIG-I and MDA-5 pathways. ANDV interferes with IRF3 phosphorylation and TANK-binding kinase 1 autophosphorylation through its N protein (Cimica et al., 2014), which also plays a role in impairs the translational shutoff by inhibiting PKR autophosphorylation (Wang & Mir, 2015). The NS protein interacts with MAVS decreasing its ubiquitination and downregulating the innate immune response (Vera-Otarola et al., 2020). Additionally ANDV, and other pathogenic hantaviruses modulate the IFN pathway through the cytoplasmic tail of the Gn protein, a feature not seen in non-pathogenic species) (Spiropoulou et al., 2007; Matthys et al., 2014). Despite the viral strategies to avoid the innate immune system, in the late stages of infection type I IFN production is enhanced in pathogenic hantavirus species (Raftery et al., 2020). Strong immune cell activation and secretion of pro-inflammatory cytokines (such as IL-1β, TNF and IL-6) and chemokines (such as CCL5 and CXCL10) (Mori et al., 1999; MacNeil et al., 2011) are likely responsible for a sustained

recruitment of mononuclear cells at the injury site, promoting a sustained pro-inflammatory state where cytokines and cytolytic activity contribute to EC damage and vascular leakage (Mori et al., 1999; Raftery et al., 2020) . Besides *in vitro* studies, clinical reports indicate that elevated pro- and anti-inflammatory Th1/Th2 cytokine responses are found in sera from HCPS cases (Borges et al., 2008; Maleki et al., 2019) and elevated numbers of cytokine-producing cells in lungs from HCPS fatalities (Mori et al., 1999). In the Syrian hamster (SH) model of lethal ANDV infection, a strong pro-inflammatory and Th1/Th2 response is observed in damaged lungs, further suggesting that immune activation is responsible for the pathology. In this line, regulatory T cell (Treg) responses have been discussed as relevant for HCPS pathology but the evidence is still inconclusive. In the SH model, Treg responses are strongly suppressed throughout the course of ANDV infection and disease (David Safronetz, et al., 2011, 2012). On the contrary, Tregs subset have been postulated as essential in limiting pathology in natural hantavirus reservoirs (Schountz & Prescott, 2014).

Despite further information and mechanistic studies addressing ANDV-induced HCPS are needed, these studies overall argue for a dysregulated immune response as a determinant of pathology in lethal HCPS infection.

1.3.4. Correlates of protection to HCPS

Hantavirus infection elicits a robust IgM response followed by an increase in serum IgG antibodies (López et al., 1997; Sjölander et al., 1997; Navarrete et al., 2007[1]. The initial antibody response seems to predominantly target the N protein (Lundkvist et al., 1993; Kallio-Kokko et al., 2001). High nAb titers have been correlated to protection in humans (Saavedra et al., 2021). High, long-lasting nAb titers have been detected years after SNV and ANDV infections,

implying a long-lasting immune response (Hörling et al., 1992; Valdivieso et al., 2006). In SHs, passive transfer of nAbs obtained from vaccinated rhesus macaques protect against lethal ANDV (Custer et al., 2003). In Chile, fresh frozen plasma from recovered individuals has been used as a therapy in patients with HCPS, however, this intervention lacks a proper standardization (Saavedra et al., 2021).

The role of T cells during hantavirus disease has not been properly elucidated. A study of deceased cases initially suggested that strong immune activation of CTLs is involved in capillary leakage during a severe clinical presentation. Immunoblasts are present in peripheral blood, and $CD3^+$ infiltrates are found in lung tissue from HCPS fatalities (Zaki et al., 1995). Furthermore, patients with severe HCPS by SNV infection might have higher specific circulating $CD8^+$ T cells than those with a mild course of the disease (Kilpatrick et al., 2004). However, these observations find no support on recent studies on the SH model of fatal ANDV infection, in which despite a $CD8^+$ T-cell increase in peripheral blood and lungs, no differences in severity or outcome are observed after T cell depletion, suggesting that T cells play neither pathological nor protective roles in the hamster model of fatal ANDV infection (Hammerbeck & Hooper, 2011). In contrast, $CD8^+$ T cells secreting granzyme B and IFN-γ have been associated with viral clearance in a case report. (Manigold et al., 2008). In HCPS and HFRS, studies suggest that CD8+ T cell responses following natural infection are strong and long-lasting (Van Epps et al., 2002; Manigold et al., 2010).

1.3.5. HCPS vaccine development

Currently, there are no FDA-approved vaccines or immunotherapeutics to prevent or treat HCPS. Considering the high fatality rate of HCPS, the development of protective vaccines for these syndromes is imperative. The high eco-epidemiological complexity of HCPS makes it

difficult to rely only on control measures to prevent virus spillover to humans (Saavedra et al., 2021). Vaccine development for hantaviruses has been troublesome due to several factors, including a lack of animal models that recapitulate critical aspects HCPS, the limited knowledge on the immunopathology and protective immunity mechanisms, and other obstacles inherent to clinical studies (Golden et al., 2015; Saavedra et al., 2021). Several strategies have been tested to develop a vaccine against one or more hantaviruses species, including purified recombinant proteins (Geldmacher et al., 2004; Maes et al., 2006), virus vectored antigens (Prescott, et al., 2014; Warner et al., 2019, 2020), virus-like particles, and nucleic acid-based vaccines (Hooper et al., 2006, 2013, 2014).

Several of those vaccine candidates elicit protection from severe disease or infection. However, evaluation of vaccine-induced long-lasting immune responses to HCPS have been scarcely evaluated (Dong et al., 2019; Saavedra et al., 2021). Since nAbs have been shown to confer protection against hantavirus-related diseases, the evaluation of immunogenicity of candidate vaccines has relied mainly upon the determination of NTs. However, evidence from diverse models and prototypes suggests that cellular immunity may be necessary for clearance and protective immunity against these viruses. During the development of a Puumala orthohantavirus (PUUV) DNA vaccine, cross-protection against DOBV and ANDV was observed without the detection of nAbs, suggesting a cell-based cross-protective mechanism. Furthermore, T cells, but not the neutralizing activity, have been associated with homologous and heterologous protection against hantavirus infection in mice, and CD8+ T cell responses seem to be required to provide protection (Nicacio et al., 2002). Regarding HCPS, protection in the absence of nAbs has been reported after immunization with adenoviral vectors encoding for Gn, Gc or NP (Safronetz et al., 2009). Moreover, a pan-hantavirus DNA vaccine

against Hantaan orthohantavirus (HTNV), PUUV, SNV and ANDV was reported to protect against ANDV infection despite low nAbs titers (Hooper et al., 2013). And recently, a study characterizing the immune response to the inactivated vaccine Hantavax revealed that vaccinated individuals presenting high NTs but not individuals with low NTs had a strong, broad, and concomitant cellular response to the vaccine (Khan et al., 2019). These observations suggest that T-cell immunity, particularly cytotoxic T-cell activity, can be important for generating protection and even cross-protection against hantavirus species. A vaccine boosting antiviral Th1-cell immunity might also be appropriate to balance the dysregulated Th1/Th2 response found after severe HCPS in humans and animal models (Borges et al., 2008; Safronetz et al., 2011; Maleki et al., 2019).

1.4. MYCOBACTERIUM BOVIS BACILLUS CALMETTE GUÉRIN: APPLICATION AS A RECOMBINANT VACCINE TARGETING RESPIRATORY VIRUSES.

The bacillus Calmette-Guérin is an attenuated *M. bovis* widely used as a vaccine against human Tuberculosis (TB) (Stover et al., 1991). It is the first vaccine routinely applied in humans and has an excellent safety profile. It can be administered at any time after birth, and is unaffected by maternal antibodies (Lotte et al., 1988; Stover et al., 1991). Upon immunization, the live bacilli persist *in vivo* without harmful effects to the recipient, potentially boosting immunity. In mice and human newborns, BCG induces Th1 immunity towards mycobacterial antigens, triggering $CD4^+$ and $CD8^+$ T cells and IFN-γ production by $CD4^+$ T cells. Several components of the BCG, including, cord factor, arabinogalactan, and arabinomannan, might play a role in inducing Th1 biased responses (Paik et al., 2010). Importantly, BCG vaccination overcomes the Th2 bias seen in newborn calves generating antigen-specific cell-mediated immunity and IL-2, IFN-γ secretion (Buddle et al., 2003; Skinner et al., 2005). Between 4 and 8 weeks following BCG vaccination, there is an increase in IgG production and induction of long-lived memory B cells (Chen et al., 2016; Sebina et al., 2012).

Remarkably, by generating recombinant BCGs (rBCG), this *M. bovis* strain has been extensively used as a vector to develop vaccines targeting viral pathogens, including measles virus (Fennelly et al, 1995), human immunodeficiency virus (Fuerst et al., 1991), influenza virus (Power et al., 2010), hRSV (Bueno et al., 2008; Cautivo et al., 2010) and hMPV (Palavecino et al., 2014). Cell-mediated immunity and Th1 responses have also been reported in experimental rBCG vaccines (Fuerst et al., 1991; Stover et al., 1991; Cautivo et al., 2010). These characteristics turn BCG into an attractive platform to elicit adaptive antiviral immunity. In this line, an rBCG

vaccine has been developed expressing the nucleoprotein of the hRSV (rBCG-N-hRSV) (Bueno et al., 2008). In a mice preclinical model, the immunization with rBCG-N-hRSV confers protection against hRSV challenge after eliciting a balanced Th1-mediated immune response, reducing both clinical pathology and neutrophil infiltration in the lungs (Bueno et al., 2008; Soto et al., 2018). Vaccination with rBCG-N-hRSV generates early recruitment of IFN-γ producing $CD4^+$, promotes isotype switching and enhances the production of nAbs, conferring protective humoral and cellular immunity (Cautivo et al., 2010; Soto et al., 2018). Interestingly, rBCG-N-hRSV vaccination promotes the secretion of antibodies targeting antigens other than the N protein upon infection likely through a linked recognition mechanism (Soto et al., 2018). which would account for a nAb response A GMP version of this vaccine has been shown to elicit Th1/Th17 immunity in mice and human adults, as recently reported (Céspedes et al., 2017; Abarca et al., 2020). This vaccine has been designed to be applied in newborns (Rey-Jurado et al., 2017). Therefore, studies on neonatal models are needed to evaluate the safety and immunogenicity of this vaccine in neonates.

Considering the current evidence regarding the immunopathology of hRSV and ANDV and the characteristics of the BCG-based vaccine model, we propose that the platform was ideal for developing an ANDV vaccine. Therefore, we aimed to create an rBCG-based vaccine that expressed the ANDV N protein (rBCG-N-ANDV). We hypothesized that the administration of the rBCG-N-ANDV vaccine elicited a Th1 cellular and humoral immunity towards ANDV antigens in a preclinical mice model without severe adverse reactions. Furthermore, based on the advances of the rBCG-N-hRSV vaccine in preclinical and clinical studies, we proposed that the rBCG-N-hRSV is a safe, efficient candidate vaccine to protect newborns and infants against

severe hRSV disease. If so, administering this vaccine in a bovine neonatal animal model should be safe, immunogenic, and protective against severe RSV disease. We, therefore, hypothesize that the administration of an rBCG-N-hRSV vaccine in neonatal calves is immunogenic and protects against bRSV disease.

2. HYPOTHESES

2.1 Hypothesis 1

Administration of an rBCG-N-ANDV vaccine elicits a long-lasting N-ANDV specific Th1 cellular and humoral response in a rodent model.

2.2 Hypothesis 2

Administration of an rBCG-N-hRSV vaccine in neonatal calves is immunogenic and protects against bRSV disease.

3. AIMS

3.1 General aims

3.1.1 To determine the safety, immunogenicity, and protective efficacy of the rBCG-N-hRSV vaccine in neonatal calves.

3.1.2 To elicit a long-lasting N-ANDV specific, Th1-biased immune response in a rodent model through the administration of an rBCG-N-ANDV vaccine.

3.2 Specific Aims for hypothesis 1

3.2.1 To determine the safety and immunogenicity of the rBCG-N-hRSV vaccine in neonatal calves.

3.2.2 To evaluate the efficacy of the rBCG-N-hRSV vaccine against bovine RSV infection in neonatal calves.

3.3 Specific Aims for hypothesis 2

3.3.1 To develop a recombinant BCG expressing ANDV nucleoprotein.

3.3.2 To evaluate cellular and humoral immunogenicity of an rBCG-N-ANDV vaccine in a mouse model.

3.3.3 To evaluate long-term cellular immunogenicity of an rBCG-N-ANDV vaccine in a mouse model.

4. CHAPTER I. A RECOMBINANT BCG VACCINE IS SAFE AND IMMUNOGENIC IN NEONATAL CALVES AND REDUCES THE CLINICAL DISEASE CAUSED BY THE RESPIRATORY SYNCYTIAL VIRUS

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4.1 ABSTRACT

The hRSV constitutes a major health burden, causing millions of hospitalizations in children under five years old worldwide due to acute lower respiratory tract infections. Despite decades of research, licensed vaccines to prevent hRSV are not available. Development of vaccines against hRSV targeting young infants requires ruling out potential vaccine-enhanced disease presentations. To achieve this goal, vaccine testing in proper animal models is essential. A recombinant BCG vaccine that expresses the Nucleoprotein of hRSV (rBCG-N-hRSV) protects mice against hRSV infection, eliciting humoral and cellular immune protection. Further, this vaccine was shown to be safe and immunogenic in human adult volunteers. Here, we evaluated the safety, immunogenicity, and protective efficacy of the rBCG-N-hRSV vaccine in a neonatal bovine RSV calf infection model. Newborn, colostrum-replete Holstein calves were either vaccinated with rBCG-N-hRSV, WT-BCG, or left unvaccinated, and then inoculated via aerosol challenge with bRSV strain 375. Vaccination with rBCG-N-hRSV was safe and well-tolerated, with no systemic adverse effects. There was no evidence of vaccine-enhanced disease following bRSV challenge of rBCG-N-hRSV vaccinated animals, suggesting that the vaccine is safe for use in neonates. Vaccination increased virus-specific IgA and virus-neutralization activity in nasal fluid and increased the proliferation of virus- and BCG-specific $CD4^+$ and $CD8^+$ T cells in PBMCs and lymph nodes at 7dpi. Furthermore, rBCG-N-hRSV vaccinated calves developed reduced clinical disease as compared to unvaccinated control calves, although neither pathology nor viral burden were significantly reduced in the lungs. These results suggest that the rBCG-N-hRSV vaccine is safe in neonatal calves and induces protective humoral and cellular immunity against this respiratory virus. These data from a newborn animal model provide

further support to the notion that this vaccine approach could be considered as a candidate for infant immunization against RSV.

4.2 INTRODUCTION

The hRSV is the leading etiological agent of acute lower respiratory tract infections in infants (Lozano et al., 2012), responsible for an estimated 3.4 million hospitalization episodes in children under 5 years of age each year (Nair et al., 2010). Clinical disease ranges from mild presentations, including rhinorrhea, coughing, and congestion, to respiratory distress, and life-threatening conditions characterized by alveolitis, bronchiolitis, and pneumonia (Collins & Melero, 2011). Importantly, hRSV is a significant cause of mortality in this age group, mostly in developing countries (Nair et al., 2010). Most children are infected by hRSV during the first three years of life, and reinfections are common (Glezen et al., 1986). Furthermore, severe hRSV disease is a predisposing factor for otitis media (Uhari et al., 1995) and has been associated with later health complications, such as development of asthma and recurrent wheezing (Sigurs et al., 2010; Silvestri et al., 2004). Besides, extrapulmonary symptoms, including central nervous system pathology and neurological signs have also been linked to hRSV infections (Sweetman et al., 2005; Bohmwald et al., 2018, 2021; Peña et al., 2020).

Despite the high health burden due to hRSV, no licensed vaccines are available to reduce or prevent the disease caused by this virus in infants (Soto et al., 2020). An early trial using a FI-RSV led to VED upon natural RSV infection in vaccinated volunteers, instead of generating protective immunity against the virus (Chin et al., 1969; Fulginiti et al., 1969; Kapikian et al., 1969; Kim et al., 1969). Increased hospitalization rate and two fatalities were observed after this

trial. Studies on different animal models have associated VED to a Th2 polarized immune response (Grahan et al., 1993; Connors et al., 1994; Waris et al., 1996; Knudson et al., 2015), a suppressed CTL response (Waris et al., 1996), and an inadequate antibody response (Polack et al., 2002; Delgado et al., 2009). However, the immunological mechanisms have been scarcely addressed in mechanistic studies. An essential role for $CD4^+$ T cells, but not eosinophils, has been recently demonstrated in *in vivo* mouse models. Interestingly, $CD4^+$ Th1 subsets appear to be responsible for airway obstruction and weight loss, while Th2 subsets account for mucus hypersecretion and airway hyperreactivity (Knudson et al., 2015). Despite that VED mechanisms are still under discussion, RSV vaccine candidates targeting infant populations require evaluating potential VED manifestations in animal models (Acosta et al., 2016). Importantly, these studies highlight the importance of a balanced cellular immunity to prevent immunopathology.

Along these lines, we have shown that a recombinant BCG expressing hRSV Nucleoprotein (N) (rBCG-N-hRSV) primes hRSV-specific $CD4^+$ T cells and $CD8^+$ CTLs that promote antiviral immunity, reduce neutrophil infiltration, and prevent lung damage in a mouse model of infection (Bueno et al., 2008; Céspedes et al., 2017). This vaccine generates a Th1/Th17 biased repertoire of virus-specific memory T cells that confer long-term immunity against hRSV (Bueno et al., 2008; Céspedes et al., 2017), with early recruitment of IFN-γ producing T cells into the lung (Cautivo et al., 2010). Furthermore, mice immunized with this vaccine developed a protective humoral response characterized by an isotype class switching towards IgG2a that correlates with viral clearance (Soto et al., 2018). Importantly, immunization with rBCG-N-hRSV manufactured under current Good Manufacturing Practices (GMP) is safe in mouse models, and induces no observable adverse effects (Céspedes et al., 2017). Moreover, recent phase I clinical

trial indicated that intradermal administration of doses up to 1 x 10^5 CFU of GMP rBCG-N-hRSV is safe in healthy adults (Abarca et al., 2020). Considering the extensively accepted safety and immunogenicity profile of the BCG vaccine in newborns (Stover et al., 1991), the rBCG-N-hRSV is intended for use on neonates to prevent severe hRSV infection (Rey-Jurado et al., 2017). However, since the mouse model is not ideal to rule out the possibility of VED (Acosta et al., 2016; Taylor, 2017), further studies employing suitable animal models are required to determine the safety of the rBCG-N-hRSV in target populations.

Bovine RSV is a significant cause of respiratory disease in cattle worldwide, as an agent of enzootic pneumonia in dairy calves and summer pneumonia in nursing beef calves (Sacco et al., 2012, 2014; Guerra-Maupome et al., 2019). Furthermore, bRSV infection is a predisposing factor to secondary bacterial infection and the development of Bovine Respiratory Disease Complex (Sacco et al., 2012, 2014; Guerra-Maupome et al., 2019). These conditions are highly prevalent and a major cause of mortality, as well as of economic losses due to reduced animal performance and costs associated with treatment and control measures (Sacco et al., 2012, 2014; Guerra-Maupome et al., 2019). Bovine and human RSV are similar at both genetic and antigenic levels, and calf bRSV infection displays many similarities to hRSV infection in humans, including seasonal periodicity, similar age-related susceptibility, gross and microscopic pathology, and innate and adaptive immune responses (Sacco et al., 2014; Guzman & Taylor, 2015). Severe RSV infection in infants and calves is characterized by an excessive, rapid neutrophil recruitment into lung, a delayed RSV specific $CD8^+$ T cells response, and a strong expression of Th2 cytokines (Heidema et al., 2007; Lukens et al., 2010; Mukherjee & Lukacs, 2010; Blodörn et al., 2015). Such an unbalanced immune response ultimately leads to lung damage and respiratory deficiency (Openshaw et al., 2017; Bohmwald et al., 2019). In addition,

RSV vaccine development needs to overcome similar challenges in humans and the bovine species, including the need to generate a robust immune response in a young population in presence MDA. Moreover, VED has been observed in calves after natural (Kimman, et al., 1989; Schreiber et al., 2000) and experimental bRSV infection (Gershwin et al., 1998; Antonis et al., 2003), characterized by a Th2-biased immune response, reduced $CD8^+$ T lymphocyte response and decreased IFN-γ production (Woolums et al., 2004).

The neonatal calf model is a relevant model for the infant immune system and has been extensively used to study antiviral and therapeutic compounds, and vaccines, including preclinical evaluation of candidate hRSV vaccines that contain proteins that are conserved between hRSV and bRSV (Taylor, 2017; Guerra-Maupome et al., 2019). Thus, RSV vaccine evaluation in a neonatal calf model might provide useful information for the study of vaccine candidates for infants (Acosta et al., 2016). Here, we evaluated the safety, immunogenicity, and protective efficacy of a GMP rBCG-N-hRSV vaccine (Céspedes et al., 2017) in a neonatal bRSV calf infection model. To parallel the newborn infant immunological scenario, we employed colostrum-replete calves that had circulating MDAs. Our results show that vaccination with rBCG-N-hRSV is safe, immunogenic, and partially protective in neonatal calves with MDA. We observed no systemic adverse reactions to the vaccine, and calves developed only minor and resolving vaccine-site reactions following rBCG-N-hRSV immunization. Calves vaccinated with rBCG-N-hRSV mounted virus-specific cellular and humoral immune responses as shown by increased virus-specific IgA and virus-neutralization activity and increased proliferative responses by $CD4^+$ and $CD8^+$ T cells. Further, rBCG-N-hRSV vaccinated calves developed significantly reduced clinical disease as compared to unvaccinated control calves; however, we did not observe differences in lung pathology or viral replication between vaccinates and

controls. Importantly, we observed no evidence of VED following bRSV challenge of rBCG-N-hRSV vaccinated animals, suggesting that the vaccine could be further evaluated for safety and efficacy in neonates.

4.3 MATERIALS AND METHODS

4.3.1 Animals and housing

Newborn (2-4 days of age), colostrum-replete, Holstein bull calves were enrolled in the study. All animals had total serum protein levels >5.7 g/dL as of one day after birth, and therefore had an early and adequate colostrum intake. Calves were obtained from a local dairy farm free of bovine TB, bovine viral diarrhea virus (BVDV) and bRSV. The animals were housed in BSL-2 climate-controlled environment rooms, at the Livestock Infectious Disease Isolation Facility, Iowa State University. Calves were fed commercial milk replacer and later starter concentrates and hay. Water was provided *ad libitum*. Animals were under supervision of a veterinarian throughout the entire study. All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC-18-232) and Institutional Biosafety Committee (IBC-18-076).

4.3.2 Vaccines

The rBCG-N-hRSV (Danish 1331 strain) vaccine (Bueno et al., 2008; Céspedes et al., 2017) used in both studies was produced under current Good Manufacturing Practices (cGMP) standards at IDT Biologika (Rockville, MD USA). A Wild type (WT) BCG, Danish 1331 strain, was used as immunization control in Study 2.

4.3.3 Immunization schemes

Calves were acclimatized for five days to the study environment prior to vaccination. For Study 1, calves were vaccinated subcutaneously (s.c.) in the right neck with 10^6 CFU of rBCG-N-

hRSV suspended in 500 µl of sterile saline (Figure 5). A control group of calves remained unvaccinated. Two weeks after the primary vaccination, a booster vaccination was administered in the right neck with 10^6 CFU of rBCG-N-hRSV. For Study 2, 8 calves were vaccinated s.c. with 10^6 CFU of rBCG-N-hRSV, while 8 calves were vaccinated with 10^6 CFU of WT BCG (Figure 5). A control group of 8 calves remained unvaccinated. In both studies, animals were monitored daily for body temperature and injection site reactions for 1 week following each vaccination.

Nasal fluid samples were collected at weekly intervals following vaccination, and on various days post challenge. Sterile 1-2-inch sponges were dampened with 1 mL of sterile saline solution, and then a single square was inserted into the nostril for 5-10 minutes. Then, sponges were removed, placed in a tube, and an additional 1 mL of sterile saline was added. Liquid was recovered from each sponge by squeezing in the barrel of a 5 mL syringe. The resulting nasal fluid was then aliquoted and frozen at -80° C for later analysis. Peripheral blood mononuclear cells (PBMCs) and sera were collected immediately before vaccination, at regular intervals following vaccination, and on days 3 and 7 after challenge.

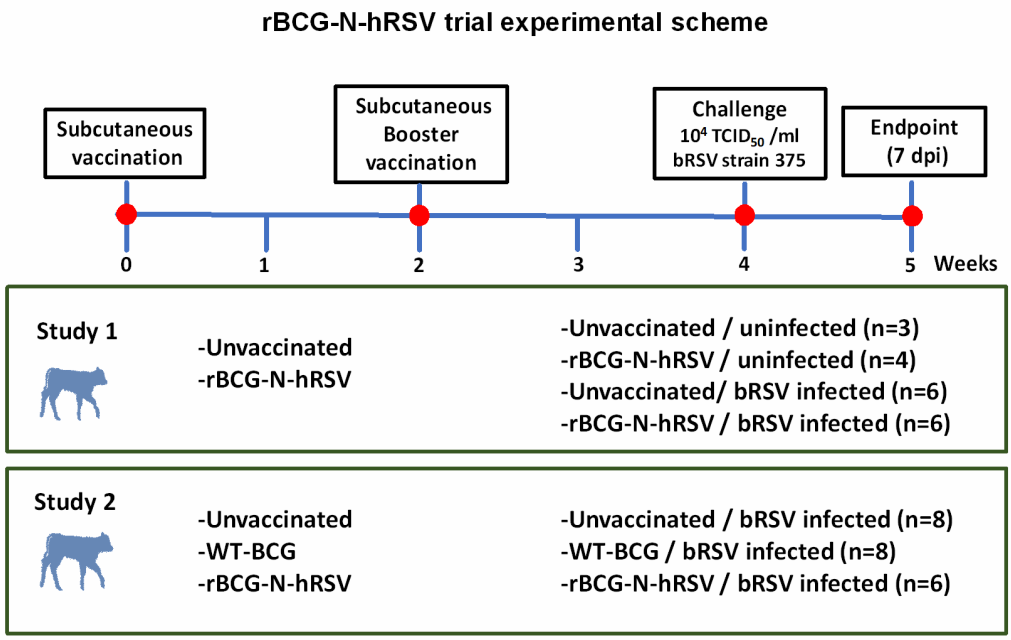

Figure 5. **Diagram for the experimental design for studies 1 and 2**. Newborn Holstein calves were vaccinated subcutaneously with GMP rBCG-N-hRSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Control calves were left unimmunized. After each immunization, animals were monitored for systemic alterations or local reactions to vaccination. Throughout the study, blood was collected weekly from the jugular vein. Fourteen days after the booster, calves were infected with bRSV strain 375 via aerosol inoculation. For Study 1, groups were: unimmunized, uninfected (n=3); rBCG-N-hRSV, uninfected (n=4); unvaccinated, bRSV infected (n=6); and rBCG-N-hRSV vaccinated, bRSV infected (n=6). For Study 2, groups were: Unvaccinated (n=8), WT-BCG vaccinated (n=8), and rBCG-N-hRSV vaccinated (n=6), being all animals bRSV-infected Animals were monitored and sampled daily after challenge to obtain blood and nasal fluid samples. All animals were euthanized 7 dpi for pathological evaluation and sampling.

4.3.4 Comparative Cervical Tuberculin tests

A Comparative Cervical Test (CCT) was performed on all animals 10 days after booster immunization. Briefly, 0.1 mL (1 mg/mL) of purified protein derivative (PPD) from *M. avium* (PPD-A) and of *M. bovis* (PPD-B) were injected in the neck skin of calves three days prior to infection. Then, the reaction size was measured with a caliper and registered as increase of skin thickness for both injection sites, 72h after antigen inoculation and before bRSV challenge. The

test and results were performed and interpreted according to the OIE Terrestrial Manual, Eight Edition.

4.3.5 bRSV inoculum and aerosol challenge model

BRSV strain 375 was prepared from virus stock re-isolated from the lung of an infected animal and passaged less than 4 times on bovine turbinate cells. The viral inoculum was determined free of contaminating BVDV by PCR. Two weeks after the booster vaccination, calves were inoculated via aerosol challenge with ~10^4 $TCID_{50}$/mL of bRSV strain 375 as previously described (Sacco et al., 2012).

4.3.6 Clinical Illness scoring

For each calf, clinical illness was scored once daily by a trained and blinded observer using an adaptation of the University of Wisconsin Calf Health Respiratory Scoring Chart, originally developed by Dr. Sheila McGuirk (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_respiratory_scoring_chart.pdf). The scoring chart assigns numbers (0-3) based upon fever and severity of clinical signs that include cough, nasal discharge, eye crusting, and ear position. For our scoring chart we included two additional categories for expiratory effort (0 = no effort to 3 = significant effort) and lung sounds by auscultation (0 = clear, 1 = wheezing and crackling). The scores for each category were totaled to determine the overall clinical score, being 18 the maximum possible score. Any calf with a score equal or over 3 in more than 3 categories for more than 72 hours was euthanized as humane endpoint. One unvaccinated calf was euthanized at 6 days post-infection (dpi) due to severe bRSV during Study 1.

4.3.7 Necropsy and Pathological Evaluation

Calves were euthanized on 7 dpi by barbiturate overdose. Draining tracheobronchial lymph nodes and lungs of were removed, and dorsal and ventral sides of lungs were photodocumented. Pathological evaluation was performed similar to previous descriptions (Sacco et al., 2012; Viuff et al., 2002). The extent of gross pneumonic consolidation was evaluated using the scoring system similar to that previously outlined (Viuff et al., 2002). A score of 0 was given to lungs free of lesions; 1 was given to lungs with 1-5% affected; 2 was given for 5-15% affected; 3 with 15-30% affected; 4 to lungs with 30-50% of consolidated tissue; and 5 for lungs >50% affected.

Bronchoalveolar lavage fluid (BAL) was obtained after introducing 500 mL of sterile, ice-cold saline solution through the trachea, and then pouring lavage fluid into a glass bottle. Samples of affected and unaffected lung tissue were collected from eight pre-designated sites for histopathological analysis. Tissues were fixed by immersion in 10% neutral buffered formalin and processed by routine paraffin-embedment and sectioning. Five μm sections were H&E stained. Microscopic lesions were evaluated by a board-certified veterinary pathologist in a blinded manner. The severity of the lung lesions was scored based upon the criteria we have previously established (McGill et al., 2018, 2019).

4.3.8 Real Time PCR analyses.

For quantification of bRSV NS2 copy number, lung samples from 2 representative gross-lesioned and 2 non-lesioned tissues from each calf were collected and stored in RNAlater (Invitrogen, Life Technologies). RNA was isolated from lung tissue samples using Trizol Reagent (Invitrogen, Life Technologies). Total RNA was placed in Qiagen RNA isolation columns for RNA clean-up and to remove any contaminating DNA using RNAse-free DNase,

per the instructions of the manufacturer (Qiagen). For nasal samples, viral RNA was isolated using MagMax Viral RNA Isolation Kit per the manufacturer's instructions (Applied Biosystem, Life Technologies. The quality and quantity of isolated RNA was verified by QuBit 4 Fluorometer (Thermofisher Scientific), and 500 ng of total RNA were used in each reaction. cDNA synthesis and quantitative rtPCR reactions were carried out using the Taqman RNA-to-CT 1-step kit (Applied Biosystems) per manufacturer's instructions using the following primers and probes: NS2 forward, 5'-GAACGACAGGCCACATTTA-3'; NS2 reverse, 5'-AGGCATTGGAAATGTACCATA-3'; NS2 probe, 5'-/56-FAM/TGAAGCTAT/ZEN/TGCATAAAGTGGGTAGCACA/3IABkFQ/-3'; RPS9 forward, 5'-GTGAACATCCCGTCCTTCAT-3'; RPS9 reverse, 5'-TCTTGGCGTTCTTCCTCTTC-3'; RPS9 probe, 5'-/56-FAM/AAGTCGATG/ZEN/TGCTTCTGCGAGTCC/3IABkFQ/-3'. The reactions were performed on a ThermoFisher QuantStudio 3 Real-Time PCR machine with the following cycling conditions: 48° C hold for 15 minutes; 95° C hold for 10 minutes; 40 cycles of 95° C for 15 seconds, then 60°C for 1 minute. Standard curves for NS2 and RPS9 genes were run in parallel with test samples, and all standards and test samples were run in triplicate. DNA sequences coding for nucleotides 1-706 of bovine RPS9, and nucleotides 524-1152 of bRSV NS2, both cloned separately into PCR2.1-TOPO vectors, were employed as templates for standard curve construction, respectively. Viral NS2 copy numbers were calculated using standard curves and normalized to RPS9 to correct for differences in lung tissue input.

4.3.9 Virus isolation

Samples from lesioned lung tissue were snap-frozen during necropsy and stored at -80° C until use. Nasal swabs were collected from each calf prior to infection and on various days post infection and placed in 500 µl virus transport media. Swabs were vortexed vigorously in the media, removed from the collection tube, and the supernatant was stored at -80° C. Samples were thawed once, and a 200 µl aliquot was removed for qPCR. The remaining volume was used for virus isolation, which were performed as previously described (Sacco et al., 2012) .

4.3.10 Serum and Nasal Fluid Neutralization assays

Serum and nasal fluid samples collected immediately before challenge and at 7dpi were submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa) for evaluation of bRSV-specific neutralization titers.

4.3.11 Antigen Recall Assays

Bovine peripheral blood was drawn from the jugular vein into syringes containing 2 × acid-citrate-dextrose solution. For isolation of PBMCs, blood was diluted 1:1 in PBS, and centrifuged for buffy coat fractions. Then, those fractions were centrifuged with Histopaque-1077 (Sigma-Aldrich) to obtain isolated PBMCs. Erythrocytes were removed incubating 5 minutes in warm RBC lysis buffer. Finally, cells were washed three times, counted, and resuspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine sera (FBS), 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 1% non-essential amino acids 2% essential amino acids, 1% sodium pyruvate, and 50 µM 2-mercaptoethanol (all from Sigma-Aldrich). Besides, BAL samples obtained during necropsies were kept on ice, filtered over sterile gauze, and centrifuged 10 minutes at 200g, 4 °C.

Erythrocytes were removed incubating 5 minutes in warm RBC lysis buffer. Also, tracheobronchial lymph nodes were collected, and cells obtained after disaggregating the tissue on cRPMI and passing the cell suspension through a 40 μm cell strainer. For all samples, cells were washed, counted, and resuspended in cRPMI. Then, PBMCs and lymph node cells were resuspended in 1 ml of PBS containing 10 μM of the CellTrace Violet (CTV) stain and incubated 20 minutes at 37 °C. Labeling was quenched by adding 4 volumes of RPMI, and washed two times with RPMI (Invitrogen, Life Technologies). For all samples, $5x10^6$ cells/mL were plated in round-bottom 96-well plates with 10 μg/mL Purified Protein Derivative from *M. bovis* (PPD-B); a cocktail of 10 μg/mL each recombinant Ag85A and TB10.4 (Lionex GmbH); 10 μg/mL each recombinant N from human RSV (N-hRSV); or a 0.1 MOI of bRSV strain 375. Negative control (mock) wells remained unstimulated. Positive control wells were stimulated with 5 μg/mL Concanavalin A (ConA). Plates were incubated for 6 days at 37° C in a 5% CO_2 incubator. Then, cell culture supernatants were stored at -80° C, and PBMCs immediately stained. PBMCs were suspended in FACS buffer (10% FBS and 0.02% NA-azide in PBS) and incubated 30 minutes at 4°C with 10 μg/mL of mouse anti-bovine CD4 and 10 μg/mL of mouse anti-bovine CD8α (clones ILA11A & BAQ111A respectively, both from Kingfisher Biotech, Inc). After washing, cells were incubated 25 minutes at 4°C with anti-mouse IgG2a-FITC and anti-mouse IgM-APC, and then fixed with BD FACS lysis buffer (BD Biosciences) for 10 min at RT, washed, and resuspended in FACS buffer. Cells were analyzed using a BD FACS Canto II (BD Biosciences) and FlowJo Software (Treestar). Percentages of cell proliferation were expressed over mock conditions.

4.3.12 ELISAs

Bovine IL-17A and IFN-γ were quantified using commercial bovine kits according to the instructions provided by manufacturer (Kingfisher Biotech, Inc). Indirect ELISAs were used to quantify IgA in the nasal fluid (Studies 1 and 2) and total IgG (Study 1) in serum. For the IgA quantification, 96-well ELISA plates were coated overnight at 4° C with 100 µl/well of bRSV stock (~10^4 $TCID_{50}$). Negative control wells were coated with 100 µl/well cell culture media prepared from uninfected BT. To disrupt mucus, nasal fluid samples were diluted 1:2 and treated with 10 mM dithiothreitol for 1 hour at 37° C prior to plating. Serum samples were diluted 1:1000. Plates were blocked using 150ul/well of 1% nonfat dry milk in PBS. All samples were plated in duplicates, incubated for 2 hours at RT, and then washed with 200 ul/well of 0.05% Tween 20 in PBS. Then, plates were incubated 1 hour at RT with either Mouse anti-bovine IgA-HRP (Bethyl Laboratories) at 0.5 µg/mL, or mouse anti-bovine IgG-HRP (Bethyl Laboratories) at 0.5 µg/mL. After incubation, plates were washed three times with 200 ul/well of 0.05% Tween 20 in PBS, and then developed using 50ul/well of Pierce 1-Step Ultra TMB Substrate (ThermoScientific Pierce). The reaction was stopped with the addition of 50ul/well 0.2 M H_2SO_4, and plates were read using a 450 nm wavelength, with a 540 nm reference wavelength, using an automated plate reader. For Study 2, bovine IgG1 was quantified using commercial Svanovir BRSV Ab kit (Svanova, Boehringer Ingelheim) according to the instructions provided by the manufacturer. Bovine IgG2 was quantified modifying Svanovir BRSV Ab kit, by incubating with sheep anti-bovine IgG2-HRP (Bethyl Laboratories) instead of provided secondary antibody reagent. All samples were plated in duplicate and included a negative control well.

4.3.13 Statistical analyses

For relative gene expression analyses, ΔΔCt values were used to calculate $2^{-\Delta\Delta Ct}$(Livak & Schmittgen, 2001), and results are shown as expression relative to uninfected control samples. Results are expressed as average ± standard error of the mean (SEM). Statistical significance was determined by two-way Analysis of Variance (ANOVA) or two-way ANOVA with repeated measures, followed by Sidak's multiple comparisons test using GraphPad Prism 7 software (GraphPad Software, Inc).

4.4. RESULTS

4.4.1. Vaccination with rBCG-N-hRSV in neonatal calves shows a good safety profile.

To evaluate the safety of the rBCG-N-hRSV vaccine in a neonatal calf model, animals were monitored daily for body temperature and injection site reactions for one week following each vaccination. Calves were vaccinated with the rBCG-N-hRSV vaccine or WT-BCG at 2-4 days of age and then boosted two weeks later. All animals were monitored daily for body temperature and injection site reactions for one week following each vaccination. During Study 1, minor injection site reactions were observed in 11 of the 12 rBCG-N-hRSV vaccinated calves and included minor swelling and hardening of the vaccination site. Vaccination site reactions resolved within 4-5 days. Following booster vaccination, injection site reactions were observed in all vaccinated calves and included thickening and hardening of the skin surrounding the injection site. Those reactions resolved within 7-10 days after vaccination. In study 2, only one rBCG-N-hRSV vaccinated calf developed minor swelling and hardening of the skin after the first immunization, which resolved within three days. No reactions were observed in WT BCG

vaccinated or unvaccinated calves. Furthermore, no significant body temperature changes were observed following booster vaccination in any animal (data not shown).

4.4.2. Vaccination with rBCG-N-hRSV ameliorates bRSV clinical symptoms without signs of enhanced disease.

Two weeks after the booster immunization, calves were challenged via aerosol inoculation with 10^4 $TCID_{50}$ bRSV strain 375. Control calves were not challenged. Following infection, all animals were monitored daily for body temperature and clinical signs, as described in the Materials and Methods section. During study 1, unvaccinated bRSV infected calves displayed significant clinical signs beginning on days 4-5 after infection, which included fever, lethargy, nasal and ocular discharge, dyspnea, and lung sounds (Figure 6A). One animal was euthanized on day six after infection due to severe clinical disease. Although calves immunized with rBCG-N-hRSV developed some signs of bRSV infection, disease and clinical scores were significantly reduced on days 4-7 pi, as compared to unvaccinated and infected calves ($p<0.05$ for day 4 and 8 pi, <0.001 for day 6pi and <0.0001 for day 5 pi) (Figure 6A). Unvaccinated and challenged calves presented fever starting day 4 pi, while rBCG-N-hRSV-vaccinated calves had no rise in body temperature (Figure 7A). During study 2, unvaccinated, bRSV infected calves also developed clinical signs, including fever, lethargy, nasal and ocular discharge, and mild dyspnea (Figures 6B, 7B) An increase in clinical score on days 6-7 was evidenced in unvaccinated calves when comparing scores at those days with day 0 (pre-challenge) ($p<0.05$). Some vaccinated calves also developed clinical signs; however, no significant rise in clinical score was observed at any day when compared to day 0. Similarly, no significant rise in clinical score was observed for WT BCG-vaccinated animals. Although some unvaccinated animals had fever, we observed

no statistically significant differences in body temperature between vaccinated and unvaccinated animals (Figure 7B). Importantly, we observed no signs of VED in calves receiving the rBCG-N-hRSV or the WT BCG vaccines.

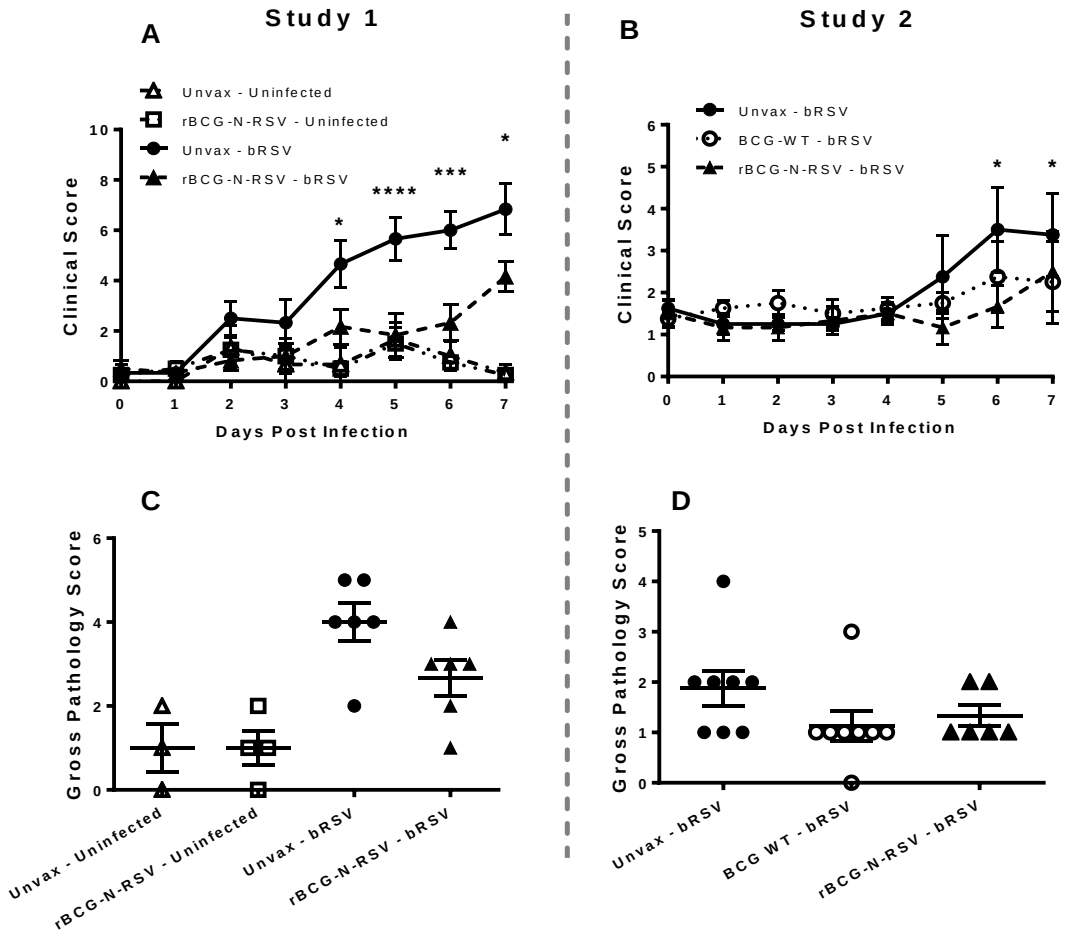

Figure 6. rBCG-N-hRSV vaccination reduces bRSV-associated disease in neonatal calves. Newborn calves were vaccinated with rBCG-N-hRSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Fourteen days after the booster, calves were infected with BRSV strain 375 via aerosol inoculation. (A, B) Clinical Scores. Calves in all four groups were monitored daily by a blinded observer and assigned a clinical score using the criteria outlined in Materials and Methods. Data represented as mean ± SEM. *$p<0.05$ ***$p<0.001$ ****$p<0.0001$ as determined by 2-way ANOVA with repeated measures and Sidak's multiple comparisons test (C, D) Gross Pathology Scores. All animals were humanely euthanized on day 7 post-infection. The extent of gross pneumonic consolidation was evaluated based upon the percent of lung affected. Aggregate gross pathology results from all groups and all animals from Study 1 and 2 are depicted in (C, D), respectively. Data represented as mean ± SEM. No statistically significant differences were observed.

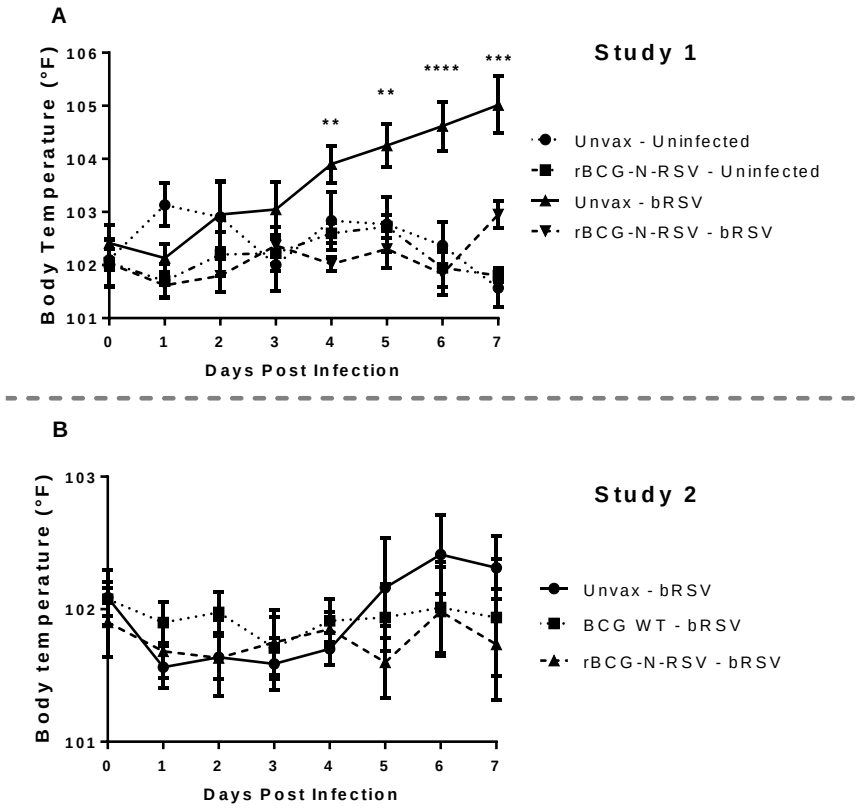

Figure 7. rBCG-N-RSV vaccination prevents fever in neonatal calves. Newborn calves were vaccinated with rBCG-N-RSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Fourteen days after the booster, calves were infected with bRSV strain 375 via aerosol inoculation. Body temperatures in F° for **(A)** Study 1 and **(B)** Study 2. Calves in all four groups were monitored daily by a blinded observer and assigned a clinical score using the criteria outlined in Materials and Methods. Data represented as mean ± SEM. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 as determined by 2-way ANOVA with repeated measures and Sidak's multiple comparisons test.

To determine the extent of macroscopic lung damage after bRSV infection, calves were euthanized seven days post-infection, and the lungs were evaluated and scored for gross pathology by a blinded veterinary pathologist, as described in the Materials and Methods section. No significant lesions were observed in the lungs of the uninfected control calves in study 1 (Figures 6C and Supp. Figure 1). Calves challenged with bRSV developed evident macroscopic lung pathology, including regional and coalescing areas of lung consolidation

(Figures 6C and Supp Figure 1). The extent of lesions in challenged calves was greater in the Study 1 as compared to Study 2. Although no significant differences in gross lung pathology were observed between unvaccinated control calves and those receiving the rBCG-N-hRSV vaccine, a clear reduction of lung damage was observed for the rBCG-N-hRSV-vaccinated calves in both studies (Figure 6C, D). Remarkably, most of the WT BCG-vaccinated animals in study 2 showed very little macroscopic pathology (Figure 6D). Additionally, samples of eight predesignated regions of the lung were collected and formalin-fixed during necropsy for histopathological evaluation. Lungs were sectioned and scored by a blinded veterinary pathologist. Few microscopic lesions were observed in the lung tissue samples collected from the uninfected calves (Figure 8A). On the other hand, and as expected, calves challenged with bRSV developed extensive histologic lesions, including airway inflammation and necrosis, bronchiolar luminal exudate, leukocyte, and lymphocyte infiltration, and pneumocyte hyperplasia. Overall, there were no significant differences in the lung histopathology scores between unvaccinated animals, WT BCG, or rBCG-N-hRSV vaccinated calves infected with bRSV (Figure 8A, B).

Severe RSV disease in newborn humans and calves is characterized by bronchointerstitial pneumonia and bronchiolitis, as well as significant airway neutrophil infiltration (Sacco et al., 2014; Altamirano-Lagos et al., 2019). The local host inflammatory reaction to the infection is a major cause of tissue damage (Zhang et al., 2002; Johnson et al., 2007; Ioannidis et al., 2012; Openshaw et al., 2017), with neutrophils pointed out as an important immunopathology source (McGill et al., 2019; Sebina & Phipps, 2020). Next, we evaluated whether vaccination with rBCG can modulate neutrophil infiltration at the site of infection. At necropsy, BAL fluid was

collected from each animal and cytospin preparations were differentially stained. The relative numbers of neutrophils, macrophages, lymphocytes, and eosinophils were then quantified by microscopy. As expected, bRSV infection increased neutrophil infiltration into the airways, and the frequency of neutrophils was increased in the BAL of challenged animals as compared to uninfected controls ($p<0.0001$) (Figure 8C). However, in both studies, rBCG-N-hRSV-vaccinated calves showed significantly reduced frequencies of neutrophils in the BAL at 7 dpi ($p<0.0001$) as compared to unvaccinated infected animals (Figure 8C, D). Remarkably, a mild, significant reduction in neutrophil frequency was observed for WT BCG-vaccinated animals when compared to unvaccinated animals ($p<0.05$) (Figure 8D). In both studies, a lower relative frequency of macrophages was observed in unvaccinated, challenged animals when compared to vaccinated calves ($p<0.05$, <0.001 for study 1 and 2, respectively) (Supp Figures 2A and E). While no differences in relative lymphocyte frequency were observed between any group in Study 1, significantly higher relative lymphocyte counts in rBCG-N-hRSV-vaccinated animals were seen in study 2 as compared to unvaccinated controls ($p<0.001$) (Supp Figure 2B and F). Finally, no differences were seen in eosinophil infiltration between groups (Supp Figure 2C and G).

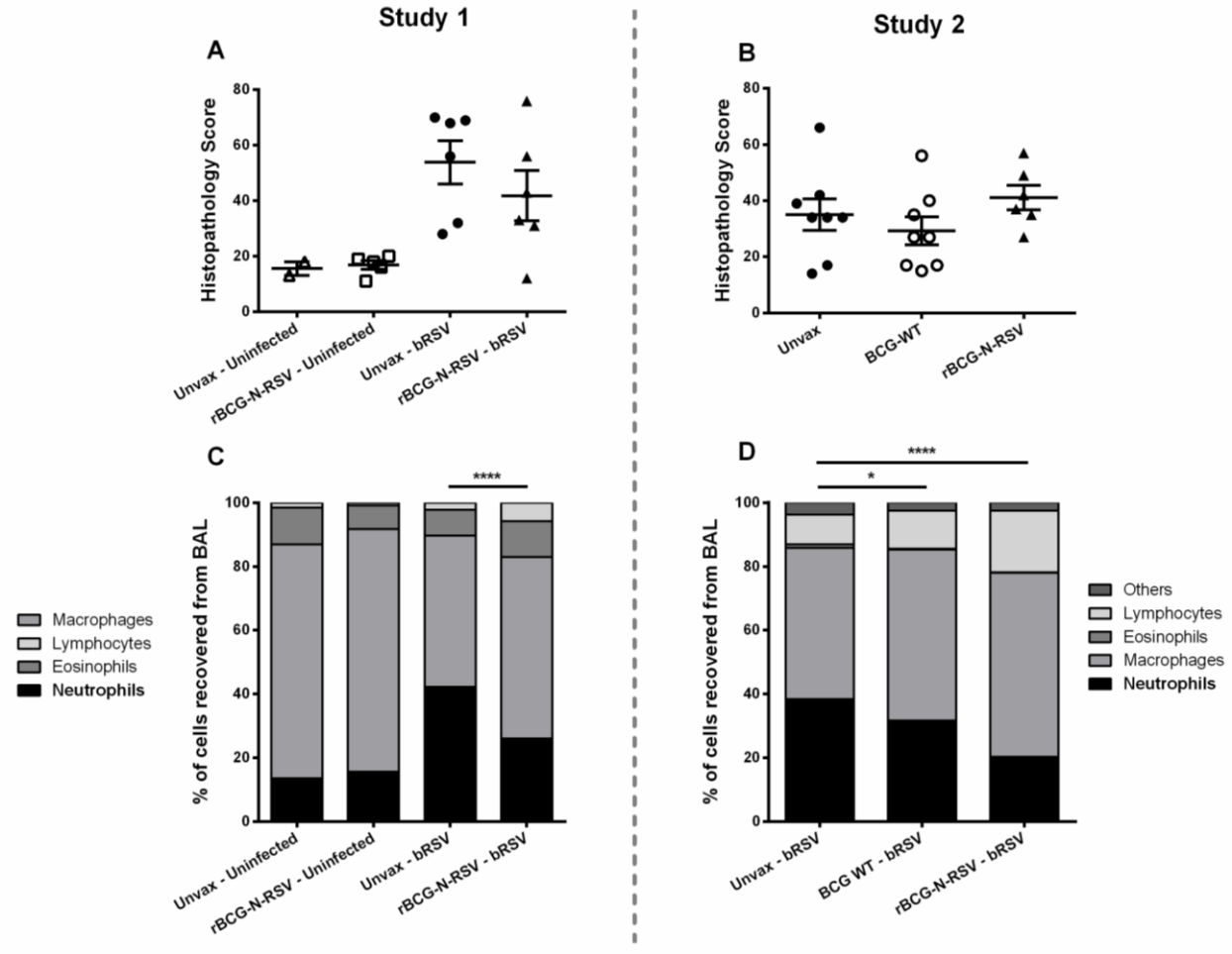

Figure 8. rBCG-N-hRSV vaccination reduces neutrophil BAL infiltration in neonatal calves after bRSV infection. (A, B) Lung Histopathology Score. Sections of lungs were collected from 8 predesignated locations and microscopic lesions were evaluated by a pathologist in a blinded manner using a scoring system we have previously described. Aggregate histopathology scores from animals in Study 1 and 2 are depicted in (A, B), respectively. Data represented as mean ± SEM. No statistically significant differences between infected groups were observed. (C, D) BAL cells relative frequency. On day 7 post-infection, BAL samples were collected, and cytospins prepared. The cells were differentially stained with Modified Wright stain. The number of neutrophils, macrophages, lymphocytes, and eosinophils were determined by microscopy. Data are depicted as mean relative frequencies of each population. Data represented as mean ± SEM. *$p<0.05$ ****$p<0.0001$ for the frequencies of neutrophils as determined by 2-way ANOVA and Sidak's multiple comparisons test.

4.4.3. Viral shedding and viral lung loads are not reduced in rBCG-N-hRSV vaccinated calves.

Nasal swabs and lung tissue were collected and immediately frozen, then processed for virus isolation as previously described (Sacco et al., 2012). As shown in Table 1, no virus was isolated from the nasal swabs of any calf prior to challenge. Following bRSV infection, virus was isolated from the nasal swabs of most infected animals throughout the infection period, regardless of vaccination status. For Study 1, bRSV was isolated from lung tissue samples in 6/6 calves in the unvaccinated infected group and from 4/6 rBCG-N-hRSV vaccinated, infected calves on day 7 after infection. Regarding Study 2, the virus was isolated from lung tissue of all unvaccinated and WT BCG-vaccinated animals and in 4/5 rBCG-N-hRSV vaccinated animals (Table 1). Quantitative PCR analyses for the bRSV NS2 gene revealed no statistically significant differences between vaccinated and unvaccinated infected calves for the copy number of NS2 neither in lesioned lung tissue (Figure 9A, B) nor in viral shedding (Figure 9C, D). Neither virus nor NS2 copies were detected, in uninfected control calves (Table 1, and Figure 9). These results suggest that neither rBCG-N-hRSV nor WT BCG significantly modulate virus replication in the lower and upper respiratory tract during neonatal calf bRSV infection.

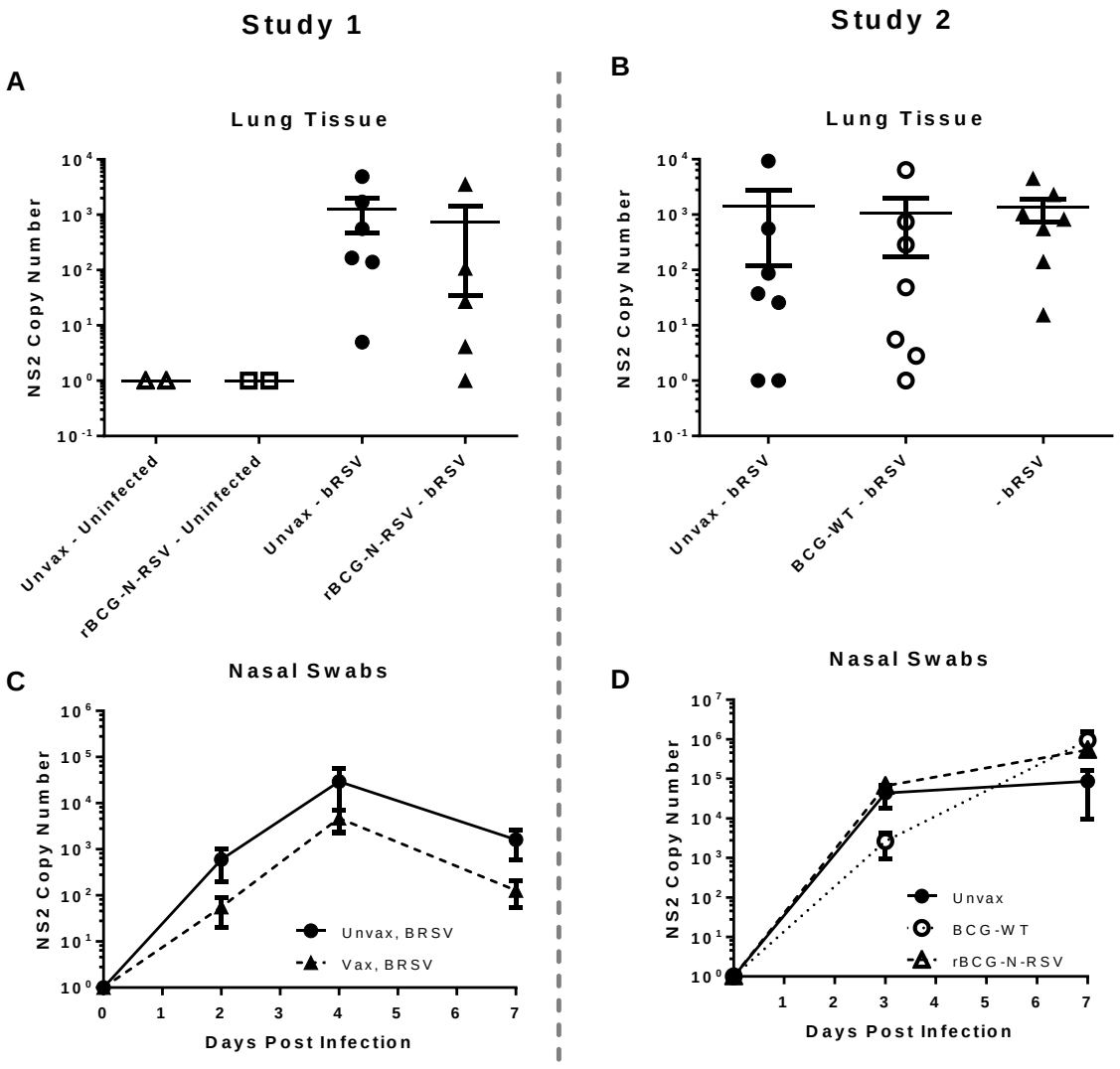

Figure 9. Viral loads and nasal shedding in lungs and nasal samples. Nasal swabs were collected from each calf prior to infection and on various days post infection. Lesioned lung tissue was collected at necropsy. Samples were divided and analyzed for virus isolation and virus quantification by qPCR for the bRSV NS2 gene. Viral NS2 copy numbers were calculated using standard curves. For nasal swabs, 500 ng of isolated RNA were used in the qPCR reactions. Lung tissue were normalized to the housekeeping gene, RPS9, to correct for differences in input material. NS2 copies presented as mean (range) of each group. Neither virus nor NS2 copies were isolated or detected, respectively, in uninfected control calves and are therefore not shown. No significant differences were observed between unvaccinated, WT BCG and rBCG-N-hRSV vaccinated animals as determined by RM 2-way ANOVA and Sidak's multiple comparisons test.

Group	Study 1					Study 2		
	Nasal Swabs				Lung	Nasal Swabs		Lung
	Day 0	Day 2pi	Day 4pi	Day 7pi	Day 7pi	Day 3pi	Day 7pi	Day 7pi
Unvaccinated - bRSV	0/6	6/6	5/6	4/6	6/6	7/7	7/7	7/7
rBCG-N-RSV - bRSV	0/6	4/6	4/6	3/6	4/6	6/7	6/6	4/5
WT BCG - bRSV	-	-	-	-		7/7	7/7	8/8

Table 1. **Virus isolation in the nasal swabs and lungs from infected calves**. Nasal swabs were collected from each calf prior to infection and on various days post infection. Lesioned lung tissue was collected at necropsy. Samples were divided and analyzed for virus isolation and virus quantification by qPCR for the bRSV NS2 gene. Virus isolations were performed as previously described (Randy E. Sacco, Nonnecke, et al., 2012). No significant differences were observed between unvaccinated, WT BCG and rBCG-N-hRSV vaccinated animals as determined by RM 2-way ANOVA and Sidak's multiple comparisons test.

4.4.4. Vaccination with rBCG-N-hRSV induces antigen-specific $CD4^+$ and $CD8^+$ T cells secreting Th1/Th17 cytokines upon bRSV challenge

To evaluate the effect of rBCG-N-hRSV vaccination on adaptive cellular immunity after calf bRSV infection, PBMCs and lymph node cell cultures were collected on day seven pi, labeled with CTV stain, stimulated with viral or mycobacterial antigens for six days, and then analyzed by flow cytometry. Antigen-specific $CD4^+$ and $CD8^+$ cells were identified by CTV dilution after proliferation in response to viral or mycobacterial antigens. Background (mock) proliferation was subtracted from all values, and results represent change over mock (Supp Figure 3). For PBMCs obtained from Study 1, we observed no statistically significant differences in $CD4^+$ or $CD8^+$ T cell proliferation between both infected groups when stimulated with viral antigens. Although trends to increased proliferative responses were observed for $CD4^+$ T (Figure 10A)

and $CD8^+$ T (Figure 10C) in response to N-hRSV and bRSV in the vaccinated/bRSV infected calves, increased T cell proliferation was also observed in samples from vaccinated uninfected animals (Figure 10A and C). Besides, $CD4^+$ T cells from calves receiving the rBCG-N-hRSV vaccine responded robustly to both PPD-B and the Ag85A/TB10.4 antigen cocktail (Figure 10A). As expected, $CD4^+$ T cells from the unvaccinated/bRSV infected calves did not divide in response to stimulation with PPD-B or Ag85A/TB10.4 (Figure 10A). Similar trends were observed for $CD8^+$ T cells, with the highest responses observed in samples from the rBCG-N-hRSV-vaccinated and challenged calves (Figure 10C). To evaluate if rBCG-N-hRSV vaccination promotes a Th1/Th17 phenotype in calves, as reported in the murine and human studies (Abarca et al., 2020; Bueno et al., 2008; Céspedes et al., 2017), cell culture supernatants from the stimulated PBMCs were analyzed by ELISA for bovine IFN-γ and IL-17A. Compared to the PBMCs from unvaccinated/bRSV infected animals, rBCG-N-hRSV-vaccinated/bRSV infected animals mounted a significant IFNγ (Figure 11A) and IL-17A (Figure 11B) response to both BCG- and RSV-associated antigens including PPD-B, Ag85A/TB10.4, N-hRSV, and BRSV strain 375. Some cytokine responses were seen in unvaccinated, bRSV infected calves, although those responses were not statistically significant when compared to unstimulated control wells (Figure 11). All samples produced robust amounts of IFNγ and IL-17A in response to conA, which was used as a positive control (not shown). Additionally, BAL samples were stimulated with mycobacterial and viral antigens for 6 days and supernatants analyzed by ELISA for bovine IFN-γ and IL-17A. As shown in Figure 12, rBCG-N-RSV-vaccinated, infected animals produced significantly higher IFN-γ in comparison to unvaccinated, infected animals ($p<0.05$), however, the enhanced IL-17A levels in response to PPD-B and AG85A/TB10.4 did

not reach statistical significance. Both infected groups produced similar levels of IFN-γ and IL-17A in response to viral antigens at 7 dpi (Figure 12).

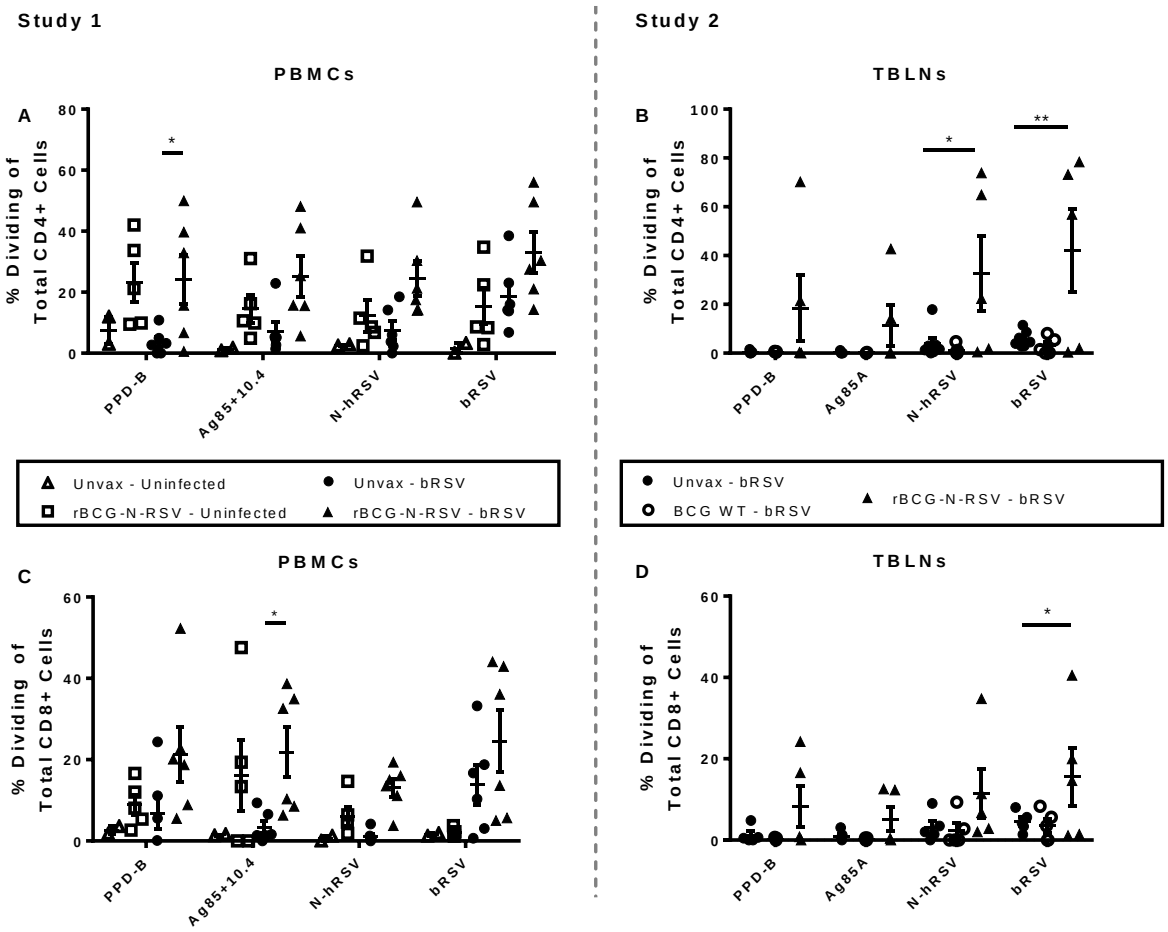

Figure 10. Vaccination with rBCG-N-hRSV elicits antigen-specific CD4 and CD8 T cell proliferative responses. (A, C) Study 1 PBMCs and (B, D) Study 2 Tracheobronchial lymph node cells (TBLNs) were isolated on day 7 after infection, labeled with Cell Trace Violet, and restimulated *in vitro* with PPD-B, Ag85A/ TB10.4, N-hRSV or bRSV strain 375. Mock stimulated cultures were used as negative controls. ConA stimulated cultures were used as positive controls (not shown). Six days later, (A, B) $CD4^+$ T and (C, D) $CD8^+$ T cell proliferation, as measured by dilution of the Cell Trace dye, was analyzed by flow cytometry. Data represented as mean ± SEM *p<0.05 **p<0.01 as determined by 2-way ANOVA and Sidak's multiple comparisons test.

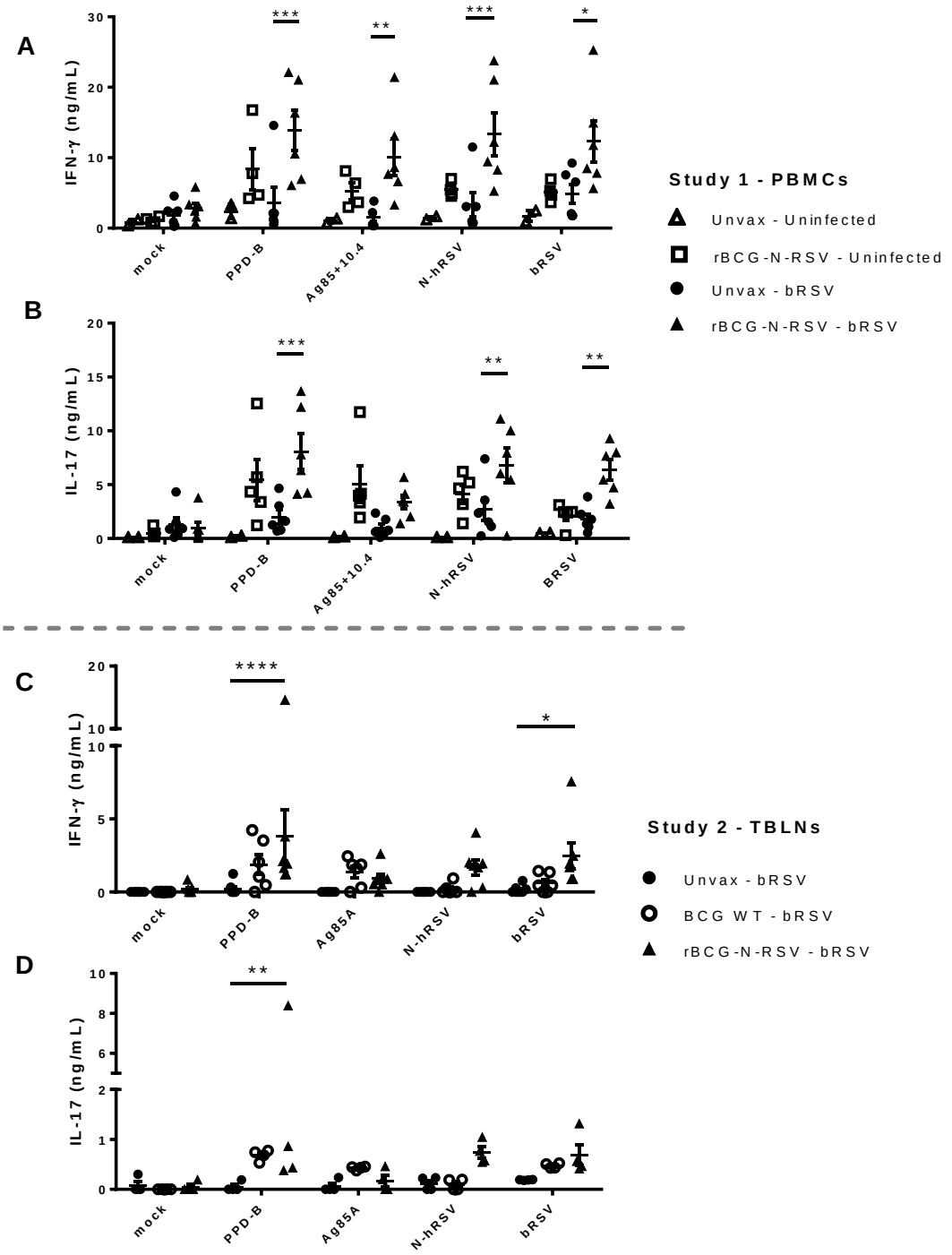

Figure 11. Vaccination with rBCG-N-hRSV increases virus and BCG-specific IFN-γ and IL-17 secretion in PBMC cultures. Assays were performed as in Figure 10. Cell culture supernatants were analyzed for bovine IFN-γ and IL-17A secretion using commercial ELISA kits. *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001 as determined by Two-way ANOVA and Sidak's multiple comparisons test. Data represented as mean ± SEM.

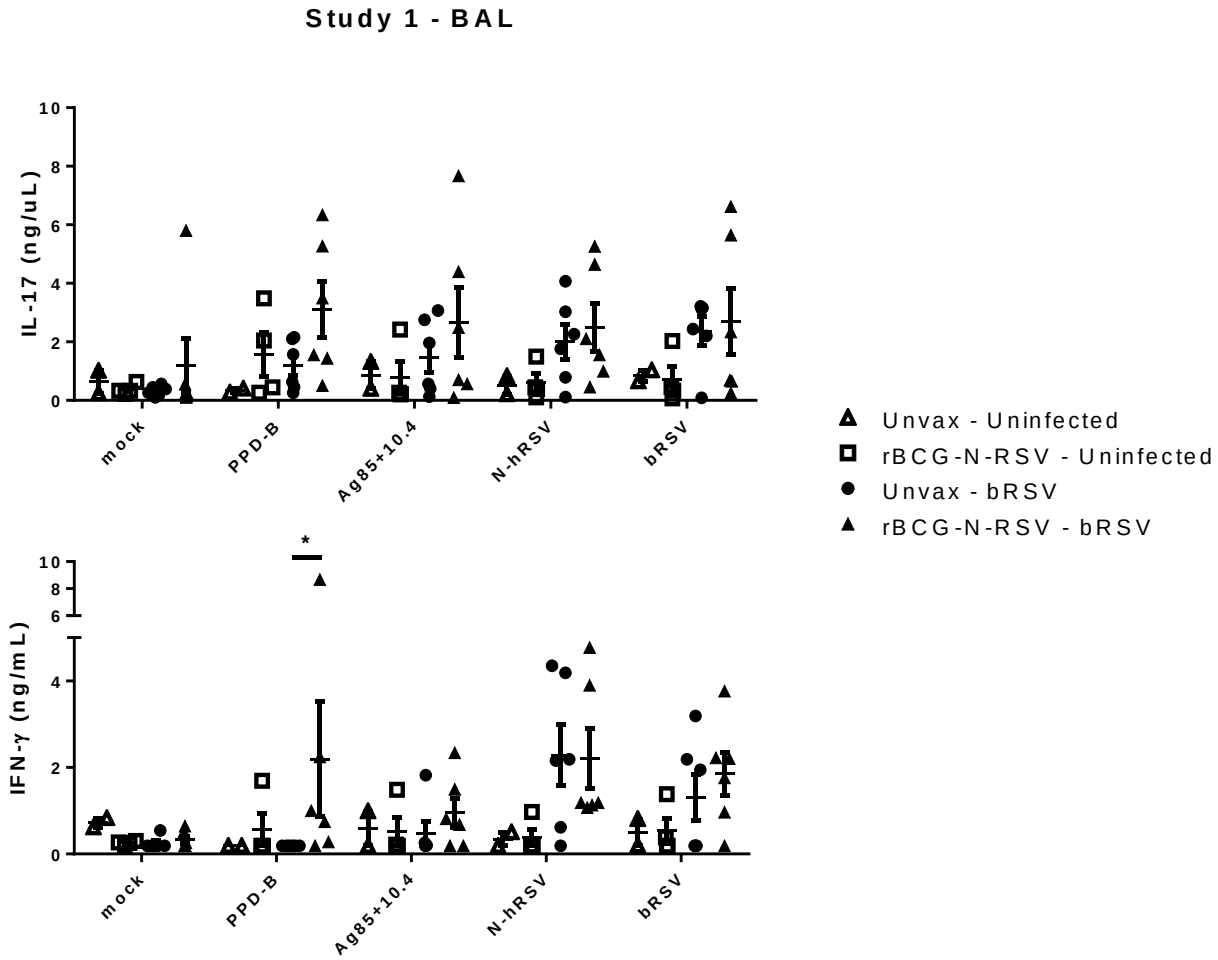

Figure 12. **Mycobacterium and virus-specific cytokine production by BAL cells**. On day 7 post infection, BAL samples were collected and stimulated with PPD-B, Ag85A/TB10.4, N-hRSV or bRSV for 72 hours. Cell culture supernatants were analyzed for concentrations of bovine IFN-γ and IL-17A. *p<0.05 as determined by 2-way ANOVA and Sidak's multiple comparisons test.

For the second study, we analyzed proliferative responses of $CD4^+$ and $CD8^+$ T cells, and IFN-γ and IL-17A secretion in tracheobronchial lymph node cell cultures, performing assays as described above. $CD4^+$ T cells from vaccinated calves showed robust proliferation after recall stimulation with N-hRSV ($p<0.05$) or bRSV ($p<0.01$) in comparison to the unvaccinated control group (Figure 10B). Differences in proliferation after PPD-B or Ag85 stimulation between vaccinated and unvaccinated control groups were not statistically significant. As expected,

$CD8^+$ T cell responses were also robustly induced after recall stimulation with bRSV in lymph node cells from rBCG-N-hRSV-vaccinated animals ($p<0.05$) but not unvaccinated or WT BCG-vaccinated control animals (Figure 10D). Although statistically significant differences were not found when comparing rBCG-N-hRSV-vaccinated calves to unvaccinated controls, a higher proliferation of $CD8^+$ T cells was observed in rBCG-N-hRSV-vaccinated calves upon recall PPD-B and N-hRSV stimulation (Figure 10D)**.** Importantly, cells from all calves included in data analyses showed a robust proliferative response to Con A positive control (not shown). Furthermore, rBCG-N-hRSV-vaccinated animals also showed a significantly higher IFN-γ response to PPD-B ($p<0.0001$) and to bRSV ($p<0.05$) stimulation (Figure 11C), which was not seen on WT BCG and unvaccinated animals (Figure 11C). A similar trend was observed in N-hRSV-stimulated wells, although differences did not reach statistical significance (Figure 11C). Regarding IL-17A, a robust response to PPD-B ($p<0.01$) was seen only in rBCG-N-hRSV-vaccinated calves (Figure 11D). In summary, these results indicate that vaccination with rBCG-N-hRSV induce antigen-specific $CD4^+$ and $CD8^+$ T cells associated with a Th1/Th17 secretory phenotype upon bRSV infection in neonatal calves with MDA.

4.4.5 Humoral immune responses to vaccination and bRSV challenge in neonatal calves.

To analyze the humoral immune responses induced by rBCG-N-hRSV vaccination, nasal fluid and serum samples were collected at several time points to analyze virus-specific IgA and IgG, respectively. In the Study 1, virus-specific IgA was undetectable in the nasal fluid from any group at baseline (prior to vaccination) or immediately prior to infection (Figure 13A). Virus-specific IgA remained below the limit of detection in uninfected control calves throughout the study. By day 7 post-infection, unvaccinated infected calves were beginning to show a virus-specific IgA response in the nasal tract. However, this increase was not statistically significant

as compared to the pre-challenge values or to uninfected controls. On the other hand, rBCG-N-hRSV-vaccinated calves developed an anamnestic virus-specific IgA response in the respiratory tract, evidenced by significantly higher levels of virus-specific IgA in the nasal fluid as compared to all other groups ($p<0.0001$) (Figure 13A). Analyses of total virus-specific IgG revealed a similar trend, but the differences between groups were not statistically significant (Figure 13B). In Study 2, an increase in IgA levels was observed in WT BCG- and rBCG-N-hRSV-vaccinated calves but not in unvaccinated controls when comparing pre-infection levels to 7dpi, however, this difference was not statistically significant (Figure 13C). Comparisons between groups at 7dpi showed no differences in IgA levels. In Study 2, virus-specific IgG1 and IgG2 were measured. No differences between groups at any time point were observed for virus-specific IgG1 serum levels. It is noteworthy that IgG1 levels were higher in the unvaccinated and WT BCG-vaccinated group at baseline, as compared to later time points, and as expected, no rise in IgG1 serum levels was seen in those groups at 7dpi (Figure 13D). Although a similar trend was observed in rBCG-N-hRSV-vaccinated calves, no significant changes between any time point were observed in this group. In contrast, rBCG-N-hRSV-vaccinated calves showed significantly higher serum levels of IgG2 at 7dpi ($p<0.01$) when compared to unvaccinated animals, whereas unvaccinated calves showed a decreasing trend in IgG2 levels when comparing baseline to 7 dpi levels (Figure 13E). Importantly, animals in both studies were colostrum replete, which is likely the main factor contributing to the higher IgG1 and IgG2 serum levels at baseline.

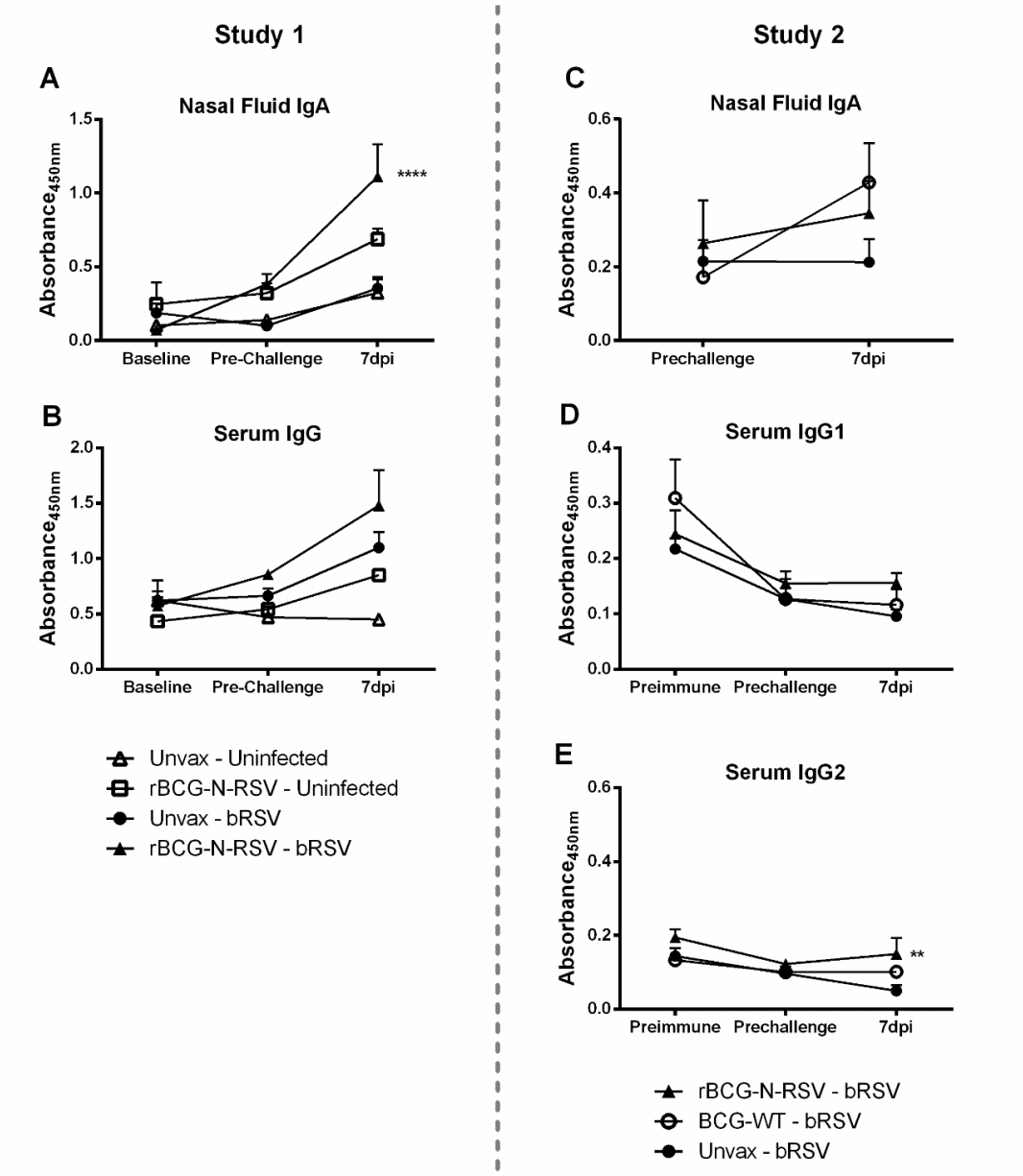

Figure 13. rBCG-N-hRSV vaccinated calves mount an amnestic virus-specific IgA and IgG2 response in the respiratory tract and peripheral blood, respectively. (A, C) Nasal fluid samples were collected and analyzed for bRSV-specific IgA by indirect ELISA prior to immunization (baseline), at pre-challenge, and on day 7 postinfection. Data represented as mean ± SEM. No significant differences were detected in IgA levels at baseline and pre-challenge between any group. ****$p<0.0001$ between rBCG-N-hRSV-vaccinated – bRSV and unvaccinated – bRSV groups on day 7 post infection as determined by 2-way ANOVA followed by Sidak's multiple comparisons test. (C, E) Serum samples were collected and analyzed for IgG (B, Study 1), IgG1, and IgG2 (D, E, Study 2). **$p < 0.01$ between rBCG-N-hRSV-vaccinated – bRSV and unvaccinated – bRSV groups on day 7 post-infection as determined by 2-way ANOVA followed by Sidak's multiple comparisons test.

Virus neutralization assays revealed that all groups, regardless of treatment, had similar pre-challenge titers of neutralizing antibodies in nasal secretions (Table 2). however, neutralizing titers (NTs) increased significantly in the nasal fluid samples from rBCG-N-hRSV-vaccinated animals following bRSV challenge in both studies ($p<0.05$) (Table 2). Interestingly, WT BCG vaccinated calves also had higher NTs from nasal samples when compared un unvaccinated animals ($p<0.01$). On the other hand, a trend in the increase in NTs in the serum of the rBCG-N-hRSV vaccinated calves was observed in Study 1, but this increase was not statistically significant, which is along the lines with virus-specific IgG serum levels. No differences in serum NTs were observed in study 2 when comparing different groups or time points.

Group mean NT (range)	Nasal Fluid NTs				Serum NTs				
	Study 1		Study 2		Study 1		Study 2		
	Day 0	7dpi	Day 0	7dpi	Day 0	7 dpi	Baseline	Day 0	7 dpi
Unvaccinated - Uninfected	3 (2-4)	2 (2)	-	-	24 (8-32)	12 (8-32)	-	-	-
rBCG-N-RSV - Uninfected	5 (2-8)	3.33 (2-8)	-	-	22 (8-32)	18 (8-32)	-	-	-
Unvaccinated - bRSV	4 (2-8)	17.3 (8-32)	2.5 (2-4)	6 (2-16)	17.3 (8-32)	48 (32-64)	52 (32-64)	42 (16-64)	66 (16-128)
rBCG-N-RSV - bRSV	5.3 (4-8)	**85.3*** (64-128)	6 (2-8)	**10.6*** (8-16)	26-6 (16-32)	74.6 (64-128)	60 (32-128)	34 (16-64)	53 (16-128)
WT BCG - bRSV	-	-	3.3 (2-8)	**12.6**** (8-16)	-	-	68 (32-128)	42 (16-64)	61.3 (32-128)

Table 2. **Virus neutralization titers measured in nasal fluid and serum on baseline (pre immune), day 0 (prior to challenge) and day 7 post infection.** Two-way ANOVA followed by Sidak's multiple comparisons test, *p<0.05, **<0.01 compared to unvaccinated - bRSV group.

4.5 DISCUSSION

Human RSV causes a high impact on health systems worldwide annually, being responsible for millions of hospitalizations and hundreds of thousands of deaths due to acute low respiratory tract infections in high-risk populations, which include infants, elderly and immunocompromised patients (Nair et al., 2010; Shi et al., 2017). Despite more than five decades of research, no vaccine has been licensed to prevent RSV infection in any age group. While several vaccine strategies are under development and clinical testing for either pregnant women, infants, and the elderly (Mazur et al., 2018; Biagi et al., 2020), the development of efficacious vaccines targeting specific age groups faces different challenges at both pre-clinical and clinical levels. RSV-associated morbidity and mortality are higher in infants under one-year-old in low-income countries; thus children remain a critical target population to implement therapeutic and preventive measures (Shi et al., 2017; Stein et al., 2017). Importantly, vaccines for this age group must be able to elicit robust, long-lasting immunity in a population that is not

well equipped to do so and might present significant levels of circulating MDA (Hurwitz et al., 2011; Blanco et al., 2018; Mazur et al., 2018). Additionally, vaccines should avoid VED after natural infections, a phenomenon described after FI-RSV vaccination and subsequent natural infection in seronegative infants, that has been linked to a Th2-biased, dysregulated immune response characterized by inadequate antibody production and weak cytotoxic $CD8^+$ T cell response (Delgado et al., 2009; Varga et al., 2009; Soto et al., 2020). Those challenges underscore the importance of rational design and proper testing of each candidate.

Our group has developed a recombinant BCG vaccine expressing hRSV Nucleoprotein (Bueno et al., 2008; Cautivo et al., 2010; Céspedes et al., 2017; Soto et al., 2018). This vaccine is aimed to prevent severe RSV infection in infants and is the sole vaccine in clinical development intended for use in neonates (Rey-Jurado et al., 2017). Intradermal administration of 10^5 CFU of a GMP rBCG-N-hRSV in healthy male adult volunteers is safe and well-tolerated, as demonstrated in phase I clinical trial (Abarca et al., 2020). To evaluate the safety and immunogenicity of the rBCG-N-hRSV in a neonatal model of RSV infection, we tested the rBCG-N-hRSV vaccine in a neonatal calf model of bRSV infection. The neonatal calf represents a tractable model of infant immunity and a homologous model of RSV infection displaying key clinical and pathological similarities to infant RSV infection, representing a suitable model to study antiviral immunity and to develop preventive strategies, and thus, several vaccines targeting hRSV have been tested in young calves, as we recently reviewed (Guerra-Maupome et al., 2019). Our results from two independent studies on newborn dairy calves suggest that a dose of 10^6 CFU of GMP rBCG-N-hRSV, administered within the first week of life and boosted 14 days after first immunization, is safe and well-tolerated (Figure 5). As expected, local reactions to the recombinant vaccine on the injection site were observed, including minor

inflammation and swelling, similar to the lesions described in mice (Céspedes et al., 2017) and healthy adults (Abarca et al., 2020), and BCG-vaccinated calves (Francis, 1947). Those reactions, which could be attributed to BCG components of the vaccine, were transient and self-resolved before five days. While those reactions were present in 11 of 12 calves during Study 1, only one calf in Study 2 showed a similar reaction. Calves vaccinated with WT BCG showed no detectable local reactions. Reactogenicity differences might be related to the different manufacturing processes of the WT and recombinant BCG vaccines, and to the outbred condition of calves. Importantly, no systemic adverse effects, such as fever, were seen in any vaccinated calf during the 28-day post-immunization period, suggesting that rBCG-N-hRSV vaccination has an adequate safety profile in neonates. Similarly, it is well known that BCG immunization of immunocompetent infants is safe, with a very low incidence of serious adverse effects (Lotte et al., 1988; Murphy et al., 2008).

Calves received an aerosol challenge with ~10^4 $TCID_{50}$/mL of bRSV strain 375, 14 days after the booster immunization (Figure 5). Clinical disease was evident in most unvaccinated challenged animals starting 4 dpi, and their condition turned more severe towards day 7 pi. A rise in the clinical score (Figure 6A and B) and a trend in increased gross pathology (Figure 6C and D) were evident in unvaccinated calves but not in rBCG-N-hRSV-vaccinated animals in both studies. Starting from 4dpi clinical score of unvaccinated animals was significantly higher in comparison to rBCG-N-hRSV-vaccinated calves. Clinical disease and elevated body temperature (were more evident in Study 1 in comparison to Study 2 (Figures 6A, B and 7), with one calf being prematurely euthanized due to severe disease. For that reason, we chose to present and analyze the results from both studies separately. Similarly, while unvaccinated infected calves had increased relative neutrophil infiltration in BAL samples in comparison to

uninfected controls, rBCG-N-hRSV vaccination decreased neutrophil infiltration, which is a recognized disease parameter in human and bovine RSV infection (Figure 8C, D) (Hägglund et al., 2017; Openshaw et al., 2017; Sebina & Phipps, 2020). Regarding eosinophils, which some studies had previously shown to be augmented in calves suffering VED (West et al., 1999; Schreiber et al., 2000; Antonis et al., 2003), we observed no significant changes between vaccinated and unvaccinated animals (Supp. Figure 2C, G). Since BAL composition, clinical score and gross pathology are used to evaluated whole-lung and systemic effects, our results suggest that rBCG-N-hRSV vaccination of neonatal calves with MDA protects against severe RSV infection. However, we found no differences in histopathology scores (Figure 8 A, B), viral loads or viral shedding in nasal secretions (Figure 9 and Table 1) when comparing representative lesioned lung samples from vaccinated and unvaccinated calves. These data suggest that protection would be partial and that the antiviral immunity elicited by the chosen vaccination scheme in this model is not optimally tuned to fully prevent virus replication and spread in calves. Further studies are required to define a more efficacious vaccination scheme.

A major challenge in development of RSV vaccines for infant and calf populations is to generate active immunity in presence of MDAs (Chase et al., 2008; Ellis, 2017). While some studies have suggested that MDA can prevent RSV infection (Glezen et al., 1981; Chu et al., 2014), it is known that RSV severe disease can occur in presence of MDA in both calves (Kimman et al., 1988; McGill et al., 2018) and humans (Yamazaki et al., 1994; Stensballe et al., 2009; Freitas et al., 2011; Jans et al., 2017). Although the outcome of vaccination is shaped by multiple factors, it is well documented that MDA can interfere with generation of active immunity in vaccinated calves (Kimman et al., 1989; Ellis et al., 2014; Chamorro et al., 2016). Here, we employed animals that received colostrum shortly after birth (total serum protein >5.7 g/dL) and

had MDAs (Table 2), and observed significantly increased N-hRSV and bRSV-specific $CD4^+$ T cells and bRSV-specific $CD8^+$ T cells in lung-draining TBLNs near the site of infection (Figure 10 B, D), as well as increasing trends in peripheral blood of rBCG-N-hRSV-vaccinated calves at 7 dpi (Figure 10A, C). These proliferative responses were associated with an IFN-γ response to bRSV and PPD-B in TBLNs (Figure 11B) and a robust peripheral IFN-γ and IL-17 response to mycobacterial and viral antigens (Figure 11A, C), showing that the rBCG-N-hRSV vaccine is immunogenic in neonatal calves with MDA, inducing a Th1/Th17 cellular response to both the N-hRSV protein and bRSV, that might be suitable to overcome Th2 bias and dysbalanced cytokine responses associated with RSV infection in infants (legg et al., 2003; Kristjansson et al., 2005) and calves (Stewart & Gershwin, 1989; Miao et al., 2004; Antonis et al., 2010). Although we did not determine specific T cells in the lung, detection of virus-specific cells at TBLNs suggests that specific responses took place in the lung as early as 7dpi. However, we found that neither IFN-γ nor IL-17 were upregulated in BAL at 7dpi in antigen recalls assays after viral antigen stimulation (Figure 12). The ability of our candidate vaccine to recruit virus-specific T cells and their ability to impact their cytokine milieu should be addressed in future studies as the early recruitment of $CD4^+$ and $CD8^+$ T cells is required to elicit antiviral immunity and prevent lung damage according to our previous studies (Cautivo et al., 2010).

Natural RSV infection in infants generates a weak, short-lived primary IgG and IgA response that returns to pre-infection levels within less than four months (McIntosh et al., 1978, 1979; Sande et al., 2014). Moreover, the generation of antibody responses might be affected by circulating maternal antibodies (Murphy et al., 1986; Shinoff et al., 2008). Neutralizing antibodies might have an important role in the prevention of RSV infection and are an important vaccination goal (Schmidt et al., 2020; Soto et al., 2020). RSV. Mucosal RSV-specific IgA has

been correlated to protection in both adults (Mills et al., 1971; Habibi et al., 2015) and infants (Tsutsumi et al., 1995). In calves, IgA and IgM can be detected since eight days after bRSV challenge (Kimman et al., 1987), and protection from respiratory disease has been achieved after vaccination with a mucosal polyanhydride nanovaccine by inducing significant levels of RSV-specific IgA in nasal secretion and BAL, as well as cellular responses in airways and peripheral blood (McGill et al., 2018). Here, parenteral vaccination with rBCG did not induce significant levels of IgA or NTs in nasal secretion prior to challenge, with all animals having little to no NTs. At 7dpi, significantly higher IgA levels were found in nasal secretions of rBCG-N-hRSV-vaccinated animals only Study 1 (Figure 13A and C), however, increased NTs were observed in nasal secretions in rBCG-N-hRSV-vaccinated animals in both studies (Table 2). These results indicate that parenteral rBCG-N-hRSV vaccination can induce mucosal and systemic immune responses to bRSV in neonatal calves with MDA. The enhanced NTs in nasal secretions were associated to reduced clinical disease (Figure 6) but not to an effect on viral shedding (Table 2). Although the induction of mucosal responses is generally sought by mucosal routes of immunization, several vaccines have demonstrated to induce mucosal immunity after parenteral administration (Clements & Freytag, 2016; Su et al., 2016), including an adjuvanted, modified-live multivalent vaccine targeting bRSV and other bovine respiratory viruses (Kolb et al., 2020). The mechanisms for these local immune priming following parenteral vaccination are not well understood (Clements & Freytag, 2016; Su et al., 2016), but might be dependent on the type of vaccine, immunization route, use and type of adjuvants, and several other factors. Considering previous evidence arguing for a protective role of mucosal IgA against RSV in infants and calves, and the results from these studies, we hypothesize that aerosol or intranasal administration of the rBCG-N-hRSV vaccine could be an efficient way to induce efficient

immunity against RSV by mounting both an early mucosal neutralizing response and a sustained systemic cellular response towards RSV able to prevent virus replication and induce protective memory responses.

Although our candidate vaccine encodes the nucleoprotein form hRSV, it was able to elicit N-hRSV and virus-specific cellular responses (Figures 10 and 11). Along these lines, the Nucleoprotein is the most conserved antigen when comparing human and bovine viral species, reaching 93% of AA identity according to previous analyses (Taylor, 2013). It is interesting to note that although RSV nucleoprotein might not be a neutralizing target, the rBCG-N-hRSV vaccine is able to elicit antibody responses to other RSV antigens through a linked recognition mechanism, including surface antigens G and F, as previously demonstrated in mice models (Soto et al., 2018). Interestingly, WT BCG vaccinated calves also exhibited increased NTs in nasal secretions at 7dpi. This observation suggests that the increased NT seen in these animals might be related to unspecific immune priming other than a linked recognition mechanism, and suggests that the increase in IgA and NTs in calves vaccinated with rBCG-N-hRSV might be explained by both unspecific and specific effects. While some studies on human infants have shown that BCG vaccination can impact heterologous antibody production (Ota et al., 2002; Ritz et al., 2013), conflicting evidence indicates that timing of vaccination and several other factors might impact on such responses, as discussed previously (Butkeviciute et al., 2018).

When measuring bRSV-specific serum IgG and NT, an increasing trend in rBCG-N-hRSV-vaccinated calves at 7dpi was observed in Study 1 (Figure 13 and Table 2). Considering that result, for Study 2 we determined serum bRSV-specific IgG1 and IgG2 levels, which display different kinetics in peripheral blood and are associated with different T helper phenotypes

(Kimman et al., 1989; Guerra-Maupome et al., 2019). IgG1 levels were higher at baseline and then decreased towards day 28 post-immunization, suggesting that rBCG vaccination did not induce significant peripheral IgG1 levels (Figure 13D). Regarding Ig2, comparisons between baseline and 7dpi levels showed a decreasing trend in unvaccinated WT BCG groups but not in rBCG-N-hRSV-vaccinated animals, which showed a modest, although significantly higher level of virus-specific IgG2 at 7dpi when compared to unvaccinated animals (Figure 13E). This suggests that vaccination with rBCG favors an IgG2 response in peripheral blood of neonatal calves, which is consistent with a Th1 phenotype in bovines and with our previous results (24, 35). Despite that difference, serum NTs from study 2 showed similar kinetics regardless of the vaccination status, with similar levels at baseline, which tended to drop before the challenge and then to slightly rise at 7dpi, without differences between groups. Since all animals received colostrum after birth, it is no surprise to find NTs as high as 128 and the higher IgG1 levels at baseline. Although baseline NTs were not measured during Study 1, it is possible that those NTs might account for the less severe disease in Study 2 calves in comparison to Study 1. The use of colostrum-replete animals allows us to test vaccine candidates in the most physiological model that resembles the scenario of vaccinating infants with MDA. More importantly, our results indicate that vaccination with rBCG-N-hRSV is immunogenic in neonatal calves even in the presence of significant virus-specific circulating antibodies. Further studies should address the duration and role of cellular and humoral responses elicited by vaccination and infection.

Previous studies evaluating the efficacy of rBCG-N-hRSV and rBCG-P-hMPV vaccines against hRSV and human Metapneumovirus infection in mice, respectively, have shown reduced disease parameters in WT BCG vaccinated control animals (Palavecino et al., 2014; Soto et al.,

2018), which might be explained by unspecific functional effects of BCG vaccination on innate immune cells (Covián et al., 2019). Here, WT BCG vaccination prevented a rise in the clinical score (Figure 6B) and slightly reduced relative neutrophil infiltration (Figure 8D), without modulating lung viral loads or viral shedding. As expected, WT BCG was not associated with N-hRSV or bRSV-specific $CD4^+$ and $CD8^+$ T cell responses in TBLNs (Figure 10 B, D). On the contrary, both vaccines increased NTs in nasal secretions (Table 2). This data suggests that BCG-related unspecific immune mechanisms might confer some degree of heterologous protection against bRSV challenge. Concurrently, increased relative macrophage frequencies were seen in WT BCG and rBCG-N-hRSV-vaccinated animals (Supp Fig2 A, E), indicating that vaccination significantly modulated BAL innate cellular composition. While unspecific effects of BCG or recombinant BCG vaccines might be beneficial against some infectious diseases (Garly et al., 2003; Biering-Sørensen et al., 2012; Kleinnijenhuis et al., 2012, 2014; Arts et al., 2018; de Bree et al., 2018), and thus might be tested as potential immunomodulatory strategies for in infants and young animals, the extent of these effects and the impact on the induction of adaptive immunity should be comprehensively studied in order to fine-tune cellular protective mechanisms on candidate vaccines.

In summary, a two-dose subcutaneous administration of 10^6 CFU of GMP rBCG-N-hRSV is safe and well-tolerated in neonatal calves with MDA, inducing mucosal humoral immunity and systemic cellular immunity against bovine RSV, skewed towards a Th1 phenotype. Besides, vaccination conferred partial protection to bRSV, reducing clinical disease severity and modulating neutrophil infiltration in the lower respiratory tract, without sings of enhanced disease. These results support further investigation on the use of the candidate vaccine for prevention of RSV in infants and calves.

4.6 CONFLICTS OF INTERESTS

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

4.7 ACKNOWLEDGEMENTS

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4.8 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JLM, AMK, FED, MGM. Performed the experiments: JLM, FD, MGM, POM. Analyzed the data: FD, JLM, MGM, DR, AMK. Contributed reagents/materials/analysis tools: AMK, JLM. Wrote the paper draft: FD. Edited the paper: FD, JLM, DR, AMK.

5. CHAPTER 2. A NEW RECOMBINANT BCG VACCINE TO PREVENT HANTAVIRUS CARDIOPULMONARY SYNDROME IS SAFE AND IMMUNOGENIC IN MICE.

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5.1 ABSTRACT.

The hantavirus cardiopulmonary syndrome (HCPS) is a severe zoonotic disease in humans, endemic in the American continent for which no efficacious preventive measures are available. Andes (ANDV) and Sin Nombre (SNV) orthohantavirus are the main HCPS etiological agents in North and South America, respectively, being responsible for lethality rates as high as 40% in affected individuals. After hantavirus infection, massive endothelial dysregulation in lungs leads to pulmonary edema, which is aggravated by a strong dysbalanced Th1/Th2 immune response that promotes inflammation without efficient viral clearance. While experimental vaccines aiming to prevent severe hantavirus-induced disease are designed to elicit high titers of nAbs, several vaccines have shown protection without a neutralizing response, suggesting that the cellular arm of immunity might be important for protective responses. We have shown that recombinant BCG vaccines are efficient inductors of protective cellular and humoral Th1 immunity against respiratory viruses such as pneumoviruses. Here, we developed a new recombinant BCG vaccine expressing ANDV nucleoprotein (rBCG-N-ANDV) and conducted safety and immunogenicity experiments in a BALB/c model. Four to six-week-old male mice were vaccinated with either rBCG-N-ANDV, WT BCG or left unimmunized. Animals received a booster vaccination 14 days after the prime dose and were euthanized at day 28. Vaccination with the recombinant vaccine showed a good safety profile, similar to WT BCG. Immunization with rBCG-N-ANDV elicited N-specific CD4+ and CD8+ T cells in spleen after *ex vivo* recall antigen assays with recombinant purified N-ANDV protein associated to an IFN-γsecretory profile. Finally, an increase in N-ANDV specific IgG serum levels was observed in mice vaccinated with the recombinant formulation after two doses in comparison to pre-immune levels. In conclusion, this preliminary data regarding this candidate vaccine supports further

studies on to explore immunogenicity and efficacy to prevent severe ANDV-induced HCPS in suitable animal models.

5.2 INTRODUCTION

Orthohantaviruses belong to *the Hantaviridae* family, *Bunyavirales* order, and correspond to -ss RNA viruses with tri-segmented genome. Worldwide, pathogenic Orthohantaviruses produce two important clinical diseases: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and Hantavirus Cardiopulmonary Syndrome (HCPS) in the Americas. HCPS case fatality rates can reach up to 20-40% (Riquelme et al., 2015; Alonso et al., 2019). Several orthohantavirus species have been recognized as etiological agents of HCPS, including Sin Nombre orthohantavirus (SNV) and Andes orthohantavirus (ANDV) as the most relevant species in North America and South America, respectively (Jonsson et al., 2010; MacNeil et al., 2011).

The hantavirus viral genome encodes for four viral proteins. A large genome (L) segment encodes for RNA-dependent RNA polymerase, a medium (M) segment encodes for Gn and Gc glycoproteins, and a Small (S) segment encodes for a nucleoprotein (N) (Vaheri et al., 2013) (Figure 2). Orthohantaviruses are transmitted to humans through inhalation of aerosolized excreta from wild infected rodents, which are natural reservoirs. Nevertheless, person-to-person transmission is also described for Andes orthohantavirus (ANDV) (Martinez et al., 2005; Martinez-Valdebenito et al., 2014).

Clinically, HCPS is characterized by an initial short prodromal period, with flu-like symptoms as fever, headache, cough, and myalgia, with gastrointestinal symptoms also described (Riquelme et al., 2015; López et al., 2019). Following the prodrome, the patient may quickly progress to a cardiopulmonary stage, with severe dyspnea, pulmonary edema, and cardiogenic shock. Patients who survive the cardiopulmonary stage enter the convalescent phase and slowly recover the pulmonary function (Llah et al., 2018). Importantly, respiratory symptoms can be detected even three years after SNV or Choclo orthohantavirus infection, suggesting that pulmonary dysfunction could be long-lasting in some individuals (Gracia et al., 2010).

The specific mechanisms involved in the pathophysiology of HCPS are not fully understood. The main targets of orthohantaviruses are vascular endothelial cells; nevertheless, the virus does not produce a cytopathic effect in infected cells (Mackow & Gavrilovskaya, 2009), but otherwise inhibits apoptosis through a mechanism involving the N protein (Solà-Riera et al., 2019). A severe endothelial dysregulation leads to increased permeability of endothelial linings, and ultimately, pulmonary edema might be the main pathogenic mechanism (Vaheri et al., 2013). Besides, different studies suggest that immune factors are implicated in the disease (Mori et al., 1999; Raftery et al., 2002, 2020; Marsac et al., 2011; Angulo et al., 2017). Mononuclear infiltrates of high cytokine-producing cells are present in the lungs of HCPS-deceased patients (Mori et al., 1999). Additionally, a cytokine storm is described by different groups, with higher levels of IL-6 in deceased individuals (Angulo et al., 2017; Maleki et al., 2019). A strong, dysbalanced Th1/Th2 immune response towards the virus has been associated to AEC dysregulation in clinical studies and animal models of HCPS infection (Borges et al., 2008; Safronetz et al., 2011). The severe immune dysregulation after ANDV infection likely

contribute to the capillary leakage and the consequent organic dysfunction reported in HCPS cases.

To date, there are neither specific treatments nor vaccines for any of the hantavirus diseases approved by the FDA (Brocato & Hooper, 2019). Hantavax, an inactivated vaccine against Hantaan orthohantavirus (HTNV), is currently applied in China, but provides a short-lived antibody response (Cho & Howard, 1999). A phase 3 clinical trial is in progress to know their effectivity in high-risk groups due to the absence of other options (clinicaltrials.gov, NCT 02553837). Additionally, several strategies are in evaluation using S or M genes or their respective N, Gn and/or Gc proteins, as previously reviewed (Brocato & Hooper, 2019; Liu et al., 2020).

Recently, our group and others have published the use of recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) as a strategy to induce protective immunity against respiratory viruses. In this line, BCG offers several advantages for to be considered a vector for the development of vaccines: it is well-tolerated, a potent adjuvant, inexpensive to produce (Stover et al., 1991), and induces immunity training, improving host defense by metabolic and epigenetic changes in innate immune cells (Covián et al., 2019; Cirovic et al., 2020). Vaccine candidates using recombinant BCGs are currently in development, showing a neutralizing antibody (nAb) response in the animal model for the following viruses: metapneumovirus (Palavecino et al., 2014; Soto et al., 2018), SARS-CoV-2 (de Queiroz et al., 2020), RSV (Bueno et al., 2008; Céspedes et al., 2017; Soto et al., 2018). More importantly, those vaccines have shown to be potent inducers of efficacious antiviral Th1 cellular immunity (Cautivo et al., 2010; Soto et al., 2018). Moreover, a phase 1 clinical trial using a nucleoprotein of hRSV with rBCG

as vector shows successful results in healthy donors, with a neutralizing antibody response and Th1 profile (Abarca et al., 2020).

Here, we tested if a new rBCG Danish strain expressing N-ANDV antigen (rBCG-N-ANDV) induces a protective response in mice. Our results show that immunization with rBCG-N-ANDV is safe and well-tolerated in vaccinated mice. Moreover, rBCG-N-ANDV vaccination induces an antigen-specific cellular response with T $CD4^+$ and $CD8^+$ activation when splenocytes are restimulated *ex vivo* with purified N-ANDV. Additionally, serum IgG antibodies against N-protein are increased in the immunized mice group in comparison to unvaccinated mice, suggesting that a humoral specific response is generated. Together, these results suggest that our vaccine prototype could be considered a safe strategy for developing an ANDV hantavirus vaccine.

5.3 MATERIALS AND METHODS

5.3.1 Generation of recombinant BCG expressing ANDV nucleoprotein.

To generate a rBCG strain expressing ANDV nucleoprotein, we defined an *in silico* cloning strategy and designed an *E. coli – Mycobacterium* shuttle vector, assembling an N-ANDV ORF into a pMV361 plasmid (Stover et al., 1991), which allows genome integration of vectored ORFs. To construct the recombinant pMV361-N-ANDV plasmid, the N-ANDV ORF was PCR amplified from a TOPO-HisMAX pCDNA4 vector containing the full N-ANDV sequence from CHI-7913 isolate (Genbank, AY228237.1) using Fwd 5' –

GCCAAGACAATTGCGATGAGCACCCTCCAAGAATTGC – 3’ and Rev 5’-GTCGATCGTACGCTAGCTACAACTTAAGTGGCTCTTGGTTG… - 3’ primers. The pMV361 vector was amplified by PCR using the following primers: Fwd 5’ – CAAGAGCCACTTAGTTGTAGCTAGCGTACGATCGACTGCC – 3’ and Rev 5’-CTTGGAGGGTGCTCATCGCAATTGTCTTGGCCATTGC - 3’, with a Phusion High Fidelity DNA polymerase (F530, ThermoFisher). PCR products were gel purified using Wizard SV Gel and PCR Clean-Up System (A9282, Promega), and DNA products were assembled into a recombinant pMV361-N-ANDV plasmid using a Gibson Assembly Protocol (E5510, New England Biolabs). Briefly, 0.01 pmol of each amplified N-ANDV ORF and pMV361 DNA were incubated at 50°C for 15 minutes with Gibson Assembly Master Mix and then transformed into dh5α competent *E. coli* using the manufacturer’s instruction. The recombinant plasmid was recovered and sequenced through the Sanger method at the Unidad de Secuenciación y Tecnologias Ómicas, PUC. Primers Fwd 5-'CGGTGAGTCGTAGGTCGGGA – 3' and Rev 5' – GAGCAAGACGTTTCCCGTTG – 3' that anneal at flanking regions of the pMV361 plasmid cloning region were used to sequence the multi-cloning site and confirm correct cloning of the ORF into the pMV361 vector. Then, BCG Danish strain was electroporated with the pMV361-N-ANDV plasmid using a Bio-Rad gene pulse electroporator at 2.5 V, 25 μF, and 1000Ω, and transformants were selected by growing the electroporation products on 7H10 agar plates (Sigma-Aldrich, M0303), supplemented with 10% OADC (Sigma-Aldrich, M0678), and Kanamycin [20 μg/mL] (Sigma-Aldrich, 60615). Finally, rBCG colonies were grown on 7H9 liquid medium (Sigma-Aldrich, M0178), supplemented with 10% OADC (Sigma-Aldrich, M0678), 0.05% Tween 80, and Kanamycin [20 μg/mL].

5.3.2 Evaluation of recombinant BCG expressing ANDV Nucleoprotein

Protein lysates were prepared from an rBCG-N-ANDV culture to evaluate N-ANDV protein expression. Briefly, liquid cultures containing mycobacteria were washed three times in 1X PBS – 0.05% Tween 80, pelleted, and resuspended in a lysis buffer (50 mM Tris, 0.6% SDS, 5 mM EDTA, and 1X cOmplete (Roche, 11697498001) protease inhibitor cocktail). Then, total lysates were obtained through ultrasonic disruption, and total protein was quantified with BCA Pierce Protein Kit (Cat. 23225, ThermoFisher). Finally, 20 μg of total protein were blotted on nitrocellulose membranes to evaluate N-ANDV protein expression in a Western Blot Assay. A purified N-ANDV protein (from *E. coli*) was used as a positive control. 1X PBS, and recombinant *E. coli* and BCG expressing irrelevant antigens were used as negative controls. A blocking incubation step with 5% BSA in PBS was performed during 2 h at RT. Then, an incubation step with monoclonal anti-N-ANDV primary mouse IgG1 antibody (Austral Biologicals, 1:10000 dilution) and a goat anti mouse-IgG (Abcam, 1:20000 dilution) as secondary Ab. Three washes with PBS – 0.05% Tween 20 were done between each incubation step. A final 1X PBS washing step was performed before membrane incubation with the Pierce™ ECL western blotting substrate (ThermoFisher, 32106). Chemiluminescence was visualized and captured in a myECL Imager (ThermoFisher, 62236).

Further, a second sequencing step was performed on rBCG DNA to confirm the correct cloning of the ORF using the pMV361 primers.

5.3.3. Preparation of WT BCG and rBCG-N-ANDV doses.

Doses of WT BCG (Danish strain) and rBCG-N-ANDV were prepared by growing the mycobacteria on 7H9 liquid medium (Sigma-Aldrich, M0178), supplemented with 10% OADC

(Sigma-Aldrich, M0678), 0.05% Tween 80. Kanamycin selection [20 μg/mL] was included for the recombinant BCG only. When the liquid culture reached an OD_{600} near to 0.8, mycobacteria were washed in sterile PBS - 0,05% Tween 80, and frozen at −80°C in sterile PBS – 20% glycerol at a final concentration of 4×10^8 CFU per vial until use.

5.3.4. Animals, immunization, and safety evaluation.

Four to 6-week-old male BALB/c mice were included in this study and kept in an animal BSL-2 room. Animals were acclimated for three days in the room before starting the experiments. Prior to immunization, 4×10^8 CFU mycobacteria were resuspended in 400 μl of sterile 1X PBS. Four to six-week-old male BALB/c mice received subcutaneous administration of 10^8 CFU of BCG WT or rBCG-N-ANDV in the central dorsal flank area in 100 μl of sterile PBS (Supplementary Figure 6A). A control group received 100μl of sterile PBS only. A booster immunization was administered to mice 14 days after prime immunization. Animals were monitored daily, and a clinical score was applied to determine the safety of the vaccine administration, including behavior, posture, and physiological data, as wells as evaluation of the inoculation site. Blood samples were collected through submandibular puncture on day 0 (pre immunization), day 14 (pre booster), and day 28 (study endpoint). Animals were euthanized on day 28 through intraperitoneal administration of ketamine/xylazine (200 and 16 mg/kg, respectively) then spleens were aseptically collected and placed on cRPMI media (Supplementary Figure 6A). A veterinarian conducted all procedures where animals were involved. All animal work was conducted according to institutional guidelines of the Pontificia Universidad Católica de Chile (PUC) and under the guidance of the "Guide for the Care and Use of Laboratory Animals", Eight Edition (NRC, 2011).

5.3.5. Ex vivo T cell stimulation and flow cytometry.

Spleens were disaggregated in sterile cRPMI media, erythrocytes were lysed, and then cells were resuspended in cRPMI media, supplemented with 10% FBS, 10 mM HEPES, 2 Mm L-glutamine, 1mM Sodium pyruvate, 50 μM 2-mercaptoethanol, non-essential amino acids, penicillin [100 UI/mL], streptomycin [100 μg/mL], and [0.25 μg/mL] amphotericin B [0.25 μg/mL). Then, an *ex vivo* antigen recall assay was performed on splenocytes (Supplementary Figure 6B). Briefly, 500,000 cells were plated in a 96-well plate with cRPMI and incubated with N-ANDV protein [10 μg/mL], PPD-B [20 μg/mL], and concanavalin A (3 μg/mL) as a positive control. After 72 hours, plates were centrifuged at 0.3 g for 5 min at 4°C, and supernatants were collected and stored. Cells were analyzed through flow cytometry to detect the expression of CD69, CD71, and CD25 activation markers on $CD4^+$ and $CD8^+$ T lymphocytes (Supplementary Figure 6B). For this, splenocytes were washed on with FACS buffer and stained with fixable viability stain AF780 diluted 1:5000 for 10 minutes at RT in the dark. After three washes, cells were incubated for 30 minutes at 4ºC in the dark with a mix of: anti-CD3 PerCP, anti-CD4 BUV496, anti-CD8 BV510, anti-CD45 APC-Cy7, anti-CD69 BV786, anti-CD71 BUV395, and anti-CD25 BV412 (all from BD Biosciences) antibodies. Fifty-thousand events were acquired on BD LSR-Fortessa-X20 (BD Biosciences) and analyzed using FlowJo v10.6.2 software (BD Biosciences).

5.3.6. Determination of cytokine secretion after *ex vivo* T cell stimulation

Supernatants collected from *ex vivo* T cell stimulation were stored at -80°C until the determination of cytokine secretion. IFN-γ, IL-4, and IL-10 ELISAs were carried out using BD

OptEIA™ Mouse IFN-γ, IL-4, and IL-10 kits (BD Biosciences), respectively, according to the manufacturer's instruction, using 1:2 dilution of supernatants tested in duplicate.

5.3.7 Quantification of serum humoral responses

Serum was obtained from blood samples at several time points; day 0 (preimmune), day 14 (prebooster) and day 28 (endpoint) (Supp. Figure 6). Immediately after serum collection, samples were stored at -80°C until processing. A 96-well ELISA plate was seeded with recombinant 0-4 ug/mL NANDV protein (GenScript) and incubated at 4°C overnight. Plates were blocked with 5% BSA in PBS for 1 h and the plates were washed three times 0.05% Tween 20 in PBS. After washing, 100 µl of serum (1:200 dilution in PBS) was added to wells and incubated 2h at RT. The plates were washed three times and incubated with HRP-goat anti-mouse IgG (H+L) (1:2000 dilution) for 1 h at RT. After three washes, plates were added 100 µL of 1 mg/ TMB (Merck) at RT. Reaction was stopped with 100 µL of H_2SO_4 and plates were read at 450 nm using Multiskan EX (Thermo Fisher Scientific).

5.3.8. Statistical Analyses

Results are expressed as average ± standard error of the mean (SEM). Data was analyzed using One-way ANOVA, One-way ANOVA with repeated measures, or Two-way ANOVA were applied followed by Tukey's multiple comparisons test. Differences were statistically significant when $p<0.05$. Analyzes were carried out using Graphpad Prism 7 software (GraphPad Software, Inc).

5.4. RESULTS

5.4.1. Development of a recombinant BCG producing N-ANDV antigen

To generate a recombinant BCG expressing N-ANDV protein, we cloned N-ANDV ORF into a pMV361 integrative vector, which allows constitutive gene transcription under the control of mycobacterial hps60 promoter. For this, we used the Gibson assembly with primers shown in Supplementary Table 2. Once the reaction was performed, we confirmed the correct assembly of the recombinant plasmid (pMV361-N-ANDV) performing sequence alignments with *in silico* designed sequences in Snapgene Software (GSL Biotech. Neither a frameshift nor potential amino acid substitutions were detected, but only six synonymous base pairs changes were observed (Supplementary Figure 5).

Then, we transformed our recombinant plasmid into electrocompetent BCG Danish strain. After antibiotic selection of transformants, colonies were grown in Middlebrook 7H9 media and tested for recombinant protein production. As shown in Figure 14 for selected colonies, recombinant BCG efficiently expresses N-ANDV protein.

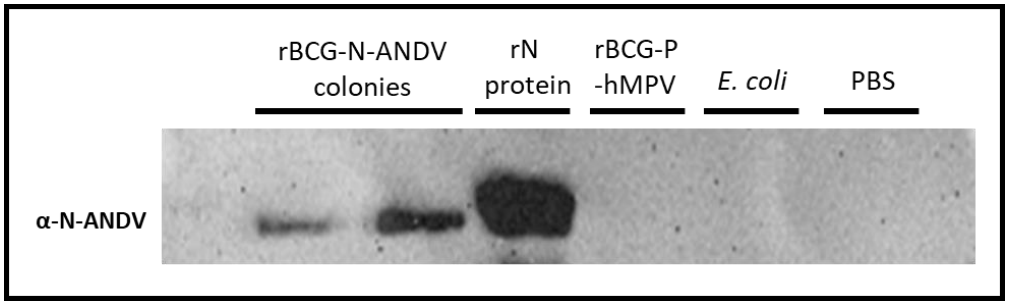

Figure 14. Expression of ANDV N protein by recombinant BCG. BCG Danish was transformed with pMV361-N-ANDV plasmid and plated on solid 7H10 medium supplemented with 20 μg/mL kanamycin for selection. For WB, a monoclonal antibody against N-ANDV was employed as primary antibody, and an HRP-conjugated as secondary antibody. Two rBCG colonies are shown, as well as a positive control (rN-ANDV protein expressed in *E. coli*) and negative controls (rBCG-P-hMPV lysate, *E. coli* lysate, and PBS).

5.4.2. Subcutaneous administration of rBCG-N-ANDV is safe in mice.

Male 4-6-week-old BALB/c mice were immunized with 10^8 CFU of WT BCG or 10^8 CFU of rBCG-N-ANDV. An unimmunized control received PBS. A booster immunization was administered 14 days after the prime immunization, and animals were euthanized 14 days after the booster, and spleen and lymph node samples were collected. Regarding vaccine safety, we observed no significant adverse reactions affecting the immunized animals, neither with BCG nor rBCG-N-ANDV vaccine, throughout the entirety of the study. Weight data from all immunized animals indicated that vaccination did not affect weight gain (Figure 15A). rBCG-N-ANDV vaccinated animals had a significantly higher score compared to unvaccinated animals ($p < 0.001$ at days 7, 14 and 28, and < 0.0001 at day 21) (Figure 15B) as early as 7 days post-immunization, but did not display any behavior or physiological alteration and had a similar clinical score compared to WT BCG immunized animals. A granuloma-like reaction was observed starting from day 4 post-immunization on rBCG-N-ANDV and WT BCG vaccinated animals and accounted for the significantly higher clinical scores (Figure 15B, C). This induration was evident on all vaccinated animals by the end of the study (Figure 15C). Mild alopecia was observed in some animals, with no additional dermal reactions. These observations suggest that rBCG-N-ANDV vaccine administration is safe in the BALB/c immunization model.

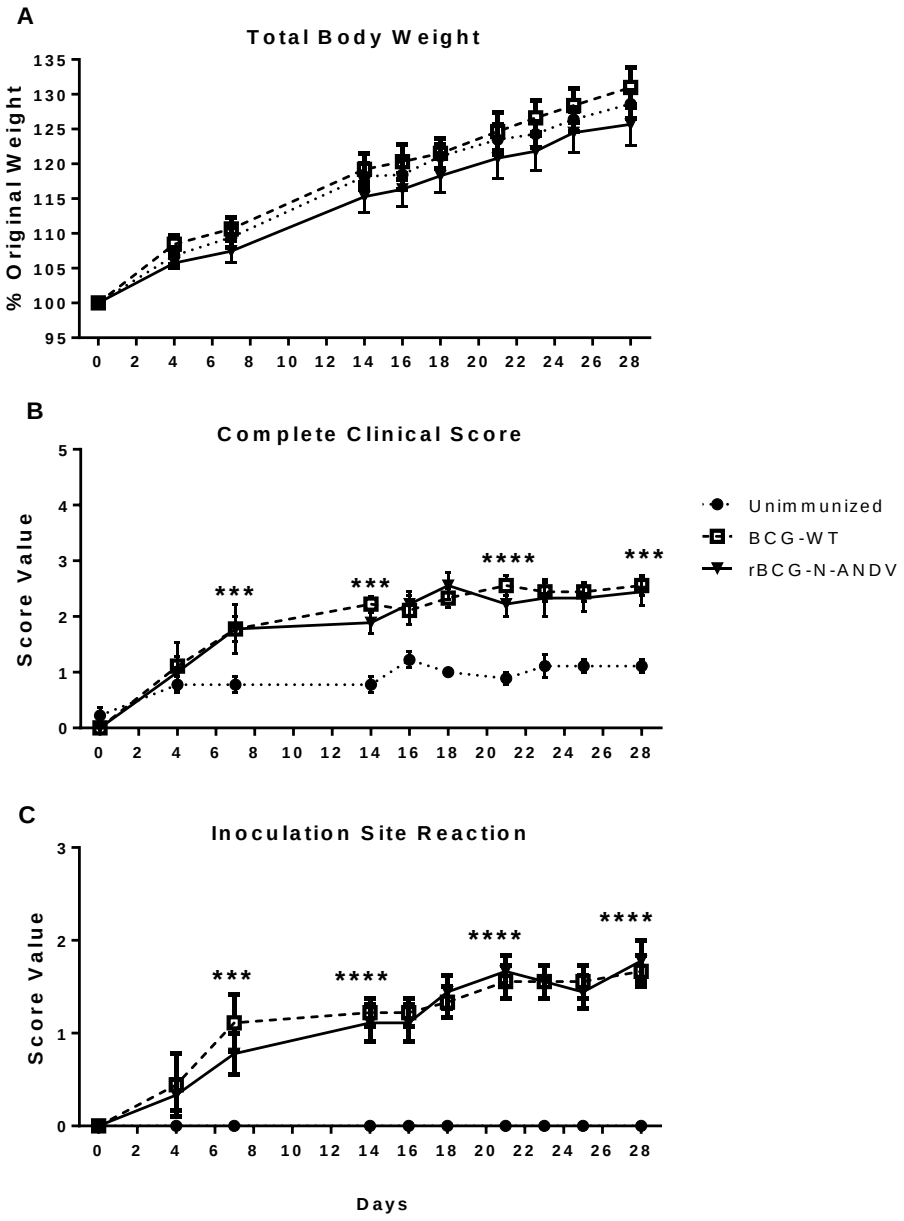

Figure 15. Subcutaneous administration of rBCG-N-ANDV is safe in mice. Male 4-6-week-old BALB/c mice were vaccinated with either WT BCG o rBCG-N-ANDV at day 0 and day 14. An unimmunized control group received PBS. A clinical score was applied three times a week during the 28-day study period. **A**. Total body weight curve, **B**: Complete clinical score. **C**. Inoculation site reaction score. *** $p < 0.001$, **** <0.0001 (One-way ANOVA with RM and Tukey's multiple comparisons test between rBCG and unimmunized group) Data presented as mean ± SEM, from three independent experiments and n=9 / group.

5.4.3. Vaccination of mice with rBCG-N-ANDV induces N-ANDV-specific cellular responses associated to IFN-γ secretion.

To determine if vaccination with rBCG-N-ANDV is able to generate antigen-specific $CD4^+$ and $CD8^+$ populations in mice, splenocytes were collected to carry out *ex vivo* antigen stimulation assays with recombinant N-ANDV and PPD-B for 72 hours and then analyzed by flow cytometry. As shown in Figure 16, both $CD4^+$ and $CD8^+$ T cells subsets present a significantly higher frequency of CD69 (p 0.0018 and <0.0001, respectively) (Figure 16A and D) and CD71 (p 0.0004 and 0.028, respectively) (Figure 16B and E) activation markers in rBCG-N-ANDV vaccinated mice after stimulation with N-ANDV antigen in comparison to unstimulated group. Additionally, results show a near 2-fold increase in $CD69^+$ for $CD4^+$ and $CD8^+$ T cell populations when rBCG-N-ANDV group is compared with the unimmunized group (Figure 16A and D). Meanwhile, CD25 was significantly upregulated in $CD8^+$ T cell population (p 0.0079) (Figure 16C), but not in $CD4^+$ T cells (p 0.0541) (Figure 16F).

These results indicate that antigen-specific activation takes place upon recall stimulation assays and suggests that rBCG-N-ANDV immunization elicits a cellular immune response against the target antigen.

To determine if the antigen-specific activation of immune cells is associated to a Th1 or Th2 phenotype, supernatants from cultures were tested for IFN-γ, IL-4, and IL-10 secretion through ELISA assays (BD OptEIA, BD Biosciences). Mice vaccinated with rBCG-N-ANDV showed increased secretion of IFN-γ in response to N-ANDV and PPD-B stimulation in comparison to unimmunized mice, whereas animals vaccinated with WT BCG showed increased expression to PPD-B only (Figure 17A). IL-10 levels were below the limit of detection for all samples (not

shown). Regarding IL-4, we found no difference in secretion between vaccinated and unvaccinated groups after N-ANDV stimulation, but higher levels in both vaccinated groups in comparison to unvaccinated animals after PPD-B stimulation (Figure 17B). However, those levels were strikingly low in comparison to IFN-γ levels overall, suggesting that the cellular response to N-ANDV antigen is predominantly biased towards Th1 phenotype. We then analyzed a potential Th bias calculating IFN-γ/IL-4 ratio between vaccinated groups for the N-ANDV stimulation conditions, and found no statistically significant differences between the unimmunized and rBCG-N-ANDV groups, but only an increasing trend (Table 3).

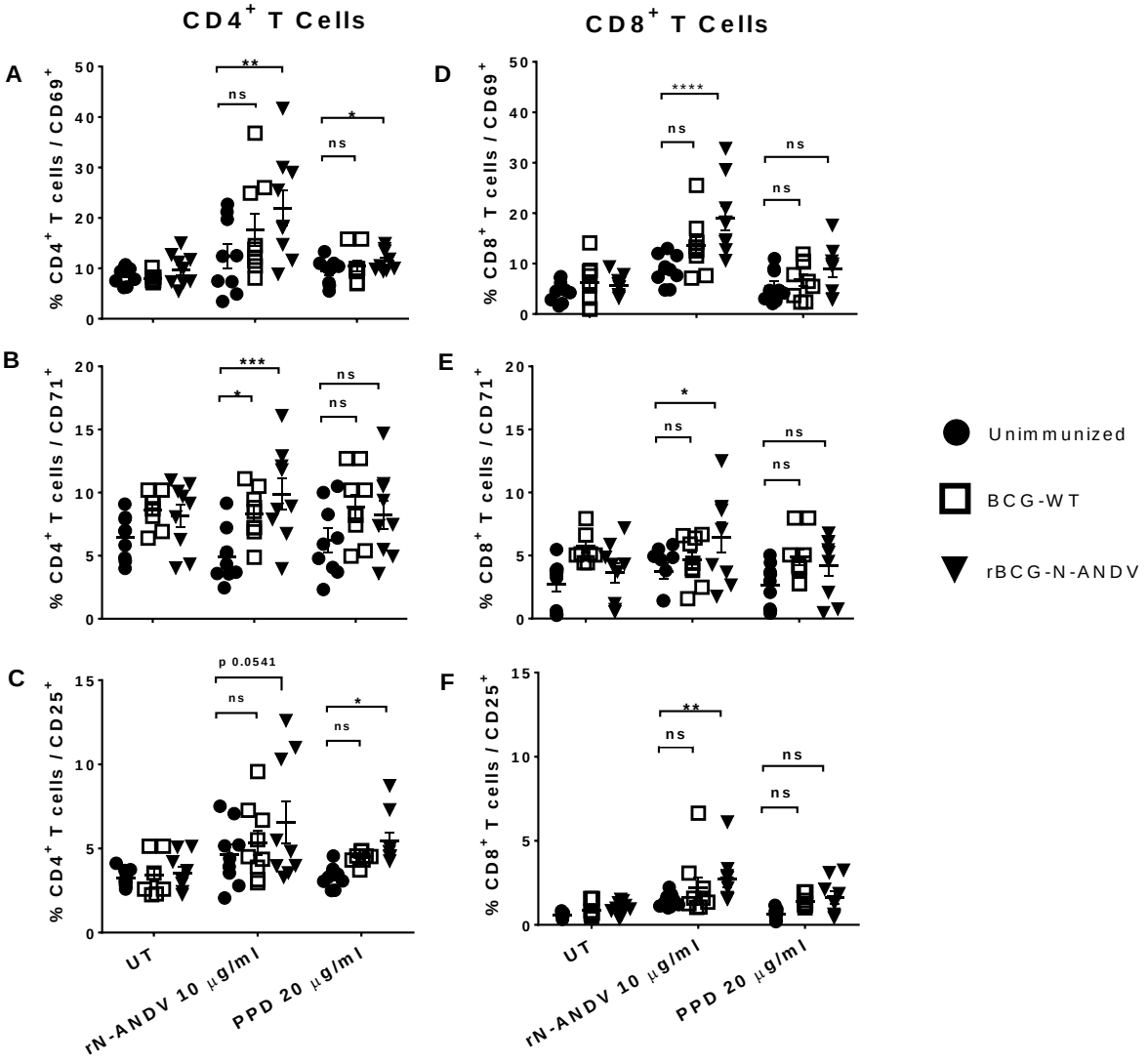

Figure 16. rBCG-N-ANDV vaccination elicits antigen-specific CD4$^+$ and CD8$^+$ T cells. Male BALB/c mice of 4-6-week-old were vaccinated with either WT BCG o rBCG-N-ANDV at day 0 and day 14. An unimmunized control group received PBS. On day 28, animals were euthanized, and splenocytes were isolated to carry out *ex vivo* stimulation assays with recombinant rN-ANDV (10 ug/mL) and PPD-B (20 ug/mL). After 72h incubation, flow cytometry analyses were performed on splenocytes using activation markers CD25, CD69, and CD71. A-C. CD69 and CD71 activation markers are upregulated in CD4$^+$ T cells after N-ANDV stimulation. D-F. CD25, CD69, and CD71 activation markers are upregulated in CD8+ T cells after N-ANDV stimulation. Data presented as mean ± SEM, from three independent experiments and n=9 / group. * p<0.5, ** <0.01, *** <0.001 **** <0.0001 as determined by 2-way ANOVA followed by Tukey's multiple comparisons test.

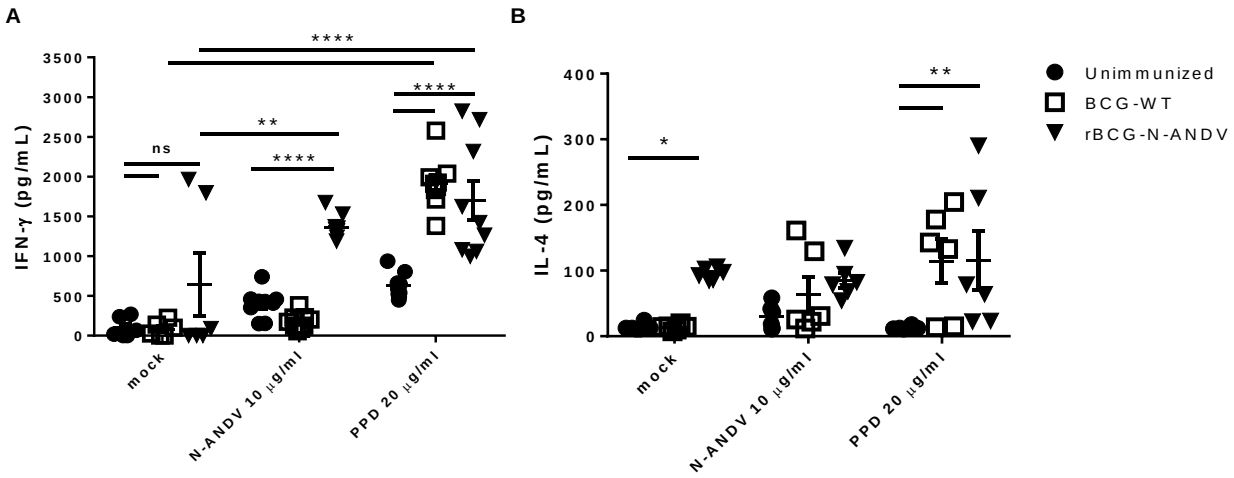

Figure 17. rBCG-N-ANDV vaccination generates a strong IFN-γ secretion in splenocytes stimulated with PPD-B and rN-ANDV. *Ex vivo* T cell stimulation was performed as in Figure 13. Then, culture supernatants were used to detect **A**. IFN-γ, **B**. IL-4 and IL-10 levels (not shown) using BD OptEIA™ Mouse ELISA kits following the manufacturer instructions. Data expressed as mean ± SEM from two independent experiments. * p<0.5, ** <0.01, **** <0.0001 as determined by 2-way ANOVA followed by Tukey's multiple comparisons test.

IFN/IL4 ratio	Unimmunized	BCG-WT	rBCG-N-ANDV
mock	**7,096526** (3,579502)	**6,067113** (2,706416)	**6,766504** (4,138758)
N-ANDV 10 ug/mL	**11,55693** (2,047232)	**2,63277** (0.774)	**15,36396** (0.310)

Table 3. IFN-γ/IL-4 ratios of different treatment and vaccination groups. *Ex vivo* T cell stimulation was performed as in Figure 13. Then, culture supernatants were used to detect IFN-γ, and IL-10 levels using BD OptEIA™ Mouse ELISA kits following the manufacturer instructions. IFN- levels of each condition was compared to the group mean IL-4 levels. Data expressed as mean ± SEM from two independent experiments. No statistically significant differences were found between unimmunized and rBCG-N-ANDV groups for neither mock nor N-ANDV conditions after performing 2-way ANOVA followed by Tukey's multiple comparisons test.

5.4.4 Subcutaneous vaccination with rBCG-N-ANDV elicits a mild increase in anti N-ANDV IgG antibodies in serum after two doses.

To determine if rBCG-N-ANDV vaccination in mice could elicit a systemic humoral response towards the recombinant antigen, blood collected during different time points was analyzed through ELISA to detect anti N-ANDV total IgG in serum. No differences in IgG levels between groups were observed in serum neither previous to immunization (pre-immune) nor 14 days after the first vaccine dose (pre-booster) (Figure 18). No rise in IgG was observed 14 days after vaccination neither with WT nor recombinant BCG. However, samples obtained during necropsy showed a mild increase of IgG levels in the rBCG group in comparison to both unimmunized ($p<0.001$) and WT BCG ($p<0.01$) groups (Figure 18). Additionally, N-specific IgG levels at necropsy time were higher in comparison to pre-immune levels in the same group ($p<0.001$), suggesting the development of an N-ANDV specific IgG response after the booster vaccination.

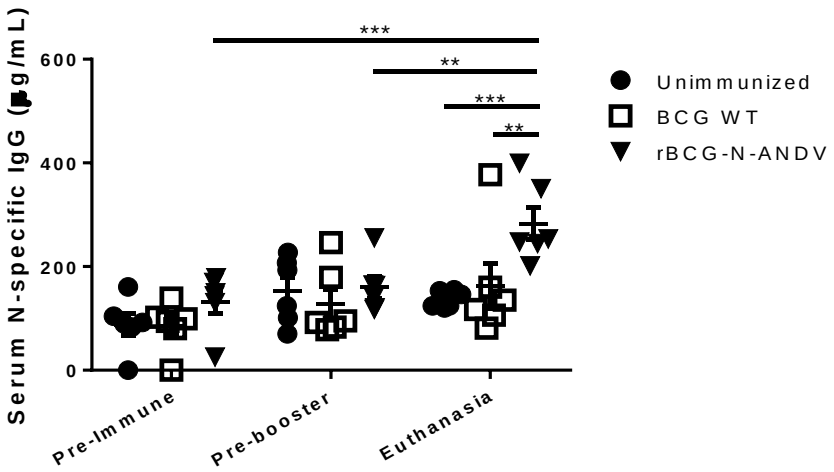

Figure 18. Subcutaneous vaccination with rBCG-N-ANDV elicits a mild increase in anti N-ANDV IgG antibodies in serum after two doses. Serum from animals vaccinated with either WT BCG or rBCG-N-ANDV and unimmunized mice collected at day 0 (pre-immune), day 14 (pre-booster) and day 28 (euthanasia) were tested through ELISA for detection of N-ANDV specific IgG antibodies as detailed in materials and methods. Data expressed as mean ± SEM from two independent experiments. ** $p<0.01$, *** <0.001 as determined by 2-way ANOVA followed by Tukey's multiple comparisons test.

5.5 DISCUSSION

In this work, we have developed a new recombinant vaccine intended to prevent severe HCPS, a highly lethal disease for which no vaccine has been licensed. Despite the pathogenesis of HCPS is still not fully understood, data from clinical samples and animal models suggest that strong, dysbalanced Th1/Th2 immune responses participate and increase AEC dysregulation which ultimately leads to pulmonary edema and cardiogenic failure (Mori et al., 1999; Borges et al., 2008; Safronetz et al., 2011). To prevent this syndrome, several vaccines are under testing, whose main mechanism of action is induction of nAbs to prevent viral engagement of target cell (Hooper et al., 2006, 2013; Brocato et al., 2013; Warner et al., 2019). Since the *Hantaviridae* family includes a phylogenetically diverse group of viruses that cause different manifestations

that are commonly categorized into two different syndrome, candidate vaccines are usually tested for cross-protection. Several reports have shown that cellular immunity, particularly CD8+ T cells are also important to generate protective and cross-protective responses to hantavirus infections (Nicacio et al., 2002; Safronetz et al., 2009; Hooper et al., 2013; Dong et al., 2019). Regarding HCPS, protection in the absence of NTs has been reported after immunization with adenoviral vectors encoding for Gn, Gc or N antigens (Safronetz et al., 2009). Similarly, a pan-hantavirus DNA vaccine against HTNV/PUUV/SNV/ANDV was reported to protect against ANDV infection in SHs despite eliciting low nAb titers (Hooper et al., 2013). For this reason, we chose to develop a BCG-based recombinant vaccine expressing a highly immunogenic, conserved hantavirus antigen, the N protein. Several reports in different hantavirus disease models have reported protection from infection or disease after immunization with recombinant nucleoprotein from hantavirus (Nicacio et al., 2002; Safronetz et al., 2009). Additionally, rBCG vaccines have been successfully used to promote Th1 immunity against several respiratory viruses such as hRSV and hMPV at preclinical and clinical phases, with promising safety, immunogenicity and efficacy results (Bueno et al., 2008; Céspedes et al., 2017; Abarca et al., 2020).

Similar to other rBCG vaccines, our trial showed that the administration of two 10^8 CFU doses of rBCG-N-ANDV was well tolerated by mice, without showing significant changes in growth, physiology, behavior or posture that might be associated to systemic adverse effects in comparison to WT BCG administered in the same dose and regime (Figure 15A, B). Likewise, local reactions to the rBCG-N-ANDV vaccine were similar to those evident after WT BCG vaccination (Figure 15C) and those reported with other rBCG vaccines (Céspedes et al., 2017). Although no histological analyses of those lesions were performed in this study, it is likely that

this reaction is of similar nature to granulomatous lesions generated after BCG and GMP rBCG-N-hRSV vaccination (Céspedes et al., 2017). These results indicate that the administration of this recombinant vaccine in a mice model has a good safety profile and suggest that could be studied in SHs and humans.

Recombinant BCG vaccines have shown to be inducers of Th1 immune response, eliciting long-term, antigen-specific $CD4^+$ and $CD8^+$ T cell priming that has been associated to efficient antiviral immunity (Bueno et al., 2008; Cautivo et al., 2010; Céspedes et al., 2017). Here, vaccination with the rBCG-N-ANDV vaccine generated antigen-specific activation of $CD4^+$ and $CD8^+$ T cells in spleen 14 days after booster administration (Supplementary Figure 6), evident as increased percentage of T cells expressing activation markers $CD69^+$, $CD71^+$ and $CD25^+$ (Figure 16) after *ex vivo* stimulation with recombinant N-ANDV antigen, in comparison to unimmunized animals. As expected, N-specific responses were not seen in WT BCG animals (Figure 16), indicating that the recombinant expression of N-ANDV was required for eliciting the antigen-specific response. Further, generation of antigen specific T cells was associated to increased IFN-γ secretion after N-ANDV stimulation in splenocytes of rBCG-vaccinated mice when compared to unimmunized mice, and also when compared to mock conditions (Figure 17A). IL-4 secretion was also increased in rBCG vaccinated group in comparison to the unimmunized group (Figure 17B), however, IFN-γ levels were higher as shown by the IFN-γ /IL-4 ratio in this group (15,36396) (Table 3). Despite this, a skew to Th1 could not be demonstrated as this ratio showed only an increasing trend without reaching statistical significance (Table 3).

As shown in Figure 18, a mild but significant increase in serum N-ANDV specific IgG levels was observed 14 days after the booster vaccination in rBCG-vaccinated animals in comparison to pre-immune and pre-booster levels, suggesting that a specific humoral response takes place only after two doses of rBCG-N-ANDV in mice. No rise was seen in unimmunized or BCG WT groups at any time. Although research on rBCG vaccines have focused mainly on cellular responses, it has been reported that other rBCG vaccines elicit a humoral response towards the recombinantly expressed antigen, such as hRSV nucleoprotein and hMPV phosphoprotein, upon experimental infection with the corresponding virus, but not 7 days after a booster administration (Soto et al., 2018). Despite not having a strong infection stimulus, the mild increase seen in our model maybe be related to the higher IFN-γ secretion associated to the vaccination status, which favors the proliferation and differentiation of the B cells into effector plasmatic cells, which might trigger antibody secretion, specifically of IgG2a isotypes (Nurieva & Chung, 2010; Soto et al., 2018). Also, IgG levels were measured 14 instead of 7 days after the booster as done in previous rBCG studies (Soto et al., 2018). It remains to be elucidated if rBCG vaccination triggers a suitable humoral immune response in a lethal HCPS animal model since nAbs have been correlated to protection. (Hooper et al., 2006; Valdivieso et al., 2006; Brocato et al., 2013; Warner et al., 2019).

In summary, our new recombinant BCG vaccine is able to produce the N-ANDV antigen in i*n vitro* culture, and to generate an N-ANDV cellular and humoral immune response after two doses of 10^8 CFU in BALB/c mice, with a good safety profile. This study provides initial data regarding this candidate vaccine, but further studies should explore if administration of this recombinant vaccine in SHs provides efficient antiviral immunity to prevent severe ANDV-induced HCPS. It remains to be elucidated if the generation of T cell immunity is able to prevent

ANDV clearance skewing the immune response towards an antiviral Th1 phenotype without causing immunopathology. In this line, studies on SH have demonstrated that T cells are not required for the lethal ANDV-induced HCPS (Prescott et al., 2013), giving weight to other hypotheses pointing out for a role of the mononuclear innate system as driver of excessive inflammation (Schönrich & Raftery, 2019; Raftery et al., 2020). Considering the long incubation period of HCPS, we hypothesize that an early memory T cell response at the site of infection would prevent immunopathology even before the symptomatic HCPS. Finally, new vaccination regimes exploring the effects of lower and GMP doses of this vaccine, which are suitable for human use, should be tested in a proper HCPS animal model.

5.6 CONFLICTS OF INTERESTS

No conflicts of interest declared.

5.7 ACKNOWLEDGEMENTS

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5.8 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AMK, FED, ARD, FS. Performed the experiments: FED, ARD, FS, CC. Analyzed the data: FED, ARD, FS, CC, AK. Wrote the paper draft: FD,

FS. Reviewed the paper: FED, AK.

6. GENERAL DISCUSSION

The control and prevention of respiratory viral infections pose a continuous challenge to biomedical science since these infections are widespread, associated with significant morbidity and mortality, and imply high economic costs, thus strongly impact public health worldwide. Efforts to develop preventive measures against highly prevalent and emergent viruses are matter of extensive research due to the intricated host, viral, and environment relationship often studied under the epidemiological triad concept. Immunization through vaccination is one of the milestones of modern medicine and has positively impacted public health by reducing the risks of infections and mortality (WHO, 2021). While several vaccines against respiratory viruses have been FDA-approved and licensed, including IAV, IVB, SARS-CoV-2 (van Doorn & Yu, 2020; Bullard & Weaver, 2021; Kyriakidis et al., 2021), the need vaccines for several endemic and emerging viruses remains a task yet to accomplish. In this work, we aimed to contribute to the development of vaccines to prevent two different severe respiratory diseases impacting public health in different ways: hRSV-induced severe disease in infants, and HCPS.

The taxonomic, epidemiology, structure, and pathogenesis, between hRSV and ANDV and their related affections can significantly differ (Jonsson et al., 2010; Openshaw et al., 2017; Bohmwald et al., 2019; Saavedra et al., 2021). However, both viruses might generate a strong, dysbalanced immune activation leading to excessive inflammation in the lungs instead of proper efficient antiviral immunity, thus causing lung damage and life-threatening conditions on certain risk groups (Krüger et al., 2011; Safronetz et al., 2014; Bohmwald et al., 2019; Carvajal et al., 2019; Schönrich & Raftery, 2019). The hRSV elicits an aberrant Th2 immunity in the infant host, with the secretion of IL-4, IL5-, IL-8 cytokines from both innate and adaptive immune

cells, an inefficient T cell response, and a suboptimal antibody production without induction of neutralizing antibodies upon natural infection (Delgado et al., 2009; Varga, 2009; Christiaansen et al., 2014; Vázquez et al., 2019). On the other hand, ANDV produces a strong Th1/Th2 immune activation without activation of regulatory components, and intensive cytokine-secreting cell infiltration in lungs after an extended incubation period, according to data derived from patients and animal models (Mori et al., 1999; Safronetz et al., 2011, 2014, Borges et al., 2008; Saavedra et al., 2021). Current knowledge on pathogenesis and vaccination against these diseases has shown us that both cellular and humoral mechanisms are implicated in protection, as extensively discussed in previous chapters, with CD8+ T cells and neutralizing antibodies as crucial parts of protective mechanisms (Hooper et al., 2006; Manigold et al., 2008; Safronetz et al., 2009; Brocato et al., 2013; Brocato & Hooper, 2019; Warner et al., 2019; Schmidt et al., 2020; Soto et al., 2020). In this regard, we have shown that the recombinant expression of viral antigens in a BCG vector can provide efficient antiviral Th1 immunity against hRSV and hMPV in rodent models, being also safe to administer (Bueno et al., 2008; Cautivo et al., 2010; Palavecino et al., 2014; Soto et al., 2018). Further, we have shown that subcutaneous vaccination of a GMP rBCG-N-hRSV is safe and immunogenic in adult volunteers in recent phase I clinical trials. These results, along with the safety profile, and adjuvant characteristics of the BCG vaccine have led us and several other investigators to explore recombinant BCG vaccines as candidates for several respiratory virus infections, including HIV and SARS-CoV2 (Fuerst et al., 1991; Power et al., 2010, de Queiroz et al., 2020), and to explore the WT BCG vaccine as means of boosting nonspecific effects and to take lessons for future rational vaccine design.

In the first chapter of this work, we took a different approach to explore the candidate rBCG-N-hRSV vaccine and moved from a semi-permissive model of RSV infection to a homologous,

fully-permissive neonate bovine model of RSV infection (Sacco et al., 2012; Altamirano-Lagos et al., 2019). Neonate colostrum-replete calves are a more physiologic model of RSV infection and allow us to explore the vaccine safety and immunogenicity in a system that parallels the human infant, and also allow us to explore potential VED episodes or mechanisms (Acosta et al., 2016; Guerra-Maupome et al., 2019), as extensively discussed in previous chapters. For this, we conducted two independent experiments, one including a WT BCG immunization control (Figure 5). Our results indicate that the subcutaneous administration of 10^7 CFU of GMP rBCG-N-hRSV was well tolerated by newborn animals, without inducing systemic adverse effects but only local, self-resolving reactions and further providing clinical protection against severe RSV disease upon experimental bRSV infection (Figures 6, 7). Clinical protection was associated with decreased relative neutrophil infiltration in immune cell composition in BAL samples (Figure 8 and Supplementary Figure 2). Importantly, vaccination also induced bRSV-specific CD4+ and CD8+ T cell responses (Figure 10) associated with IFN-γ secretion (Figure 11), mucosal NTs in nasal samples (Table 2), in calves that had MDAs, and an IgG2 response (Figure 13E) at 7dpi, indicating that the vaccine is immunogenic in newborn animals with circulating of MDAs (Table 2). BCG immunogenicity seems to be unaffected by MDAs (Stover et al., 1991) and tends to overcome neonatal Th2 immune bias (Marchant et al., 1999), and our results further support those previous observations. Moreover, our safety and immunogenicity results are in line with earlier observations in mice (Céspedes et al., 2017; Soto et al., 2018). Despite the immunogenicity of the rBCG-N-hRSV vaccine, no efficient antiviral immunity was observed, since neither viral loads in the lung nor viral shedding were reduced in rBCG vaccinated animals (Figure 9). Only a trend in reduction of gross pathology was seen in rBCG vaccinated animals (Figure 8A, B). To gain insight into this phenomenon, the analysis of WT

BCG vaccinated animals provides valuable data, although only one experiment included this group with a low sample size (Figure 5). WT BCG vaccination also prevented fever and clinical signs of RSV infection (Figure 6B), modulated neutrophil BAL infiltration (Figure 8D) in the absence of effects on viral load or shedding (Figure 9B, D and Table 1) or bRSV-specific immunity (Figures 10B, D and 11C, D). These results suggest that unspecific mechanisms of protection might be partially responsible for attenuated clinical disease following rBGC and WT BCG vaccination.

The ability of BCG and recombinant BCG vaccines to elicit long-lasting Th1 immunity and IFN-γ secretion (Stover et al., 1991; Marchant et al., 1999; Cautivo et al., 2010; Siddiqui et al., 2012), a pivotal cytokine of antiviral immunity (Kang et al., 2018), led us to develop a new recombinant BCG vaccine to target ANDV-induced HCPS. While several vaccines - notoriously DNA and viral-vectored vaccines - have proven to be protective against several hantaviruses through eliciting nAbs, evidence suggests that optimal protective immunity requires the cellular arm to be triggered alongside (Dong et al., 2019; Khan et al., 2019). After *in silico* design (Supplementary Figure 4), we were able to generate a new recombinant vaccine expressing N-ANDV antigen *in vitro* (Figure 14). The subcutaneous administration of a 10^8 CFU dose of the rBCG-N-ANDV vaccine had similar reactogenicity to WT BCG and a good safety profile, being well-tolerated by mice (Figure 15 A-C). Further, expression of the recombinant antigen *in vivo* is likely since we observed the development of N-ANDV specific immunity: First, a systemic response was detected through *ex vivo* antigen recall assays with recombinant N-ANDV protein, where antigen-specific CD4+ and CD8+ T cells were detected as a higher percentage of cells positive for activation markers CD69, CD25 and CD71 in comparison to control groups after 72 h of stimulation (Figure 16 A-E). The upregulation of the three activation markers after 72 h

of stimulation was not obvious since CD69 expression is considered an early indicator of activation while CD71 is usually upregulated at later times (Johannisson et al., 1995; Cibrián & Sánchez-Madrid, 2017), though more notorious differences might be seen in future experiments exploring CD69+ expression at earlier splenocyte harvesting time. Bueno *et al*. reported similar upregulation levels as percentage of cell counts expressing CD69 after 72h for the rBCG-N-hRSV (Bueno et al., 2008). The relevance of CD4+ and CD8+ T cells for HCPS protection is still under discussion (Prescott et al., 2013; Maleki et al., 2019). Thus, further immunization experiments should be carried in SHs to explore rBCG-N-ANDV immunogenicity and potential protective effects in the lethal ANDV model.

Although an association to a Th1-bias was not demonstrated with our experiments after calculating an IFN-/IL-4 ratio (Table 3), the trend in a higher upregulation of IFN-γ over IL-4 (Figure 17) suggests that the Th1 response might be predominant. Previous reports on rBCG vaccines targeting pneumoviruses, have not explored IFN-/IL-4 ratios to elucidate a Th bias but consistent evidence on antigen-specific IFN-γ upregulation in splenocyte culture and IFN-γ expression by CD4+ T cells, and antibody class switching towards IgG2a isotype argues for a Th1 bias (Bueno et al., 2008; Cautivo et al., 2010; Soto et al., 2018). Similarly, our results from the first chapter of this work also show a strong IFN-γ upregulation in TBLN cell culture and PBMCs upon specific stimulation with either hRSV nucleoprotein, bRSV or PPD-B antigen in calves vaccinated with the rBCG-N-hRSV vaccine (Figure 11 A, C). Moreover, a rBCG vaccine harboring HIV antigens have shown to elicit Env protein specific Th1 immunity and CD4+ T cell responses in spleen and mucosal sites in mice after intraperitoneal injection (Yu et al., 2007). On the contrary, a rBCG expressing the HIV-1 principal neutralizing determinant epitope elicited Th2 immunity in mice when using chaperonin-10 as a carrier antigen (Aravindhan et

al., 2006), evidencing that Th polarization after rBCG immunization is not obvious but dependent at least on the antigen and delivery system, and thus further experiments should be carried out to elucidate if a Th bias is evident upon rBCG-N-ANDV vaccination.

Regarding the humoral immunogenicity of the new rBCG-N-ANDV vaccine, a preliminary analysis of N-ANDV specific IgG levels in serum was performed. A higher level of N-ANDV specific IgG was detected only 14 days after the booster administration of the recombinant vaccine, being not even a two-fold rise. These results suggest that our candidate vaccine might elicit a specific humoral response towards the recombinant antigens but might not be the main immunogenic effect. Previous observations with recombinant BCG and WT BCG vaccines are in line with this, with a rBCG vaccine expressing HIV-1 antigens showing a rise in humoral response only 6 weeks after a primary immunization. Other rBCG vaccines have shown no rise in serum IgG 7 days after booster immunization in mice but only after a viral challenge (Soto et al., 2018). In a phase I clinical trial a rBCG vaccine expressing *Borrelia burgdorferi* outer surface protein A lipoprotein failed to induce a primary humoral response (Edelman et al., 1999). Nasal immunization with rBCG seems to be a better approach in comparison to parenteral immunization to elicit humoral response (Stover et al., 1994). Therefore, our candidate rBCG-N-ANDV vaccine should be tested through different routes. Our results from pre-challenge time points from the calf model of RSV infection are in line with our results showing no upregulation of bRSV specific IgG and IgA in serum and nasal samples (Figure 10 A-D), but a rise in IgG2, and IgA and NTs in serum and nasal fluid only 7dpi (Figure 10 A, E and Table 2). Humoral responses to BCG in cattle have been described as rare and in general BCG vaccination studies show inconsistent results when analyzing humoral response towards bovine TB (Tanner et al., 2019). Overall, this suggest our rBCG vaccines do not induce a primary humoral response but

might prime the cellular immunity to secrete antibodies during a viral challenge, as seen with the rBCG-N-hRSV in mice (Soto et al., 2018) and calves. Thus, an important limitation of our study is the lack of immunogenicity and efficacy data on the SH model of lethal ANDV infection, being those studies a mandatory step to evaluate the candidate vaccine.

While the safety and immunogenicity results drawn from this work and our previous work support further exploration of the rBCG-N-hRSV vaccine in neonate models, and ultimately in human infants, exploring different administration routes and elucidating the relative weight of both heterologous and antigen specific effects might be crucial to promote a more efficient protective response against both human and bovine RSV. Although not evaluated here, *in vitro* and *in vivo* innate training has been reported in the bovine species after administration of heat-killed *M. bovis* (Juste et al., 2016) and BCG (Guerra-Maupome et al., 2019b), and the immunomodulatory effects of BCG administration on innate cells have been numerously reported on humans (Kleinnijenhuis et al., 2012, 2014; Arts et al., 2018; Walk et al., 2019; Moorlag et al., 2020). With intense debate about the potential benefits and limitations of BCG-induced non-specific effects is being discussed nowadays (de Bree et al., 2018; Covián et al., 2019; Gonzalez-Perez et al., 2021), recent transcriptomic data of COVID-19 patients have shown that BCG vaccination induces very long-lasting changes that mimic viral infections by upregulation of antiviral defense response and downregulation of myeloid cell immunity (Sharma, 2021). Regarding our new rBCG-N-ANDV vaccine, our preliminary immunogenicity experiments showed no upregulation of N-ANDV-specific responses at neither cellular nor humoral levels, however exploring potential cross-protective effects of BCG on a lethal ANDV infection model of HCPS might be very informative and thus further exploration of BCG effects on HCPS progression in SHs is warranted.

7. CONCLUSIONS

Based on the results obtained from this doctoral thesis, the following conclusions are drawn:

The administration of a subcutaneous 10^7 CFU dose of a GMP rBCG-N-hRSV vaccine in a prime and booster regime shows a good safety profile in a newborn neonatal model of RSV infection, generating only self-resolving injection site reactions with no systemic compromise of calf health, and no signs of VED following experimental RSV infection during the first 7 dpi. Besides, this vaccination approach is immunogenic in the presence of MDAs (Table 2), generating an anamnestic humoral response upon RSV infection in the upper respiratory tract and a CD4+, CD8+ RSV specific T cell response near the site of infection at 7dpi. These results support further testing of the rBCG-N-hRSV in infants to investigate its safety profile and immunogenicity. Interestingly, vaccination provided clinical protection to severe RSV disease in calves, supporting further trials in calves to prevent bRSV infection.

On the other hand, we observed that the N-ANDV antigen was efficiently produced by a new rBCG vaccine after inserting an integrative plasmid harboring the antigen CDS. The subcutaneous administration of a 10^8 CFU dose of rBCG-N-ANDV in a prime and booster regime showed a similar safety profile to WT BCG, generating only injection site reactions in the form of skin induration. Also, it generated a systemic CD4+ and CD8+ N-ANDV-specific T cell response associated with higher secretion of IFN-γ, suggestive of Th1 immunity. An anamnestic anti N-ANDV IgG response was detected in the serum of animals vaccinated with rBCG-N-ANDV 14 days after the booster. These results indicate that the candidate vaccine is safe and immunogenic and therefore suitable to be tested in further animal models to prevent lethal HCPS.

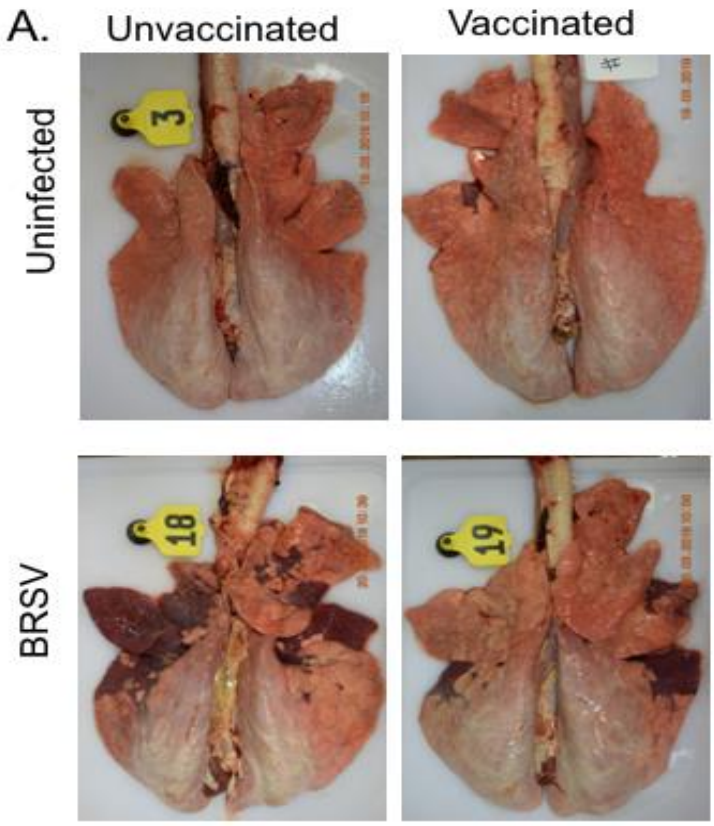
8. SUPPLEMENTARY MATERIAL

Group	Study 1				Study 2			
	Negative	Suspect	Positive	Total	Negative	Suspect	Positive	Total
Unvaccinated	8	0	0	8	8	0	0	8
WT BCG	--	--	--	-	6	0	2	8
rBCG-N-hRSV	4	2	5	11	2	2	4	8

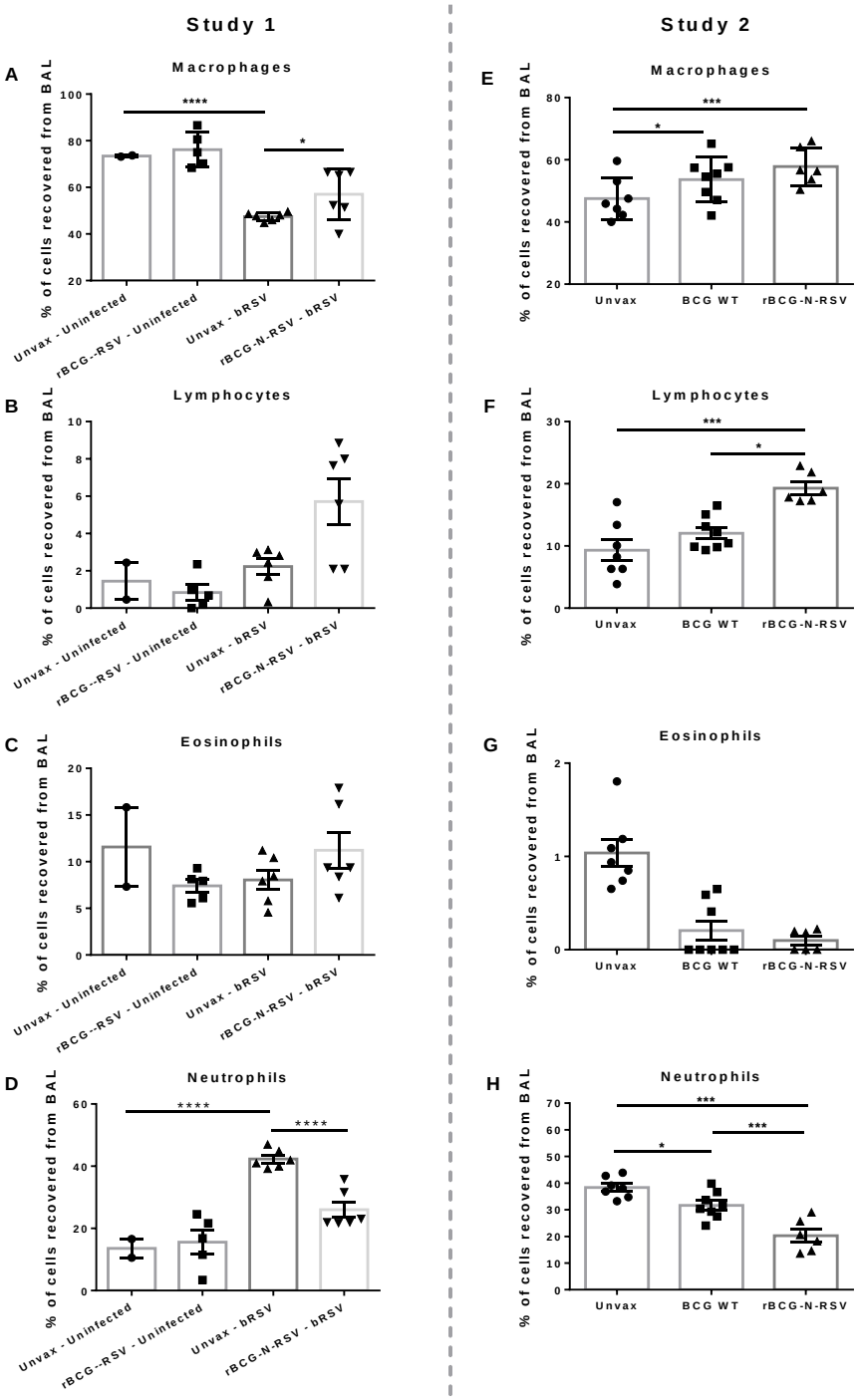
Supplementary Table 1. **Summary of Comparative Cervical Test Results**. Newborn calves were vaccinated with rBCG-N-RSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Then, 0.1 mL (1 mg/mL concentration) of PPD-A and of PPD-B were injected into the neck skin of calves. Local reactions were registered as increase of skin thickness for both injection sites, 72h after antigen inoculation and before bRSV challenge. Negative: No reaction or no increase of PPD-B over PPD-A; Suspect: Increase of 1-3mm of PPD-B over PPD-A; Positive: Increase ≥ 4mm of PPD-B over PPD-A. The test and results were performed and interpreted according to the OIE Terrestrial Manual, Eight Edition.

Primer	5’ – 3’ sequence	Use
VECTOR.FOR	CAAGAGCCACTTAAGTTGTAGCTAGCG TACGATCGACTGCC	Gibson assembly cloning, plasmid PCR
VECTOR.REV	CTTGGAGGGTGCTCATCGCAATTG TCTTGGCCATTGC	Gibson assembly cloning, plasmid PCR
FRAGMENT.FOR	GCCAAGACAATTGCGATGAGCAC CCTCCAAGAATTGC	Gibson assembly cloning, CDS PCR
FRAGMENT.REV	GTCGATCGTACGCTAGCTACAAC TTAAGTGGCTCTTGGTTG	Gibson assembly cloning, CDS PCR
pMV 361 FW	CGGTGAGTCGTAGGTCGGGA	Confirmation of CDS integration into plasmid
pMV 361 RV	GAGCAAGACGTTTCCCGTTG	Confirmation of CDS integration into plasmid
N FW	TACGTGAATTCATGAGCACCCTCCAAG	Colony PCR detection of CDS
N RV	AGTGAATTCCTACAACTTAAGTGGCTCT TGG	Colony PCR detection of CDS

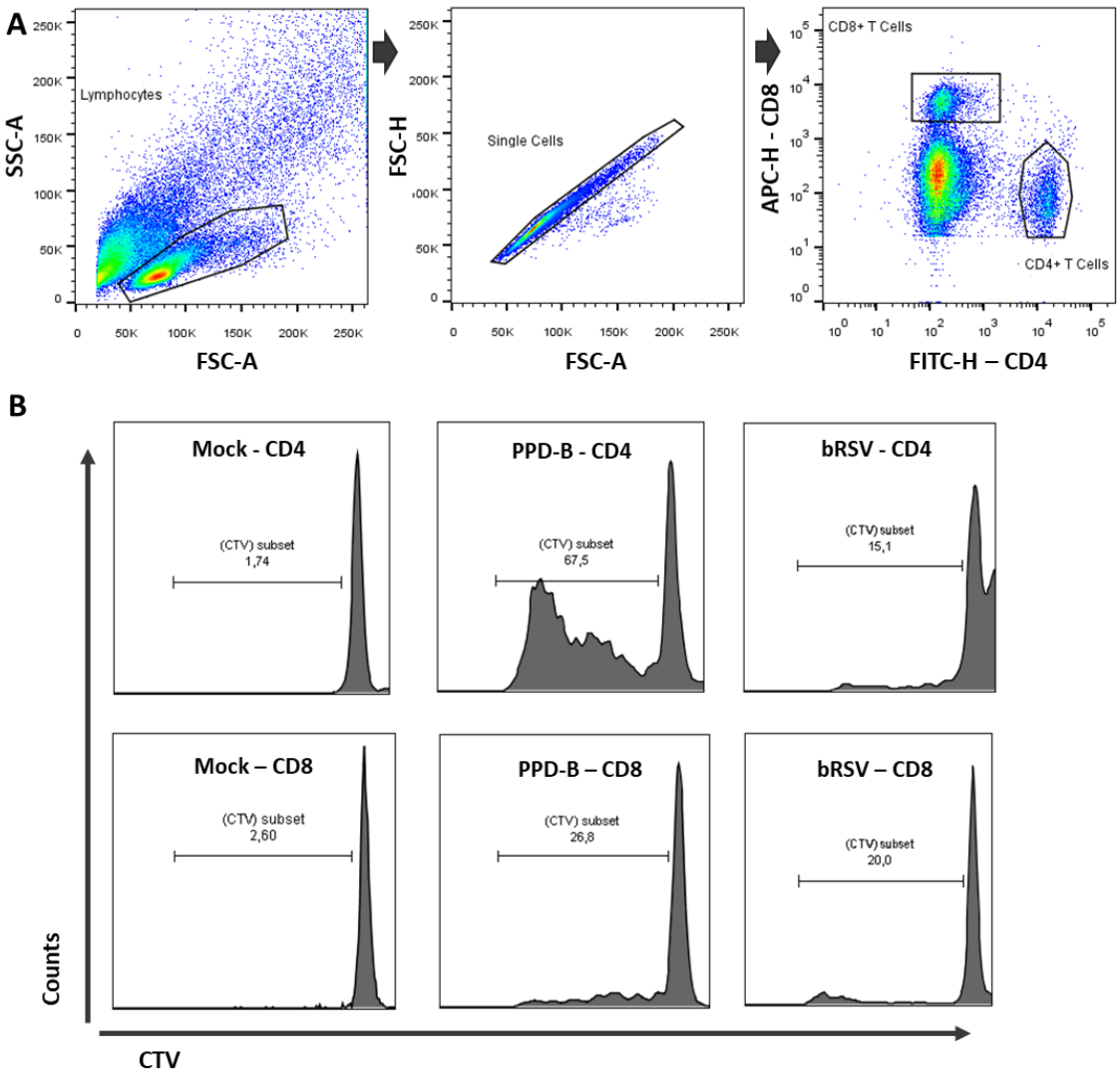
Supplementary Table 2. Primers used for construction and confirmation of pMV361-N-ANDV.



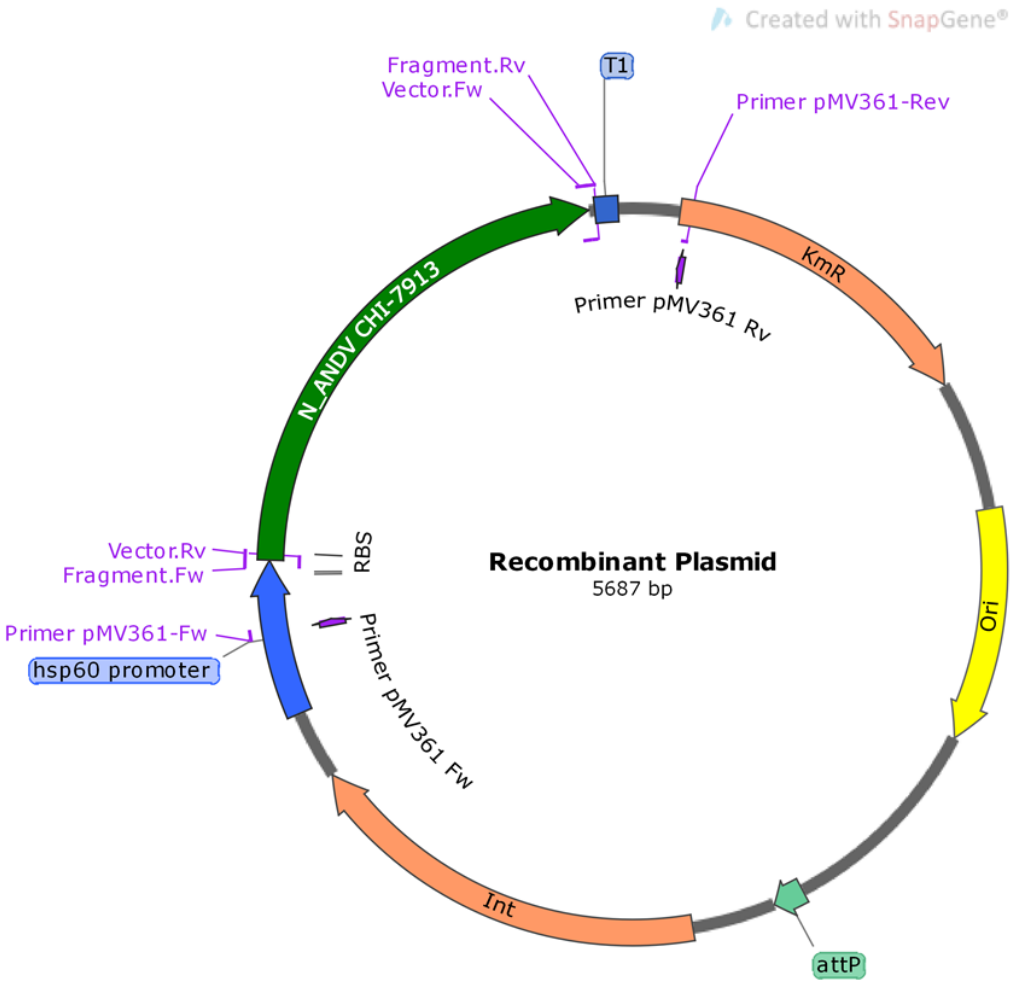
Supplementary Figure 1. Gross pathology images of representative calves from Study 1. Newborn calves were vaccinated with rBCG-N-hRSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Fourteen days after the booster, calves were infected with BRSV strain 375 via aerosol inoculation. All animals were humanely euthanized on day 7 post-infection. **(A)** Lungs were removed, and the dorsal and ventral sides of the lungs were photodocumented. A pathological evaluation was performed similarly to previous descriptions (Randy E. Sacco, Nonnecke, et al., 2012; Viuff et al., 2002).



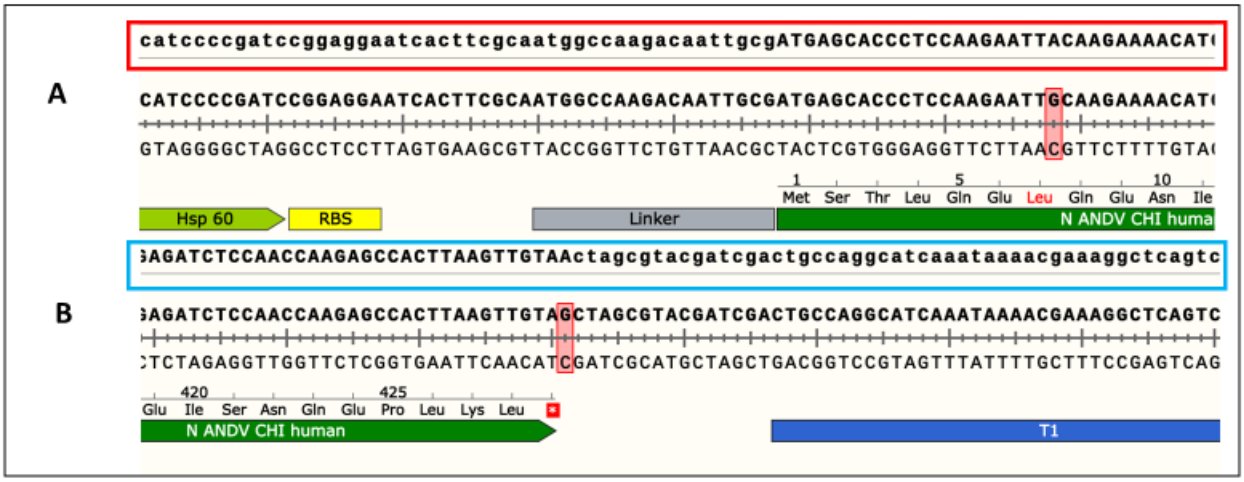
Supplementary Figure 2. rBCG-N-RSV vaccination modifies BAL cells relative frequency after bRSV infection. On day 7 post-infection, BAL samples were collected, and cytospins prepared. The cells were differentially stained with Modified Wright stain. The number of neutrophils, macrophages, lymphocytes, and eosinophils were determined by microscopy. Data are depicted as mean relative frequencies of each population ± SEM. *p<0.05 ***p<0.001 ****p<0.0001 as determined by 2-way ANOVA and Sidak's multiple comparisons test.



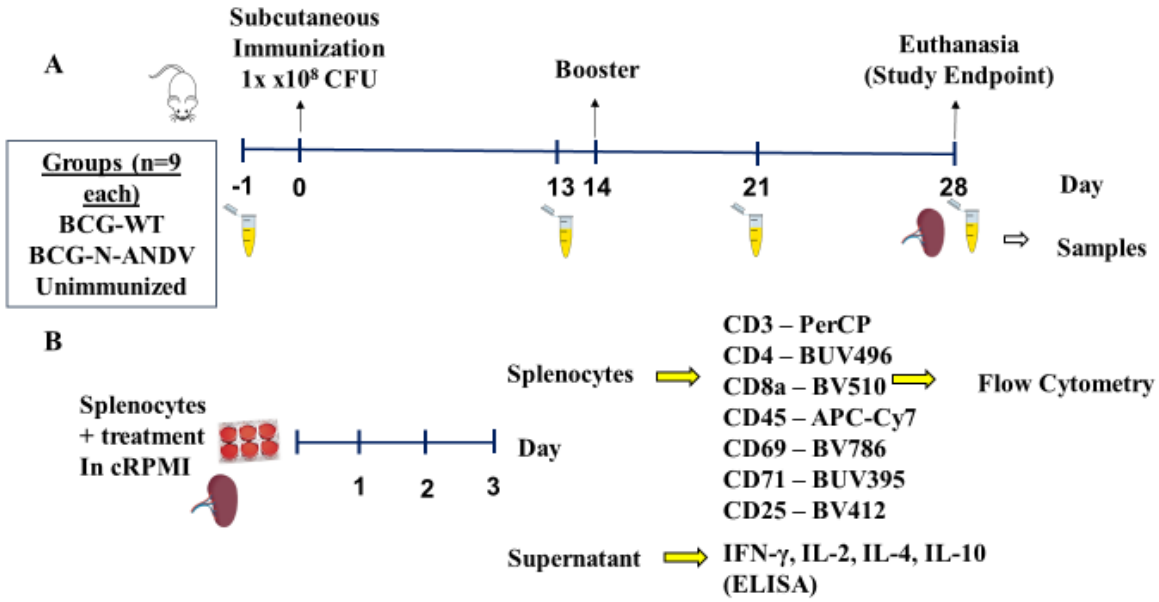
Supplementary Figure 3. Representative gating plots for identification of dividing CD4$^+$ and CD8$^+$ T cells. Study 1 PBMCs and Study 2 Tracheobronchial lymph node cells (TBLNs) were isolated on day 7 after infection, labeled with Cell Trace Violet, restimulated *in vitro* as in Figure 5, and analyzed by flow cytometry for determining dividing CD4$^+$ and CD8$^+$ cells. **(A)** Gating hierarchy as depicted by arrows: lymphocytes (first gate), single cells (second gate), and CD4$^+$ and CD8$^+$ cells (third gate). **(B)** Determination of dividing subsets: Results from each stimulation condition were calculated as change of CTV subset over mock. Representative samples of mock, PPD-B and bRSV conditions from a rBCG-N-RSV-vaccinated, bRSV-infected animal are shown.



Supplementary Figure 4. *In silico* design of recombinant plasmid for N-ANDV expression in BCG. *In silico* design of the recombinant plasmid was made with SnapGene software. Ubication of primers used for plasmid assembly (Fragment.Fw, Vector.Rv, Fragment.Rv, Vector.Fw) and diagnostic sequencing (Primer pMV361-Fw and pMV361-Rev) are shown. Functional structures are shown with arrows. The hsp60 promoter is depicted as a blue arrow. RBS: Ribosome binding site; attP: recombination site; Int Integrase CDS; Ori: origin of replication; KmR: kanamycin resistance gene; N_ANDV CHI-7913: N-ANDV CDS; T1: terminator.



Supplementary Figure 5. Confirmation of correct plasmid asssembly through Sanger Sequencing. The recombinant plasmid was assembled using primers shown in Supp Table 2, and after the Gibson Assembly reactions, products were submitted to Sanger sequencing using primers shown in Supp Table 2. **A.** Expected sequence at 5' end of the N-ANDV ORF (red box, above), according to in silico design of the recombinant plasmid and sequenced product at 5' end of the N-ANDV ORF, according to Sanger sequencing (below). **B**. Expected sequence at 3' end of the N-ANDV ORF (light blue box, above), according to in silico design of the recombinant plasmid and sequenced product at 5' end of the N-ANDV ORF, according to Sanger sequecing (below). Hsp60: promoter region; RBS: ribosome binding site; linker: remanent codifying sequence of pMV361 plasmid; N-ANDV CHI human: N-ANDV CDS; T1: terminator. Stop codon shown in the red box at the end of N-ANDV CDS.



Supplementary Figure 6. Diagram of experimental procedures to test the immunogenicity of rBCG-N-ANDV in BALB/c mice. A. Four to six-week-old male BALB/c mice received subcutaneous administration of 10^8 CFU of BCG WT or rBCG-N-ANDV in the central dorsal flank area in 100µl of sterile PBS. A control group received 100µl of sterile PBS only. A booster immunization was administered to mice 14 days after prime immunization. Blood samples were collected through submandibular puncture on day 0 (pre immunization), day 14 (pre booster), and day 28 (study endpoint). Animals were euthanized on day 28 with a combination of ketamine/xylazine, then spleens were aseptically collected and placed on cRPMI media. **B.** Splenocytes were aseptically collected and placed in cRPMI media. 500,000 cells were plated in a 96-well plate with cRPMI and incubated with N-ANDV protein [10 µg/mL], PPD-B [20 µg/mL], and concanavalin A (3 µg/mL) as a positive control. After 72 hours, plates were centrifuged at 0.3 g for 5 min at 4°C, and cells were analyzed through flow cytometry to detect the expression of CD69, CD71, and CD25 activation markers on $CD4^+$ and $CD8^+$ T lymphocytes. Supernatants were evaluated through ELISA for the secretion of cytokines.

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10. APPENDIX

10.1 Original articles generated during this thesis and doctoral formation.

Díaz F.E.; Guerra-Maupome M.; McDonald P.O.; Rivera-Pérez D., Kalergis A.M.; McGill, J.L. A recombinant BCG vaccine is safe and immunogenic in neonatal calves and reduces the disease caused by the Bovine Respiratory Syncytial Virus. Front. Immunol. 12:664212. doi: 10.3389/fimmu.2021.664212

10.2 Participation as first or second author in revision articles during this thesis and doctoral formation.

Saavedra, F, **Díaz, F.E**.; Retamal-Díaz, A.; Covián, C; González, P.A.; Kalergis, A.M. Immune response during hantavirus diseases. Implications for immunotherapies and vaccine design. doi: 10.1111/imm.13322

Altamirano-Lagos, M.J; **Díaz, F.E**.; Mansilla, M.A.; Rivera-Pérez, D.B.; Soto, D.A.; Vásquez, A.E.; Kalergis, A.M. 2018. Contribution of Animal Models to Understanding the Pathology Caused by the Respiratory Syncytial Virus. Front Microbiol. 2019 May 3;10:873 doi: 10.3389/fmicb.2019.00873.

Acevedo O.A, **Díaz F.E**, Beals T.E, Benavente F.M, Soto J.A, Escobar-Vera J, González P.A, Kalergis A.M. Contribution of Fcγ Receptor-Mediated Immunity to the Pathogenesis Caused by the Human Respiratory Syncytial Virus. Front Cell Infect Microbiol. 2019 Mar 29;9:75. doi: 10.3389/fcimb.2019.00075.

Díaz F.E., Abarca K., Kalergis A.M. An Update on Host-Pathogen Interplay and Modulation of Immune Responses during Orientia tsutsugamushi Infection. Clin Microbiol Rev. 2018 Jan 31;31(2). pii: e00076-17. doi: 10.1128/CMR.00076-17.

10.3. Other contributions to revision articles during this thesis and doctoral formation.

Covián C, Fernández-Fierro A, Retamal-Díaz A, **Díaz FE**, Vasquez AE, Lay MK, Riedel CA, González PA, Bueno SM, Kalergis AM. BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design. Front Immunol. 2019. Nov 10:2806. doi: 10.3389/fimmu.2019.02806.

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10.3 Participation in academic events during this thesis and doctoral formation

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A Recombinant BCG Vaccine Is Safe and Immunogenic in Neonatal Calves and Reduces the Clinical Disease Caused by the Respiratory Syncytial Virus

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The human respiratory syncytial virus (hRSV) constitutes a major health burden, causing millions of hospitalizations in children under five years old worldwide due to acute lower respiratory tract infections. Despite decades of research, licensed vaccines to prevent hRSV are not available. Development of vaccines against hRSV targeting young infants requires ruling out potential vaccine-enhanced disease presentations. To achieve this goal, vaccine testing in proper animal models is essential. A recombinant BCG vaccine that expresses the Nucleoprotein of hRSV (rBCG-N-hRSV) protects mice against hRSV infection, eliciting humoral and cellular immune protection. Further, this vaccine was shown to be safe and immunogenic in human adult volunteers. Here, we evaluated the safety, immunogenicity, and protective efficacy of the rBCG-N-hRSV vaccine in a neonatal bovine RSV calf infection model. Newborn, colostrum-replete Holstein calves were either vaccinated with rBCG-N-hRSV, WT-BCG, or left unvaccinated, and then inoculated *via* aerosol challenge with bRSV strain 375. Vaccination with rBCG-N-hRSV was safe and well-tolerated, with no systemic adverse effects. There was no evidence of vaccine-enhanced disease following bRSV challenge of rBCG-N-hRSV vaccinated animals, suggesting that the vaccine is safe for use in neonates. Vaccination increased virus-specific IgA and virus-neutralization activity in nasal fluid and increased the proliferation of virus- and BCG-specific CD4+ and CD8+ T cells in PBMCs and lymph nodes at 7dpi. Furthermore, rBCG-N-hRSV vaccinated calves developed reduced clinical disease as compared to unvaccinated control calves, although neither pathology nor viral burden were significantly reduced in the lungs. These results suggest that the rBCG-N-hRSV vaccine is safe in neonatal calves and induces protective humoral and cellular immunity against this respiratory virus. These data from a newborn animal model provide further

support to the notion that this vaccine approach could be considered as a candidate for infant immunization against RSV.

Keywords: RSV, neonatal calf model, vaccines, BCG, recombinant BCG

INTRODUCTION

The human Respiratory Syncytial Virus (hRSV) is the leading etiological agent of acute lower respiratory tract infections in infants (1), responsible for an estimated 3.4 million hospitalization episodes in children under 5 years of age each year (2). Clinical disease ranges from mild presentations, including rhinorrhea, coughing, and congestion, to respiratory distress, and life-threatening conditions characterized by alveolitis, bronchiolitis, and pneumonia (3). Importantly, hRSV is a significant cause of mortality in this age group, mostly in developing countries (2). Most children are infected by hRSV during the first three years of life, and reinfections are common (4). Furthermore, severe hRSV disease is a predisposing factor for otitis media (5) and has been associated with later health complications, such as development of asthma and recurrent wheezing (6, 7). Besides, extrapulmonary symptoms, including central nervous system pathology and neurological signs have also been linked to hRSV infections (8–11).

Despite the high health burden due to hRSV, no licensed vaccines are available to reduce or prevent the disease caused by this virus in infants (12). An early trial using a formalin-inactivated RSV vaccine (FI-RSV) led to vaccine enhanced disease (VED) upon natural RSV infection in vaccinated volunteers, instead of generating protective immunity against the virus (13–16). Increased hospitalization rate and two fatalities were observed after this trial. Studies on different animal models have associated VED to a Th2 polarized immune response (17–20), a suppressed cytotoxic lymphocyte (CTL) response (19), and an inadequate antibody response (21, 22). However, the immunological mechanisms have been scarcely addressed in mechanistic studies. An essential role for $CD4^+$ T cells, but not eosinophils, has been recently demonstrated in *in vivo* mouse models. Interestingly, CD4+ Th1 subsets appear to be responsible for airway obstruction and weight loss, while Th2 subsets account for mucus hypersecretion and airway hyperreactivity (20). Despite that VED mechanisms are still under discussion, RSV vaccine candidates targeting infant populations require evaluating potential VED manifestations in animal models (23). Importantly, these studies highlight the importance of a balanced cellular immunity to prevent immunopathology.

Along these lines, we have shown that a recombinant *Mycobacterium bovis* Calmette-Guerin (BCG) expressing hRSV Nucleoprotein (N) (rBCG-N-hRSV) primes hRSV-specific $CD4^+$ T cells and $CD8^+$ CTLs that promote antiviral immunity, reduce neutrophil infiltration, and prevent lung damage in a mouse model of infection (24, 25). This vaccine generates a Th1/Th17 biased repertoire of virus-specific memory T cells that confer long-term immunity against hRSV (24, 25), with early recruitment of IFN-γ producing T cells into the lung (26). Furthermore, mice immunized with this vaccine developed a protective humoral response characterized by an isotype class switching towards IgG2a that correlates with viral clearance (27). Importantly, immunization with rBCG-N-hRSV manufactured under current Good Manufacturing Practices (GMP) is safe in mouse models, and induces no observable adverse effects (25). Moreover, recent phase I clinical trial indicated that intradermal administration of doses up to 1×10^5 CFU of GMP rBCG-N-hRSV is safe in healthy adults (28). Considering the extensively accepted safety and immunogenicity profile of the BCG vaccine in newborns (29), the rBCG-N-hRSV is intended for use on neonates to prevent severe hRSV infection (30). However, since the mouse model is not ideal to rule out the possibility of VED (23, 31, 32), further studies employing suitable animal models are required to determine the safety of the rBCG-N-hRSV in target populations.

Bovine RSV (bRSV) is a significant cause of respiratory disease in cattle worldwide, as an agent of enzootic pneumonia in dairy calves and summer pneumonia in nursing beef calves (33–35). Furthermore, bRSV infection is a predisposing factor to secondary bacterial infection and the development of Bovine Respiratory Disease Complex (33–35). These conditions are highly prevalent and a major cause of mortality, as well as of economic losses due to reduced animal performance and costs associated with treatment and control measures (34, 35). Bovine and human RSV are similar at both genetic and antigenic levels, and calf bRSV infection displays many similarities to hRSV infection in humans, including seasonal periodicity, similar age-related susceptibility, gross and microscopic pathology, and innate and adaptive immune responses (34, 36). Severe RSV infection in infants and calves is characterized by an excessive, rapid neutrophil recruitment into lung, a delayed RSV specific $CD8^+$ T cells response, and a strong expression of Th2 cytokines (37–40). Such an unbalanced immune response ultimately leads to lung damage and respiratory deficiency (41, 42). In addition, RSV vaccine development needs to overcome similar challenges in humans and the bovine species, including the need to generate a robust immune response in a young population in presence of maternally derived antibodies (MDA). Moreover, VED has been observed in calves after natural (43, 44) and experimental bRSV infection (45, 46), characterized by a Th2-biased immune response, reduced $CD8^+$ T lymphocyte response and decreased IFN-γ production (47).

The neonatal calf model is a relevant model for the infant immune system and has been extensively used to study antiviral and therapeutic compounds, and vaccines, including preclinical evaluation of candidate hRSV vaccines that contain proteins that are conserved between hRSV and bRSV (31, 35). Thus, RSV vaccine evaluation in a neonatal calf model might provide useful information for the study of vaccine candidates for infants (23).

Here, we evaluated the safety, immunogenicity, and protective efficacy of a GMP rBCG-N-hRSV vaccine (25) in a neonatal bRSV calf infection model. Our results show that vaccination with rBCG-N-hRSV is safe, immunogenic, and partially protective in neonatal calves with MDA. We observed no systemic adverse reactions to the vaccine, and calves developed only minor and resolving vaccine-site reactions following rBCG-N-hRSV immunization. Calves vaccinated with rBCG-N-hRSV mounted virus-specific cellular and humoral immune responses as shown by increased virus-specific IgA and virus-neutralization activity and increased proliferative responses by $CD4^+$ and $CD8^+$ T cells. Further, rBCG-N-hRSV vaccinated calves developed significantly reduced clinical disease as compared to unvaccinated control calves; however, we did not observe differences in lung pathology or viral replication between vaccinates and controls. Importantly, we observed no evidence of VED following bRSV challenge of rBCG-N-hRSV vaccinated animals, suggesting that the vaccine could be further evaluated for safety and efficacy in neonates.

MATERIAL AND METHODS

Animals and Housing

Newborn (2-4 days of age), colostrum-replete, Holstein bull calves were enrolled in the study. The calves were obtained from a local dairy farm free of bovine Tuberculosis, bovine viral diarrhea virus (BVDV) and bRSV. The animals were housed in BSL-2 climate-controlled environment rooms, at the Livestock Infectious Disease Isolation Facility (LIDIF), Iowa State University. Calves were fed commercial milk replacer and later starter concentrates and hay. Water was provided *ad libitum*. Animals were under supervision of a veterinarian throughout the entire study. All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC-18-232) and Institutional Biosafety Committee (IBC-18-076).

Vaccines

The rBCG-N-hRSV (Danish 1331 strain) vaccine (24, 25) used in both studies was produced under current Good Manufacturing Practices (cGMP) standards at IDT Biologika (Rockville, MD USA). A Wild type (WT) BCG, Danish 1331 strain, was used as immunization control in Study 2.

Immunization Schemes

Calves were acclimatized for five days to the study environment prior to vaccination. For Study 1, calves were vaccinated subcutaneously (s.c.) in the right neck with 10^6 CFU of rBCG-N-hRSV suspended in 500 µl of sterile saline (**Figure 1**). A control group of calves remained unvaccinated. Two weeks after the primary vaccination, a booster vaccination was administered in the right neck with 10^6 CFU of rBCG-N-hRSV. For Study 2, 8 calves were vaccinated s.c. with 10^6 CFU of rBCG-N-hRSV, while 8 calves were vaccinated with 10^6 CFU of WT BCG (**Figure 1**). A control group of 8 calves remained unvaccinated. In both studies,

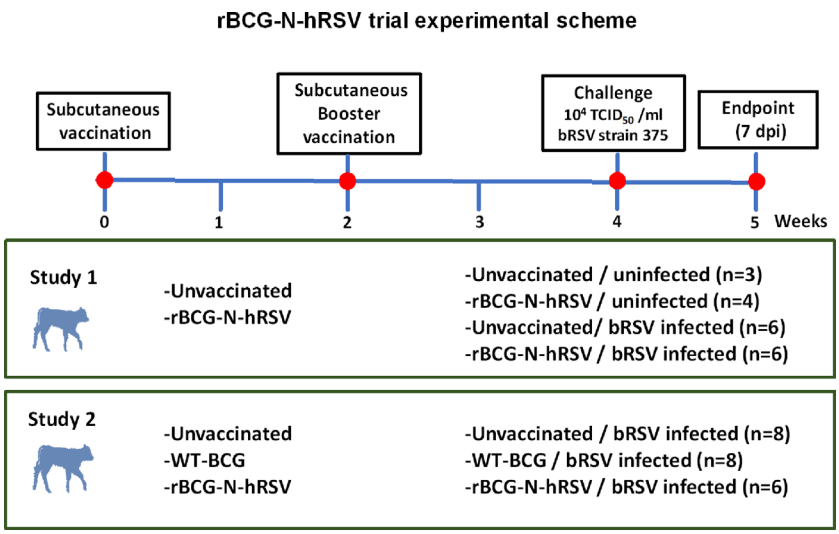

FIGURE 1 | Diagram for the experimental design for studies 1 and 2. Newborn Holstein calves were vaccinated subcutaneously with GMP rBCG-N-hRSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Control calves were left unimmunized. After each immunization, animals were monitored for systemic alterations or local reactions to vaccination. Throughout the study, blood was collected weekly from the jugular vein. Fourteen days after the booster, calves were infected with bRSV strain 375 *via* aerosol inoculation. For Study 1, groups were: unimmunized, uninfected (n=3); rBCG-N-hRSV, uninfected (n=4); unvaccinated, bRSV infected (n=6); and rBCG-N-hRSV vaccinated, bRSV infected (n=6). For Study 2, groups were: Unvaccinated (n=8), WT-BCG vaccinated (n=8), and rBCG-N-hRSV vaccinated (n=6), being all animals bRSV-infected Animals were monitored and sampled daily after challenge to obtain blood and nasal fluid samples. All animals were euthanized 7 dpi for pathological evaluation and sampling.

animals were monitored daily for body temperature and injection site reactions for 1 week following each vaccination.

Nasal fluid samples were collected at weekly intervals following vaccination, and on various days post challenge. Sterile 1-2-inch sponges were dampened with 1 mL of sterile saline solution, and then a single square was inserted into the nostril for 5-10 minutes. Then, sponges were removed, placed in a tube, and an additional 1 mL of sterile saline was added. Liquid was recovered from each sponge by squeezing in the barrel of a 5 mL syringe. The resulting nasal fluid was then aliquoted and frozen at -80° C for later analysis. Peripheral blood mononuclear cells (PBMCs) and sera were collected immediately before vaccination, at regular intervals following vaccination, and on days 3 and 7 after challenge.

Comparative Cervical Tuberculin Tests

A Comparative Cervical Test (CCT) was performed on all animals 10 days after booster immunization. Briefly, 0.1 mL (1 mg/mL concentration) of purified protein derivative (PPD) from M. avium (PPD-A) and of *M. bovis* (PPD-B) were injected in the neck skin of calves three days prior to infection. Then, the reaction size was measured with a caliper and registered as increase of skin thickness for both injection sites, 72h after antigen inoculation and before bRSV challenge. The test and results were performed and interpreted according to the OIE Terrestrial Manual, Eight Edition.

bRSV Inoculum and Aerosol Challenge Model

BRSV strain 375 was prepared from virus stock re-isolated from the lung of an infected animal and passaged less than 4 times on bovine turbinate (BT) cells. The viral inoculum was determined free of contaminating BVDV by PCR. Two weeks after the booster vaccination, calves were inoculated *via* aerosol challenge with ~10^4 $TCID_{50}$/mL of bRSV strain 375 as previously described (48).

Clinical Illness Scoring

For each calf, clinical illness was scored once daily by a trained and blinded observer using an adaptation of the University of Wisconsin Calf Health Respiratory Scoring Chart, originally developed by Dr. Sheila McGuirk (https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_respiratory_scoring_chart.pdf). The scoring chart assigns numbers (0–3) based upon fever and severity of clinical signs that include cough, nasal discharge, eye crusting, and ear position. For our scoring chart we included two additional categories for expiratory effort (0 = no effort to 3 = significant effort) and lung sounds by auscultation (0 = clear, 1 = wheezing and crackling). The scores for each category were totaled to determine the overall clinical score, being 18 the maximum possible score. Any calf with a score equal or over 3 in more than 3 categories for more than 72 hours was euthanized as humane endpoint. One unvaccinated calf was euthanized at 6 days post-infection (dpi) due to severe bRSV during Study 1.

Necropsy and Pathological Evaluation

Calves were euthanized on 7 dpi by barbiturate overdose. Draining tracheobronchial lymph nodes and lungs of were removed, and dorsal and ventral sides of lungs were photodocumented. Pathological evaluation was performed similar to previous descriptions (48, 49). The extent of gross pneumonic consolidation was evaluated using the scoring system similar to that previously outlined (49). A score of 0 was given to lungs free of lesions; 1 was given to lungs with 1-5% affected; 2 was given for 5-15% affected; 3 with 15-30% affected; 4 to lungs with 30-50% of consolidated tissue; and 5 for lungs >50% affected.

Bronchoalveolar lavage fluid (BAL) was obtained after introducing 500 mL of sterile, ice-cold saline solution through the trachea, and then pouring lavage fluid into a glass bottle. Samples of affected and unaffected lung tissue were collected from eight pre-designated sites for histopathological analysis. Tissues were fixed by immersion in 10% neutral buffered formalin and processed by routine paraffin-embedment and sectioning. Five μm sections were H&E stained. Microscopic lesions were evaluated by a board-certified veterinary pathologist in a blinded manner. The severity of the lung lesions was scored based upon the criteria we have previously established (50, 51).

Real Time PCR Analyses

For quantification of bRSV NS2 copy number, lung samples from 2 representative gross-lesioned and 2 non-lesioned tissues from each calf were collected and stored in RNAlater (Invitrogen, Life Technologies). RNA was isolated from lung tissue samples using Trizol Reagent (Invitrogen, Life Technologies). Total RNA was placed in Qiagen RNA isolation columns for RNA clean-up and to remove any contaminating DNA using RNAse-free DNase, per the instructions of the manufacturer (Qiagen). For nasal samples, viral RNA was isolated using MagMax Viral RNA Isolation Kit per the manufacturer's instructions (Applied Biosystem, Life Technologies. The quality and quantity of isolated RNA was verified by QuBit 4 Fluorometer (Thermofisher Scientific), and 500 ng of total RNA were used in each reaction. cDNA synthesis and quantitative rtPCR reactions were carried out using the Taqman RNA-to-CT 1-step kit (Applied Biosystems) per manufacturer's instructions using the following primers and probes: NS2 forward, 5'-GAACGACAGGCCACATTTA-3'; NS2 reverse, 5'-AGGCATTGGAAATGTACCATA-3'; NS2 probe, 5'-/56-FAM/TGAAGCTAT/ZEN/TGCATAAAGTGGGTAGCACA/3IABkFQ/-3'; RPS9 forward, 5'-GTGAACATCCCGTCCTTCAT-3'; RPS9 reverse, 5'-TCTTGGCGTTCTTCCTCTTC-3'; RPS9 probe, 5'-/56-FAM/AAGTCGATG/ZEN/TGCTTCTGCGAGTCC/3IABkFQ/-3'. The reactions were performed on a ThermoFisher QuantStudio 3 Real-Time PCR machine with the following cycling conditions: 48° C hold for 15 minutes; 95° C hold for 10 minutes; 40 cycles of 95° C for 15 seconds, then 60°C for 1 minute. Standard curves for NS2 and RPS9 genes were run in parallel with test samples, and all

standards and test samples were run in triplicate. DNA sequences coding for nucleotides 1-706 of bovine RPS9, and nucleotides 524-1152 of bRSV NS2, both cloned separately into PCR2.1-TOPO vectors, were employed as templates for standard curve construction, respectively. Viral NS2 copy numbers were calculated using standard curves and normalized to RPS9 to correct for differences in lung tissue input.

Virus Isolation

Samples from lesioned lung tissue were snap-frozen during necropsy and stored at -80° C until use. Nasal swabs were collected from each calf prior to infection and on various days post infection and placed in 500 µl virus transport media. Swabs were vortexed vigorously in the media, removed from the collection tube, and the supernatant was stored at -80° C. Samples were thawed once, and a 200 µl aliquot was removed for qPCR. The remaining volume was used for virus isolation, which were performed as previously described (48).

Serum and Nasal Fluid Neutralization assays

Serum and nasal fluid samples collected immediately before challenge and at 7dpi were submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa) for evaluation of bRSV-specific neutralization titers.

Antigen Recall Assays

Bovine peripheral blood was drawn from the jugular vein into syringes containing 2 × acid-citrate-dextrose solution. For isolation of PBMCs, blood was diluted 1:1 in PBS, and centrifuged for buffy coat fractions. Then, those fractions were centrifuged with Histopaque-1077 (Sigma-Aldrich) to obtain isolated PBMCs. Erythrocytes were removed incubating 5 minutes in warm RBC lysis buffer. Finally, cells were washed three times, counted, and resuspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine sera (FBS), 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 1% non-essential amino acids 2% essential amino acids, 1% sodium pyruvate, and 50 µM 2-mercaptoethanol (all from Sigma-Aldrich). Besides, BAL samples obtained during necropsies were kept on ice, filtered over sterile gauze, and centrifuged 10 minutes at 200g, 4 °C. Erythrocytes were removed incubating 5 minutes in warm RBC lysis buffer. Also, tracheobronchial lymph nodes were collected, and cells obtained after disaggregating the tissue on cRPMI and passing the cell suspension through a 40 µm cell strainer. For all samples, cells were washed, counted, and resuspended in cRPMI. Then, PBMCs and lymph node cells were resuspended in 1 ml of PBS containing 10 µM of the CellTrace Violet (CTV) stain, and incubated 20 minutes at 37 °C. Labeling was quenched by adding 4 volumes of RPMI, and washed two times with RPMI (Invitrogen, Life Technologies). For all samples, $5x10^6$ cells/mL were plated in round-bottom 96-well plates with 10 µg/mL Purified Protein Derivative from *M. bovis* (PPD-B); a cocktail of 10 µg/mL each recombinant Ag85A and TB10.4 (Lionex GmbH); 10 µg/mL each recombinant N from human RSV (N-hRSV); or a 0.1 MOI of bRSV strain 375. Negative control (mock) wells remained unstimulated. Positive control wells were stimulated with 5 µg/mL Concanavalin A (ConA). Plates were incubated for 6 days at 37° C in a 5% CO_2 incubator. Then, cell culture supernatants were stored at -80° C, and PBMCs immediately stained. PBMCs were suspended in FACS buffer (10% FBS and 0.02% NA-azide in PBS) and incubated 30 minutes at 4°C with 10 µg/mL of mouse anti-bovine CD4 and 10 µg/mL of mouse anti-bovine CD8α (clones ILA11A & BAQ111A respectively, both from Kingfisher Biotech, Inc). After washing, cells were incubated 25 minutes at 4°C with anti-mouse IgG2a-FITC and anti-mouse IgM-APC, and then fixed with BD FACS lysis buffer (BD Biosciences) for 10 min at RT, washed, and resuspended in FACS buffer. Cells were analyzed using a BD FACS Canto II (BD Biosciences) and FlowJo Software (Treestar). Percentages of cell proliferation were expressed over mock conditions.

ELISAs

Bovine IL-17A and IFNγ were quantified using commercial bovine kits according to the instructions provided by manufacturer (Kingfisher Biotech, Inc). Indirect ELISAs were used to quantify IgA in the nasal fluid (Studies 1 and 2) and total IgG (Study 1) in serum. For the IgA quantification, 96-well ELISA plates were coated overnight at 4° C with 100 µl/well of bRSV stock (~10^4 $TCID_{50}$). Negative control wells were coated with 100 µl/well cell culture media prepared from uninfected BT. To disrupt mucus, nasal fluid samples were diluted 1:2 and treated with 10 mM dithiothreitol for 1 hour at 37° C prior to plating. Serum samples were diluted 1:1000. Plates were blocked using 150ul/well of 1% nonfat dry milk in PBS. All samples were plated in duplicates, incubated for 2 hours at RT, and then washed with 200 ul/well of 0.05% Tween 20 in PBS. Then, plates were incubated 1 hour at RT with either Mouse anti-bovine IgA-HRP (Bethyl Laboratories) at 0.5 µg/mL, or mouse anti-bovine IgG-HRP (Bethyl Laboratories) at 0.5 µg/mL. After incubation, plates were washed three times with 200 ul/well of 0.05% Tween 20 in PBS, and then developed using 50ul/well of Pierce 1-Step Ultra TMB Substrate (ThermoScientific Pierce). The reaction was stopped with the addition of 50ul/well 0.2 M H_2SO_4, and plates were read using a 450 nm wavelength, with a 540 nm reference wavelength, using an automated plate reader. For Study 2, bovine IgG1 was quantified using commercial Svanovir BRSV Ab kit (Svanova, Boehringer Ingelheim) according to the instructions provided by the manufacturer. Bovine IgG2 was quantified modifying Svanovir BRSV Ab kit, by incubating with sheep anti-bovine IgG2-HRP (Bethyl Laboratories) instead of provided secondary antibody reagent. All samples were plated in duplicate and included a negative control well.

Statistical Analyses

For relative gene expression analyses, ΔΔCt values were used to calculate $2^{-\Delta\Delta Ct}$ (52), and results are shown as expression relative to uninfected control samples. Results are expressed as average ± standard error of the mean (SEM). Statistical significance was determined by two-way Analysis of Variance (ANOVA) or two-way ANOVA with repeated measures,

followed by Sidak's multiple comparisons test using GraphPad Prism 7 software (GraphPad Software, Inc).

RESULTS

Vaccination With rBCG-N-hRSV in Neonatal Calves Shows a Good Safety Profile

To evaluate the safety of the rBCG-N-hRSV vaccine in a neonatal calf model, animals were monitored daily for body temperature and injection site reactions for one week following each vaccination. Calves were vaccinated with the rBCG-N-hRSV vaccine or WT-BCG at 2-4 days of age and then boosted two weeks later. All animals were monitored daily for body temperature and injection site reactions for one week following each vaccination. During Study 1, minor injection site reactions were observed in 11 of the 12 rBCG-N-hRSV vaccinated calves and included minor swelling and hardening of the vaccination site. Vaccination site reactions resolved within 4-5 days. Following booster vaccination, injection site reactions were observed in all vaccinated calves and included thickening and hardening of the skin surrounding the injection site. Those reactions resolved within 7-10 days after vaccination. In study 2, only one rBCG-N-hRSV vaccinated calf developed minor swelling and hardening of the skin after the first immunization, which resolved within three days. No reactions were observed in WT BCG vaccinated or unvaccinated calves. Furthermore, no significant body temperature changes were observed following booster vaccination in any animal (data not shown).

Vaccination With rBCG-N-hRSV Ameliorates bRSV Clinical Symptoms Without Signs of Enhanced Disease

Two weeks after the booster immunization, calves were challenged *via* aerosol inoculation with 10^4 $TCID_{50}$ bRSV strain 375. Control calves were not challenged. Following infection, all animals were monitored daily for body temperature and clinical signs, as described in the Materials and Methods section. During study 1, unvaccinated bRSV infected calves displayed significant clinical signs beginning on days 4-5 after infection, which included fever, lethargy, nasal and ocular discharge, dyspnea, and lung sounds (**Figure 2A**). One animal was euthanized on day six after infection due to severe clinical disease. Although calves immunized with rBCG-N-hRSV developed some signs of bRSV infection, disease and clinical scores were significantly reduced on days 4-7 pi, as compared to unvaccinated and infected calves ($p<0.05$ for day 4 and 8 pi, <0.001 for day 6pi and <0.0001 for day 5 pi) (**Figure 2A**). Unvaccinated and challenged calves presented fever starting day 4 pi, while rBCG-N-hRSV-vaccinated calves had no rise in body temperature (**Supplementary Figure 1A**). During study 2, unvaccinated, bRSV infected calves also developed clinical signs, including fever, lethargy, nasal and ocular discharge, and mild dyspnea (**Figure 2B**). An increase in clinical score on days 6-7 was evidenced in unvaccinated calves when comparing scores at those

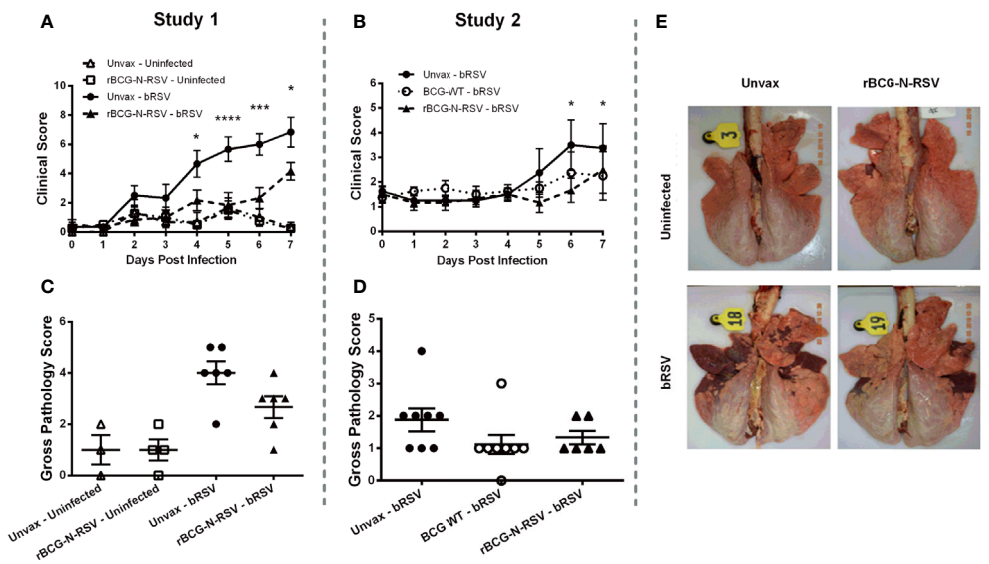

FIGURE 2 | rBCG-N-hRSV vaccination reduces bRSV-associated disease in neonatal calves. Newborn calves were vaccinated with rBCG-N-hRSV (Studies 1 and 2) or WT BCG (Study 2) and boosted 14 days after prime immunization. Fourteen days after the booster, calves were infected with BRSV strain 375 *via* aerosol inoculation. **(A, B)** Clinical Scores. Calves in all four groups were monitored daily by a blinded observer and assigned a clinical score using the criteria outlined in Materials and Methods. Data represented as mean ± SEM. *$p<0.05$ ***$p<0.001$ ****$p<0.0001$ as determined by 2-way ANOVA with repeated measures and Sidak's multiple comparisons test **(C–E)** Gross Pathology Scores. All animals were humanely euthanized on day 7 post-infection. The extent of gross pneumonic consolidation was evaluated based upon the percent of lung affected (0=free of lesions; 1 = 1-5% affected; 2 = 5-15% affected; 3 = 15-30% affected; 4 = 30-50% affected; 5 = >50% affected). Aggregate gross pathology results from all groups and all animals from Study 1 and 2 are depicted in **(C, D)**, respectively. Data represented as mean ± SEM. No statistically significant differences were observed. **(E)** Representative images from one animal from each group from Study 1.

days with day 0 (pre-challenge) ($p<0.05$). Some vaccinated calves also developed clinical signs; however, no significant rise in clinical score was observed at any day when compared to day 0. Similarly, no significant rise in clinical score was observed for WT BCG-vaccinated animals. Although some unvaccinated animals had fever, we observed no statistically significant differences in body temperature between vaccinated and unvaccinated animals (**Supplementary Figure 1B**). Importantly, we observed no signs of VED in calves receiving the rBCG-N-hRSV or the WT BCG vaccines.

To determine the extent of macroscopic lung damage after bRSV infection, calves were euthanized seven days post-infection, and the lungs were evaluated and scored for gross pathology by a blinded veterinary pathologist, as described in the Materials and Methods section. No significant lesions were observed in the lungs of the uninfected control calves in study 1 (**Figures 2C, E**). Calves challenged with bRSV developed evident macroscopic lung pathology, including regional and coalescing areas of lung consolidation (**Figures 2C, E**). The extent of lesions in challenged calves was greater in the Study 1 as compared to Study 2. Although no significant differences in gross lung pathology were observed between unvaccinated control calves and those receiving the rBCG-N-hRSV vaccine, a clear reduction of lung damage was observed for the rBCG-N-hRSV-vaccinated calves in both studies (**Figures 2C, D**). Remarkably, most of the WT BCG-vaccinated animals in study 2 showed very little macroscopic pathology (**Figure 2D**). Additionally, samples of eight predesignated regions of the lung were collected and formalin-fixed during necropsy for histopathological evaluation. Lungs were sectioned and scored by a blinded veterinary pathologist. Few microscopic lesions were observed in the lung tissue samples collected from the uninfected calves (**Figure 3A**). On the other hand, and as expected, calves challenged with bRSV developed extensive histologic lesions, including airway inflammation and necrosis, bronchiolar luminal exudate, leukocyte and lymphocyte infiltration, and pneumocyte hyperplasia. Overall, there were no significant differences in the lung histopathology scores between unvaccinated animals, WT BCG, or rBCG-N-hRSV vaccinated calves infected with bRSV (**Figures 3A, B**).

Severe RSV disease in newborn humans and calves is characterized by bronchointerstitial pneumonia and bronchiolitis, as well as significant airway neutrophil infiltration (32, 34). The local host inflammatory reaction to the infection is a major cause of tissue damage (41, 53–55), with neutrophils pointed out as an important immunopathology source (50, 56). Next, we evaluated whether vaccination with rBCG can modulate neutrophil infiltration at the site of infection. At necropsy, BAL fluid was collected from each animal and

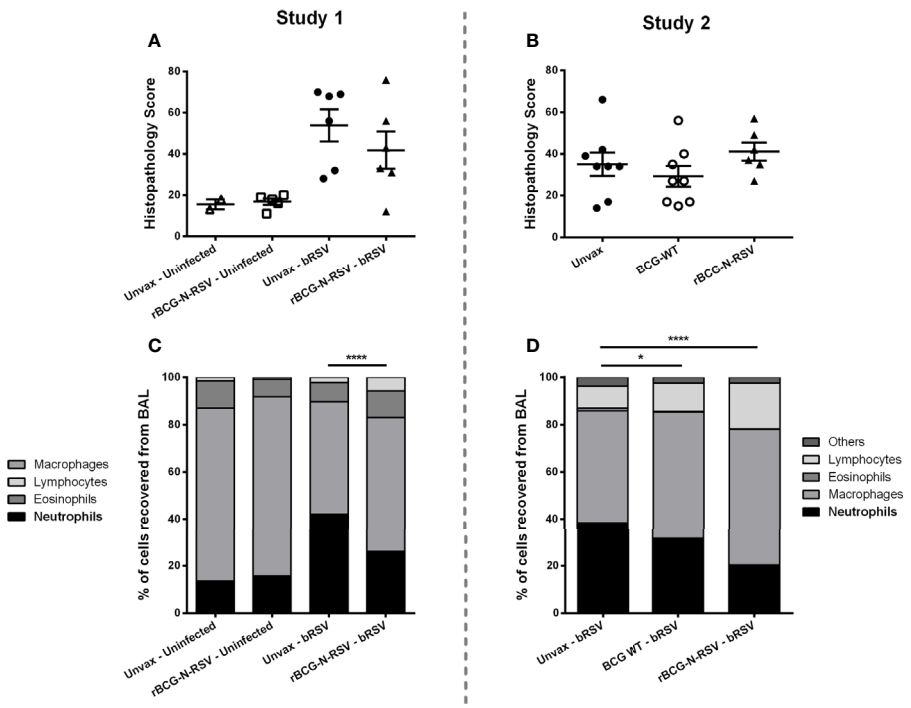

FIGURE 3 | rBCG-N-hRSV vaccination reduces neutrophil BAL infiltration in neonatal calves after bRSV infection. **(A, B)** Lung Histopathology Score. Sections of lungs were collected from 8 predesignated locations and microscopic lesions were evaluated by a pathologist in a blinded manner using a scoring system we have previously described. Aggregate histopathology scores from animals in Study 1 and 2 are depicted in **(A, B)**, respectively. Data represented as mean ± SEM. No statistically significant differences between infected groups were observed. **(C, D)** BAL cells relative frequency. On day 7 post-infection, BAL samples were collected, and cytospins prepared. The cells were differentially stained with Modified Wright stain. The number of neutrophils, macrophages, lymphocytes, and eosinophils were determined by microscopy. Data are depicted as mean relative frequencies of each population. Data represented as mean ± SEM. *$p<0.05$ ****$p<0.0001$ for the frequencies of neutrophils as determined by 2-way ANOVA and Sidak's multiple comparisons test.

cytospin preparations were differentially stained. The relative numbers of neutrophils, macrophages, lymphocytes, and eosinophils were then quantified by microscopy. As expected, bRSV infection increased neutrophil infiltration into the airways, and the frequency of neutrophils was increased in the BAL of challenged animals as compared to uninfected controls ($p<0.0001$) (**Figure 3C**). However, in both studies, rBCG-N-hRSV-vaccinated calves showed significantly reduced frequencies of neutrophils in the BAL at 7 dpi ($p<0.0001$) as compared to unvaccinated infected animals (**Figures 3C, D**). Remarkably, a mild, significant reduction in neutrophil frequency was observed for WT BCG-vaccinated animals when compared to unvaccinated animals ($p<0.05$) (**Figure 3D**). In both studies, a lower relative frequency of macrophages was observed in unvaccinated, challenged animals when compared to vaccinated calves ($p<0.05$, <0.001 for study 1 and 2, respectively) (**Supplementary Figures 2A, E**). While no differences in relative lymphocyte frequency were observed between any group in Study 1, significantly higher relative lymphocyte counts in rBCG-N-hRSV-vaccinated animals were seen in study 2 as compared to unvaccinated controls ($p<0.001$) (**Supplementary Figures 2B, F**). Finally, no differences were seen in eosinophil infiltration between groups (**Supplementary Figures 2C, G**).

Viral Shedding and Viral Lung Loads Are Not Reduced in rBCG-N-hRSV Vaccinated Calves

Nasal swabs and lung tissue were collected and immediately frozen, then processed for virus isolation as previously described (48). As shown in **Table 1**, no virus was isolated from the nasal swabs of any calf prior to challenge. Following bRSV infection, virus was isolated from the nasal swabs of most infected animals throughout the infection period, regardless of vaccination status. For Study 1, bRSV was isolated from lung tissue samples in 6/6 calves in the unvaccinated infected group and from 4/6 rBCG-N-hRSV vaccinated, infected calves on day 7 after infection. Regarding Study 2, the virus was isolated from lung tissue of all unvaccinated and WT BCG-vaccinated animals and in 4/5 rBCG-N-hRSV vaccinated animals (**Table 1**). Quantitative PCR analyses for the bRSV NS2 gene revealed no statistically significant differences between vaccinated and unvaccinated infected calves for the copy number of NS2 in lesioned lung tissue (**Table 1**). Neither virus nor NS2 copies were detected, in uninfected control calves. These results suggest that neither rBCG-N-hRSV nor WT BCG significantly modulate virus replication in the lower and upper respiratory tract during neonatal calf bRSV infection.

Vaccination With rBCG-N-hRSV Induces Antigen-Specific $CD4^+$ and $CD8^+$ T Cells Secreting Th1/Th17 Cytokines Upon bRSV Challenge

To evaluate the effect of rBCG-N-hRSV vaccination on adaptive cellular immunity after calf bRSV infection, PBMCs and lymph node cell cultures were collected on day seven pi, labeled with CTV stain, stimulated with viral or mycobacterial antigens for six days, and then analyzed by flow cytometry. Antigen-specific $CD4^+$ and $CD8^+$ cells were identified by CTV dilution after proliferation in

TABLE 1 | Virus shedding and lung viral loads in the nasal swabs and lungs.

Group		Study 1					Study 2				
		Nasal Swabs				Lung	Nasal Swabs				Lung
		Day 0	Day 2pi	Day 4pi	Day 7pi	Day 7pi	Day 3pi	Day 7pi			Day 7pi
Unvaccinated - bRSV	Virus isolation	0/6	6/6	5/6	4/6	6/6	7/7	7/7			7/7
	NS gene copies/10^4 RPS9 copies		602 (0-2,544)	29,448 (0-164,817)	1,604 (0-4,986)	122 (1-488)	43,560 (0-190,000)	86,189 (0-547,000)			1,427 (0-9,274)
rBCG-N-hRSV - bRSV	Virus isolation	0/6	4/6	4/6	3/6	4/6	6/7	6/6			4/5
	NS gene copies/10^4 RPS9 copies		56 (0-176)	4,684 (0-14,790)	129 (0-449)	73 (1-351)	66,354 (315-210,000)	540,880 (0-2,470,000)			1,335 (15-4,489)
WT BCG - bRSV	Virus isolation	–	–	–	–		7/7	7/7			8/8
	NS gene copies/10^4 RPS9 copies						2,640 (0-12,700)	946,478 (176-4,460,000)			1,069 (0-6,399)

Nasal swabs were collected from each calf prior to infection and on various days post infection. Lesioned lung tissue was collected at necropsy. Samples were divided and analyzed for virus isolation and virus quantification by qPCR for the bRSV NS2 gene. Virus isolations were performed as previously described Viral NS2 copy numbers were calculated using standard curves. For nasal swabs, 500 ng of isolated RNA were used in the qPCR reactions. Lung tissue were normalized to the housekeeping gene, RPS9, to correct for differences in input material. NS2 copies presented as mean (range) of each group. Neither virus nor NS2 copies were isolated or detected, respectively, in uninfected control calves. No significant differences were observed between unvaccinated, WT BCG and rBCG-N-hRSV vaccinated animals as determined by RM 2-way ANOVA and Sidak's multiple comparisons test.

response to viral or mycobacterial antigens. Background (mock) proliferation was subtracted from all values, and results represent change over mock (**Supplementary Figure 3**). For PBMCs obtained from Study 1, we observed no statistically significant differences in $CD4^+$ or $CD8^+$ T cell proliferation between both infected groups when stimulated with viral antigens. Although trends to increased proliferative responses were observed for $CD4^+$ T (**Figure 4A**) and $CD8^+$ T (**Figure 4C**) in response to N-hRSV and bRSV in the vaccinated/bRSV infected calves, increased T cell proliferation was also observed in samples from vaccinated uninfected animals (**Figures 4A, C**). Besides, $CD4^+$ T cells from calves receiving the rBCG-N-hRSV vaccine responded robustly to both PPD-B and the Ag85A/TB10.4 antigen cocktail (**Figure 4A**). As expected, $CD4^+$ T cells from the unvaccinated/bRSV infected calves did not divide in response to stimulation with PPD-B or Ag85A/TB10.4 (**Figure 4A**). Similar trends were observed for $CD8^+$ T cells, with the highest responses observed in samples from the rBCG-N-hRSV-vaccinated and challenged calves (**Figure 4C**). To evaluate if rBCG-N-hRSV vaccination promotes a Th1/Th17 phenotype in calves, as reported in the murine and human studies (24, 25, 28), cell culture supernatants from the stimulated PBMCs were analyzed by ELISA for bovine IFN-γ and IL-17A. Compared to the PBMCs from unvaccinated/bRSV infected animals, rBCG-N-hRSV-vaccinated/bRSV infected animals mounted a significant IFNγ (**Figure 5A**) and IL-17A (**Figure 5C**) response to both BCG- and RSV-associated antigens including PPD-B, Ag85A/TB10.4, N-hRSV, and BRSV strain 375. Some cytokine responses were seen in unvaccinated, bRSV infected calves, although those responses were not statistically significant when compared to unstimulated control wells. All samples produced robust amounts of IFNγ and IL-17A in response to conA, which was used as a positive control (not shown). Additionally, BAL samples were stimulated with mycobacterial and viral antigens for 6 days and supernatants analyzed by ELISA for bovine IFN-γ and IL-17A. As shown in **Supplementary Figure 4**, rBCG-N-hRSV-vaccinated, infected animals produced significantly higher IFN-γ in comparison to unvaccinated, infected animals ($p<0.05$), however, the enhanced IL-17A levels in response to PPD-B and AG85A/TB10.4 did not reach statistical significance. Both infected groups produced similar levels of IFN-γ and IL-17A in response to viral antigens at 7 dpi.

For the second study, we analyzed proliferative responses of $CD4^+$ and $CD8^+$ T cells, and IFNγ and IL-17A secretion in tracheobronchial lymph node cell cultures, performing assays as described above. $CD4^+$ T cells from vaccinated calves showed robust proliferation after recall stimulation with N-hRSV ($p<0.05$) or bRSV ($p<0.01$) in comparison to the unvaccinated control group (**Figure 4B**). Differences in proliferation after PPD-B or Ag85 stimulation between vaccinated and unvaccinated control groups were not statistically significant. As expected, $CD8^+$ T cell responses were also robustly induced after recall stimulation with bRSV in lymph node cells from rBCG-N-hRSV-vaccinated animals ($p<0.05$) but not unvaccinated or WT BCG-vaccinated control animals (**Figure 4D**). Although statistically significant differences were not found when comparing rBCG-N-hRSV-vaccinated calves to unvaccinated controls, a higher proliferation of $CD8^+$ T cells was observed in rBCG-N-hRSV-vaccinated calves upon recall PPD-B and N-hRSV stimulation (**Figure 4D**). Importantly, cells from all calves included in data analyses showed a robust proliferative response to Con A positive control (not shown). Furthermore, rBCG-N-hRSV-vaccinated animals also showed a significantly higher IFN- γ response to PPD-B ($p<0.0001$) and to bRSV ($p<0.05$) stimulation (**Figure 5B**), which was not seen on WT BCG and unvaccinated animals (**Figure 5B**). A similar trend was observed in N-hRSV-stimulated wells, although differences did not reach statistical significance (**Figure 5B**). Regarding IL-17A, a robust response to PPD-B ($p<0.01$) was seen only in rBCG-N-hRSV-vaccinated calves (**Figure 5D**). In summary, these results indicate that vaccination with rBCG-N-hRSV induce antigen-specific $CD4^+$ and $CD8^+$ T cells associated with a Th1/Th17 secretory phenotype upon bRSV infection in neonatal calves with MDA.

Humoral Immune Responses to Vaccination and bRSV Challenge in Neonatal Calves

To analyze the humoral immune responses induced by rBCG-N-hRSV vaccination, nasal fluid and serum samples were collected at several time points to analyze virus-specific IgA and IgG, respectively. In the Study 1, virus-specific IgA was undetectable in the nasal fluid from any group at baseline (prior to vaccination) or immediately prior to infection (**Figure 6A**). Virus-specific IgA remained below the limit of detection in uninfected control calves throughout the study. By day 7 post-infection, unvaccinated infected calves were beginning to show a virus-specific IgA response in the nasal tract. However, this increase was not statistically significant as compared to the pre-challenge values or to uninfected controls. On the other hand, rBCG-N-hRSV-vaccinated calves developed an anamnestic virus-specific IgA response in the respiratory tract, evidenced by significantly higher levels of virus-specific IgA in the nasal fluid as compared to all other groups ($p<0.0001$) (**Figure 6A**). Analyses of total virus-specific IgG revealed a similar trend, but the differences between groups were not statistically significant (**Figure 6B**). In Study 2, an increase in IgA levels was observed in WT BCG- and rBCG-N-hRSV-vaccinated calves but not in unvaccinated controls when comparing pre-infection levels to 7dpi, however, this difference was not statistically significant (**Figure 6C**). Comparisons between groups at 7dpi showed no differences in IgA levels. In Study 2, virus-specific IgG1 and IgG2 were measured. No differences between groups at any time point were observed for virus-specific IgG1 serum levels. It is noteworthy that IgG1 levels were higher in the unvaccinated and WT BCG-vaccinated group at baseline, as compared to later time points, and as expected, no rise in IgG1 serum levels was seen in those groups at 7dpi (**Figure 6D**). Although a similar trend was observed in rBCG-N-hRSV-vaccinated calves, no significant changes between any time point were observed in this group. In contrast, rBCG-N-hRSV-vaccinated calves showed significantly higher serum levels of IgG2 at 7dpi ($p<0.01$) when compared to unvaccinated animals, whereas unvaccinated calves showed a decreasing trend in IgG2 levels when comparing baseline to 7 dpi levels (**Figure 6E**). Importantly, animals in both studies were colostrum replete, which is likely the main factor contributing to the higher IgG1 and IgG2 serum levels at baseline.

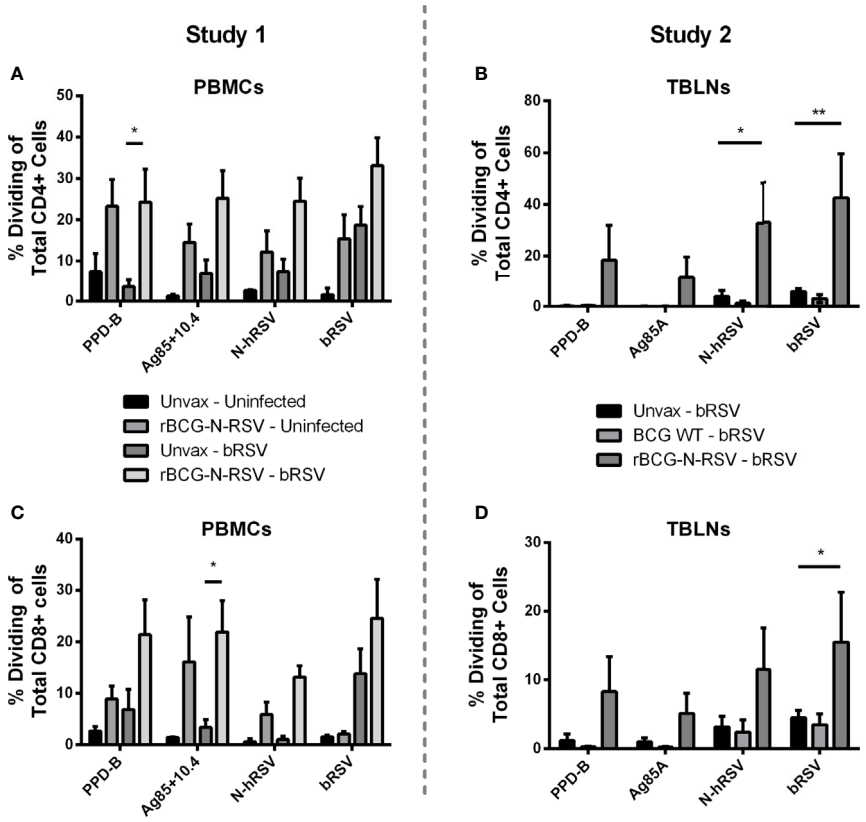

FIGURE 4 | Vaccination with rBCG-N-hRSV elicits antigen-specific CD4 and CD8 T cell proliferative responses. **(A, C)** Study 1 PBMCs and **(B, D)** Study 2 Tracheobronchial lymph node cells (TBLNs) were isolated on day 7 after infection, labeled with Cell Trace Violet, and restimulated *in vitro* with PPD-B, Ag85A/TB10.4, N-hRSV or bRSV strain 375. Mock stimulated cultures were used as negative controls. ConA stimulated cultures were used as positive controls (not shown). Six days later, **(A, B)** $CD4^+$ T and **(C, D)** $CD8^+$ T cell proliferation, as measured by dilution of the Cell Trace dye, was analyzed by flow cytometry. Data represented as mean ± SEM *p<0.05 **p<0.01 as determined by 2-way ANOVA and Sidak's multiple comparisons test.

Virus neutralization assays revealed that all groups, regardless of treatment, had similar pre-challenge titers of neutralizing antibodies in nasal secretions (**Table 2**). however, neutralizing titers (NTs) increased significantly in the nasal fluid samples from rBCG-N-hRSV-vaccinated animals following bRSV challenge in both studies ($p<0.05$) (**Table 2**). Interestingly, WT BCG vaccinated calves also had higher NTs from nasal samples when compared un unvaccinated animals ($p<0.01$). On the other hand, a trend in the increase in NTs in the serum of the rBCG-N-hRSV vaccinated calves was observed in Study 1, but this increase was not statistically significant, which is along the lines with virus-specific IgG serum levels. No differences in serum NTs were observed in study 2 when comparing different groups or time points.

DISCUSSION

Human RSV causes a high impact on health systems worldwide annually, being responsible for millions of hospitalizations and hundreds of thousands of deaths due to acute low respiratory tract infections in high-risk populations, which include infants, elderly and immunocompromised patients (2, 57). Despite more than five decades of research, no vaccine has been licensed to prevent RSV infection in any age group. The only prophylactic tool to prevent severe infection is Palivizumab, a humanized monoclonal antibody that is used only in high-risk infants due to its high cost (58, 59). While several vaccine strategies are under development and clinical testing for either pregnant women, infants, and the elderly (58, 60), the development of efficacious vaccines targeting specific age groups faces different challenges at both pre-clinical and clinical levels. RSV-associated morbidity and mortality are higher in infants under one-year-old in low-income countries; thus children remain a critical target population to implement therapeutic and preventive measures (57, 61). Importantly, vaccines for this age group must be able to elicit robust, long-lasting immunity in a population that is not well equipped to do so and might present significant levels of circulating MDA (60, 62, 63). Additionally, vaccines should avoid VED after natural infections, a phenomenon described after FI-RSV vaccination and subsequent natural infection in seronegative infants, that has been linked to a Th2-biased, dysregulated immune response characterized by inadequate antibody production and weak cytotoxic $CD8^+$ T cell response

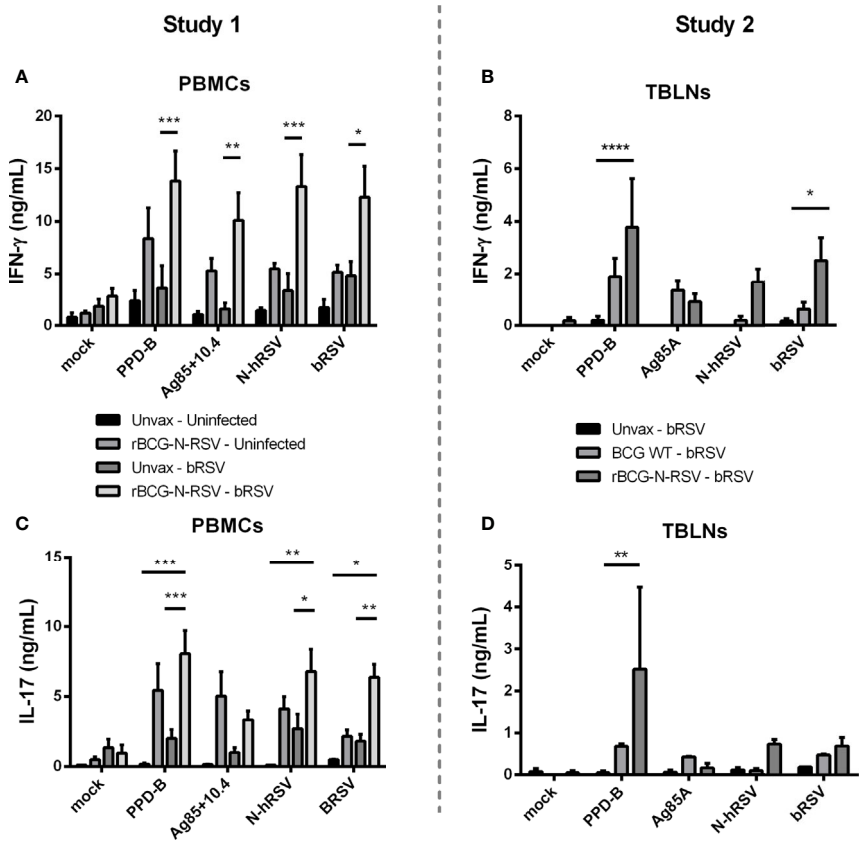

FIGURE 5 | Vaccination with rBCG-N-hRSV increases virus and BCG-specific IFN-γ and IL-17 secretion in PBMC cultures. **(A, C)** Study 1 PBMCs and **(B, D)** Study 2 Tracheobronchial lymph node cells (TBLNs) were isolated on day 7 post-infection, labeled with Cell Trace Violet, and restimulated *in vitro* with PPD-B, Ag85A/TB10.4, N-hRSV or bRSV strain 375. Mock stimulated cultures were used as negative controls. ConA stimulated cultures were used as positive controls (not shown). Six days later, cell culture supernatants were analyzed for bovine IFN-γ and IL-17A secretion using commercial ELISA kits. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ ****$p < 0.0001$ as determined by Two-way ANOVA and Sidak's multiple comparisons test. Data represented as mean ± SEM.

(12, 22, 64). Those challenges underscore the importance of rational design and proper testing of each candidate.

Our group has developed a recombinant BCG vaccine expressing hRSV Nucleoprotein (24–28). This vaccine is aimed to prevent severe RSV infection in infants and is the sole vaccine in clinical development intended for use in neonates (30). Intradermal administration of 10^5 CFU of a GMP rBCG-N-hRSV in healthy male adult volunteers is safe and well-tolerated, as demonstrated in phase I clinical trial (28). To evaluate the safety and immunogenicity of the rBCG-N-hRSV in a neonatal model of RSV infection, we tested the rBCG-N-hRSV vaccine in a neonatal calf model of bRSV infection. The neonatal calf represents a tractable model of infant immunity and a homologous model of RSV infection displaying key clinical and pathological similarities to infant RSV infection, representing a suitable model to study antiviral immunity and to develop preventive strategies, and thus, several vaccines targeting hRSV have been tested in young calves, as we recently reviewed (35). Our results from two independent studies on newborn dairy calves suggest that a dose of 10^6 CFU of GMP rBCG-N-hRSV, administered within the first week of life and boosted 14 days after first immunization, is safe and well-tolerated (**Figure 1**). As expected, local reactions to the recombinant vaccine on the injection site were observed, including minor inflammation and swelling, similar to the lesions described in mice (25) and healthy adults (28), and BCG-vaccinated calves (65). Those reactions, which could be attributed to BCG components of the vaccine, were transient and self-resolved before five days. While those reactions were present in 11 of 12 calves during Study 1, only one calf in Study 2 showed a similar reaction. Calves vaccinated with WT BCG showed no detectable local reactions. Reactogenicity differences might be related to the different manufacturing processes of the WT and recombinant BCG vaccines, and to the outbred condition of calves. Importantly, no systemic adverse effects, such as fever, were seen in any vaccinated calf during the 28-day post-immunization period, suggesting that rBCG-N-hRSV vaccination has an adequate safety profile in neonates. Similarly, it is well known that BCG immunization of immunocompetent infants is safe, with a very low incidence of serious adverse effects (66, 67).

Calves received an aerosol challenge with ~10^4 $TCID_{50}$/mL of bRSV strain 375, 14 days after the booster immunization

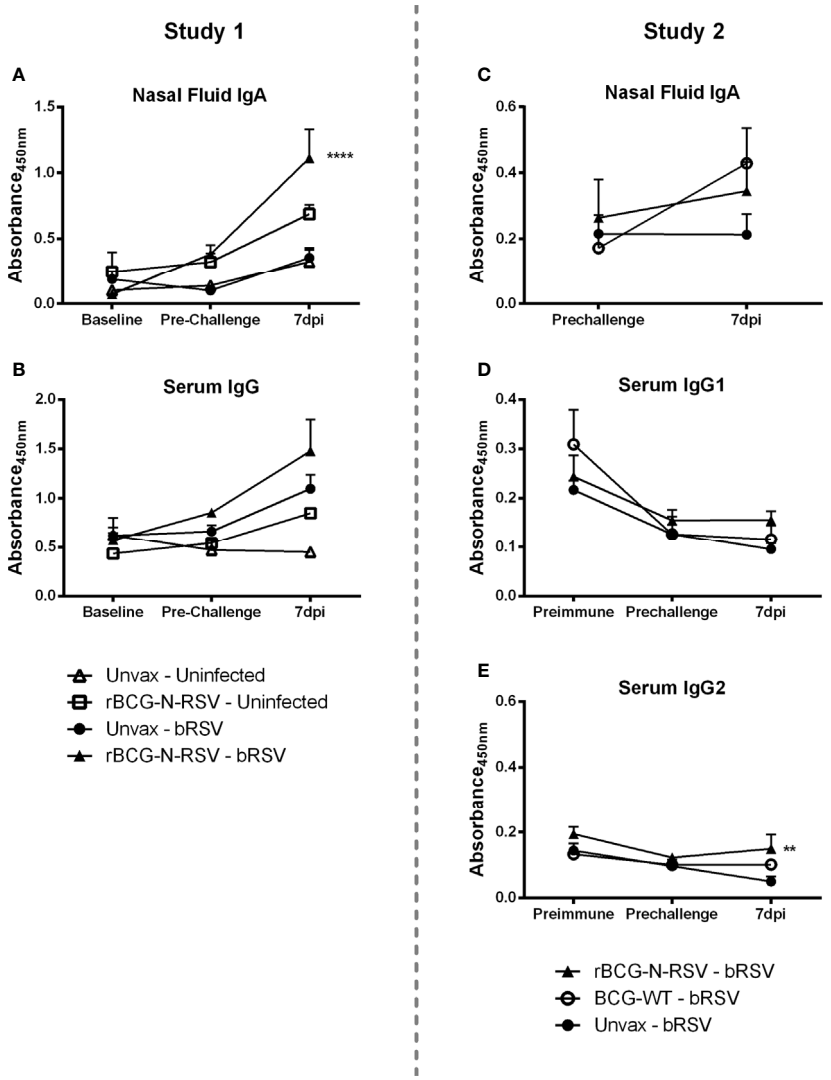

FIGURE 6 | rBCG-N-hRSV vaccinated calves mount an amnestic virus-specific IgA and IgG2 response in the respiratory tract and peripheral blood, respectively. **(A, C)** Nasal fluid samples were collected and analyzed for bRSV-specific IgA by indirect ELISA prior to immunization (baseline), at pre-challenge, and on day 7 post-infection. Data represented as mean ± SEM. No significant differences were detected in IgA levels at baseline and pre-challenge between any group. ****p<0.0001 between rBCG-N-hRSV-vaccinated – bRSV and unvaccinated – bRSV groups on day 7 post infection as determined by 2-way ANOVA followed by Sidak's multiple comparisons test. **(C, E)** Serum samples were collected and analyzed for IgG (**B**, Study 1), IgG1, and IgG2 (**D, E**, Study 2). **p < 0.01 between rBCG-N-hRSV-vaccinated – bRSV and unvaccinated – bRSV groups on day 7 post-infection as determined by 2-way ANOVA followed by Sidak's multiple comparisons test.

(**Figure 1**). Clinical disease was evident in most unvaccinated challenged animals starting 4 dpi, and their condition turned more severe towards day 7 pi. A rise in the clinical score (**Figures 2A, B**) and a trend in increased gross pathology (**Figures 2C, D**) were evident in unvaccinated calves but not in rBCG-N-hRSV-vaccinated animals in both studies. Starting from 4dpi clinical score of unvaccinated animals was significantly higher in comparison to rBCG-N-hRSV-vaccinated calves. Clinical disease and elevated body temperature (**Supplementary Figure 1**) were more evident in Study 1 in comparison to Study 2 (**Figures 2A, B**), with one calf being prematurely euthanized due to severe disease. For that reason, we chose to present and analyze the results from both studies separately. Similarly, while unvaccinated infected calves had increased relative neutrophil infiltration in BAL samples in comparison to uninfected controls, rBCG-N-hRSV vaccination decreased neutrophil infiltration, which is a recognized disease parameter in human and bovine RSV infection (**Figures 3C, D**) (41, 56, 68). Regarding eosinophils, which some studies had previously shown to be augmented in calves suffering VED (44, 45, 69), we observed no significant changes between vaccinated and unvaccinated animals (**Supplementary Figures 2C, G**). Since BAL composition, clinical score and gross pathology are used to evaluated whole-lung and systemic effects, our results suggest that rBCG-N-hRSV vaccination of neonatal calves with MDA protects against severe RSV infection. However, we found no differences in

histopathology scores (**Figures 3A, B**), viral loads or viral shedding in nasal secretions (**Table 1**) when comparing representative lesioned lung samples from vaccinated and unvaccinated calves. These data suggest that protection would be partial and that the antiviral immunity elicited by the chosen vaccination scheme in this model is not optimally tuned to fully prevent virus replication and spread in calves. Further studies are required to define a more efficacious vaccination scheme.

A major challenge in development of RSV vaccines for infant and calf populations is to generate active immunity in presence of maternally derived antibodies (70, 71). While some studies have suggested that MDA can prevent RSV infection (72, 73), it is known that RSV severe disease can occur in presence of MDA in both calves (51, 74) and humans (75–78). Although the outcome of vaccination is shaped by multiple factors, it is well documented that MDA can interfere with generation of active immunity in vaccinated calves (79–81). Successful strategies to overcome this major hurdle include mucosal vaccination and triggering cell-mediated immune mechanisms, i.e., by adjuvanted parenteral vaccines (70, 82) The BCG vector employed in this formulation is well recognized as a highly immunogenic vaccine or adjuvant, being a potent stimulator of Th1 immunity in adults and newborns, triggering antigen-specific $CD4^+$ and $CD8^+$ T cells (83–85) Efficient cell-mediated immunity and IFN-γ secretion have been observed after s.c. BCG vaccination of calves as early as 8 h after birth (86), and one-week-old BCG-vaccinated calves show cellular and IFN-γ responses to PPD-B comparable to adult animals (87). The rBCG-N-hRSV vaccine elicits efficient cellular and humoral Th1 immunity against hRSV in mice, promoting the early recruitment of $CD4^+$ and $CD8^+$ T cells in the lung, but also a specific antibody response against several viral proteins and increased serum neutralizing activity (24–27). Here, we observed significantly increased N-hRSV and bRSV-specific $CD4^+$ T cells and bRSV-specific $CD8^+$ T cells in lung-draining TBLNs near the site of infection (**Figures 4B, D**), as well as increasing trends in peripheral blood of rBCG-N-hRSV-vaccinated calves at 7 dpi (Figure A,C). These proliferative responses were associated with an IFN-γ response to bRSV and PPD-B in the TBLN (**Figure 5B**) and a robust peripheral IFN-γ and IL-17 response to mycobacterial and viral antigens (**Figures 5A, C**), showing that the rBCG-N-hRSV vaccine is immunogenic in neonatal calves with MDA, inducing a Th1/Th17 cellular response to both the N-hRSV protein and bRSV, that might be suitable to overcome Th2 bias and dysbalanced cytokine responses associated with RSV infection in infants (88, 89) and calves (90–92). Although we did not determine specific T cells in the lung, detection of virus-specific cells at TBLNs suggests that specific responses took place in the lung as early as 7dpi. However, we found that neither IFN-γ nor IL-17 were upregulated in BAL at 7dpi in antigen recalls assays after viral antigen stimulation (**Supplementary Figure 4**). The ability of our candidate vaccine to recruit virus-specific T cells and their ability to impact their cytokine milieu should be addressed in future studies as the early recruitment of CD4+ and CD8+ T cells is required to elicit antiviral immunity and prevent lung damage according to our previous studies (26).

Natural RSV infection in infants generates a weak, short-lived primary IgG and IgA response that returns to pre-infection levels within less than four months (93–95). Moreover, the generation of antibody responses might be affected by circulating maternal antibodies (96, 97). Neutralizing antibodies might have an important role in the prevention of RSV infection and are an important vaccination goal (12, 98). RSV. Mucosal RSV-specific IgA has been correlated to protection in both adults (99, 100) and infants (101). In calves, IgA and IgM can be detected since eight days after bRSV challenge (102), and protection from respiratory disease has been achieved after vaccination with a mucosal polyanhydride nanovaccine by inducing significant levels of RSV-specific IgA in nasal secretion and BAL, as well as cellular responses in airways and peripheral blood (51). Here, parenteral vaccination with rBCG did not induce significant levels of IgA or NTs in nasal secretion prior to challenge, with all animals having little to no NTs. At 7dpi, significantly higher IgA levels were found in nasal secretions of rBCG-N-hRSV-vaccinated animals only Study 1 (**Figures 4A, C**), however, increased NTs were observed in nasal secretions in rBCG-N-hRSV-vaccinated animals in both studies (**Table 2**). These results indicate that parenteral rBCG-N-hRSV vaccination can induce mucosal and systemic immune responses to bRSV in neonatal calves with MDA. The enhanced NTs in nasal secretions were associated to reduced clinical disease (**Figure 2**) but not to an effect on viral shedding (**Table 1**). Although the induction of mucosal responses is generally sought by mucosal routes of immunization, several vaccines have demonstrated to induce mucosal immunity after parenteral administration (103, 104),

TABLE 2 | Virus neutralization titers.

Group mean NT (range)	Nasal Fluid NTs				Serum NTs				
	Study 1		Study 2		Study 1		Study 2		
	Day 0	7dpi	Day 0	7dpi	Day 0	7 dpi	Baseline	Day 0	7 dpi
Unvaccinated - Uninfected	3 (2-4)	2 (2)	–	–	24 (8-32)	12 (8-32)	–	–	–
rBCG-N-hRSV - Uninfected	5 (2-8)	3.33 (2-8)	–	–	22 (8-32)	18 (8-32)	–	–	–
Unvaccinated - bRSV	4 (2-8)	17.3 (8-32)	2.5 (2-4)	6 (2-16)	17.3 (8-32)	48 (32-64)	52 (32-64)	42 (16-64)	66 (16-128)
rBCG-N-hRSV -bRSV	5.3 (4-8)	85.3* (64-128)	6 (2-8)	10.6* (8-16)	26.6 (16-32)	74.6 (64-128)	60 (32-128)	34 (16-64)	53 (16-128)
WT BCG - bRSV	–	–	3.3 (2-8)	12.6** (8-16)	–	–	68 (32-128)	42 (16-64)	61.3 (32-128)

*Virus neutralization titers measured in nasal fluid and serum on baseline (pre immune), day 0 (prior to challenge) and day 7 post infection. Two-way ANOVA followed by Sidak's multiple comparisons test, *p<0.05, **<0.01 compared to unvaccinated - bRSV group.*

including an adjuvanted, modified-live multivalent vaccine targeting bRSV and other bovine respiratory viruses (105). The mechanisms for these local immune priming following parenteral vaccination are not well understood (103, 104), but might be dependent on the type of vaccine, immunization route, use and type of adjuvants, and several other factors. Considering previous evidence arguing for a protective role of mucosal IgA against RSV in infants and calves, and the results from these studies, we hypothesize that aerosol or intranasal administration of the rBCG-N-hRSV vaccine could be an efficient way to induce efficient immunity against RSV by mounting both an early mucosal neutralizing response and a sustained systemic cellular response towards RSV able to prevent virus replication and induce protective memory responses.

Although our candidate vaccine encodes the nucleoprotein form hRSV, it was able to elicit N-hRSV and virus-specific cellular responses (**Figures 4** and **5**). Along these lines, the Nucleoprotein is the most conserved antigen when comparing human and bovine viral species, reaching 93% of AA identity according to previous analyses (106). It is interesting to note that although RSV nucleoprotein might not be a neutralizing target, the rBCG-N-hRSV vaccine is able to elicit antibody responses to other RSV antigens through a linked recognition mechanism, including surface antigens G and F, as previously demonstrated in mice models (27). Interestingly, WT BCG vaccinated calves also exhibited increased NTs in nasal secretions at 7dpi. This observation suggests that the increased NT seen in these animals might be related to unspecific immune priming other than a linked recognition mechanism, and suggests that the increase in IgA and NTs in calves vaccinated with rBCG-N-hRSV might be explained by both unspecific and specific effects. While some studies on human infants have shown that BCG vaccination can impact heterologous antibody production (107, 108), conflicting evidence indicates that timing of vaccination and several other factors might impact on such responses, as discussed previously (109).

When measuring bRSV-specific serum IgG and NT, an increasing trend in rBCG-N-hRSV-vaccinated calves at 7dpi was observed in Study 1. Considering that result, for Study 2 we determined serum bRSV-specific IgG1 and IgG2 levels, which display different kinetics in peripheral blood and are associated with different T helper phenotypes (35, 43). IgG1 levels were higher at baseline and then decreased towards day 28 post-immunization, suggesting that rBCG vaccination did not induce significant peripheral IgG1 levels. Regarding Ig2, comparisons between baseline and 7dpi levels showed a decreasing trend in unvaccinated WT BCG groups but not in rBCG-N-hRSV-vaccinated animals, which showed a modest, although significantly higher level of virus-specific IgG2 at 7dpi when compared to unvaccinated animals. This suggests that vaccination with rBCG favors an IgG2 response in peripheral blood of neonatal calves, which is consistent with a Th1 phenotype in bovines and with our previous results (24, 35). Despite that difference, serum NTs from study 2 showed similar kinetics regardless of the vaccination status, with similar levels at baseline, which tended to drop before the challenge and then to slightly rise at 7dpi, without differences between groups. Since all animals received colostrum after birth, it is no surprise to find NTs as high as 128 and the higher IgG1 levels at baseline. Although baseline NTs were not measured during Study 1, it is possible that those NTs might account for the less severe disease in Study 2 calves in comparison to Study 1. The use of colostrum-replete animals allows us to test vaccine candidates in the most physiological model that resembles the scenario of vaccinating infants with MDA. More importantly, our results indicate that vaccination with rBCG-N-hRSV is immunogenic in neonatal calves even in the presence of significant virus-specific circulating antibodies. Further studies should address the duration and role of cellular and humoral responses elicited by vaccination and infection.

Previous studies evaluating the efficacy of rBCG-N-hRSV and rBCG-P-hMPV vaccines against hRSV and human Metapneumovirus infection in mice, respectively, have shown reduced disease parameters in WT BCG vaccinated control animals (27, 110), which might be explained by unspecific functional effects of BCG vaccination on innate immune cells (111). Although not evaluated here, *in vitro* and *in vivo* innate training has been reported in the bovine species after administration of heat-killed *M. bovis* (112) and BCG (113), and the immunomodulatory effects of BCG administration on innate cells have been numerously reported on humans (114–118). Here, WT BCG vaccination prevented a rise in the clinical score (**Figure 2B**) and slightly reduced relative neutrophil infiltration (**Figure 3D**), without modulating lung viral loads or viral shedding. As expected, WT BCG was not associated with N-hRSV or bRSV-specific CD4+ and CD8+ T cell responses in TBLNs (**Figures 4B, D**). On the contrary, both vaccines increased NTs in nasal secretions (**Table 2**). This data suggests that BCG-related unspecific immune mechanisms might confer some degree of heterologous protection against bRSV challenge. Concurrently, increased relative macrophage frequencies were seen in WT BCG and rBCG-N-hRSV-vaccinated animals (Supp Fig2 A,E), indicating that vaccination significantly modulated BAL innate cellular composition. While unspecific effects of BCG or recombinant BCG vaccines might be beneficial against some infectious diseases (114–116, 119–121), and thus might be tested as potential immunomodulatory strategies for in infants and young animals, the extent of these effects and the impact on the induction of adaptive immunity should be comprehensively studied in order to fine-tune cellular protective mechanisms on candidate vaccines.

In summary, a two-dose subcutaneous administration of 10^6 CFU of GMP rBCG-N-hRSV is safe and well-tolerated in neonatal calves with MDA, inducing mucosal humoral immunity and systemic cellular immunity against bovine RSV, skewed towards a Th1 phenotype. Besides, vaccination conferred partial protection to bRSV, reducing clinical disease severity and modulating neutrophil infiltration in the lower respiratory tract, without sings of enhanced disease. These results support further investigation on the use of the candidate vaccine for prevention of RSV in infants and calves.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Iowa State University Institutional Animal Care and Use Committee (IACUC-18-232) and Institutional Biosafety Committee (IBC-18-076).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JM, AK, FD, and MG-M. Performed the experiments: JM, FD, MG-M, and PM. Analyzed the data: FD, JM, MG-M, DR-P, and AK. Contributed reagents/materials/analysis tools: AK and JM. Wrote the paper draft: FD. Edited the paper: FD, JM, DR-P, and AK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664212/full#supplementary-material

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Conflict of Interest: A patent for the rBCG-N-hRSV vaccine has been filled and issued by Pontificia Universidad Catolica de Chile in several countries.

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REVIEW

An Update on Host-Pathogen Interplay and Modulation of Immune Responses during *Orientia tsutsugamushi* Infection

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SUMMARY The obligate intracellular bacterium *Orientia tsutsugamushi* is the causative agent of scrub typhus in humans, a serious mite-borne disease present in a widespread area of endemicity, which affects an estimated 1 million people every year. This disease may exhibit a broad range of presentations, ranging from asymptomatic to fatal conditions, with the latter being due to disseminated endothelial infection and organ injury. Unique characteristics of the biology and host-pathogen interactions of *O. tsutsugamushi*, including the high antigenic diversity among strains and the highly variable, short-lived memory responses developed by the host, underlie difficulties faced in the pursuit of an effective vaccine, which is an imperative need. Other factors that have hindered scientific progress relative to the infectious mechanisms of and the immune response triggered by this bacterium in vertebrate hosts include the limited number of mechanistic studies performed on animal models and the lack of genetic tools currently available for this pathogen. However, recent advances in animal model development are promising to improve our understanding of host-pathogen interactions. Here, we comprehensively discuss the recent advances in and future perspectives on host-pathogen interactions and the modulation of immune responses related to this reemerging disease, highlighting the role of animal models.

KEYWORDS *Orientia tsutsugamushi*, scrub typhus, immunity, immune evasion

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INTRODUCTION

Orientia tsutsugamushi is a Gram-negative, obligate intracellular bacterium known as the etiological agent of scrub typhus, a mite-borne disease with endemic presentation in vast regions of Asia and insular territories of the Pacific and Indian Oceans (1, 2). While areas of endemicity continue to expand in Asian regions (3, 4), strong molecular, serological, and clinical evidence of transmission of *O. tsutsugamushi* in Africa and South America has been reported (5–7), suggesting that the global health burden of this severe infection may currently be underestimated. The *Orientia* genus belongs to the *Rickettsiales* order, *Rickettsiaceae* family, and is therefore closely related to the *Rickettsia* genus. Both genera have in common that infectious particles are located free in the cell cytoplasm; however, the absence of peptidoglycan, lipopolysaccharide (LPS), and genes involved in the lipid A biosynthetic pathway is a feature that differentiates this pathogen from the *Rickettsia* genus. Interestingly, the presence of peptidoglycan-like structures in *Orientia* was recently reported, turning this into an interesting field of debate (8–11). These molecular features may shape the immune response to different *O. tsutsugamushi* strains, since the presence of classical LPS or peptidoglycans usually contributes to the generation of cross-protective responses. According to the antigenic variation of the major outer membrane protein 56-kDa type-specific antigen (TSA56), several strains of *O. tsutsugamushi* have been described (12, 13). Nevertheless, to date, the genomes of only two strains, Ikeda and Boryong, have been reported, revealing high levels of sequence variation among surface genes. The circular genome of *O. tsutsugamushi* has a length of 2.0 to 2.1 Mb, showing high levels of sequence repeat density and complexity, making this species one of the most remarkable species, in genomic terms, within the *Rickettsiaceae* family. Genes encoding proteins that mediate interactions with host cells and constituents of the conjugative type IV secretion system (T4SS) have undergone massive proliferation, while the intensive amplification of several mobile elements reveals evolutionarily recent genome shuffling (9, 12, 14).

Scrub typhus is described as an acute affection, usually febrile, with a wide range of clinical manifestations, including nonspecific signs such as headache, fever, rash, breathlessness, cough, nausea, vomiting, myalgia, and regional lymphadenopathy, which are observable after a 6- to 21-day incubation period. A highly variable percentage of patients may develop a distinctive eschar at the inoculation site. Despite self-limiting clinical conditions being commonly reported, fatal multiple-organ involvement may be observed in an important percentage of cases (7 to 15%). In these cases, a syndrome of acute respiratory distress may occur, related to lung injury, hepatitis, renal failure, myocarditis, encephalitis, and central nervous system involvement. Usually, these severe manifestations develop 2 weeks after infection and are frequently related to inappropriate or absent antibiotic treatment (13, 15–20). Moreover, antibiotic resistance has been reported, which sums to the natural resistance of the pathogen to fluoroquinolones and beta-lactam drugs (1, 21–23). The broad range of unspecific manifestations may lead to the late establishment of a diagnosis, contributing to aggravation of the clinical condition. Several other factors may be involved in misdiagnosis or delayed diagnosis, such as the suboptimal capacity of available serological tests, the high number of strains of the pathogen, the presence of serum IgG and IgM from previous infections, the time of generation of new detectable antibodies, and the difficult access to rural medical attention within zones of endemicity (24, 25). To complement serological assays and overcome the difficulties in diagnosis, the routine use of serum- or biopsy specimen-based molecular assays for scrub typhus diagnosis, including PCR, quantitative PCR (qPCR), and loop-mediated isothermal PCR (26–34), is hopefully increasing.

Although the physiopathology of this disease is not fully understood, immune-mediated processes are thought to contribute, along with direct bacterial damage, to cause local or disseminated vasculitis that drives the pathological mechanism (20, 35). Humans acquire the pathogen during the feeding of trombiculid mite larvae (chiggers),

which host *Orientia tsutsugamushi* in salivary glands (36). Humans are incidental hosts of *Leptotrombidium* species and dead-end hosts of *O. tsutsugamushi*. Transovarian transmission between mites and parasitism of the larvae on infected animals maintain the bacterium in its natural cycle (36–38). In rural areas of endemicity, more than 20% of hospitalized patients that present with acute undifferentiated fever are diagnosed with this infection, and 1 million cases are detected every year, with an estimated 1 billion people being at risk (24, 39, 40). Outbreaks of scrub typhus and *Orientia* reinfections are frequent, possibly due to the heterogeneity of the organism and the suboptimal development of immunity from natural infections (15, 41). Despite decades of research efforts, the development of an effective vaccine eliciting long-lasting protection against *O. tsutsugamushi* has not been achieved. The urgent need for this vaccine is supported by several reasons, such as (i) the high public health burden caused by this pathogen, (ii) the expansion of anthropic environmental modifications that impact the epidemiology of the disease, (iii) the ever-growing geographical areas of endemicity where cases are reported, (iv) the deficient natural immune response that lacks cross-protection, (v) the challenges underlying the achievement of early diagnosis, (vi) reports of antibiotic resistance, and (vii) the mortality rate related to systemic infections, among others (24, 42, 43). Currently, in order to achieve a comprehensive understanding of host-pathogen interactions and immune responses, promising advances are opening alternatives to carry out profound mechanistic studies, including the development of accurate animal models that resemble the natural inoculation routes, the dissemination of the pathogen into the vertebrate host, or the human-pathological characteristics of the disease (43–49). Thus, the aim of this review is to present an update on studies of the *O. tsutsugamushi*-host interplay and immunological responses to this pathogen, which constitute a basis for the development of disease control strategies, highlighting the current state of the art and future perspectives on animal model employment.

HOST-PATHOGEN INTERPLAY IN *ORIENTIA TSUTSUGAMUSHI* INFECTION

Target Cells

To accomplish its replicative cycle, *Orientia tsutsugamushi* may infect a wide range of cells, displaying differential tropism depending on the phase of infection and the affected tissue. After initial inoculation in the dermal layer, dermal dendritic cells (DCs) and activated monocytes constitute the main target cells, as reported in an *ex vivo* study of human eschar biopsy specimens (50). Infection of monocytes has also been observed in studies based on isolated cells from healthy human donors both *ex vivo* and *in vitro* (51). Activated DCs and monocytes may constitute a rapid, potential dissemination vehicle for the pathogen while circulating to lymph nodes (50). The accumulation of *O. tsutsugamushi* in these organs in a sublethal, footpad-injected BALB/c mouse model has been reported. Posterior systemic dissemination along with macrophage tropism are characteristic of this model, instead of disseminated endothelial infection (44), which constitutes a hallmark of lethal infection in humans (52). Endothelial infection involves several organs, including skin, heart, lung, kidney, pancreas, and brain, with additional infection of macrophages in the liver and spleen, as reported in postmortem analyses of human patients (52). In addition, endothelial cell (EC) infection has been observed in several rodent models of *Orientia* infection, including intradermal (i.d.) and intravenous (i.v.) models (45, 46, 48, 49). Also, mouse fibroblasts (51, 53), cultured polymorphonuclear leukocytes (PMNs) of guinea pigs, and neutrophils in intraperitoneal (i.p.) infection in BALB/c mice are reported target cells (54, 55).

Cell Invasion and Intracellular Life Cycle

Although a precise understanding of host cell invasion mechanisms remains to be elucidated, some molecular interactions concerning the attachment and entry of *Orientia* have been described. The internalization of intracellular bacteria requires initial attachment to the extracellular matrix (ECM) and host cells, mediated by high-affinity

binding to host receptors. Fibronectin, a glycoprotein of the ECM involved in the binding and entry of several intracellular bacteria, takes part in this process by interacting with the *Orientia* TSA56 antigen (56) and potentially with one of the autotransporter proteins, ScaC (57). The interaction of fibronectin with an extracellular binding region of TSA56, formed by amino acids (aa) 312 to 341, decreases the invasion of the bacteria in L929 cells (56, 58). Moreover, the potential interaction of ScaC with fibronectin may be responsible for the demonstrated adherence to nonphagocytic cells mediated by this antigen (57). Additionally, *Orientia* ScaA, another autotransporter protein, mediates bacterial adhesion to nonphagocytic cells, since adhesion is enhanced by the recombinant expression of this antigen in *Escherichia coli*, while treatment with anti-ScaA antibody neutralizes it. However, an interacting host protein has not been reported for this antigen (59). Heparan sulfate (HS) proteoglycans (HSPGs), which are molecules expressed on cell surfaces and the ECM, are also involved in the attachment of viruses and the uptake of intracellular bacteria. Low expression levels of HSPGs in mutant Chinese hamster ovarian cell lines lead to a diminished infection capacity of *O. tsutsugamushi*. Specifically, HS and heparin may be involved in the infectious process, since invasion of L929 cells is inhibited with HS or heparin pretreatment (60).

Other HSPGs involved in *Orientia* interaction are syndecans, molecules widely distributed in mammalian cells. Syndecan-4 is the most ubiquitous (61) and participates in the early attachment of *Orientia*, as demonstrated by experiments with rat embryo fibroblasts, where bacterial invasion is correlated with the expression levels of this transmembrane molecule and is specifically reduced by the addition of a recombinant core protein of syndecan-4 (62). However, a bacterial ligand for syndecan-4 has not yet been reported, and the role of this molecule in the invasion mechanism requires further investigation (62).

Integrins, a family of transmembrane glycoproteins that usually associate with fibronectin in the bacterial entry process, are also involved in cell invasion by *O. tsutsugamushi*. The early colocalization of *O. tsutsugamushi* with integrin $\alpha5\beta1$ has been related to the activation of integrin-activated signal transduction pathways (56). Focal adhesion (FAK) and Src kinases as well as RhoA GTPase act as integrin downstream signaling molecules in nonphagocytic cells, promoting the local induction of focal adhesion points and the subsequent induction of actin cytoskeleton rearrangement. Talin and paxillin are also upregulated and mediate this process (56). The role of integrin $\alpha5\beta1$ is also supported by a report showing that the administration of anti-integrin $\alpha5\beta1$ antibodies decreased L929 cell invasion (58).

In summary, it seems that the *O. tsutsugamushi* strategy of manipulating these widely distributed cell receptor families and molecules has allowed attachment to host cells not specialized in phagocytosis, thereby providing a mechanism of active internalization in target cells.

The internalization of *Orientia* in human epithelial cells and fibroblasts exploits the endocytic pathway, particularly clathrin-mediated endocytosis. This has been shown by the colocalization of the bacterium with clathrin and adaptor protein 2 and confirmed by inhibition assays with chlorpromazine hydrochloride, monodansylcadaverine, and sucrose. A similar approach, this time with filipin III inhibition assays, discarded the utilization of caveola-mediated endocytosis in human epithelial cells and fibroblasts. Colocalization with early and late endosomal markers has been observed in nonphagocytic cells at between 1 and 2 h postinfection (hpi). After this time, visualization of free *Orientia* bacteria in the cytoplasm revealed the early escape of the bacteria from the phagosomal compartment.

Once in the cytoplasm, *Orientia* can replicate by binary fusion (36, 63), after moving to a perinuclear region. This is accomplished within the first 2 hpi through specific interactions with microtubules and the dynein-dynactin motor protein complex (64). Evidence of replication at this region has been observed in nonphagocytic cells (64) and phagocytic cells, including macrophages and dermal DCs (50, 65). Although the whole replicative cycle can be fulfilled within the cytoplasm, an intranuclear location has been reported (53).

The early escape from the phagosomal compartment allows this bacterium to subvert an important innate defense mechanism as well as to establish its intracytoplasmic life cycle. Unfortunately, the mechanisms underlying this process are poorly characterized. Blocking the acidification of the endocytic compartment through NH_4Cl or bafilomycin A treatment impairs *Orientia* escape, thereby suggesting the requirement of this metabolic acidification process (66). However, a detailed mechanism involved in this escape is lacking. Studies have revealed the presence of a hemolysin gene and a potential phospholipase D gene in the *Orientia* genome, along with the expression of the corresponding protein in cultured mouse fibroblasts, but whether they have a role in the *Orientia* replicative cycle or not has not been explored (9, 67). Considering that the *Listeria monocytogenes* listeriolysin and phospholipase C proteins have a role in phagosomal escape by this intracellular bacterium, some authors have speculated about their potential involvement (68–70). In accordance with the early escape from phagosome observed for *Orientia*, it has been reported that nitric oxide (NO), a potent free radical involved in the killing of engulfed microbes, enhances the cytosolic replication of *O. tsutsugamushi* in RAW264.7 murine macrophages through a mechanism that is not currently elucidated (71).

Infected cells may harbor a high density of *Orientia* bacteria despite the slow growth reported at 37°C, reaching a density of 10^3 bacteria in a single cell (72–74). The release of infective bacteria from host cells involves budding from the cell surface with the acquisition of a membrane coat (63). Membrane lipid rafts may participate in this process, since an *Orientia* protein, 47-kDa high-temperature requirement A, colocalizes at 72 hpi with the caveolin raft protein in an endothelial cell line. At this time, *Orientia* moves to the cell surface and starts the release process (75). The main events reported in *Orientia* infection of nonphagocytic cells are depicted in Fig. 1. Despite reports of budding-like release, BHK-21 cell line infection results in few extracellular membrane-coated *Orientia* bacteria (54). Previous studies suggested that after being released from an infected cell, *Orientia* may employ different mechanisms to infect subsequent cells, which may require the bacteria either to detach from the surrounding membrane to contact further cells or to involve phagocytosis of membrane-coated *Orientia* bacteria by host cells (54, 63, 70), as reported for mouse peritoneal mesothelial cells (63).

Apoptosis Modulation and Subversion of Autophagy

In a cell infection context, apoptosis may be a host defense mechanism aimed at limiting pathogen replication. Whether this process is detrimental or advantageous to the host depends on several factors, such as the identity of the infected cell, the stage of infection, and the nature of the pathogen. This highly regulated cell death process has a significant role in the infected cell-pathogen interplay, especially in the life cycle of obligate intracellular pathogens. Interestingly, events taking part in this process are often the target of extensive modulation by the infectious agent (76, 77). Studies on *O. tsutsugamushi* have revealed either the induction or inhibition of cell apoptosis after infection, with this duality being relative to the strain or host cell/animal model studied. Most importantly, mechanisms of modulation of apoptosis have been scarcely explored to date. On the human monocyte cell line THP-1, apoptosis induction by beauvericin treatment was inhibited by both heat-killed and live *Orientia* bacteria, with the former showing greater antiapoptotic activity, thereby suggesting a role for heat-stable molecules. In general, extensive calcium release from the endoplasmic reticulum (ER) mediates apoptosis induction triggered by this drug; however, in *O. tsutsugamushi*-infected cells, a reduced, retarded cytosolic calcium redistribution may be responsible for the inhibition of apoptosis (78). Experimental modulation of NF-κB levels did not influence apoptotic effects, suggesting that this transcription factor is not implicated in this inhibition pathway (78). That observation contrasts with the reported NF-κB-dependent antiapoptotic effect on cells elicited by *Rickettsia rickettsii* (79).

In contrast, apoptotic effects of *Orientia* have been observed for various cell types and organs. In *O. tsutsugamushi*-infected BALB/c mice, nucleus fragmentation has been observed in a strain-specific manner in lymphocytes located in spleens and lymph

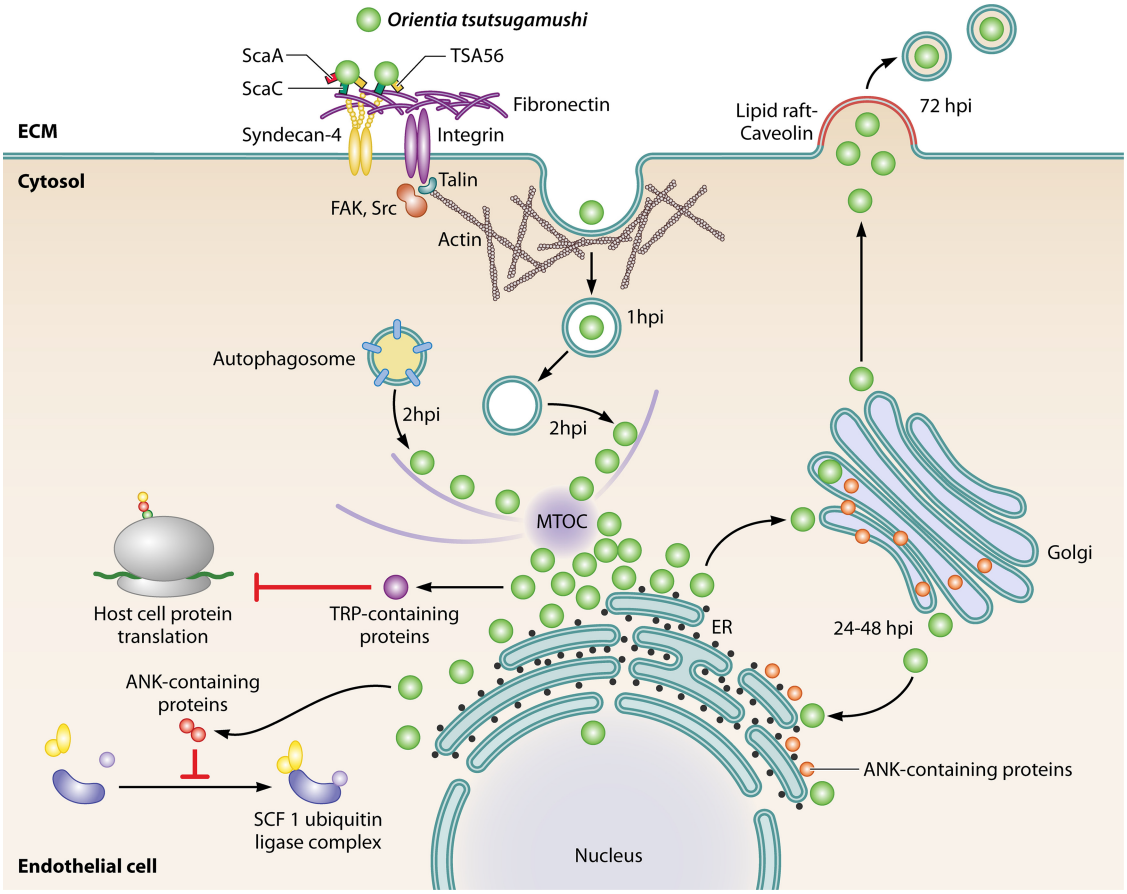

FIG 1 Reported events related to the replication cycle of *Orientia tsutsugamushi* in nonphagocytic cells. Fibronectin in the ECM interacts with the *Orientia* antigen TSA56, and potentially with ScaC, for the attachment of bacteria to the host cell. This results in integrin-mediated signaling, involving FAK, Src, and Rho GTPase, with consequent actin cytoskeleton rearrangement, talin/paxillin recruitment, and clathrin-mediated endocytosis. The *Orientia* antigen ScaA and transmembrane syndecan-4 also mediate bacterial adhesion to nonphagocytic cells. At 1 hpi, *Orientia* colocalizes with early endosomal markers but at 2 hpi escapes from phagosomes through an unknown mechanism that requires compartment acidification and also evades cellular autophagy. It reaches the perinuclear region after moving through microtubules associated with the dynein-dynactin protein complex, where it replicates via binary fission. The secretion of effector cells participates as a virulence factor, probably via the T1SS and T4SS, disrupting the SCF1 ubiquitin ligase complex, degrading EF1α, and impairing protein translation. Also, *Orientia* colocalizes with the Golgi complex and moves backwards to the ER, producing membrane instability mediated by the effector protein Ank9. The release of *Orientia* particles occurs in a way similar to virus budding at 72 hpi and has been associated with lipid rafts and caveolin colocalization. MTOC, microtubule organizing center.

nodes. This effect was observed 10 days after lethal Karp strain challenge but not after nonlethal Gilliam strain challenge (80). Moreover, apoptosis of the cultured endothelial cell line ECV304 has been reported (81, 82). Detachment from the substrate and DNA fragmentation were observed following infection with the Boryong strain at a multiplicity of infection (MOI) of 20:1, accompanied by the disruption of focal adhesions and changes in the cytoskeleton, which may be related to the apoptotic mechanism (81). It has been suggested that the replicative capacity of *Orientia* is associated with the extent of apoptosis induction, since live bacteria produced apoptosis of 18% and 8% $\pm$ 1% of human monocyte-derived macrophages and naive monocytes, respectively, with naive monocytes being less suitable for *Orientia* strain Kato replication (83, 84). More recently, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) immunochemistry performed on mouse kidneys revealed apoptotic effects on ECs after lethal infection with the Karp strain. A large number of apoptotic cells along with the reduced transcription of antiapoptotic Bcl-2 were observed in wild-type (WT) mice in comparison to interleukin-33 (IL-33)$^{-/-}$ mice, suggesting a role for this interleukin in promoting EC stress and apoptosis (49).

Considering these results, it may be hypothesized that the existence of differential pro- and antiapoptotic effects may vary within different stages of target cell infection or host dissemination to decrease the spread of *O. tsutsugamushi*. However, to unveil the mechanisms and objectives of apoptosis modulation in an infection context, integrative studies on susceptible cell lines and animal models should be performed.

Another defense strategy that counters intracellular pathogen growth is autophagy, a regulated catabolic process aimed at degrading cytosolic components. To accomplish this, endomembrane-based organelles, called autophagosomes, engulf components targeted for degradation, which is achieved by the fusion of the organelle with other endosomal and lysosomal compartments (85, 86). One of the first studies addressing the role of autophagy showed increased autophagosomal formation on *Orientia tsutsugamushi*-infected PMNs (87). Subsequently, immunoblot and immunofluorescence confocal microscopy studies employing phagocytic and nonphagocytic cell lines revealed the formation of autophagosomes within 1 h after Boryong strain infection, as identified by LC3B marker recruitment (86). Later, at 2 hpi, escape from autophagosomes was observed in HeLa cells, revealed by the scarce colocalization of the bacterial particles with LC3B. This evasion was evident even in the presence of rapamycin, an autophagy inducer. It is noteworthy that the induction of autophagy did not impair bacterial growth (86). Moreover, phagosome formation also takes place with live and UV-inactivated Boryong strain bacteria in bone marrow-derived DCs (bmDCs), with only live bacteria showing an ability to evade the autophagic system (65). An elucidation of virulence factors that impair the phagosome degradation of *O. tsutsugamushi* has not yet been described, but it has been proposed that this bacterium may carry several factors involved in this process. Finally, since autophagy may have a role in inflammasome activation and naive T cell antigen presentation (85, 88), the extent and mechanisms of autophagy subversion should be further explored in order to understand the mechanisms underlying the impaired adaptive responses related to human *O. tsutsugamushi* infection.

Secretion Systems and Effectors

Genome sequencing of the Boryong strain of *O. tsutsugamushi* revealed the presence of 79 genomic sites, encompassing 359 *tra* genes related to constituents of conjugative T4SSs, a strikingly high number in comparison to those of other intracellular bacteria and the *Rickettsia* genus (9, 89, 90). Several bacteria and some archaea use T4SSs, which are large protein complexes that allow the translocation of DNA and protein substrates through a channel assembly that connects two cells. These structures participate in horizontal gene transfer and in delivering virulence factors to the cytosol of infected cells, enabling bacteria to subvert host cell processes (91, 92). Strain Ikeda also exhibits an important set of genes of the T4SS. Genome analysis of T4SSs in *Rickettsia* and *Orientia* revealed a highly conserved gene organization, as observed for the *Anaplasma* genus, which suggests an essential role for this secretion system in the replicative cycle of these intracellular pathogens (14). The *Orientia* genome also harbors a high number of genes encoding host cell interaction and signaling proteins, such as histidine kinases, ankyrin repeat proteins, and tetratricopeptide repeat proteins (TRPs) (9).

The tetratricopeptide repeat structural motif, present in prokaryote and eukaryote organisms, functions as a scaffold for assembling multiprotein complexes. Every TPR fold structure may participate in several protein-protein interactions and therefore impact different facets of cellular metabolism, including the functional modulation of steroid receptors, gene regulation, cell cycle control, protein folding, and protein transport (93, 94). Importantly, they serve as virulence factors in several bacterial pathogens, such as *Francisella tularensis*, *Francisella novicida*, *Mycobacterium tuberculosis*, *Shigella* spp., *Pseudomonas*, and *Yersinia* (95–103). In *O. tsutsugamushi*, this family of proteins may interfere with host cell metabolism through interactions with DDX3, an RNA helicase that belongs to the multifunctional DEAD box family of proteins (104, 105). Proteins encoded by *trp* genes are expressed after L929 cell infection and interact with DDX3, as revealed by pulldown assays with ECV304 cell lysates and immunoblot

assays with specific antibodies. *In vitro* translation assays employing reticulocyte cell lysates have shown dose-dependent translation impairment of a reporter by the TRP43 and TRP46 proteins (94).

Another important family of molecules employed by intracellular organisms to accomplish their infectious cycle is the family of Ank-containing proteins. These proteins mediate various protein-protein interactions aimed at impeding normal processes of eukaryotic cells (106–108). In this regard, the Ikeda strain carries 38 *ank* open reading frames and 9 *ank* pseudogenes (14). L929 cells respond to *Orientia* infection by upregulating the transcription of type I secretion systems (T1SSs), T4SSs, and several *ank* genes (109, 110). The products of these *ank* genes may be T1SS substrates, as revealed by the examination of C termini of Ank-containing proteins. It is noteworthy that after their ectopic expression in HeLa cells, Ank-containing proteins are distributed in diverse cellular locations, suggesting a vast potential to modulate many host cellular functions (109, 110). Particularly, the interaction of these proteins with core components of the SCF1 ubiquitin ligase complex has been documented in two reports. Interactions with Cullin-1 in ECV304 cell lysates have been revealed by using glutathione *S*-transferase (GST) pulldown assays with *E. coli*-purified GST fusion proteins, while immunoblot analysis revealed interactions with Skp1 (109, 111). F-box-like motifs, homologous to those found in eukaryotes and poxvirus, are present in Ank-containing proteins, as revealed by *in silico* and manual sequence analyses (109, 111). This F-box motif is required for the interaction of the Ank-containing proteins with Skp1, as demonstrated in transfected HeLa cells by coimmunoprecipitation of FLAG-tagged Ank-containing proteins with GST-tagged Skp1 and GST-Skp1 pulldown analyses performed with FLAG-tagged Ank-containing proteins with a deletion of their F-box motifs. In addition to this recombinant-protein approach, the expression of the Ank9 protein and interaction with Skp1 also occur after *Orientia* infection of mammalian host cells, which were observed by antiserum recognition of the Ank9 protein coprecipitated with GST-Skp1. This interaction is due to residues L384, I392, and E400 in the F-box-like motif of Ank9 (111).

Furthermore, an *Orientia* mechanism for targeting the eukaryotic secretory pathway was recently proposed, where Ank9 plays a key role (112). Experiments with Ank9 ectopic expression in HeLa cells allowed the recognition of a novel GRIP-like motif in Ank9, which is necessary and sufficient for the protein to localize within the Golgi complex, to reach the ER by retrograde trafficking, and to generate instability in both compartments. The binding of coatomer protein complex subunit beta 2, involved in vesicular trafficking, Golgi budding, and Golgi-to-ER trafficking, may be a factor contributing to the instability of the Golgi complex. As a result of the interaction of Ank9 and coatomer protein complex subunit beta 2, ER stress and the impairment of eukaryotic cell protein secretion are generated. In addition, Golgi instability, the impairment of host cell protein secretion, and the enhanced replication of bacteria have also been observed during *O. tsutsugamushi* infection (112), highlighting the importance of Ank-containing proteins as virulence factors in *O. tsutsugamushi*. Moreover, GST pulldown assays and immunoblot analyses have confirmed the interaction of several of these proteins with eukaryotic elongation factor 1α (EF1α). In a functional analysis of transfected HeLa cells expressing of AnkU5 and AnkD, colocalization of AnkU5 with Cullin and EF1α was observed mainly in nuclei by using immunofluorescence confocal microscopy. This interaction mediated a reduction of the host protein level via polyubiquitination, as revealed by *in vitro* ubiquitination reactions. Following *Orientia* infection, a reduction in EF1α levels in endothelial, epithelial, and monocytic cell lines was observed at 2 days postinfection (dpi) by using a specific anti-EF1α antibody, with this reduction being triggered by posttranscriptional proteosomal degradation (109). Although the EF1α function is canonically related to the cytosolic loading of aminoacyl-tRNAs, there may be several other functions related to this protein in physiological and pathological scenarios (113, 114). Nonetheless, the impact of EF1α degradation on *O. tsutsugamushi* infection has not yet been assessed.

Unveiling the potential roles of virulence factors and pathways related to secretion systems, repeat-containing proteins, and effector proteins in general constitutes an exciting area of study that should be promptly explored. Considering the range of effects of a unique Ank-containing protein, Ank9, it may be speculated that the entire repertoire of Ank-containing proteins may potentially exert a vast effect on host cell biology.

INNATE IMMUNITY CELLS: EARLY RESPONSES AND EFFECTOR ROLES

Dendritic Cell Responses to *Orientia* Infection

With the objective of surviving and perpetuating in a host, several pathogens prevent or modulate the normal function of cells involved in innate immune responses (115, 116). Considering that the main infection targets of *O. tsutsugamushi* are DCs, monocytes, and ECs (50, 52), it is of major importance to understand the response of these cells and host immunity to infection.

In vitro studies have revealed that *Orientia* infection of monocyte-derived DCs (moDCs) or bmDCs drives the activation of these cells, but some of their functions may be severely impaired (65, 117, 118). Upregulated expression of major histocompatibility complex (MHC) class II and CD40, CD80, CD86 (65), and CD83 costimulatory molecules (117, 118) has been reported prior to 24 hpi, revealing DC activation. Interestingly, no differences were found in the expression levels of costimulatory molecules between live and heat-inactivated bacteria, suggesting the involvement of heat-stable molecules in the activation of DCs (117). A reduction of endocytic activity is also associated with the maturation of DCs and has been reported for *Orientia*-infected moDCs in an *in vitro* assay of fluorescein isothiocyanate (FITC)-coupled albumin uptake (65). The upregulation of a proinflammatory profile, including tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-12p70, and the chemoattractant molecules CCL3 and CCL5, provides additional evidence of *Orientia*-infected DC activation (65, 117) and suggests that these sentinel cells participate in lymphocyte and monocyte recruitment. *Orientia* infection also upregulates the antiviral type I interferon (IFN) pathway, enhancing the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and IFN-β production, a feature also observed in other intracellular pathogens after being sensed in the cytosolic compartment of dendritic cells, such as *Listeria* and *Francisella* (119–121). Once activated, DCs must move toward lymph nodes to prime naive T cells in a tightly regulated event required for the proper development of adaptive immunity (122, 123). *In vitro* assays indicate that infected moDCs retain activity to activate $CD4^+$ T cells, as revealed by lymphocyte IFN-γ secretion (117). However, the migration process seems to be impaired in *O. tsutsugamushi* infection, as demonstrated by a migration assay in a three-dimensional collagen matrix using the chemoattractant CCL19, where *Orientia*-infected DCs exhibited a diminished chemotactic response, in a way similar to that exhibited by immature DCs. This diminished chemotactic response was not related to decreased levels of CCR7 (65), which is the receptor for the CCL19 and CCL21 chemoattractant molecules (124), and was confirmed by *ex vivo* and *in vivo* assays. Therefore, *Orientia* exhibits the potential to interfere with the migration of DCs while harnessing this niche for replication.

Taking these data in consideration, along with the reported accumulation of DCs in eschars of infected human patients (50), we recommend performing future *in vivo* studies with rodent animal models for characterizing dendritic cell dynamics employing i.d. (47, 48, 125) or laboratory-reared chigger infection models that closely resemble the natural acquisition of this pathogen (126, 127).

Monocyte/Macrophage Responses to *Orientia* Infection

As mentioned above, monocytes are either present at or recruited to the inoculation site in dermal tissue, where they contribute to early inflammatory responses and serve as a niche for bacterial replication (Fig. 2) (50, 83, 84, 128–131). Moreover, they may contribute to *Orientia* dissemination toward the bloodstream and lymph nodes as well as mononuclear cell recruitment at the infection site (50). Early insights into the

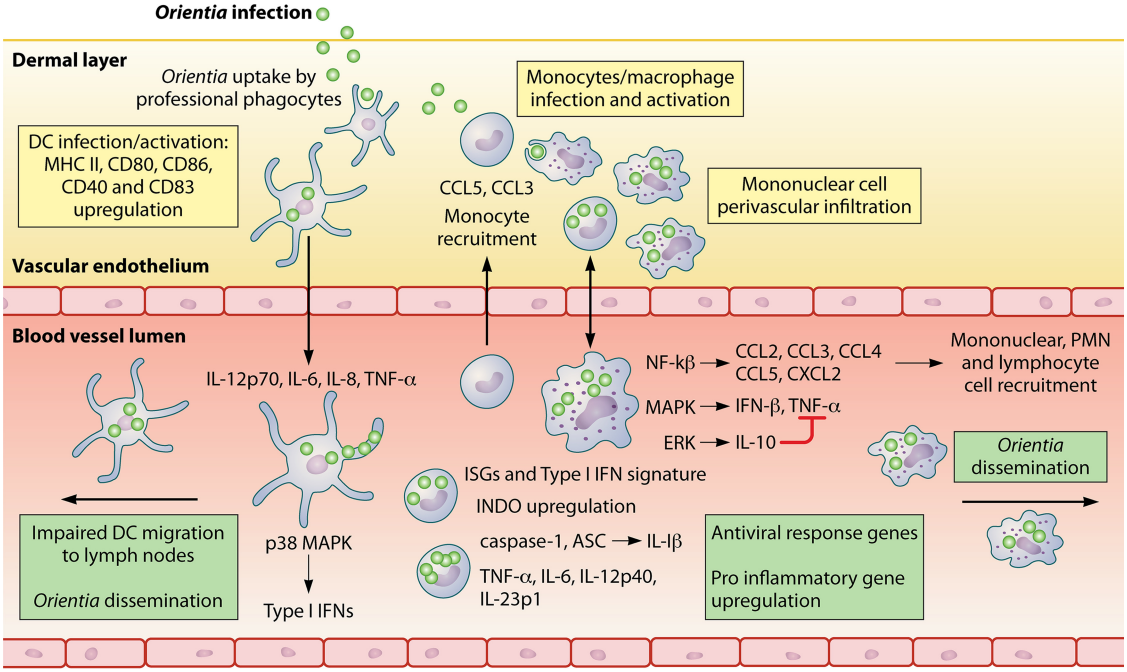

FIG 2 Early events following *Orientia tsutsugamushi* infection. An initial tropism for dendritic cells (DCs) and monocytes at the inoculation zone induces infection and maturation of these cell populations. Infection of DCs impairs their migration, but this promotes rickettsial dissemination nonetheless. Additionally, DCs secrete proinflammatory cytokines and activate the type I IFN response. Monocyte recruitment occurs, which results in mononuclear cell perivascular infiltration and the activation of several transcriptional programs and pathways ending in the upregulation of proinflammatory genes and of the antiviral response and the recruitment of other cell subsets. In summary, by exploiting immune cells, early infection events may favor rickettsial dissemination and an inflammatory profile in *Orientia tsutsugamushi* infection.

importance of monocytes and macrophages in combating initial phases of *Orientia* infection were reported in challenges of mice by i.p. inoculation (132, 133), a route that does not mimic natural inoculation but nevertheless may enlighten us about the activities of these cells during infection. Additional evidence of monocyte/macrophage activation comes from markers detected in South Indian scrub typhus patients. Some of these markers have been associated with severe disease presentation, including sCD163 and sCD14, and even with host lethality in the case of macrophage migration inhibitory factor (MIF) and tyrosine-lysine-leucine-40 (YKL-40) (134).

Except for those reports, most subsequent studies have been performed by employing murine cell lines. Infection of the J774A.1 mouse macrophage cell line with either live or heat-inactivated *Orientia* bacteria triggered similar expression levels of CCL2, CCL3, CCL4, CCL5, and CXCL2 transcripts without the need for *de novo* protein synthesis and mostly within 0.5 hpi. The NF-κB transcription factor may be responsible for the induction of these chemokines, since NF-κB nuclear translocation was observed, while the inhibition of its activation resulted in the downregulation of chemokine expression (128). Additionally, live, but not heat-inactivated, *Orientia* bacteria also upregulated IFN-β. MAPK pathways were required for this upregulation, as suggested by the reduced levels of IFN-β transcripts resulting from extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 phosphorylation inhibition (129). Moreover, the MAPK pathways are also involved in the upregulation of TNF-α at the transcriptional and posttranscriptional levels (131). Consistent with the lack of LPS (8, 14), both pharmacological blockade of LPS, which did not affect TNF-α production, and Toll-like receptor 4 (TLR4) mutation, which rescued its production only slightly, suggest that TNF-α production may be independent of TLR4 and LPS (131). In contrast, a very weak stimulation of TNF-α expression, along with a repression of its production in LPS-stimulated J774A.1 cells, has been reported after infection with the Boryong strain, without affecting NF-κB activation. Similar inhibitory effects were seen with culture

medium stimulation of J774A.1 cells, which contained high levels of an uncharacterized, potent IL-10-inducing factor, suggesting that *Orientia* increases the production of this immunosuppressive molecule in order to inhibit TNF-α production and enhance bacterial survival inside phagocytic cells (135). Therefore, *Orientia* may exploit a strategy similar to those observed for several bacterial intracellular pathogens (136, 137), including *Mycobacterium tuberculosis* (138), *Coxiella burnetii* (139), and *Yersinia enterocolitica* (140).

The discrepancies among data from studies of TNF-α expression in monocytes may be attributed to several factors, including the strain and dose of *Orientia* as well as the nature of the infected cell line, tissue, or animal model employed. A recent study revealed differential responses of THP-1 macrophages to different doses of the highly virulent Karp strain (141), in contrast to previous reports, where a low-virulence Boryong strain was used on murine macrophages (131, 135, 142, 143). NF-κB/TNF-α pathway inhibition via STAT3 as well as high-level IL-10 expression via ERK/IL-10 were observed with low-dose infection, promoting bacterial replication. An opposite response was observed with high-dose infection, characterized by the upregulation of NF-κB/TNF-α along with diminished IL-10 production. Moreover, pretreatment of macrophages with this cytokine completely suppressed IL-1β, IL-6, and TNF-α production, even during high-dose infection, suggesting that this interleukin contributes to the regulation of the inflammatory response. MicroRNAs (miRNAs) may participate in this process, with miRNA-155 being a molecule that acts as a fine-tuner of the inflammatory response, acting with IL-10 to prevent cytokine hyperproduction. In response to Karp strain challenge, peripheral blood mononuclear cells obtained from patients who recovered from severe scrub typhus and a cytokine storm were low producers of IL-10 and miRNA-155 but high producers of proinflammatory TNF-α, IL-1β, and IL-6 in comparison to patients who recovered from mild scrub typhus. These observations support the role of IL-10 and miRNA-155 cross talk in modulating cytokine production (141). In addition, an early peak from day 9 postinfection, followed by elevated IL-10 production for 8 days, was observed in a sublethal i.d. mouse model, which was characterized by bacterial persistence in several tissues and enhanced, but nonlethal, pathology (48). In this model, lung and spleen histological lesions did not resolve before 60 dpi, suggesting that early, sustained IL-10 production contributed to ameliorating tissue injury (48). In summary, these results suggest a significant role for this cytokine in the regulation of widespread tissue damage and in the modulation of proinflammatory cytokine levels.

Moreover, cytokine overproduction has also been observed in rodent models of i.p. infection, where the susceptibility of C3H/HeN and BALB/c mice to Gilliam and Karp strain challenges, respectively, was accompanied by the overexpression of chemokines and cytokines that clearly correlated with inflammatory cell infiltration. This was otherwise not observed in BALB/c mice resistant to the Gilliam strain (130). Moreover, analyses of human clinical samples revealed increased IFN-γ, IL-1β, IL12p40, TNF-α, and IL-10 levels and, interestingly, a significant correlation between TNF-α and disease severity in early phases of infection (144–146). Those results highlight the inflammatory response program elicited after *Orientia* infection and its impact on disease susceptibility and development. Gene expression analysis following *Orientia* strain Kato infection in human naive monocytes revealed a massive modification of transcriptional programs, with both downregulation and upregulation of several groups of genes. Particularly, immune response, inflammatory response, cell-cell signaling, and chemotaxis-related genes showed 20% transcriptional enrichment, while genes involved in the antiviral state reached up to 40% upregulation (84). The upregulation of genes that encode IFN-β, IFN-α, and several interferon-stimulated genes has been observed after inoculation with live, but not heat-killed, bacteria, along with a marked type I IFN signature characterized by IFN-α8, IFN-β, MX1, and OAS1 expression. In contrast, proinflammatory cytokine genes were upregulated by both live and heat-killed bacteria, including IL-1β, IL-6, IL-12p40, IL-23p19, and TNF-α. However, infected cells showed reduced TNF-α production and an inability to produce IL-1β when infected with

heat-killed bacteria. Indoleaminepyrrole 2,3-dioxygenase (INDO), a protein involved in the killing of intracellular bacteria and several other functions, was upregulated, and an M1-specific prolife of monocyte activation was, in summary, evident in the presence of both live and heat-killed bacteria. These *in vitro* results correlated with IFN-related genes and certain characteristics of M1 polarization that occur in human patients (84). Furthermore, Kato strain infection of macrophage-derived monocytes may also trigger the enhanced transcription of genes that participate in inflammation, the type I IFN response, and M1 activation. Differences regarding bacterial viability were also reported, with a type I IFN response being present only in response to live organisms and a reduced inflammatory response being observed after infection by heat-killed bacteria (83, 84).

The generation of active IL-1β requires a first signal derived from the activation of pattern recognition receptors (PRRs) and a second one involving nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor (NLR) inflammasomes. These signals lead to pro-IL-1β transcription and the cleavage of the IL-1β precursor, respectively, to produce active interleukin (147, 148). In *Orientia* infection of bone marrow-derived macrophages, the secretion and processing of IL-1β require the internalization of live bacteria and phagosome maturation, including the acidification of the endosomal compartment. The NLR involved in the recognition of live cytosolic *Orientia* bacteria and the triggering of this process remains to be characterized, since LPS-stimulated macrophages from *Nlrp3*-, *Nlrc4*-, or *Aim2*-deficient mice challenged with *Orientia tsutsugamushi* were able to produce IL-1β and the caspase-1 p10 subunit, which is a hallmark of caspase-1 activation (149), in a way similar to that of macrophages from WT C57BL/6 mice (143). Furthermore, *rip2*-deficient macrophages, which do not produce the RIP2 downstream effector of NOD1 or NOD2, also showed IL-1β production similar to that of WT mice, suggesting that caspase-1 production does not depend on signaling from these PRRs (143). The apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein is present in all inflammasomes, connecting with the sensor molecule of the inflammasome via a pyrin domain. It possess an activation-and-recruitment domain, which participates in the activation of caspase-1 (150). The inability of *Asc*-deficient macrophages to produce active IL-1β after *Orientia* infection supports the role of this adaptor molecule in the activation of caspase-1 (143). Finally, data from *in vivo* studies with C57BL/6 mice support evidence of the role of the downstream signaling of IL-1 receptors (IL-1Rs) in protection against *O. tsutsugamushi* infection, as revealed by an elevated pathogen load within blood and spleen of IL-1R-deficient animals at 6 and 19 dpi in comparison to WT mice (143). However, supporting data for the role of this receptor family in human scrub typhus, from either clinical samples or *in vitro* studies, are scarce, and a study of scrub typhus patient samples revealed that neither a correlation between IL-1β and serum bacterial loads nor differences in bacterial loads between the acute and posttreatment phases were evident (146).

Data from studies addressing the impact of the type I IFN response do not support a clear protective role for this innate mechanism. The administration of IFN-α and IFN-β showed a modest or even absent capacity to reduce the bacterial replication of the Karp, Gilliam, and TA716 strains on fibroblasts of C3H and BALB/c mice, suggesting that the inhibitory effect elicited by the type I IFN response is strain and host cell specific but may not be a strong factor in bacterial clearance (151). It is noteworthy that the effects of type I IFN induction may be related to several factors, which include the time after infection, the specific intracellular pathogen involved, and the local levels of mediators produced, thereby showing various outcomes of analyses of these variables (152). Also, various and negative effects on host cells and *in vivo* models of intracellular pathogen infections have been widely documented in several reviews (152–154). Thus, elucidating the impact of this innate defense mechanism requires exhaustive research using different models of infection.

Endothelial Cell Responses to *Orientia* Infection

Endothelial activation plays a substantial role in processes leading to pathogen clearance, through the development of self-proinflammatory, prothrombotic, and per-

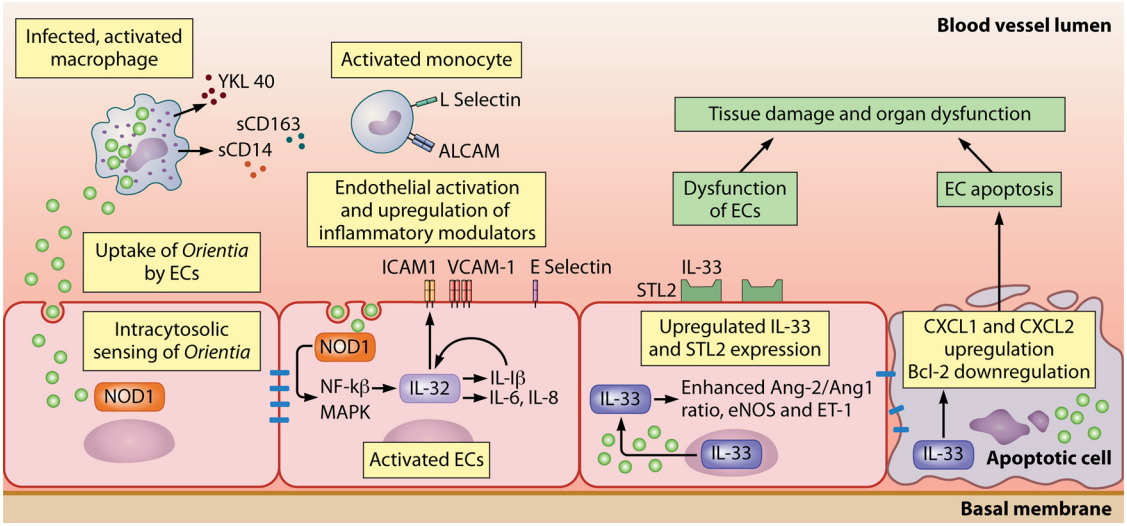

FIG 3 Model for endothelial infection and renal injury in severe, disseminated *O. tsutsugamushi* infection. *Orientia tsutsugamushi* uptake in ECs is followed by cytosolic sensing, where a confirmed role for NOD1 has been described. This leads to the activation of the infected endothelium, with the IL-32-mediated upregulation of inflammatory (IL-1β, IL-6, and IL-8) and adhesion (ICAM-1, VCAM-1, and E selectin) molecules. The increased expression of adhesion molecules in other cell subsets may be involved, as suggested by studies of human patients. The activation of ECs may present a dysfunctional phenotype, with an increased Ang2/Ang1 ratio and altered levels of endothelial nitric oxide synthase (eNOS) and endothelin (ET-1), in which the intranuclear expression and liberation of the alarmin IL-33, along with the upregulated expression of its receptor STL2, play a significant role. Renal tissue damage is enhanced by endothelial apoptosis, where the recruitment of cellular components by chemokine expression and the downregulation of antiapoptotic Bcl-2 may also be involved.

meable conditions. Endothelial cells are greatly influenced by cytokine signaling, which controls most of the physiological and pathological processes in which they participate. The expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and other adhesion molecules mediates leukocyte adhesion and trafficking in cooperation with chemokines. The expression of procoagulant factors participates in the containment of the pathogen in local foci. In addition, the detection of molecules involved in endothelial activation may serve as biomarkers of clinical prognosis and disease severity. However, as a negative effect of these molecules, sustained, widespread endothelial activation and dysregulation may enhance the severity of disease (155, 156).

Once the pathogen has disseminated, ECs constitute an important target in human *Orientia* infection (Fig. 3), as observed in human autopsy tissues (52) and diverse rodent models of lethal and sublethal infection recently reported. These models include i.v. and i.d. C57BL/6 mouse challenge models of Gilliam and Karp strain infections that, interestingly, reflect several aspects of *Orientia* infection in humans, including *Orientia* dissemination to several organs, namely, brain, lung, liver, and kidney (43, 45, 46, 48, 49). Activation markers of ECs, including activated leukocyte cell adhesion molecule (ALCAM) and VCAM-1, have been linked to scrub typhus disease severity (134). Moreover, early activation and dysregulation of ECs have been observed in a mechanistic study of a mouse model of lethal i.v. inoculation of the Karp strain, suggesting a role of EC activation in the pathogenesis of scrub typhus (46). In this model, two regulators of EC function were analyzed: angiopoietin 1 (Ang-1), a constitutively expressed regulator of activation, and Ang-2, which is normally kept in ECs but is released after inflammatory signals to trigger endothelial activation (155, 156). An augmented Ang-2/Ang1 ratio due to altered levels of both molecules was observed in this model (46), a pattern that was previously observed for septic shock, streptococcal toxic shock, and cerebral malaria (155, 157–160). This elevated ratio along with the enhanced production of endothelin 1 and endothelial nitric oxide synthase (eNOS) were observed at 2 dpi in a lethal model of Karp strain inoculation in C57BL/6 mice, where renal pathology, including cellular infiltration in and around the glomeruli and in the intertubular region,

was evident. Interestingly, the upregulation of IL-33 and its receptor was observed in livers and kidneys of challenged WT mice, whereas IL-33$^{-/-}$ mice showed marked decreases in endothelium stress and renal pathology (49). This interleukin is a nuclear factor belonging to the IL-1 family that may modulate inflammatory responses in either a pro- or an anti-inflammatory way and has a well-supported role in the development of acute or chronic kidney injury and disease (161–163). In this lethal Karp strain infection model, a tissue-specific pathogenic role was attributed to IL-33 promoting EC activation and dysregulation as well as extensive tissue damage (49). Additionally, an apoptotic response of ECs that was present in WT mice but greatly diminished in IL-33$^{-/-}$ mice provided further evidence of the proapoptotic effects of *Orientia* observed previously (81, 82) but suggested that apoptotic effects may also be dependent on the tissue and infection model, highlighting the importance of dissecting its underlying mechanisms.

Another interleukin that may be involved in the response of ECs to *Orientia* infection is IL-32, a constitutively expressed regulator of ICAM-1 expression and endothelial inflammation, along with IL-1β. This interleukin has been related to a protective effect in bacterial control, especially against *Mycobacterium*, but also to the immunopathogenesis of some infectious and noninfectious diseases (164, 165). Via NF-κB and p38 MAPK pathways, IL-32 induces several proinflammatory molecules. The role of IL-32 in EC activation has been studied by Boryong strain infection of the ECV304 cell line, where the production and secretion of ICAM-1, IL-1β, IL-6, and IL-8 occur after the activation of an upstream NOD1–IL-32 pathway, as revealed by the diminished production of IL-32 and these cytokines after treatment with specific NOD1 short interfering RNA and by the reversion of this phenotype after exogenous IL-32 treatment (142). This role has not yet been addressed in mechanistic studies, which are required to draw conclusive results.

In summary, the studies discussed above highlight the known importance of ECs in their cross talk with immune cells and as key mediators of inflammatory responses and immunopathology. Most importantly, these studies strongly suggest that ECs and the induced vasculitis are protagonists in *Orientia*-induced pathogenesis and provide evidence of the importance of mouse models in characterizing molecular interactions regarding *Orientia* infection.

Bacterial Recognition by PRRs and Activation of Inflammatory Responses

A major gap in understanding the *Orientia*-host interplay and immune responses to this infection is the limited knowledge of bacterial antigens and molecules sensed by PRRs. This information gap, partially explained by the unavailability of appropriate genetic tools to manipulate *Orientia* and the scarce mechanistic studies performed on animal models, has been delaying the development and testing of efficient, long-lasting, cross-protective vaccines. The recognition of ligands, receptors, and pathways involved in the host response to *Orientia* infection is of vital importance for developing therapeutic and vaccine formulations. For instance, the identification of immunodominant antigens and epitopes is the basis of epitope-focused vaccinology, aimed at designing safe and effective vaccines through appropriately delivering selected molecules that are able to generate a protective response in the host (166, 167). Finding conserved epitopes is particularly relevant for pathogens that display antigens with high genetic diversity, such as *O. tsutsugamushi*. Likewise, the selection of proper vaccine adjuvants, which are molecules that act as agonists of PRRs, may enhance the magnitude and quality of innate and adaptive responses and therefore may shape an improved response to vaccination (168, 169).

There is no clarity about the ligand-receptor interactions that elicit a type I IFN response in either DCs or monocytes. Moreover, the *Orientia* molecules involved in NOD1 activation are still not identified, but current genomic and structural data about this pathogen are shedding light on the possible mechanism. Considering (i) that γ-D-glutamyl-*meso*-diaminopimelic acid of peptidoglycan is responsible for NLR activation (170, 171), (ii) the existence of metabolic pathways that may be able to synthesize

peptidoglycan from UDP *N*-acetyl-D-glucosamine revealed by genome analysis (172), and (iii) the presence of both diaminopimelic acid and peptidoglycan-like structures in *O. tsutsugamushi* (11), it is possible that a unique, still undetermined ligand of this peptidoglycan-like structure may be responsible for triggering some PRRs or at least the NOD1 receptor. Furthermore, the role for TLR signaling in human *Orientia* infection remains poorly characterized, but initial insights come from studies using murine and human cell lines and rodent models of infection. Toll-like receptors 2 and 4 have been related to protective roles in mouse bacterial and fungal infections, including rickettsial (*Rickettsia akari* and *R. conorii*) infections. On the other hand, some studies have linked the activity of TLR2 to increased susceptibility to infection or enhanced pathology (173, 174). Recently, a role for TLR2 in recognizing an *Orientia* ligand and enhancing susceptibility to disease in a mouse model has been described (47). In HEK293 cells, heat-stable *Orientia* molecules are recognized by TLR2 but not TLR4, while TNF-α and IL-6 secretion in infected bmDCs obtained from C57BL/6 mice requires TLR2 (47), results which are in accordance with data from a previous study suggesting that TNF-α production was independent of ligand recognition by TLR4 (131). Interestingly, TLR2-deficient and WT C57BL/6 mice showed similar abilities to restrict bacterial growth after i.d. challenge with the Karp strain and also were more resistant than WT mice during the convalescent phase of a lethal i.p. challenge, suggesting that TLR2 is not necessary for protective immunity against *O. tsutsugamushi* in this model. Accelerated clearance of *Orientia* in affected tissues and sustained TNF-α and IL-6 production at between 15 and 18 dpi in peritoneal tissue, which is the primary target following i.p. inoculation, were observed in TLR2-competent mice, suggesting that neither elevated transcription levels of proinflammatory molecules nor a diminished ability to minimize bacterial growth was associated with a lethal outcome in this model. The differential responses in the convalescent phase suggest that adaptive immunity responses enhanced by TLR2 signaling may be responsible for these differences in susceptibility (47).

Possible Role for Mucosa-Associated Invariant T Cells

Mucosa-associated invariant T (MAIT) cells are a unique, abundant subset of T cells expressing a semi-invariant T cell receptor restricted to interactions with the MHC-related protein, or MR1 (175–177). Due to the highly conserved and nonpolymorphic nature of MR1, these cells are referred to as T cells with an innate-like phenotype. They sense infection through the riboflavin synthesis pathway, which is present only in bacteria and yeast, and have unique, important roles in antibacterial immunity against pulmonary pathogens and *E. coli*. After the recognition of antigens, they secrete proinflammatory cytokines and directly kill infected cells. Regarding this, a reduced count of circulating MAIT cells has been observed in pneumonia and tuberculosis sufferers (175, 176, 178–181). Indirect activation after chronically established viral infections through the IL-12/IL-18 pathway has also been reported (182).

The role of this cell subset in rickettsial infection has barely been explored, but a recent study of scrub typhus patients reported MAIT cell activation after *Orientia* infection and suggested that this cell subset constitutes an important arm of cell-mediated immunity (CMI). Moreover, MAIT cells may be related to disease severity after their dysregulation or deficiency (183). Diminished MAIT cell levels in peripheral blood were revealed when scrub typhus patients (0.69%) were compared to healthy controls (1.37%). These cells also show diminished production of TNF-α in comparison to those of healthy donors but increased production in the remission stage of the disease (183). Performing future studies on MAIT cells may shed light on their role in rickettsial infections and their potential for the development of immunotherapies based on cell immunity. Although they display phenotypic differences, mouse MAIT cells closely resemble human ones (184), and therefore, mouse models have interesting potential for the study of the physiological and pathological roles of these cells.

ADAPTIVE IMMUNITY: PROTECTIVE ROLES AND KEY LIMITATIONS

Acquisition of Natural Heterologous and Homologous Immune Responses

One of the most remarkable traits of adaptive immunity is the development of highly specific immunological memory after encountering a pathogen. This specificity aims to safeguard the host from successive infections by the same pathogen, a feature known as homologous protection. Nevertheless, the generated immunological memory may influence immune responses to other pathogens, which is known as heterologous immunity. Although usually associated with closely related pathogens (i.e., different strains of the same bacterial species), it is also observed between unrelated taxa and may either result in boosting protective responses or favor immunopathology (185). Observations from studies performed with human volunteers more than 50 years ago revealed that natural heterologous immunity acquired after *O. tsutsugamushi* infection is strikingly short-lived, whereas homologous protection may last some years (186, 187), observations which are further supported by data from studies performed with non-human primates (NHPs) (188). Experimental subcutaneous (s.c.) inoculation of the Gilliam strain in human volunteers who were naturally infected with *Orientia* 11 to 24 months before the experiment resulted in clinical disease. On the contrary, infection of patients who were naturally infected 1 to 2 months previously did not result in clinical disease (186). As discussed by those authors, most of those challenges were probably heterologous. A different picture comes from homologous challenges, where only an erythematous lesion surrounding the Gilliam strain inoculation site was observed in a patient who was infected with the same strain 3 years prior to the study. Additionally, complete protection was observed in another homologous challenge with a volunteer who was naturally infected more than 3 years earlier (186, 187). Similar results were observed in NHPs, where susceptibility to experimental homologous reinfection was observed 6 years after the initial infection, in contrast to the resistance otherwise observed when reinfection was performed 8 months after the initial infection (188). Previous evidence for the lack of heterologous protection came from studies with formalin-killed vaccine preparations, where lethal i.p. homologous challenge resulted in the survival of mice, whereas heterologous challenge resulted in various outcomes, from an absolute lack of protection to significant protection. This was observed only when immunization was done by the same administration route and not when the subcutaneous route was used, which resulted in a lack of protection even with homologous challenge (189). However, when live *O. tsutsugamushi* bacteria were used for s.c. immunization, homologous protection was observed even when mice were challenged by a different route (190). Interestingly, those early studies suggested high antigen diversity characterizing *Orientia* strains. Additionally, those studies revealed that both the viability of vaccine organisms and the immunization/challenge routes differentially influence the host immune response.

Antigen Diversity

More recent studies on *O. tsutsugamushi* surface molecules and antigens confirmed the previously suspected heterogeneity of surface antigens. Despite these studies, we are still far from having a comprehensive understanding of the diversity of *Orientia* antigens and their relevance in immunity. To date, five immunodominant proteins have been detected by Western blotting, with molecular masses of 22, 47, 56, 58, and 110 kDa (191–195). Some of these antigens show discrete variability: the 22-kDa antigen is well conserved among several strains (>95% similarity), whereas the 47-kDa antigen is a conserved transmembrane protein that also presents little contribution to the antigenic variability of *Orientia* (196, 197). On the other hand, higher variability is observed for the sequences of the 56- and 110-kDa antigens. TSA56 is the most well-characterized antigen and the major variable immunogenic surface protein; it contains four major variable domains (VDs) but also conserved epitopes and is the most frequently recognized antigen in human and animal infections (192, 198–201). This protein has been the subject of several vaccine studies due to its high immunogenicity; some of these studies are discussed below. The 110-kDa antigen is

less abundant in outer membranes but also presents strain-specific epitopes and is recognized in natural human infection. Its study as an immunogen is limited to one report, where recombinant plasmid expression of this antigen provided partial protection in a homologous challenge of Swiss CD-1 mice (42).

In many studies performed in several regions of endemicity in Asia examining rodent, arthropod, and human samples, high antigenic diversity is indeed observed. However, analysis of *Orientia* genetic diversity reveals a more complex diversity, where a variable frequency of genotypes is frequently found either within or between regions of endemicity (202–211). Moreover, molecular analyses of field samples, which are commonly based on analyses of outer membrane proteins, mainly the 56-kDa protein, reveal that common genotypes found in human patients do not reliably represent the prototype strains widely used in diagnostic assays and vaccine studies but fall into strain-related genotypes that show higher genotypic diversity (205–207, 209, 210, 212, 213). Thus, the *O. tsutsugamushi* diversity observed in regions of endemicity represents a major barrier that is difficult for the development of accurate diagnostic tools and adequate cross-protection through vaccination. This heterogeneity leads to the ineffective development of both effector and memory cross-protective immunity due to the antigen-specific nature of humoral and cell-mediated responses. Although testing of either heat-killed, irradiated, or live *Orientia* vaccines revealed that several subunit vaccines may provide strong homologous protection (42), on the contrary, the development of heterologous protection remains a major challenge. Since immunodominant, variable antigens have failed to provide long-lasting heterologous protection, it has been proposed that sustained, cross-protective immunization may be reached by testing vaccines that include a combination of nonimmunodominant, widely conserved antigens displayed in an adequate context that favors the development of both humoral and cellular responses (24). However, candidate antigens remain to be identified.

Humoral Immunity: Protective Roles and Related Problems

In human patients, levels of specific antibodies against *O. tsutsugamushi* are maintained during a short time, as observed in serological surveillance studies. *Orientia*-specific titers rise from the second week postinfection (214), reaching a mean peak titer of 1:499, but quickly decrease afterwards, with a mean reversion time of ~49 weeks, reaching undetectable levels after 40 months (215). The short duration of serum antibodies has also been observed in NHPs, where detectable levels disappear within 1 year after generation (188). Complement fixation studies on human strain-specific antisera show that sample titration results in significantly higher endpoint titers when tested against homologous strains than when tested against heterologous strains (189, 216).

Those observations are clearly reflected in the characteristics of the natural immune response acquired by naturally infected individuals, which are discussed above. The kinetics of antibody production have scarcely been reported in mouse inoculation studies that resemble human disease, where the duration of significant serum antibody levels was not measured. In a Karp strain i.v. infection model, animals suffering from acute lethal disease presented higher levels of IgG2c at 10 dpi than did their uninfected counterparts, whereas levels of IgG1 antibodies did not differ between the two groups. Additionally, a marked Th1 cytokine profile was evident in animals presenting serious disease, giving hints of the importance of this secretory profile in disease development. Additionally, higher IgM levels were detected by an enzyme-linked immunosorbent assay (ELISA) at 10 dpi in the lethal challenge group than in uninfected control mice (46). That same research group reported antibody responses in a sublethal challenge with the Gilliam strain, where the kinetics of seroconversion were dependent on the dose of the pathogen and the inoculation route. An overall predominance of IgG2c over IgG1 isotypes was reported, suggesting a more significant Th1 response. However, significant levels of IgG1 were also reported, suggesting that a Th2 response was present, possibly contributing to the improvement of immune homeostasis leading to

a sublethal outcome rather than a lethal one (43). When considering studies of human sera, one study reported that the kinetics and levels of antibody responses differ between conserved and variable antigens; specifically, the variable 56-kDa protein produced early, robust, and long-lasting antibody responses, while those elicited by the 47-kDa antigen were detectable later and were less pronounced (191).

Although a complete understanding of the robustness of the antibody response requires further research efforts, the results discussed above suggest that the kinetics and isotypes implicated in the humoral response may be modulated by several factors and may impact the general immune response to *Orientia* and the pathogenesis of scrub typhus. Despite the short duration of antibody levels in human patients, protective roles of antibodies were observed in studies reported decades ago, through the passive transfer of sera from immunized animals to challenged mice. In one study, complete protection or delayed death was observed after a lethal i.p. challenge when serum transfer was performed within the first week of infection (217), whereas complete protection against acute disease and enhanced mouse survival were reported in another study after the transfer of immune sera previously incubated with *Orientia in vitro* (218). Protection by antibody neutralization was observed in another report where strain-specific differences were studied. Particularly, complete protection was achieved only with homologous strain challenges, whereas no protection was observed with heterologous strain challenges (219). Hyperimmune serum inhibited infection by *Orientia* in suspended chicken cells by blocking a strain-specific surface antigen. Additional studies have shown that strain-specific antibodies enhance the intracellular uptake of the bacteria by professional phagocytes (54, 133). Guinea pig PMNs enhanced the uptake of antibody-bound *Orientia* bacteria, which were subsequently released from phagosomes to the cytosol, where these antibodies inhibited the translocation of *Orientia* to areas where free bacteria usually move (54). When the effect of immune serum on Gilliam strain infection of resident BALB/c mouse peritoneal macrophages was evaluated, a 50% reduction in the number of infected macrophages was achieved. This was improved to a 75% reduction when infection was evaluated in cytokine-activated macrophages, which also presented enhanced growth suppression of opsonized *Orientia* bacteria (133). Nonetheless, complete suppression of infection by macrophages was not observed when considering these treatments separately, indicating that the humoral response may not be able to fully subvert the infective process.

Antigens inducing neutralizing antibodies were not identified in those previous studies. However, other studies reported that sera obtained from human patients show reactivity with the 56-kDa protein (220, 221), and some important epitopes of this protein have been identified. These epitopes were mapped in a study where mouse monoclonal antibodies and sera obtained from naturally infected humans and mice immunized with the 56-kDa antigen from the Boryong strain (Bor56) were used to characterize antibody-binding domains through the expression of deletion constructs of Bor56 recombinantly expressed in *E. coli*. Antigenic domains (ADs) I (aa 19 to 103), II (aa 142 to 203), and III (aa 243 to 328) were identified as being important immunogenic regions (222). Moreover, in a similar study, sera obtained from Boryong-, Gilliam-, Karp-, or Kato-immunized mice were employed to analyze homotypic and heterotypic antibody responses, revealing that different epitopes on the 56-kDa antigen were involved in distinct antibody responses. Particularly, it was concluded that ADs II and III and VD IV were relevant for heterotypic antibody responses, whereas VD I defined homotypic antibody responses (223). Those results suggested that the 56-kDa antigen plays a significant role in generating protective humoral responses. In fact, C3H/HeDub mice immunized with recombinant Bor56, but not control mice, showed a triggering of the secretion of neutralizing antibodies, which conferred enhanced resistance to lethal challenge. These immunized mice also secreted Th1 cytokines when stimulated with irradiated strain Boryong bacteria and displayed dose-dependent lymphocytic proliferation, thereby shedding light on the protective responses achieved by targeting this antigen (224). Additionally, high antibody titers were also observed, which, in other studies, showed the ability to neutralize infection in an i.p. challenge with a homolo-

gous strain (222, 225). Also, immunization of cynomolgus monkeys 1 month prior to challenge with a recombinant truncated fragment of this antigen elicited significant humoral and cellular immune responses and diminished clinical disease but did not prevent rickettsemia (42).

The reports discussed above support the idea that humoral immunity has a significant role in providing protection against homologous infections. On the contrary, a protective role in the development of heterologous protection is otherwise less well supported. Several aspects of the humoral response require urgent research, including the detection of conserved antigens and characterization of their immunogenic potential. Also, specific isotype responses and their kinetics should be further studied, along with their effect on scrub typhus pathogenesis and diverse aspects of humoral and cellular responses. The development of accurate animal models may constitute a powerful tool for the characterization and modulation of these responses.

Contribution of T Cells and Cytotoxic Lymphocytes to Protection and Pathogenesis

According to reports based on human patients and experimental animal studies, CMI has a significant role in protective responses to *Orientia tsutsugamushi*, being more important in the development of cross-protective responses than humoral immunity (132, 226–233). One study found that mice that survived a first Gilliam strain challenge were able to survive a subsequent lethal i.p. Karp challenge, despite a lack of cross-reactive antibodies. Moreover, complete protection of naive mice was observed after the passive transfer of splenocytes from Gilliam-inoculated immune mice, a protective effect that was otherwise absent when transferred cells were T cell depleted. Interestingly, no protection against lethal Karp challenge was observed when only sera of Gilliam-immunized animals were transferred to naive mice, confirming that humoral responses had a marginal role in these protective responses (226). In another study, gamma-irradiated Karp strain bacteria, which are able to enter the cells but are unable to replicate, were employed to perform i.p. immunization of mice, resulting in a nonlethal outcome. These irradiated immunogens yielded complete cell-mediated protection against subsequent lethal challenges performed 24 days after immunization with the Karp and Kato strains, providing further support for the role of CMI in homologous and heterologous protection (227). When formalin-inactivated *Orientia* bacteria were employed instead, neither homologous nor heterologous protection was achieved, which may be related to a decrease in antigen immunogenicity (227). In accordance to those previous results, lymphocyte proliferation triggered by both homologous and heterologous antigens was observed in an *in vitro* mouse lymphocyte proliferation assay performed after immunization with the Karp, Kato, or Gilliam strain (229). It was later reported that proliferating T cells were able to secrete MIF and interferon (234). A previously reported study using an *in vitro* assay indicated that $CD4^+$ T cells are activated after encountering infected moDCs, as revealed by IFN-γ secretion (117). Nevertheless, patients with acute scrub typhus present with peripheral $CD4^+$ lymphopenia and regulatory T cell downregulation, along with massive T cell apoptosis, which may contribute to the poor generation of memory responses and to an unregulated cytotoxic T lymphocyte (CTL) response (235).

To date, several reports have yielded robust data indicating that protection against *Orientia* infection results after IFN-γ upregulation. High IFN-γ levels were seen in C3H/He mice previously s.c. immunized with the Gilliam strain, after i.p. challenge with viable *Orientia* bacteria. The correlation of these levels with rickettsemia strongly suggested that this cytokine has an important role in protective responses (230). A protective role was observed in another study, where an IFN-γ-producing immune T cell line previously exposed to specific antigens provided protection after being transferred to challenged naive mice. Interestingly, this cell line, which was suggested to be of a T helper phenotype, showed partial reactivity to Karp but not Kato strain antigens in a lymphocyte proliferation assay (236). In NHPs, one report indicated that T cells respond with IFN-γ secretion after *Orientia* infection (188), results which were recently supported by a study carried out in a rhesus macaque model where *Orientia-*

specific cellular IFN-γ secretion was observed at between 7 and 28 dpi through an *ex vivo* IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay (237). The detection of the production of this cytokine in peripheral blood mononuclear cells has been related to the improved control of bacteremia in NHPs vaccinated with a DNA vaccine (pKarp47 DNA) (238). In human scrub typhus patients, an early response of IFN-γ and the IFN-γ-inducing cytokines IL-15 and IL-18 occurs early during the acute phase of infection (144–146). Additionally, a proinflammatory-shaped response is evident, and T cell recruitment may be enhanced by the release of the chemokines CXCL9 and CXCL10. Furthermore, increased levels of granzymes A and B indicate the upregulation of cytotoxic activity (144–146, 232). These responses are likely due to the cytosolic sensing of *Orientia*, as discussed above, and support the importance of CMI and cytotoxicity in early responses against this infection.

Cytotoxicity is an important mechanism that is usually required to eliminate cytosolic pathogens. The major effectors of these response are CTLs, which are mainly $CD8^+$ cells. These responses are less studied for *Orientia* than for the *Rickettsia* genus (239). Early reports observing a cytotoxicity effect showed that splenocytes derived from Gilliam strain-infected C3H/He mice are able to lyse L929 fibroblasts *in vitro*. Recently, using a BALB/c mice footpad inoculation model with the Karp strain, it was demonstrated that the spleen and lung are highly infiltrated by active $CD8^+$ T cells at 21 dpi. The $CD8^+$ response is required for the protection of mice, since its depletion results in elevated bacterial loads from 14 dpi onwards and in mouse mortality, while the adoptive transfer of these cells to i.p. challenged naive mice prevents lethality. Complementary roles were addressed in a C57BL/6 mice model, where an increased percentage of $CD8^+$ T cells was present in the pulmonary lymphocyte compartment for several weeks, and their depletion at 84 dpi caused the reactivation of bacterial growth. Interestingly, the activity of $CD8^+$ T cells was required to control infection and protect mice from lethality even when increased IFN-γ levels and activated macrophage responses were observed in lung and liver. Also, the cytotoxic activity of $CD8^+$ T cells was responsible for liver and lung injury. Those results underscore the relevance of these cells in orchestrating a protective response that requires several mediators and in restricting the growth of *Orientia* in acute and persistent infection. Moreover, those results demonstrate the role of this cell subset in tissue injury observed in mouse models that resemble human pathology (231).

Finally, it is interesting to note that humoral immunity also requires T cells for proper development, as concluded in a study performed with athymic mice, where antibody responses were dependent on the transfer of T cells (233). Summarizing the data from recent studies, and considering the characteristics of the infective cycle and systemic dissemination of *Orientia tsutsugamushi* in scrub typhus, is clear that a protective response requires efficient cross talk between humoral and cellular immunity, with a tight control of effector cells, in order to successfully achieve pathogen clearance without compromising the host due to exacerbated inflammatory responses. The role of T cells and cytotoxicity against *Orientia* infection seems to be crucial for several aspects of adaptive immunity; however, functional studies unveiling the contribution of CTLs, apart from the ones mentioned above, as well as studies of other T cell subpopulations in *O. tsutsugamushi* infection are scarce, particularly in the context of infections that resemble human pathology. This branch of the immune response should be of high priority in future studies, since it may constitute an essential component of the response required to achieve the complete elimination of the pathogen from infected tissues, acting as a scaffold for humoral and cytotoxic responses and as a basis for the development of robust cross-protective immunological memory, which is a crucial goal to reach.

CONCLUDING REMARKS

Scrub typhus remains a neglected disease despite the substantial health burden that it generates annually. The development of an effective and cross-protective vaccine constitutes an imperative goal for the prevention and control of this severe infection,

but current efforts have yielded ineffective results. This bacterium has a complex infectious cycle that involves several hosts and cell types, where it displays a plethora of molecular mechanisms required for successful infection and cycle consummation. In this context, great research progress has been achieved regarding details of the *Orientia*-host cell interplay, which have shown that this bacterium extensively modulates human innate immune responses. However, important aspects of innate response subversion are still not elucidated, including apoptosis modulation and phagosome/autophagosome escape mechanisms as well as enhanced cytosolic replication withstanding nitric oxide production. The availability of the complete genomes of two *Orientia* strains may help in an understanding of these processes and furthermore has given evidence of several potential pathogenic mechanisms. For example, the massive existence of secretion systems and effectors proteins, along with some recent discoveries of their roles as virulence factors, has raised an interesting hypothesis about the extent and mechanisms of the interaction network mediated by *Orientia* soluble molecules, making this a fertile field for future investigations. The incorporation of new methodologies may be useful for expanding current knowledge, as reported for other intracellular pathogens, where chromatin immunoprecipitation (ChIP) studies have shed light on the direct DNA-binding capacity of Ank proteins in *Anaplasma phagocytophilum* (240, 241) and *Ehrlichia chaffeensis* (242, 243). Similar improved methodologies for intracellular organisms were recently reported (244), and efforts from research groups will be required to expand the tools needed for dissecting *Orientia* modulation of host cells. Additionally, new releases of draft *Orientia* genomes (245) may also be employed to facilitate an understanding of the molecular complexity of different *Orientia* strains and the genetic bases of pathogenicity, as exemplified by recent work addressing strain differences in antibiotic resistance (246). Genomic information should also be exploited for the detection of conserved subdominant antigens, which may be crucial for the generation of vaccines that elicit cross-protective responses, as discussed above.

The bacterial ligands and host cell receptors that participate in the specific recognition of *Orientia* remain largely unknown, with some exceptions. However, it is well documented that infection and activation of phagocytic and nonphagocytic innate cells induce the upregulation of cytokines and chemokines that drive a proinflammatory state, which has been consistently studied by employing *in vitro* systems. It seems that these responses are insufficient to control infection and additionally may be involved in scrub typhus immunopathogenesis. It is interesting to analyze IL-10 secretion, which has been consistently observed in several *in vitro* and *in vivo* experiments, as well as studies carried out in scrub typhus patients (48, 130, 135, 141, 144, 146). Although not addressed by mechanistic studies, the role of this cytokine seems to be critical in shaping disease outcomes, possibly being involved in the modulation of the hypersecretion of inflammatory cytokines and bacterial persistence. Further research, integrating new molecular techniques and employing recently established mouse models, will have a fundamental role in determining the impact of key molecules and actors in the inflammatory response, including ligands and receptors involved in infection sensing.

Fluorescent chemical probes that allow the tracking and live imaging of cell compartments are promising tools that are expanding our knowledge of the basic biology of *Orientia* and revealing striking characteristics, such as the presence of peptidoglycan-like structures that may be involved in host cell recognition by unique patterns, which should be further characterized (11, 247). Moreover, studies based on TLR2-deficient mice have revealed interesting roles for this molecule (47), and it is expected that future mechanistic studies performed with models that closely resemble human infection will greatly improve our understanding of defense and pathogenic mechanisms. The establishment of these models constitutes a unique advancement, since in the past, several studies addressing immune responses were performed by using either i.p. or i.v. inoculation, displaying responses that do not reflect those observed in mite-borne natural human infection. Further diversification of inoculation

routes is necessary to explore different aspects of the pathology and immune responses triggered by *Orientia* infection. Laboratory-reared chigger infection models may be the most appropriate approach to study several aspects of *Orientia* infection, without neglecting vector-associated factors. Although successful *Orientia* challenges employing this inoculation route in mice have been reported (126, 248), it has not been routinely employed in the latest studies. Furthermore, to better understand natural human *Orientia* infection, and to address gaps regarding human immune responses, this laboratory-reared chigger inoculation approach may be employed on humanized mice, which constitute an interesting tool that has been unexplored to date. These mice display a functional human immune system, since they carry a mutation in the IL-2 receptor γ chain locus that renders them severely immunodeficient, a condition that permits the subsequent engrafting of primary hematopoietic cells and tissues from humans (249, 250). This combined approach would allow the assessment of several factors that are frequently bypassed, such as the immunomodulatory effects that arthropod saliva might exert (251). It would also provide a unique platform to investigate the infective process of *Orientia* and human immune responses. We expect that efforts will be made to overcome technical difficulties related to the establishment of the laboratory-reared chigger inoculation model.

Currently, interesting studies are unveiling the role of T cell subsets in defense against *Orientia*, particularly cytotoxic cells (231), but additional progress is required and expected to be addressed soon to understand the impact of each cell subset participating in CMI. The role of $CD4^+$ cells requires urgent characterization due to their impact on immunological memory development, especially considering reports of $CD4^+$ human lymphopenia (235). Finally, for achieving an accurate understanding of immune responses, it is necessary to conduct studies on the contribution of other less-studied cell subsets, including MAIT and regulatory T cells, which may be achieved via mechanistic studies thanks to the current availability of mouse models and diverse genetic backgrounds.

Several factors of this pathogen and the immune response elicited in human hosts turn the development of an effective cross-protective vaccine into a complex challenge for scientists. One of the greatest barriers is derived from the high genetic diversity and antigenic diversity among strains and their major surface proteins. However, other factors, such as (i) the natural response targeting nonconserved, immunodominant antigens; (ii) deficient naturally acquired immunity; (iii) the involvement of several immune cells in the replicative cycle of *Orientia tsutsugamushi*; (iv) the lack of established animal models for the realization of mechanistic studies; and (v) the limited knowledge on correlates of protective infection, also hinder the development of therapeutic modulation strategies and of a protective vaccine providing long-lasting and cross-protective responses. To date, it is well understood that effective immunity against *Orientia* requires the synergy of humoral responses and cellular components of both innate and adaptive responses, but a more realistic study approach will lead to a more precise understanding. We expect that future mechanistic studies based on animal models will allow researchers to dissect several branches of immunity against this pathogen, toward overcoming difficulties the underlie vaccine development. Moreover, other important goals to be achieved, such as the development of diagnostic and immunogenicity assays for field use as well as the evaluation of therapeutic agents and candidate vaccines, also require well-established animal models. Determining correlates of protection is therefore required, but this has not been achieved in humans. Interesting reports based on NHP models are providing a fertile field for future protection correlate studies and investigations into vaccine-induced immune responses (238). It is noteworthy that the recent establishment of a high-sensitivity IFN-γ-based *ex vivo* ELISPOT assay validated in both humans and a NHP model may provide a remarkable link between animal models and human studies and promises to improve field immunogenicity and candidate vaccine evaluation (237).

Finally, as stated above, this bacterium remains genetically intractable. Genetic manipulation of intracellular bacteria has been a challenging goal, mostly due to

their mandatory intracellular lifestyle. It is noteworthy that during the last decade, genetic manipulation strategies have been successfully applied to *Rickettsia*, specially spotted fever group and typhus group rickettsiae (252, 253). Research groups should direct efforts toward overcoming this genetic intractability, since genetic manipulation tools, once protocolized, may notoriously improve the knowledge of *Orientia* pathogenesis and immunity.

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REVIEW
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Current Animal Models for Understanding the Pathology Caused by the Respiratory Syncytial Virus

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The human respiratory syncytial virus (hRSV) is the main etiologic agent of severe lower respiratory tract infections that affect young children throughout the world, associated with significant morbidity and mortality, becoming a serious public health problem globally. Up to date, no licensed vaccines are available to prevent severe hRSV-induced disease, and the generation of safe-effective vaccines has been a challenging task, requiring constant biomedical research aimed to overcome this ailment. Among the difficulties presented by the study of this pathogen, it arises the fact that there is no single animal model that resembles all aspects of the human pathology, which is due to the specificity that this pathogen has for the human host. Thus, for the study of hRSV, different animal models might be employed, depending on the goal of the study. Of all the existing models, the murine model has been the most frequent model of choice for biomedical studies worldwide and has been of great importance at contributing to the development and understanding of vaccines and therapies against hRSV. The most notable use of the murine model is that it is very useful as a first approach in the development of vaccines or therapies such as monoclonal antibodies, suggesting in this way the direction that research could have in other preclinical models that have higher maintenance costs and more complex requirements in its management. However, several additional different models for studying hRSV, such as other rodents, mustelids, ruminants, and non-human primates, have been explored, offering advantages over the murine model. In this review, we discuss the various applications of animal models to the study of hRSV-induced disease and the advantages and disadvantages of each model, highlighting the potential of each model to elucidate different features of the pathology caused by the hRSV infection.

Keywords: human respiratory syncytial virus, bovine respiratory syncytial virus, lower respiratory tract infections, rodent model, non-human primate model

INTRODUCTION

Human respiratory syncytial virus (hRSV) is an enveloped, single-stranded, and negative-sense RNA virus belonging to order *Mononegavirales*, family *Pneumoviridae*, genus *Orthopneumovirus*, species *Human orthopneumovirus* (Hacking and Hull, 2002; Borchers et al., 2013; Afonso et al., 2016; Snoeck et al., 2018). This virus is a human pathogen that causes a major burden in public health, both in developing and in industrialized countries (Simoes, 2003; Zang et al., 2015; Kuhdari et al., 2018). Noteworthy, hRSV is the leading cause of acute respiratory infection in newborns and of severe lower tract respiratory disease (LTRD) in children, with an estimation of 33.8 million of RSV-associated acute LTRD episodes in children less than 5 years old in 2005 (Nair et al., 2010). Estimations indicate that this virus is responsible for up to 3.4 million of hospital admission due to severe acute LTRD (Nair et al., 2010) and constitutes the leading cause of acute bronchiolitis and subsequent hospital admissions in industrialized countries (Bush and Thomson, 2007). Importantly, this virus is an important cause of mortality in young children in developing countries. In 2015, it was estimated that 59,600 hospitalized infants younger than 5 years old have died from hRSV-related LTRD worldwide (Shi et al., 2017; Scheltema et al., 2018).

Several attempts to develop safe and protective vaccines for the high-risk groups have been ineffective, and currently, there is no licensed vaccine for this pathogen (Hurwitz, 2011). Therefore, there is an urgent need for the development of a hRSV vaccine. In addition, the efficacy of the single licensed therapeutic option remains controversial, raising interest in the development of alternative therapeutic approaches against this pathogen (Canziani et al., 2012; Ispas et al., 2015; Muñoz-Durango et al., 2018; Simon et al., 2018). Therefore, the implementation of functional animal models for studying this virus has emerged as a critical and indispensable aspect underlying the development of immunotherapies and vaccines against hRSV (Hurwitz, 2011). For this reason, the development of different animal models for studying several aspects of hRSV has been very important and is still a field where research is focused. Since no animal model reflects all aspects of this viral infection and disease (Taylor, 2017), several models have been used in the study of hRSV, ranging from rodents and small mammals to large animals and non-human primates. This results from high specificity of hRSV for the human host, lacking an animal reservoir in nature (Collins and Graham, 2008). This feature has greatly hindered the development of an exclusive animal model, and therefore, the choice of the more suitable animal model required for each researcher will depend strongly on the aspect of the infection that needs to be studied and the investigative hypothesis proposed (Jorquera et al., 2016). The most commonly used animals have been rodents, such as mice (Graham et al., 1988; Bueno et al., 2008) and cotton rats (Prince et al., 1978, 1983; Sawada and Nakayama, 2016); ruminants (Elvander, 1996; Woolums et al., 1999, 2004; Meyerholz et al., 2004; Derscheid and Ackermann, 2012; Ackermann, 2014); and non-human primates (Kakuk et al., 1993; Szentiks et al., 2009), but at the present, the diversification of animal models is a requirement for addressing the diverse problematics of this viral infection and the development of vaccines and treatments. For this reason, the objective of this article is to review the several animal models used and their applications and to discuss their pros and cons. Finally, and based on the current information, recommendations of use are made, besides highlighting the role of the use of the murine model as a first approximation in the recent preclinical studies of new vaccines and antiviral treatments.

NON-HUMAN PRIMATE MODELS OF hRSV

Mainly with the objective of vaccine efficacy and safety testing, several species of non-human primates (NHPs) have been used as animal models for hRSV infection, including chimpanzees (*Pan troglodytes*) (Szentiks et al., 2009), African green monkeys (*Chlorocebus sabaeus*), macaques (*Macaca* spp.) (Simoes et al., 1999; De Swart et al., 2002; Patton et al., 2015), and owl monkeys (*Aotus* spp.) (Prince et al., 1979). This group of species shares several anatomic and physiologic similarities to humans, but its application as an animal model of infection is challenging due to economical, technical, and ethical issues. Only chimpanzees are fully permissive to hRSV infection (Belshe et al., 1977; Crowe et al., 1993; Teng et al., 2000) and develop almost exclusively upper respiratory tract disease (URTD) symptoms in experimental studies.

The Chimpanzee

hRSV was isolated for the first time not from humans, but from a group of chimpanzees naturally infected by the virus, which displayed signs of URTD (Morris et al., 1956). The experimental infection on this species has shown varying results, but high viral replication has been reported with viral doses lower than 10^4 plaque-forming units (PFUs; Belshe et al., 1977). Viral replication measured in nasopharyngeal and tracheal samples indicated that peak viral loads reached were 1–2 order of magnitude higher than the initial inoculum, and persistence of virus was shown to last 6–10 days post-infection (DPI; Belshe et al., 1977; Crowe et al., 1993; Teng et al., 2000). Although infected chimpanzees show clear URTD signs (Blount et al., 1956), no low respiratory tract disease (LTRD) has been reported under experimental conditions; therefore, human clinical disease condition is not completely replicated in this species. However, naturally infected chimpanzees with fatal LTRD have been reported, including viral antigen presence in lungs and histopathological changes including high neutrophil infiltration, edema, and the deposition of hyaline membranes (Belshe et al., 1977; Szentiks et al., 2009; Unwin et al., 2013). Natural co-infections with *Streptococcus pneumoniae* have also been reported and may be enhancing those naturally occurring infections (Szentiks et al., 2009; Nguyen et al., 2015).

Applications of this NHP model have included the evaluation of protective efficacy and safety of live-attenuated and recombinant vaccine candidates (Richardson et al., 1978; Collins

et al., 1990; Crowe et al., 1993; Teng et al., 2000). Similar body temperatures between chimpanzees and humans have allowed studies with candidate temperature-sensitive vaccines (Council, 2011). Vaccine candidates consisting of the recombinant expression of F and G glycoproteins by modified vaccinia viruses have shown to induce low levels of neutralizing antibodies (NAs) and protection to viral challenge in chimpanzees (Collins et al., 1990; Crowe et al., 1993). On the contrary, temperature-sensitive mutant hRSV vaccines that are highly restricted at replication but highly immunogenic in chimpanzees have induced protective responses against hRSV infection in this species, although protective mechanisms have not been defined in detail (Richardson et al., 1978; Crowe et al., 1995). Permissive replication of hRSV is the most remarkable advantage of this model, being the unique species reflecting the viral replication in humans (Belshe et al., 1977; Crowe et al., 1993). Anatomical, physiological, and genetic similarities also represent a unique advantage of the use of this model.

However, several disadvantages and limitations apply to this model, and the appearance of several other useful models has diminished the utilization of chimpanzees. The economical, logistical, and ethical cost of research on chimpanzees is extremely high, and therefore, this NHP is not commonly available to research groups (Tardif et al., 2013). Published studies to date mostly yielded inconclusive results due to small sample sizes and genetic heterogeneity of animals. Lack of inbred strains, immunological tools, and reagents are also an important restriction to the use of this model (Bem et al., 2011; Jorquera et al., 2016). Finally, specialized housing, handling, and caring of this species are mandatory (Tardif et al., 2013).

The National Research Council has established that this model may be necessary exclusively for testing the candidate vaccines, based on the suitability of this model to serve as surrogate model for seronegative infants (Council, 2011).

The Macaque

Permissiveness of species within *Macaca* spp. to hRSV infection is low, with high dose intranasal (IN) or intratracheal (IT) inocula reaching low or moderate viral replication capacity, despite the age of individuals (Simoes et al., 1999; De Swart et al., 2002; McArthur-Vaughan and Gershwin, 2002; Grunwald et al., 2014; Grandin et al., 2015). Although clinical disease is rare, a report has shown evidence for mild respiratory disease with fever and increased respiratory rate in juvenile rhesus macaques associated to bronchiolitis, bronchitis, and interstitial pneumonia (McArthur-Vaughan and Gershwin, 2002). The virus has been re-isolated from lungs but at low viral titers (Simoes et al., 1999; McArthur-Vaughan and Gershwin, 2002). Studies using the formalin-inactivated hRSV vaccine (FI-hRSV) have shown enhanced disease with cellular peribronchiolar, perivascular infiltration, and high viral titers, but that enhanced response might be affected by immune responses to non-viral antigens present in the viral challenge preparations (Ponnuraj et al., 2001; De Swart et al., 2002). A vaccine candidate based on the Fusion (F) protein of hRSV adjuvanted with GLA-SE was evaluated in cynomolgus macaques and induced a robust humoral and Th1-biased cellular immunity (Patton et al., 2015). Further, lack of protection and evidence of enhanced disease were observed in infant macaques after immunization with a modified vaccinia virus Ankara vector expressing the hRSV F and G proteins (de Waal et al., 2004). Also, a heterologous adenovirus vector vaccine with the F subunit of hRSV have been evaluated in rhesus macaques (Grunwald et al., 2014). A mucosal booster immunization showed to be effective at reducing viral replication, with an immune response that included a high and persistent expansion of $CD4^+$ and $CD8^+$ T cells in the lower respiratory tract mucosal sites. These data suggest that this vaccine scheme is effective for eliciting protective immune responses at mucosal effector sites in this animal species (Grunwald et al., 2014). The pros and cons for using this animal model are equivalent to those previously mentioned for chimpanzees.

The African Green Monkey

African green monkeys (AGMs) are less susceptible to hRSV infection than chimpanzees (Kakuk et al., 1993), often lack clinical manifestations after hRSV infection, and develop only minor histopathological changes (Kakuk et al., 1993; Ispas et al., 2015). Conversely, the viral load constitutes a more reliable disease parameter in this animal model (Kakuk et al., 1993; Eyles et al., 2013; Ispas et al., 2015). Mild signs, including rhinorrhea, coughing, sneezing, and wheezing, have been described in AGMs following IN and IT administration of the hRSV A2 strain AGMs. Further, viral shedding in the oropharynx has been detected for 8 days in these animals (Kakuk et al., 1993). Studies on AGMs have focused on vaccine candidate testing, including recombinant vaccines (Kakuk et al., 1993; Tang et al., 2004; Jones et al., 2012; Wang et al., 2017) and adjuvanted subunit vaccines (Eyles et al., 2013). Vaccines that had shown efficacy in rodent challenge studies have failed to elicit appropriate responses in AGMs, which could be due to the immunological response itself or to the different experimental design relative to the vaccination and challenge (Taylor, 2017). These observations underscore the difficulty of translating results from rodent species to primates (Eyles et al., 2013). Additionally, antiviral effects of TMC353121, a fusion inhibitor, have been successfully tested in AGMs as a preclinical model (Ispas et al., 2015). The first primate model for FI-hRSV-induced enhanced respiratory disease was developed on this species, with airway and parenchymal inflammatory changes in the vaccinated animals, which could not be evidenced by viral titers (Kakuk et al., 1993). Although less expensive than chimpanzees, utilization of this species has most of the general disadvantages in which NHP models display, as those previously discussed.

The Owl Monkey

Only a few studies have explored the use of this species as an animal model for hRSV infection (Prince et al., 1979; Koff et al., 1983; Hemming et al., 1995). Prince and collaborators have reported that an IN inoculation of 10^4 PFU of hRSV can result

in high viral titers, rhinorrhea, and the development of low levels of neutralizing antibody (NA) virus titers appearing at 14 days post-infection and reaching a peak at 28 days post-infection. Viral infection was observed until 8 to 17 days (Prince et al., 1979; Koff et al., 1983). Antibody-dependent cellular cytotoxicity has been studied in owl monkeys against human epithelial HEp-2 cells infected with hRSV, with peripheral mononuclear blood cells in the presence of hRSV antibodies causing lysis of infected HEp-2 cells in an *in vitro* assay (Koff et al., 1983). Reduction of viral shedding from nose and trachea has been reported after intravenous administration of human immunoglobulin (IVIG) in owl monkeys that previously received an intratracheal (IT) hRSV challenge (Hemming et al., 1995). Response to vaccination in this species has been scarcely studied; a single dose of recombinant vaccinia virus expressing the F or G glycoprotein of hRSV has been reported to be effective in controlling hRSV-induced LRT (Olmsted et al., 1988).

RODENT MODELS FOR hRSV DISEASE

The order *Rodentia* includes a variety of small mammals that have been widely used as models of human pathogens in immunological and infection studies, including vaccine testing studies, and elucidation of immunopathogenic mechanisms (Andersen and Winter, 2017). Despite differences at the infective cycle developed by hRSV infection, permissiveness for viral replication, and host immune system differences between mice and humans, the laboratory mouse model remains to date as one of the most prevalent animal models used in hRSV studies. However, the use of the cotton rat has allowed the modeling of several aspects on this infection that the mouse model fails to resemble, including viral replication permissiveness, which is improved in cotton rats. These two species are systematically used nowadays and have allowed researchers to make significant progress in vaccine and immunotherapy development. Further, other rodent models have also been utilized, as described later.

The Mouse Model

The laboratory mouse (*Mus musculus*) is the most frequently used animal as a model in the field of biomedical sciences and is the model of choice in immunological and infection research, as well as for the evaluation of vaccines and therapies worldwide (Lee et al., 2012; Ehret et al., 2017). Although the development of vaccines and new therapeutic approaches against hRSV has been a challenge for scientists to date (Salazar et al., 2017; Mazur et al., 2018), mouse models have been very important to achieve past and recent advances in preclinical tests of new vaccines and antiviral treatments. Further, these models have contributed significantly to elucidate their mechanisms of action (Hurwitz, 2011; Heylen et al., 2017). Noteworthy, the clinical symptomatology of the hRSV disease after mouse infection has certain differences when compared to human disease. Besides, clinical differences are also observed between different strains of mice (Openshaw and Tregoning, 2005). Another feature of the murine model for hRSV infection is that these animals must be challenged with large doses (~10^7 PFU) to detect viral replication in the lungs (Graham et al., 1988). Among mice strains available, the BALB/c mouse is commonly used to study the immunopathology caused by hRSV infection, since it is semi-permissive to lung viral replication. The disease manifests with piloerection, reduced activity, and weight loss (Graham et al., 1988; Kong et al., 2005). Furthermore, moderate bronchiolitis is observed in these mice (Bueno et al., 2008). Importantly, loss of body weight within the first 3 days of infection and infiltration of lungs with neutrophils are two well-established disease parameters to assess protection against this serious disease (Bueno et al., 2008).

It has been reported that cell-mediated immunity decreases as individuals get older. Therefore, studying $CD8^+$ T-lymphocyte populations in different age groups is a relevant topic to address. (Fulton et al., 2013; Mosquera et al., 2014). The aged BALB/c mice generate a weak primary response of $CD8^+$ T cells specific for hRSV in the lung, which is associated with a delay in viral clearance. When evaluating the maximum magnitudes of the responses of virus-specific $CD8^+$ T cells in lung and airways, significant decreases have been seen in aged BALB/c mice when compared with young BALB/c mice (Fulton et al., 2013). Infected aged BALB/c mice develop severe inflammatory changes with a predominance of neutrophils and lymphocytes, in addition to diffuse alveolar damage 4 days post-infection (Mosquera et al., 2014). It has been shown that both the kinetics and the magnitude of antiviral gene expression decrease as a result of advanced age. In addition to the delay in cytokine signaling and the induction of the receptor pattern recognition, it has been found that TLR 7/8 signaling is altered in alveolar macrophages in elderly mice, and this shows that there are inherent differences in response to hRSV infection in pneumonia models of young BALB/c mice versus the model of aged BALB/c mice (Wong et al., 2014). Experimentally, it has been described that a senescence prone SAM-P1 mouse strain (H-2 K), which shares the genetic background of AKR/J mice, is more susceptible to infection by hRSV, and shows a deficient $CD8^+$ T cell response, as well as a lower gamma-interferon (INF-γ) production, which contrasts to high interleukin (IL)-4 production (Liu and Kimura, 2007). Noteworthy, this animal model allows for the generation of humanized mice or mice with a human immune system (HIS mice; Sharma et al., 2016), which has been also used to evaluate vaccine efficacy (Sharma et al., 2016). During the first 3 days of infection, the HIS mice lost more weight and eliminated hRSV faster than did NOD-scid gamma mice (NSG mice). These mice were generated by the introduction of an adeno-associated virus serotype 9 (AAV9) vector carrying human cytokine genes into highly immunodeficient NOD-scid gamma (NSG). The pathological characteristics induced by hRSV infection in HIS mice include peribronchiolar inflammation, neutrophil predominance in the bronchoalveolar lavage fluid, and enhanced production of mucus in the respiratory tract (Sharma et al., 2016). In addition, the mouse model has been extensively used to perform preclinical studies for vaccine and therapies for this virus (Hurwitz, 2011). Recently, vaccine efficacy studies were performed in mice to evaluate safety and immunogenicity of a vaccine consisting of a recombinant strain

of bacillus Calmette-Guérin (rBCG) expressing the nucleoprotein antigen of hRSV (Cautivo et al., 2010; Céspedes et al., 2017). A single immunization with the rBCG vaccine elicited protective Th1 type immunity against hRSV in mice, preventing weight loss due to infection, as well as reducing viral replication and disease severity in these animals (Céspedes et al., 2017). Vaccination with rBCG promotes the elimination of the virus at the pulmonary level and prevents development of interstitial pneumonia without evident adverse effects (Céspedes et al., 2017). Moreover, subunit vaccines against hRSV based on a novel genome replication-deficient Sendai virus (SeV) vector expressing the F protein as a genetically stable antigen have been studied (Wiegand et al., 2017). After IN or intramuscular (IM) immunization of BALB/c mice, a robust hRSV-specific immune response was induced, consisting of serum IgG and neutralizing antibodies, as well as cytotoxic T cells. Further, IN immunization was also able to stimulate hRSV-specific mucosal IgA in the upper and lower respiratory tract of these animals (Wiegand et al., 2017). The mouse is also a useful model for the study of basic aspects of mechanisms related to the pathogenesis of asthma as a consequence of hRSV infection (Peebles and Graham, 2005). It is well established that the mouse model has multiple comparative advantages in relation to other animals, such as genetic homogeneity between consanguineous animals; availability of humanized, knockout, and transgenic stains; very good reproductive performance; ease of maintenance in confined spaces; non-complex nutritional requirements; and wide variety of reagents to study the immune response. Additionally, the similarities between the mouse and human immunological response, as well as the docile nature of the laboratory mice and non-complex management, make this species one of the most used for studies relative to infections and immunity worldwide (Mestas and Hughes, 2004; Drake, 2013; Sharma et al., 2016).

The Cotton Rat Model

The cotton rat (*Sigmodon hispidus*) is currently among the most extensively used animal models of hRSV and other human infectious respiratory diseases, in part due to the semi-permissive replication of several respiratory viral agents and the developing industry of genetic and immunological tools applicable on this model. Up to date, this model has been successfully used for several studies in hRSV, including antibody prophylaxis (Ottolini et al., 2002), vaccine testing (Widjojoatmodjo et al., 2010; Garlapati et al., 2012; Kamphuis et al., 2013; Rostad et al., 2016; Stobart et al., 2016; Widjaja et al., 2016; Fuentes et al., 2017), FI-RSV enhanced respiratory disease (Prince et al., 1986; Sawada and Nakayama, 2016; Widjaja et al., 2016), maternally induced immunity (Prince et al., 1983; Blanco et al., 2015), and susceptibility of high-risk human groups (Boukhvalova and Blanco, 2013). In fact, the main advantage of this model is its greater permissiveness to hRSV infection than inbred mice, other rodents, and animals in general, reaching a replication that is 50–1,000 fold higher than mouse strains (Prince et al., 1979). Infection with hRSV undergoes active replication in lungs and nasal tissue, lasting about 6–9 days, respectively. However, tracheal replication reaches only low titers (Prince et al., 1978). Histopathological examinations have revealed mild lesions as early as day 2, including bronchitis, bronchiolitis, and exudative rhinitis (Prince et al., 1978). Interestingly, with higher doses (10^6 PFU), cotton rats develop alveolitis, peribronchiolitis, and interstitial pneumonitis, therefore reflecting a direct relationship between viral replication, histopathology, and the infectious dose inoculated (Prince et al., 1978). Although the quality and timing of pathological changes observed in cotton rats after hRSV infection share several features with the natural human hRSV infection, bronchiolitis is notoriously less severe in cotton rats (Prince et al., 1978, 1986). The absence of clinical signs, such as cough, rhinorrhea, and fever, constitutes a disadvantage that should be considered for certain studies. Although cotton rats remain susceptible through the whole life, viral replication and persistence are major in infant animals. Reinfection of this species leads to an inflammatory response in lungs that is characterized by the absence of viral production due to an abortive viral replication (Prince et al., 1979; Boukhvalova et al., 2007a), which is paralleled by an early upregulation of interferon response and expression of interferon-inducible MX genes (Pletneva et al., 2008). The presence of MX genes is noteworthy, since Mx1 and Mx2 are functionally absent in inbred mouse strains C57BL/6, BALB/c, and CBA/J, and is an important component of the antiviral innate response (Staeheli et al., 1993). In terms of the immune response to experimental infection, the arachidonic acid pathway is upregulated after cotton rat infection with hRSV and has been associated to the severity of disease (Richardson et al., 2005). Cytokine synthesis in lung after hRSV infection reveals an increase in IFN-γ, IL-10, IL-6, CCL-2, and growth-regulated oncogene, reaching a peak at day 4 after infection in infant cotton rats, which is delayed to day 6 in aged individuals (Boukhvalova et al., 2007b).

Response to repetitive exposures to hRSV has been evaluated in cotton rats, showing that during the first infection there is an absence of neutralizing antibody production, which results in a lack of protection to a second infection. During a second infection, a neutralizing antibody response and a $CD8^+IFN\text{-}\gamma^+$ T cell response take place, which seem to be nevertheless unable to clear the virus from the lungs (Yamaji et al., 2016).

The study of maternal transferred immunity is one of the earliest applications for this model (Blanco et al., 2015). Both colostrum feeding and transplacental immunity can provide protection in lungs and nasal tissue in infant cotton rats gestated by immunized mothers challenged with hRSV prior to litter birth. Unfortunately, this passively transferred protection is transient and significantly reduced after 4 weeks from birth (Prince et al., 1983; Blanco et al., 2015). This protective effect is correlated to serum neutralizing antibodies, with titers >1:380 being protective in infant cotton rats, which is similar to human infant passively-acquired immunity, which results in reduced susceptibility to infection with antibody titers equal or higher to 1:400 (Prince et al., 1983, 1985). However, the lack of correlation in some studied animals suggests that other immune mediators might be contributing to maternally transmitted protective immunity (Prince et al., 1983). Interestingly, a strong correlation between hRSV-specific neutralizing antibody titers

in cotton rat mothers and their litters has been described, and the protection provided by those antibodies appears to be inversely associated to cytokine expression in lungs (Blanco et al., 2015). Also, the maternally acquired humoral immunity is responsible for suppressing vaccine immunogenicity in infant cotton rats (Prince et al., 1979, 1982), thereby highlighting the difficulties of developing a vaccine for human infants.

The mentioned correlation between antibody levels as a way of providing protection in cotton rats and human infants led to several studies (Saez-Llorens et al., 1998; Johnson et al., 1999), and to the development of a commercial antibody formulation for prophylactic use in human infants in high risk of severe hRSV disease (Olchanski et al., 2018). The ability of predicting the success of immunoprophylaxis in infants and of assessing the dose of antibody required to elicit protective serum levels is one of the most remarkable milestones of this animal model (Prince et al., 1985; Group, 1998). It is important to mention that those studies advanced to clinical phases without the need of intermediate studies in NHPs and are currently available from commercial use in patients (Niewiesk and Prince, 2002). On the other hand, research on immunotherapeutic use of antibodies against hRSV has been conducted, but has yielded dissimilar results. Several antibodies tested have been useful for controlling viral replication but not for ameliorating lung pathology and disease severity (Rodriguez et al., 1997; Malley et al., 1998; Prince et al., 2000). More recently, a novel Ig formulation, RI-002, was successfully tested in cotton rats for treating immunocompromised patients. This formulation, containing high quantities of hRSV-specific neutralizing antibodies, inhibited the prolonged hRSV replication, which is typical in immunocompromised rats (Johnson et al., 1982) and reduced dissemination of replicative virus. Pulmonary interstitial inflammation and epithelial hyperplasia were also reduced by RI-002 administration, therefore providing evidence of a beneficial therapy for hRSV disease outcome in immunocompromised cotton rats (Boukhvalova et al., 2016) results that should be further investigated on its ability to reduce disease symptoms in humans.

Research on the pathology of vaccine-enhanced respiratory disease, described decades ago in human infants, also finds a suitable model in cotton rats. Knowledge on the mechanisms of this manifestation is critical for future studies on vaccine development. FI-hRSV vaccinated cotton rats subsequently challenged with hRSV present an increased pulmonary pathology, with severe alveolitis as a hallmark of histopathological changes, as well as a rise in neutrophil and lymphocyte infiltration (Prince et al., 1986). Additionally, association of Th2 polarization as a known mechanism of vaccine-enhanced respiratory disease has been studied in this model in which an upregulation of Th2 cytokines (IL-4, IL-10, IL-13, and CCL5) has been observed (Boukhvalova et al., 2006; Sawada and Nakayama, 2016). However, an increase in Th1 chemokines and cytokines has also been reported, and it has been suggested that those pro-inflammatory cytokines and chemokines may play a role in enhanced pulmonary inflammation (Prince et al., 2001; Boukhvalova et al., 2006). Immunodominance of post-fusion-specific site I of F protein elicits weak neutralizing antibody responses against that site in FI-RSV-immunized cotton rats and an absence of an efficient neutralizing response by other antibodies (Widjaja et al., 2016). This situation contrasts with immunization response to experimentally infected or vaccinated cotton rats, which showed a high virus neutralizing capacity from pre-fusion specific antibodies binding antigenic site Ø and other parts of the F protein, and may account for the mechanism on FI-hRSV-enhanced respiratory disease (Widjaja et al., 2016). Noteworthy, limitations of some studies performed in cotton rats have been revealed, including observations on the role of non-viral products in vaccine or challenge inoculated medium as drivers of alveolitis, which appears to be mediated chiefly by T cell specific responses to non-viral antigens, having the hRSV antigens a limited role as co-factors in the inflammation and disease manifestation (Shaw et al., 2013).

Despite limitations, cotton rats have been the main model in research on development of efficient and secure vaccine candidates, and several vaccine candidates have been recently tested in this model including subunit vaccines (Garlapati et al., 2012), recombinant vaccines (Widjojoatmodjo et al., 2010, 2015; Stobart et al., 2016; Fuentes et al., 2017), VLPs and virosomes with viral subunits (Kamphuis et al., 2013; Cullen et al., 2015), as well as live attenuated vaccines (Luongo et al., 2013; Stobart et al., 2016), therefore confirming the relevance of this animal model for those studies. However, prediction capacity of this model on protective responses after vaccination in humans is still unknown, and further investigations are required to elucidate this question.

Increased susceptibility to severe hRSV infection occurring in immunosuppressed infants and older people has been successfully modeled in the cotton rat model. Late clearance of virus is classical in immunosuppressed cotton rats, therefore impacting in disease pathogenesis (Ottolini et al., 1999). Also, this model has been used to test prophylactic and therapeutic schemes in immunosuppressed rats, reflecting the need of multiple administrations of Ig to diminish viral replication (Ottolini et al., 1999; Boukhvalova et al., 2016). Unlike mice, infant cotton rats permit a greater viral replication with a greater persistence in URT than 4-week-age rats and mount a less efficient NA response than adult animals (Prince et al., 1978). In elderly, immunosenescent cotton rats (>6-month age), prolonged viral persistence is present (Curtis et al., 2002), and peak expression of cytokines is delayed in older rats (>6 month age) than young age rats (<2-month age) (Boukhvalova et al., 2007b).

Technical advantages on the use of this model include the availability of commercial and laboratory-owned inbred strains, as well as the increasing quantity of immunological reagents and assays, which, added to similarities between cotton rats and human innate immune system (Boukhvalova and Blanco, 2013), make this species an interesting and useful model for pathogenesis and immune responses to infection. On the other hand, the lack of clinical manifestations after hRSV infection limits the use of this model in this field. Additionally, the natural progression of infection from the URT to the LRT taking place in humans is not observed in this species (Boukhvalova and Blanco, 2013). For laboratories looking

forward to including this animal model, it must be noted that handling, maintenance, and breeding of this species are different from mice strains, demanding specific facilities and protocols. Handling might be difficult to inexperienced scientists and students, and these animals may bite if not held properly. Also, blood withdrawal and drug administration are different; the retro-orbital plexus is the best site for blood collection, which requires training of personal for ensuring animal welfare (Niewiesk and Prince, 2002).

Other Rodent Models

Beyond inbred mice and cotton rats, several other rodents have been used for hRSV studies, including vaccine efficacy studies. However, to date, robust and consistent data from established research lines are lacking, and the application of these studies to vaccine development and testing is very limited. The use of these other rodent models in general and the scope of the findings are limited by the disadvantages of being not fully permissive for hRSV replication, the use of outbred animals, and the lack of immunological reagents and inbred animals. However, recent observations highlight the importance of expanding the animal model repertoire.

Experimental infections of hamsters have been reported in 3-week-age hamsters inoculated with high hRSV doses ($10^{4.6}$–$10^{6.5}$ PFU), which allow for viral replication in URT and LRT but lacking histopathological and clinical manifestations of lung compromise (Wright et al., 1970). Remarkably, Syrian hamsters (*Mesocricetus auratus*) have been used for the development and preclinical evaluation of live attenuated recombinant human parainfluenza virus 1 vectors expressing the hRSV F protein. This attenuated vaccine has provided protection in these rodents against an IN challenge on day 30 post immunization, with a dose of 10^6 PFU, protection that well correlated with the production of serum NAs against hRSV (Mackow et al., 2015).

Studies on Chinchillas (*Chinchilla lanigera*) have shown that this species is semi-permissive for hRSV infection, but only in the upper airways following high-dose intranasal challenge ($1x10^6$ – $1x10^7$ PFU). It has been proposed as a suitable model for studies of hRSV infection in upper airways, including the role of hRSV infection in the development of otitis media, based on observations of viral replication in nasal cavities and Eustachian tubes (Gitiban et al., 2005; Grieves et al., 2010), as well as a model for the understanding of mucosal immunity responses (McGillivary et al., 2013); however, this model has not been employed enough to draw robust conclusions of its application.

Guinea pig (*Cavia porcellus*) is another rodent that is semi-permissible for hRSV infection, as observed in a single study to date. Acute bronchiolitis, including bronchiolar epithelial necrosis, and either mononuclear and polymorphonuclear (PMN) lymphocyte infiltrates, have been observed at 6 days post-infection, with a remission at 14 days post-infection, in one-month age outbred guinea pigs, intranasally infected with 4×10^3 PFU. This was accompanied by the absence of clinical signs. The histopathological lesions at 6 days post-infection were paralleled with low viral titers, indicative of limited viral replication (Hegele et al., 1993). Although these observations support the use of this model for the understanding of acute hRSV infections, it has not been extensively studied, and the very limited availability of inbred strains and immunological reagents restricts its use. Noteworthy, an advantage on the use of this model is that guinea pig is more similar to humans than other small animal models in terms of physiology and immune system responses, which has been observed in several studies reviewed elsewhere (Padilla-Carlin et al., 2008).

MUSTELID MODEL

Ferrets (*Mustela putorius furo*) are small carnivores that belong to the *Mustelidae* family, which have been used for the study of different infectious diseases, including those hRSV infection. Their small size, and similarities shared with humans at an anatomical and physiopathological level are key advantages of the use of this species for investigation of infectious diseases.

In 1976, it was shown that ferrets allow for viral replication in nasal tissue of all age animals (Prince and Porter, 1976), but replication in lung tissue was observed only in infant ferrets; therefore, it was proposed as an interesting model for the age dependence studies on severe hRSV infection (Byrd and Prince, 1997). However, a recent study further highlighted the advantages of ferret models, showing that adult ferrets are highly susceptible to hRSV infection, since IT infection of immunocompetent individuals resulted in productive virus replication in the upper and lower respiratory tract, with the presence of viral antigens in tracheal and bronchial epithelial cells, reaching viral loads in throat, trachea, and lungs with similar order of magnitude in comparison to cotton rats (Stittelaar et al., 2016). This seems to be a major advantage of this model, along with the ease of handling the animals. Interestingly, recent viral interference studies have been carried out in ferrets to elucidate mechanisms that explain separate seasonal peak incidence of influenza and hRSV, showing that replication of a pandemic influenza virus strain (A[H1N1] pdm09) prevents subsequent infection with laboratory hRSV strains by antigen-independent mechanisms (Chan et al., 2018). Besides, studies on immunocompromised ferrets have shown hRSV replication in bronchiolar epithelial cells and reduced/delayed viral clearance (de Waal et al., 2018). This would allow the development of a specific animal model for a certain population at risk, such as immunocompromised patients, who experience severe pulmonary manifestations after hRSV infection (de Waal et al., 2018). Remarkably, viral transmission studies in immunocompetent or immunocompromised animals have been recently carried out, which suggest the suitability of ferrets in studying interventions aimed to limit hRSV transmission (Chan et al., 2017; de Waal et al., 2018).

Some disadvantages regarding the use of this model include the lack of clinical manifestations, the requirement of a more specialized husbandry facility and caging system, and the limited availability of immunological reagents and genetically modified mutants for immunological investigation. Also, the limited

development of inbred ferrets restricts the application for mechanistic studies (Enkirch and Von Messling, 2015; Stittelaar et al., 2016).

LARGE ANIMALS MODELS: LAMBS

Sheep (*Ovis aries*) are susceptible to natural infection by ovine respiratory syncytial virus and bovine respiratory syncytial virus (bRSV; Masot et al., 1995, 1996). The last mentioned is responsible for severe infection in preterm lambs, with an age-dependent clearance of the virus, being this preterm lamb model a useful model of severe hRSV disease in the preterm infants (Meyerholz et al., 2004), and also in exploring disease mechanisms, therapeutic regimens, and risk factors (Derscheid and Ackermann, 2012). Lambs are also susceptible to experimental infection by high doses of hRSV, which results in URTD and LTRD (Olivier et al., 2009; Sow et al., 2011; Larios Mora et al., 2015). Intratracheal inoculation of lambs with 10^8 PFU of the A2 strain induces a pulmonary pathology that shares similarities to pathology observed after human infant hRSV infection, including bronchiolitis with neutrophil infiltration, mild peribronchiolar interstitial neumonia. A peak of viral replication in airway epithelium occurs at 6 days post-infection, with virus clearance at 14 days post-infection (Olivier et al., 2009; Sow et al., 2011). Nebulization of 7.6×10^7 PFU of Memphis 37 hRSV strain results in increased expiratory effort in lambs at 4, 6, and 8 days post-infection, a viral titer peak in bronchoalveolar lavage fluid (BALF) at 3 days post-infection and viral antigen in lung tissue, both reduced at 8 days post-infection. Further, infected animals develop histopathologic lesions including bronchitis, bronchiolitis, necrosis, and hyperplasia of epithelia, peribronchial lymphocyte infiltration, and syncytial cells, resembling those described for lambs and infants (Larios Mora et al., 2015).

Lamb respiratory tract allows for a more suitable comparison with the respiratory tract of human infants than rodent model, in terms of development, structure, susceptibility to hRSV strains, pathology, immune response, and clinical manifestations (Plopper et al., 1983; Lindgren et al., 1996; Derscheid and Ackermann, 2012). Similar developmental and structural features include the size of the nasal cavity and airways, the presence of airway submucosal glands that express lactoperoxidase, prenatal alveolar development, airway branching patterns, percentage of Club cells, and development of type II cell (Scheerlinck et al., 2008; Derscheid and Ackermann, 2012; Ackermann, 2014). Additionally, lamb model offers the possibility to evaluate pulmonary functions using the same principles and techniques that are applicable for children and adults (Kirschvink and Reinhold, 2008). These advantages, along with the reported findings, establish the preterm and neonatal lamb as a model with key features that mimics hRSV infection in preterm and neonatal infants.

Additionally, induction of resistance to hRSV infection and development of hRSV-specific antibodies by neonatal and maternal immunization has been recently studied in the sheep model. Immunization of neonates with hRSV F protein in adjuvant provides effective immunization even in the presence of maternal antibodies (MatAbs; Garg et al., 2015). Moreover, pregnant ewes immunized with the same vaccine resulted in transfer of MatAbs to the newborn lambs through the colostrum. Those newborn lambs receiving MatAbs challenged with hRSV at 3 days of age showed a reduction of 70% in viral load, less lung pathology, and higher virus neutralizing titers (VNTs) than in control animals without passive immunization. This newborn lamb model of hRSV is therefore suitable for studies on maternal immunization and evaluation of the ability and safety of a vaccine to induce MatAbs and protection following hRSV challenge (Garg et al., 2016).

Studies on FI-RSV-vaccinated lambs revealed that the immune response triggered by this immunization reduced RSV titers in bronchoalveolar lavage fluids and lungs, as well as histopathology scores, but increased peribronchiolar and perivascular lymphocyte infiltration as compared to lambs undergoing either an acute RSV infection or naïve controls. Furthermore, no evident disease exacerbation was observed (Derscheid et al., 2013). These observations suggest that the lamb FI-RSV infection differs from what has been observed in children presenting FI-RSV enhanced disease (Kim et al., 1969; Prince et al., 2001). However, further studies need to be performed to understand the altered response of lambs to FI-RSV vaccination and to establish the scope of this model.

Technical advantages regarding the use of the lamb model include the ease of repeated sampling and manipulation, a long lifespan that facilitates realization and interpretation of studies on elder population. Unfortunately, there are some significant disadvantages regarding the use of the lamb model, including limited availability of molecular tools for immunologic and genetic studies, and additional efforts and requirements in housing and handling of this species (Bem et al., 2011; Gerdts et al., 2015).

NATURAL HOST PNEUMOVIRUS INFECTIONS

Pneumoviruses (family *Paramyxoviridae*, subfamily *Pneumovirinae*) include several pathogens of veterinary concern, including avian metapneumovirus, bovine respiratory syncytial virus (bRSV), ovine and caprine RSVs, pneumonia virus of mice (PVM), and canine pneumovirus (Easton et al., 2004; Renshaw et al., 2011). A pneumoviral infection in its natural host is associated with a fully permissive replication and a persistent inflammatory response. Interest in using some of those natural host pneumoviral infections as a model of hRSV infection has increased, since this approach of study offers several advantages over less permissive models, which are discussed later.

bRSV in Calves

The bRSV infection in cattle (*Bos primigenius taurus*) occurs naturally and is one of the main pathogens causing pneumonia in the cattle industry along with other viral agents such as parainfluenza type 3. In addition, bRSV infection predisposes

animals to secondary bacterial infections with agents such as *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma bovis,* producing a synergism between viral and bacterial agents known as bovine respiratory disease (van der Sluijs et al., 2010; Ellis, 2017). Therefore, bRSV infection has been extensively studied with the objective of reducing its major health and economic burden. Vaccines for bRSV are commercially available, showing variable efficacy in disease reduction (Bem et al., 2011; Ellis, 2017). Cattle can also be experimentally infected by hRSV passaged in bovine cell lines (Thomas et al., 1984). Interestingly, antigenic similarities, epidemiology, and viral pathogenesis have increased interest in studying bRSV infection in calves as an animal model of hRSV infection. Calves reproduce many of the clinical signs associated with hRSV infection in human infants, with clinical signs such as fever, rhinorrhea, cough, serous or mucopurulent nasal discharge, abnormal pulmonary auscultation, tachypnea, and hypoxia (Sacco et al., 2012a, 2015; Taylor, 2013). After bRSV infection, an incubation period of 2–5 days is followed by a range of clinical signs, from asymptomatic to URTD and severe LTRD. The severity of disease is age dependent; most severe disease occurs in young calves in the first 1–6 months of life; however, older animals are also susceptible when subjected to stressful conditions (Elvander, 1996; Hägglund and Valarcher, 2016). Experimental infection of calves results in macroscopic and microscopic lesions resembling natural infection. Lung pathology peaks at 7–8 days post-infection and includes lung consolidation, atelectasis, and sporadic deposition of hyaline membranes, with microscopic lesions of bronchointerstitial pneumonia, epithelial necrosis, alveolitis, occasional syncytial cells, debris obstruction of lumen of the bronchi and bronchioles, and extensive apoptosis of lung epithelial cells (van der Poel et al., 1996; Woolums et al., 1999; Valarcher and Taylor, 2007; Blodorn et al., 2015).

Increased levels of pro-inflammatory cytokines and chemokines, including CCL5, CCL3, IL-8, IL-6, IL-12, TNF-α, and IFN-γ, are observed after bRSV infection, as summarized from *in vitro* studies on dendritic cells (Werling et al., 2002) and *in vivo* calf studies that analyzed lung samples (Antonis et al., 2010; Sacco et al., 2012b). Calves recovering from bRSV infection demonstrate significant neutrophil accumulation in the BALF – as seen in children with hRSV bronchiolitis – as well as a predominance of $CD8^+$ T cells (Taylor et al., 1989; McInnes et al., 1999; Antonis et al., 2010). Neutrophils have a role in clearance of virus that is excreted to the lumen of the respiratory tract (Viuff et al., 2002). On the other hand, recent proteomic analyses performed in bRSV-infected calves support an immunopathologic role for neutrophils in RSV disease, demonstrating a positive correlation between disease severity and neutrophil presence in alveolar septa, as well as upregulation of neutrophil-associated proteins in the BALF during infection. Increased neutrophil degranulation and diminished anti-oxidant activity were also observed in calves with bRSV disease. Noteworthy, several histones were associated with the disease, and citrullinated histone 3, an indicator of neutrophil extracellular traps (NET), was only present in unimmunized, infected animals (Hägglund et al., 2017). Considering previous studies that identified NET formation in the lungs and airways of RSV infected children presenting with severe LTRD (Cortjens et al., 2016), these results suggest that dysregulated neutrophil responses and NET activity contribute to severe immunopathology leading to LTRD after RSV infection (Afonso et al., 2016; Hägglund et al., 2017) and evidence the advantages of using a bovine model to study neutrophil-related pathological mechanisms.

Results from several studies suggest that cellular responses are crucial for clearance of bRSV. $CD8^+$ T cells are the predominant lymphocyte subpopulation in the respiratory tract of calves recovering from bRSV infection (McInnes et al., 1999), and their appearance in the lungs on days 7–10 post-infection coincides with viral clearance (Gaddum et al., 1996; McInnes et al., 1999). Additionally, $CD8^+$ depletion in calves results in increased severity of pneumonic consolidation and prolonged nasopharyngeal excretion of the virus (Taylor et al., 1995). Interestingly, however, rapid viral clearance has been observed in bRSV-infected calves that might be related to apoptotic mechanisms rather than T cell cytotoxicity. Apoptosis of the bronchial epithelium and phagocytosis of bRSV antigen-containing apoptotic cells were evident in the lungs at 6 days post-infection, prior to a significant recruitment of $CD8^+$ cells (Viuff et al., 2002).

γδ T cells have been implicated in disease pathogenesis and the development of airway hyperreactivity following hRSV infection in humans and rodent models (Aoyagi et al., 2003; Dodd et al., 2009; Huang et al., 2015); however, it has been difficult to study their role in the disease due to their rare frequency in circulation. By contrast, γδ T cells comprise around 40% of the circulating lymphocyte pool in ruminants (Mackay and Hein, 1989), making the bovine an important model for studying the role of non-conventional T cells in bRSV infection. Bovine γδ T cells produce multiple proinflammatory cytokines and chemokines in response to *in vitro* and *in vivo* bRSV infection (McGill et al., 2013) and are a major source of IL-17 in *in vitro* bRSV antigen recall assays (McGill et al., 2016). However, their importance in this infection remains unclear, as depletion of γδ T cells does not significantly alter the course of bRSV disease (Taylor et al., 1995).

The calf model is useful for the evaluation of several aspects of vaccine development. The calf is scalable in size to human infants and is a tractable model of the neonatal immune system. Furthermore, vaccine development for bRSV faces many of the same challenges as vaccine development for hRSV, including the need to immunize infant populations and to induce long-lived and balanced immune responses, often in the face of maternal or preexisting immunity. The calf is also useful for studying vaccine-enhanced respiratory disease, as this phenomenon has been observed following natural bRSV infection of Belgian White Blue calves 3–4 months after vaccination, with fatal outcome (Kimman et al., 1989). Studies designed to experimentally induce FI-RSV enhanced respiratory disease in calves have shown mixed results regarding the onset and development of pulmonary disease (West et al., 1999; Antonis et al., 2003; Kalina et al., 2004). The differences in disease presentation may be attributable to different vaccine antigen doses, levels of serum antibodies at the time of vaccination, and the time of subsequent challenge (Taylor, 2017).

Several vaccines have been developed and tested in calves, including recombinant bRSV vaccines, modified live viruses, DNA and subunit vaccines, immunostimulating complexes, and novel adjuvants, such as CpG oligodeoxynucleotides (Oumouna et al., 2005; Letellier et al., 2008; Ellis et al., 2010; Riffault et al., 2010; Hagglund et al., 2011; Kavanagh et al., 2013; Woolums et al., 2013; Blodorn et al., 2014; Taylor et al., 2014, 2015). The calf model has also been used in preclinical safety and immunogenicity studies for vaccine candidates containing conserved proteins between both viruses, leading to phase I clinical trials in healthy human adults (Green et al., 2015; Taylor et al., 2015). The recent description of the pre-fusion form of the hRSV F protein has changed our understanding of hRSV antigenicity (McLellan et al., 2013a,b). Similarly, the recent description of the pre-fusion form of the F protein of bRSV (Zhang et al., 2017) has led to major advancements in bRSV vaccine development. Immunization of seronegative calves with a recombinant pre-fusion F protein in Montanide, an oil-in-water adjuvant, induces neutralizing antibody responses nearly 100-fold greater than similar vaccination with post-fusion form of the F protein and induces sterilizing immunity to bRSV challenge (Zhang et al., 2017). Vaccination with recombinant pre-fusion F was also recently shown to enhance neutralizing antibody responses in adult, seropositive cows (Steff et al., 2017).

Recent studies have also shown that the bovine RSV model constitutes a recommendable *in vivo* system to study therapeutic compounds aimed to control hRSV replication and airway obstruction. It has been reported that administration of GS1, a fusion inhibitor, to infected calves, provides therapeutic improvement in clinical manifestations, as well as attenuation of lung pathology and viral load (Jordan et al., 2015). More recently, Cortjens and collaborators have demonstrated that therapeutic administration of local dornase alfa reduces NET formation and airway occlusion in 4-week-old bRSV infected calves, highlighting the relevance of targeting NETs to treat severe airway obstruction induced by bRSV (Cortjens et al., 2018). Housing and handling of cattle require more expertise and specialized housing facilities; however, for veterinary personnel accustomed to working with large animals, this model has the advantage of offering a rapid and repeated sampling of the URT, in comparison to mice. Similarities in bovine and human palatine and nasopharyngeal tonsils (Rebelatto et al., 2000) provide an additional sampling zone for cattle and mark a difference from rodent models, which lack this tissue (Velin et al., 1997). Airway submucosal glands are common in humans and cattle. It has been suggested that inflammatory processes in lung traduce in only a brief detrimental effect in mice, because of the relatively large airway lumen and lack of mucosal gland (Irvin and Bates, 2003). Studies on lung function are more easily performed in calves, and the similarities in structure between calves and humans permit a more credible interpretation of those tests (Kirschvink and Reinhold, 2008). These features, added to the natural replication of the virus and clinical manifestations in calves, make this model highly relevant for studies of clinical lung disease by hRSV. The availability of reagents and tools for immunological, genetic, and molecular studies in cattle is reduced when compared to mice. However, an important number of immunological reagents, including monoclonal antibodies to cell surface molecules, recombinant cytokines and chemokines, and cross-reactive anti-human mAbs, is currently available for the cattle (Sacco et al., 2015).

PVM in Mice

The pneumonia virus of mice (PVM) was first detected in mouse lungs (Horsfall and Hahn, 1940). This virus usually causes natural infections in mouse colonies, especially in immunodeficient mice. In immunocompetent mice, infections are short lived and mostly asymptomatic (Homberger and Thomann, 1994).

Previously described as a rodent-specific pathogen (Parker and Richter, 1982), its host range remains largely unknown, but has been reported as a cause of disease in hedgehogs (Madarame et al., 2014). PVM is the only pneumovirus that is full permissible in the rodent model, with experimental low dose infections leading to clinical disease (Cook et al., 1998; Bonville et al., 2006). C57BL/6 mice show a higher resistance to infection than BALB/c mice when challenged with lethal and sub-lethal doses, probably due to the increased ability of C57BL/6 mice to control viral replication and the immune response elicited by PVM (Watkiss et al., 2013). However, higher protective responses have been observed in a second infection challenge in C57BL/6 mice, several weeks after a previous IN inoculation with 300 PFU of PVM-15 strain (Shrivastava et al., 2015). This protective response appears to be related to an innate pro-inflammatory response and IgA in the lungs after the first inoculation, leading to a higher capacity of generating VNT during the second challenge (Shrivastava et al., 2015). Virus replication capacity is high and occurs in alveolar and bronchial epithelial cells. BALF analyses post-infection have shown an increase in eosinophil and neutrophil infiltration that progresses to an inflammatory state with almost 100% of neutrophil component (Domachowske et al., 2000). This inflammatory state induces local production of proinflammatory mediators including CCL3, CXCL2, and CCL2 (Bonville et al., 2006) consistent with those detected in lung and nasal washes in severe hRSV disease in human infants (Domachowske et al., 2004). High morbidity and mortality are associated to this infection, with clinical signs including difficult breathing, and cyanosis. Histopathological analyses show alveolar epithelial cell apoptosis, bronchial, epithelial necrosis, multifocal acute alveolitis, intraalveolar edema, multifocal hemorrhage, and granulocytic infiltration (Cook et al., 1998). A correlation between pathologic abnormalities and clinical signs has been reported with an IN inoculation of 120 PFU, showing a virus clearance at 10 days post-infection (Cook et al., 1998). A $CD8^+$ T cell response with high levels of TNF-α and IFN-γ in a cytokine storm context has a major role in the pathology induced by this infection, as it has been described in C57BL/6 mice at 5 days post-infection (Walsh et al., 2014). The role of these cells in virus clearance and lung pathology was previously seen in other models of hRSV infection in mice (Cannon et al., 1988; Munoz et al., 1991). However, there are clear differences between PVM and RSV models; acute inflammatory responses to PVM infection vary in anage-dependent form, with scarce recruitment of PMN

cells and inflammatory mediators in neonatal mice when compared to 3- or 4-week-age mice (Bonville et al., 2010).

Human respiratory syncytial virus vaccine development studies have been made on this model (Maunder et al., 2015; Martinez et al., 2016) including a model of FI-PVM enhanced respiratory disease, mediated by a Th2-biased response and eosinophil infiltration in lung (Percopo et al., 2009). Most recently, a replication deficient recombinant human adenovirus serotype 5 expressing the M or N protein of PVM pathogenic strain J3666, provided protection in mice, mediated mainly by $CD8^+$ T cells (Maunder et al., 2015). However, there is still a limited number of studies in this field. Moreover, immunological and pathological studies are scarce too, and differences in mice and virus strain and infective doses have resulted in different clinical outcomes; therefore, drawing conclusions on this topic requires further studies. An advantage of rodent models in general is the availability of reagents and tools for immunological studies, including gene knockout mice facilitate mechanistic studies of PVM disease (Moore and Peebles, 2006) making this model useful for elucidation of inflammatory mechanisms associated with pneumovirus infection (Dyer et al., 2012).

Disadvantages of the use of this model include the high genetic distance between PVM and hRSV, which traduces in gene products with low aminoacidic sequence identity, ranging from 10 to 60%, and lack of direct cross-reactivity (Krempl et al., 2005; Dyer et al., 2012). Also, inherent to every mouse model of human pathogens, there are several differences in immune response between species and lung anatomy. Therefore, extrapolations of this model to hRSV studies should be made carefully.

CONCLUDING REMARKS

The contribution of animal models to the study of human respiratory syncytial virus has been a fundamental part for the development of vaccines and alternative therapies. Each animal model reviewed in this document presents advantages or disadvantages (**Table 1**) highlighting the importance of the correct choice of the model according to the hypothesis to be evaluated or the aspect of infection that requires investigation (**Figure 1**).

Within the complexity of the natural development of hRSV infection, we emphazise that there is no animal model that recapitulates morbidity and mortality trends, since high viral doses are needed to establish infection and/or manifest disease, as is the case of mice or cotton rats. Among other models of hRSV infection, we highlight the potential of ruminant models to understand pathology aspects of hRSV infection, since calves

TABLE 1 | Advantages and disadvantages of different animal species suitable for modeling different aspects of hRSV-induced disease.

Animal models	Advantages	Disadvantages
Natural host pneumovirus models		
Cattle – bRSV	- Permissive model. - Manifest respiratory signs and symptoms. - Reliable lung function testing and interpretation due to anatomical similarities. - Viral antigenic similarities.	- Heterologous model. - Limited availability of molecular tools for immunologic and genetic studies. - Requires large space and specialized veterinary maintenance. - Frequent co-infections naturally occurring.
Mice – PVM	- Permissive model. - Inbred strains and transgenic lines available. - Small size and low cost of maintenance.	- Heterologous model and viral antigenic differences. - Anatomical and immunological differences. - Requirement of special biosafety facilities.
Heterologous host hRSV models		
Mice	- Several inbred strains and transgenic lines available. - Small size, ease of handling and housing, and low cost of maintenance. - Proper sample size easy to achieve. - Extensive laboratory use, allowing for results comparison.	- Lung anatomical differences. - Several immune system differences. - Moderate natural viral replication.
Cotton rats	- More permissive than other models. - More immunological similarities to humans than inbred mice. - Increasing genetic and immunological molecular tools available. - Inbreed animals commercially available.	- Requires expert handling. - Less availability of molecular tools for immunologic and genetic studies, when compared to inbred mice. - Lack of transgenic lines. - Lack of clinical signs resembling human disease.
Ferrets	- Easy handling and sampling. - High susceptibility to infection.	- Lack of clinical disease. - Requires specialized housing, caring, and maintenance. - Limited availability of molecular tools for immunologic and genetic studies. - Lack of inbreed animals commercially available.
Lambs	- Reliable lung function testing and interpretation due to anatomical similarities. - Large size and docility allow for repeated and easy sampling.	- Requires specialized housing, caring, and maintenance. - Limited availability of molecular tools for immunologic and genetic studies.
Chimpanzees	- Permissive replication of hRSV - Anatomical, physiological, and genetic similarities to humans.	- High economical cost, logistically demanding. - Strong ethical and emotional compromise. - Lack of inbred strains, few available immunological tools, and reagents. - Requires extensive caring and highly specialized housing.

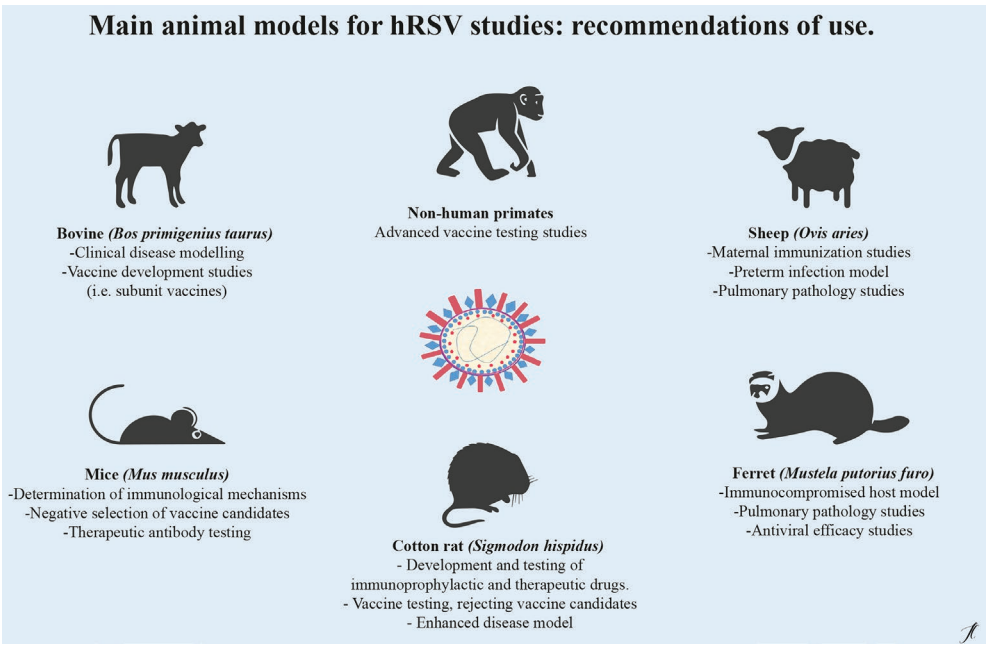

FIGURE 1 | Main animal models for hRSV preclinical studies: recommendations for use. For the study of respiratory syncytial virus, there are different animal models of infection, since there is not a single animal that replicates in an integral way the human pathology and the response to treatment and vaccine candidates. The choice of the model will depend on the investigative hypothesis and should considerate the advantages and disadvantages inherent to every model.

display a physiopathology very similar to infected human infants, showing very similar histopathological lesions. In case of non-human primates, it is noteworthy that they allow for viral replication with doses lower than those used in other models such as rodents, being a more permissive model for the study of hRSV. However, its use should be restricted exclusively to vaccine testing studies, due to ethical and technical issues. The antecedents reviewed here lead us to conclude that each animal model for the study of hRSV is fundamental for the understanding of the viral pathogenesis and all of them have to a greater or lesser extent to the development of biomedical tools against this agent. Expanding the availability of sophisticated models might be crucial to overcome information gaps and developing safe, effective therapies such as monoclonal antibodies or vaccines. To date, within all existing preclinical models, the murine model is the first preclinical approach for the development of new drugs, being a versatile tool that allows sophisticated immunological studies. Even considering its limitations, this model allow us to advance towards in other animal models that have more complex and expensive requirements in their management, maintenance, and infrastructure, as described in this work.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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

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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

INTRODUCTION

The Human Respiratory Syncytial Virus (hRSV) is a single-stranded RNA enveloped virus belonging to the *Pneumoviridae* family (Amarasinghe et al., 2018). The viral particle has a filamentous structure, which consists in a nucleocapsid surrounded by a lipid bilayer envelope obtained from the plasma membrane of the host cell (El Omari et al., 2011). Importantly, infection by hRSV is the most frequent cause of severe acute lower respiratory tract infections (ALRTIs) in children younger than 5 years old (Scheltema et al., 2017) and infection during the first year of life is the main cause of hospitalization in infants (Song et al., 2016). According to epidemiological studies, during the past decade, nearly 33 million cases of new ALRTIs episodes affect children during the first months of life are due to hRSV infection each year (Shi et al., 2017). Therefore, infection by this virus represents a major health and socio-economic burden worldwide (Diez-Domingo et al., 2014; Amand et al., 2018).

Clinical manifestations caused by hRSV infection range from mild symptoms, such as rhinitis, to more severe consequences, which include bronchiolitis, and pneumonia (Pickles and DeVincenzo, 2015). Besides, extra-pulmonary manifestations of hRSV infection have also been reported to occur, such as acute neurological symptoms with seizures and ataxia observed in hRSV-infected children (Eisenhut, 2006; Bohmwald et al., 2015) and long-term behavioral and cognitive impairments in animal models (Espinoza et al., 2013).

Remarkably, it is known that most children become infected with hRSV during the first 2 years of life (Domachowske and Rosenberg, 1999), likely because hRSV can efficiently pass on from one individual to another, but also because of the capacity of this virus to negatively modulate both, T cell and B cell responses upon infection allowing frequent re-infections (PrabhuDas et al., 2011; Cespedes et al., 2014; Zhivaki et al., 2017). These features are thought to be mediated by host and viral factors. For instance, it is known that infants show reduced capacity to produce neutralizing antibodies against hRSV, as compared to adults making the former more susceptible to recurrent infections (Siegrist and Aspinall, 2009). Although maternally-delivered antibodies (matAbs) are reported to delay the onset of primary hRSV infection, their presence in the blood of infants is not associated with the development of less severe disease symptoms (Jans et al., 2017). These observations suggest that antibody-mediated neutralization of hRSV may not be sufficient by itself to limit hRSV infection and disease severity. Furthermore, hRSV encodes several proteins that have the ability to negatively modulate or impair the host antiviral immune response, therefore contributing to re-infections (Mason et al., 2003; Cespedes et al., 2014; Saint et al., 2015; Bohmwald et al., 2016; Gomez et al., 2016; Canedo-Marroquin et al., 2017; Ward et al., 2017). Such knowledge is relevant for designing novel vaccines and therapeutic approaches that can prevent the pathology caused by hRSV. As a result, several clinical trials are currently in progress to assess the safety and effectiveness of different hRSV vaccine candidates (Cautivo et al., 2010; Rey-Jurado and Kalergis, 2017; Rezaee et al., 2017). Among them, we have developed a unique approach to be administered to newborns and young infants. Immunization in the mouse model with a recombinant bacillus of Calmette-Guérin (BCG) that expresses the nucleoprotein (N) of hRSV (rBCG-N-hRSV) induce the production of neutralizing antibodies against hRSV and a T helper 1 (Th1) cellular immunity that protects from hRSV associated-lung pathology by decreasing the infiltration of inflammatory immune cells into the lungs and reduce viral loads in the airways of hRSV-infected mice (Bueno et al., 2008; Cautivo et al., 2010; Leyrat et al., 2014) Furthermore, a single low dose of this vaccine produced using current good manufacturing practices (cGMP), conferred protection against hRSV infection in the mouse model (Cespedes et al., 2017). Given these results, this recombinant-based vaccine arises as a promising candidate to prevent lung damage caused by this virus (Cespedes et al., 2017). In this context, it is possible that a mechanism that contributes to the prevention of hRSV pathology following rBCG-N-hRSV vaccination is the induction of antibodies that recognize the hRSV N protein, which is necessary for viral replication and the inhibition of the immunological synapse (IS) between DCs and T cells that promote T- cell activation (Cespedes et al., 2014). Therefore, if the hRSV N protein becomes neutralized by antibodies during infection it cannot contribute to viral replication, but also will fail in its ability to impair the formation of the IS between DCs and T cells, thus hampering a proper immune response against hRSV. Furthermore, a recent publication from our group shows that immunization with rBCG-N-hRSV can induce the production of antibodies against other hRSV proteins, such as F and G which can serve to neutralize infection, therefore reducing hRSV associated pathology (Soto et al., 2018).

In contrast, vaccine candidates from other groups use the F protein as a target antigen to confer immunity. For example, Novavax Inc. is currently performing a clinical trial based on the use of nanoparticles linked with hRSV F protein to induce the production of neutralizing antibodies against hRSV (Mazur et al., 2018).

Similarly, Janssen is currently testing adenovirus based vector vaccines, encoding pre-fusion forms of the hRSV F protein that also induce the production of anti-hRSV neutralizing antibodies (Mazur et al., 2018).

Finally, other live attenuated vaccines as is the case of rBCG-N-hRSV are based in attenuated hRSV that lack some particular proteins such as M2-2, NS2, or both (Mazur et al., 2018).

Together, these data indicate that it is of vital importance to delineate the mechanisms contributing to hRSV induced pathology in order to prevent or treat infection.

At this latter point, recurrent hRSV re-infection episodes which are common thorough life have encouraged the generation of studies that seek to define the mechanisms responsible for what is considered an impaired or non-optimal immune response elicited against hRSV upon infection to account for re-infection episodes (Openshaw and Chiu, 2013; Cespedes et al., 2014; Shao et al., 2015). Along these lines, a role for the interaction between immune complexes consisting of IgGs and hRSV (ICs) with Fc gamma receptors (FcγRs) could be a process contributing to both, re-infection episodes, and enhancement of hRSV-disease elicited by vaccination with formalin-inactivated

hRSV (FI-hRSV) and later hRSV natural infection (Kim et al., 1969). This hypothesis is supported by the fact that high amounts of antibodies with low neutralizing activity can be induced by immunization with FI-hRSV, which correlates with enhancement of the hRSV-induced disease (Kapikian et al., 1969; Kim et al., 1969). Therefore, it is possible that these low affinity antibodies promote the infection of FcγR-bearing cells through a phenomena called antibody dependent enhancement (ADE), as previously observed for other viruses (Yip et al., 2014; Gu et al., 2015; Flipse et al., 2016). Furthermore, *in vitro* and *in vivo* studies have shown that the blockade or absence of particular FcγRs expressed on the surface of immune cells can modulate the immune response against this virus and the onset of hRSV-induced disease (Osiowy et al., 1994; Kruijsen et al., 2013; Gomez et al., 2016; van Erp et al., 2018). In this article, we review and discuss the current understanding on the contribution of FcγRs to infection and the modulation of the immune response against hRSV both, *in vitro* and *in vivo* and their impact on hRSV-induced pathology.

The Family of Fc Receptors for IgG (FcγRs)

Fc-gamma receptors (FcγRs) bind to immunoglobulin G (IgG) antibodies (Ab), by recognizing the Fc region of the IgG, which promotes receptor clustering on the cell surface and the phosphorylation of tyrosine residues present on signaling motifs within the intracellular region of these receptors. FcγRs engagement ultimately leads to signaling cascades in the cell that can result in the expression of surface molecules and secretion of soluble mediators to modulate the host immune responses (Getahun and Cambier, 2015; Renner et al., 2016); (Soto et al., 2018).

Importantly, these types of receptors are expressed on the surface of immune cells, such as neutrophils, dendritic cells (DCs) and macrophages, among others (Zhang et al., 2004). In general, classic members of this family of proteins were classified according to their immune-modulatory properties, which either promote or inhibit inflammatory responses (Nimmerjahn and Ravetch, 2008; Guilliams et al., 2014). However, FcγRs can also be classified as type-I or type-II, based on their capacity to interact with the two (open or closed) conformational states of the IgG Fc domain (Banegas Banegas et al., 1987). Type-I FcγRs include the classic FcγRs and can only be engaged by the IgG Fc domain in the open conformation state (Banegas Banegas et al., 1987). In contrast, type-II (non-canonical FcγRs), include C-type lectin receptors CD23 and Dendritic Cell-specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin (DC-SIGN), which preferentially bind IgG Fcs in a closed conformation (Banegas Banegas et al., 1987).

In humans, the so-called classic FcγRs are known as: FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a), and FcγRIIIb (CD16b) (**Table 1**) (Tripp et al., 2002; Guilliams et al., 2014). Among them, a study performed during 2002 indicates that the expression of FcγRIIIa is increased in Natural Killer cells (NK cells) from patients with severe hRSV associated pathology. Thus, suggesting that this receptor and this particular cell population could be contributing to hRSV disease (**Table 2**, Tripp et al., 2002). Nevertheless, there are two more non-classic human Fc-gamma receptors: neonatal Fc-receptor (FcRn) and cytosolic tripartite motif (TRIM) 21 that bind IgG once internalized into the cells (Guilliams et al., 2014). However, there is no study about it contribution to hRSV induced pathology in hRSV positive patients (**Table 2**). Importantly, all canonical FcγRs with the exception of FcγRIIb are involved in activating functions, such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory cytokines following FcγR-crosslinking by IgG-opsonized complexes (Guilliams et al., 2014). The activation of such processes relies on the Src-family kinase-mediated phosphorylation of an Immunoreceptor Tyrosine-based Activating Motif (ITAM) that is located in the cytoplasmic portion of these activating Fc-receptors (Nimmerjahn and Ravetch, 2008). Subsequently, phosphoinositide 3-kinase (PI3K) is activated, which generates phosphatidylinositol trisphosphates (PIP3s), leading to the recruitment of Bruton's tyrosine kinase (BTK) and the activation of phospholipase Cγ (PLCγ), which promotes the release of calcium (Ca^{2+}) from the endoplasmic reticulum (ER) that in turn activates cell effector functions (Nimmerjahn and Ravetch, 2008).

In contrast, FcγRIIb which is able to terminate the activation cascades associated with the engagement of activating FcγRs (Malbec et al., 1998), is also known as the inhibitory FcγR. During this process FcγRIIb becomes engaged by ICs and it co-aggregates with activating receptors. Following that, different recruited kinases phosphorylate a conserved tyrosine within an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) located in the cytoplasmic tail of FcγRIIb (Malbec et al., 1998). This phosphorylation step leads to the recruitment of tyrosine phosphatases SHP-1 and SHP-2, as well as the inositol phosphatases SHIP-1 and SHIP-2 that suppress the activating signals derived from activating FcγRs (D'Ambrosio et al., 1995; Ono et al., 1996).

In the mouse, there are four different canonical FcγRs expressed on the cell surface: FcγRI, FcγRIIb, FcγRIII, and FcγRIV (**Table 2**) (Nimmerjahn and Ravetch, 2008). Among them, FcγRI, FcγRIII, and FcγRIV are activating, whereas FcγRIIb is the only one that is inhibitory. Of interest, a pro-inflammatory role for the FcγRIII receptor has been reported during hRSV infection in the mouse model (Gomez et al., 2016), whereas the inhibitory FcγRIIb has been shown to hamper inflammatory reactions during allergic-like rhinitis (Malbec et al., 1998), allergic asthma (D'Ambrosio et al., 1995), and hRSV infection (Gomez et al., 2016). Therefore, such receptors appear as attractive targets for novel therapeutic approaches against this kind of diseases.

Contribution of FcγRs to Neutrophil Recruitment, Viral Replication, and Lung Damage During hRSV-Induced Pathology

Based on animal studies, neutrophils have been described to promote inflammation and tissue damage during hRSV infection (Yasui et al., 2005). In addition, other studies in mice that evaluated the role of FcγRs on the lung damage produced by neutrophils in models of acute lung injury (ALI), which

TABLE 1 | Classification of currently described human Fcγ Receptors, and evidences of their role in hRSV-induced pathogenesis.

Type	Receptor	Alternative name/CD	Main function	Evidence after hRSV infection	Suggested role	References indicating a role during hRSV infection
Classical FcγRs (Recognize ICs on the cell surface)	FcγRI	CD64	Activating	—[a]	—	—
	FcγRIIa	CD32a	Activating	—	—	—
	FcγRIIb	CD32b	Inhibitory	—	—	—
	FcγRIIc	CD32c	Activating	—	—	—
	FcγRIIIa	CD16a	Activating	Increased presence of FcγRIIIA^{+} NK cells, and lung damage in patients with severe hRSV infections	The expression of FcγRIIIA on NK cells negatively influences the immune response during hRSV infection	Tripp et al., 2002
	FcγRIIIb	CD16b	Activating	—	—	—
Non-classical FcγRs (C-type lectins that recognize ICs on cell surface or non-classic FcγRs that recognize ICs inside the cell)	CD23	CD23		—	—	—
	DC-SIGN	CD209	Recognition of glycans through a carbohydrate recognition domain (CRD)	*In vitro*: mAb-blockade of DC-SIGN increases human DC maturation markers (CD80, CD86) after hRSV infection.	hRSV-DC interaction through DC-SIGN might impair DC maturation	Johnson et al., 2012
	FcRn	—	Control of endosomal routing	—	—	—
	TRIM 21	—	Elimination of ICs via recruitment of the proteasomal machinery	—	—	—

[a] *No data are available.*

resembles those caused by hRSV infection (Zhang et al., 2016), have suggested that animals lacking activating FcγRs (FcRγ$^{-/-}$ mice) can be protected from ALI triggered by administration of IgG mAbs that recognize self-antigens, such as MHC-I molecules (Looney et al., 2006). Supporting a role for neutrophils and activating FcγRs in this model of lung injury, the same study showed that ALI was observed when *FcRγ*$^{-/-}$ mice were adoptively transferred with wild-type neutrophils followed by the administration anti-MHC-I mAbs (Looney et al., 2006). Taken together, these results suggest that lung disease in this model is dependent on the expression of activating FcγRs by neutrophils.

For the case of hRSV infection, it has been shown that the recruitment of neutrophils to the lungs of infected mice is modulated by the presence of different FcγRs (**Figure 1**) (Gomez et al., 2016). For instance, it was reported that animals lacking the activating FcγRIII (FcγRIII$^{-/-}$) showed decreased neutrophil recruitment and higher viral loads (Gomez et al., 2016), suggesting that FcγRIII could play a pro-inflammatory role during hRSV primary infection and promotes viral clearance. Consistent with the results described above, mice lacking the inhibitory FcγRIIb (FcγRIIb$^{-/-}$) showed increased neutrophil infiltration in lungs due to hRSV infection but decreased viral loads (Gomez et al., 2016), thus suggesting that this receptor can play an anti-inflammatory role during hRSV-induced disease despite it contributes to viral replication (Gomez et al., 2016).

An *in vitro* study using human neutrophils showed that hRSV-ICs, established with hRSV and anti-hRSV autologous serum, but not free hRSV or antibodies alone, could promote the release of reactive oxygen species (ROS) by neutrophils, which could contribute to lung tissue damage (**Figure 1**) (Kaul et al., 1981; Winterbourn et al., 2016). Therefore, it is possible that the activation of neutrophils, mediated by the engagement of FcγRs likely occurs under physiological conditions, when individuals become infected. This notion, is further supported by a study showing increased release of IL-8 by human neutrophils challenged with opsonized hRSV (Arnold et al., 1994). This cytokine is relevant, as it has been described that secreted IL-8 works as a chemotactic signal for neutrophils that induces their activation leading to pro-inflammatory responses (Henkels et al., 2011). This *in vitro* evidence suggests that the engagement of FcγRs can activate neutrophils and therefore contribute to lung inflammation and the progression of hRSV disease (**Figure 1**).

TABLE 2 | Classification of currently described mouse Fcγ Receptors, and evidences of their role in hRSV-induced pathogenesis.

Type	Receptor	Main function	Evidence after hRSV infection	Suggested role	References
Classical FcγRs (Recognize ICs on the cell surface)	FcγRI	Activating	—	—	—
	FcγRIIb	Inhibitory	*In vivo*: FcγRIIb$^{-/-}$ mice display increased lung neutrophil infiltration but decreased viral loads	Anti-inflammatory role	Gomez et al., 2016
			In vitro: WT mice-derived BMDCs loaded with hRSV-ICs were not able to induce the production of IL-2 by CD4^{+} T cells as compared with FcγRIII$^{-/-}$ mice-derived BMDCs	The engagement of FcγRIII by hRSV-ICs impairs DC-mediated T cell activation	Gomez et al., 2016.
			In vitro: FcγRIIb$^{-/-}$mice-derived BMDCs loaded with hRSV-ICs showed unaltered capacity to induce the secretion of IFNγ by CD4^{+} T cells	DC-mediated stimulation of IFN-γ secretion by CD4^{+} T cells does not depend on the presence of the inhibitory FcγRIIb	Kruijsen et al., 2010
	FcγRIII	Activating	*In vivo*: FcγRIII$^{-/-}$ mice display decreased neutrophil recruitment and higher viral loads	Pro-inflammatory role, promotion of viral clearance	Gomez et al., 2016
			In vitro: FcγRIII$^{-/-}$ mice-derived BMDCs loaded with hRSV-ICs showed restored capacity to induce the production of IL-2 by CD4^{+} T cells	The engagement of FcγRIII by hRSV-ICs impairs DC-mediated T cell activation	Gomez et al., 2016
	FcγRIV	Activating	—[b]	—	—
Non-classical FcγRs (FcγRs that recognize ICs inside the cell)	FcRn	IgG recycling	*In vitro: FcγRn*$^{-/-}$ mice-derived BMDCs loaded with hRSV-IC display unaltered capacity to induce IFN-γ production by CD4+ T cells. *In vivo*: FcRn$^{-/-}$ and WT mice display similar CD4^{+} IFN-γ production after hRSV-IC challenge	FcRn does not modulate DC-mediated CD4^{+} T cell activation	Kruijsen et al., 2013
	TRIM 21	Elimination of ICs via recruitment of the proteasomal machinery	—	—	—

[b] *No data are available.*

Modulation of Dendritic Cell Function by FcγRs and ICs Containing hRSV: Implications for T Cell Immunity

Dendritic cells (DCs) can modulate the immune response during viral infections after capturing ICs through either, activating or inhibitory FcγRs (Guilliams et al., 2014). Along these lines, IgG–antigen complexes can trigger activating signals in human DCs (hDCs) after binding to FcγRIII and promote an inflammatory response (Bandukwala et al., 2007). In contrast, binding of ICs to the inhibitory FcγRIIb trigger inhibiting signals that can lead to reduced inflammation (Boruchov et al., 2005). In addition, it has been described that hRSV-ICs containing either, neutralizing or non-neutralizing antibodies can modulate DC function and subsequent T cell responses elicited by the antigen presentation of these cells (Kruijsen et al., 2013; Gomez et al., 2016).

In the context of hRSV infection, it has been reported that DC-mediated T cell activation and IFN-γ production by these cells, is modulated by the presence of activating FcγRs on the DC surface (Kruijsen et al., 2013). In this case, it was observed that DCs derived from WT adult mice were able to induce the production of IFN-γ by CD4^{+} T cells in the presence of anti-hRSV immune serum obtained from mice being challenged with hRSV (Kruijsen et al., 2013). Nevertheless, this observed increase in the IFN-γ response by CD4^{+} T cells was reduced when the DCs were derived from FcRγ$^{-/-}$ mice. Therefore, the expression of all activating FcγRs on the DC surface is required to promote the production of this cytokine by CD4^{+} T cells. Remarkably, unaltered secretion of IFN-γ by CD4^{+} T cells was observed in DCs derived from FcγRIIb$^{-/-}$ mice, when compared to WT mice (Kruijsen et al., 2013), indicating that DC-mediated stimulation of IFN-γ secretion by CD4^{+} T cells does not depend on the presence of the inhibitory FcγRIIb (Kruijsen et al., 2013).

Interestingly, another report indicates that CD4^{+} T cells represent an important source of IFN-γ during neonatal hRSV infection in the murine model, which is required to prevent re-infection and disease severity in adult mice (Lee et al., 2008).

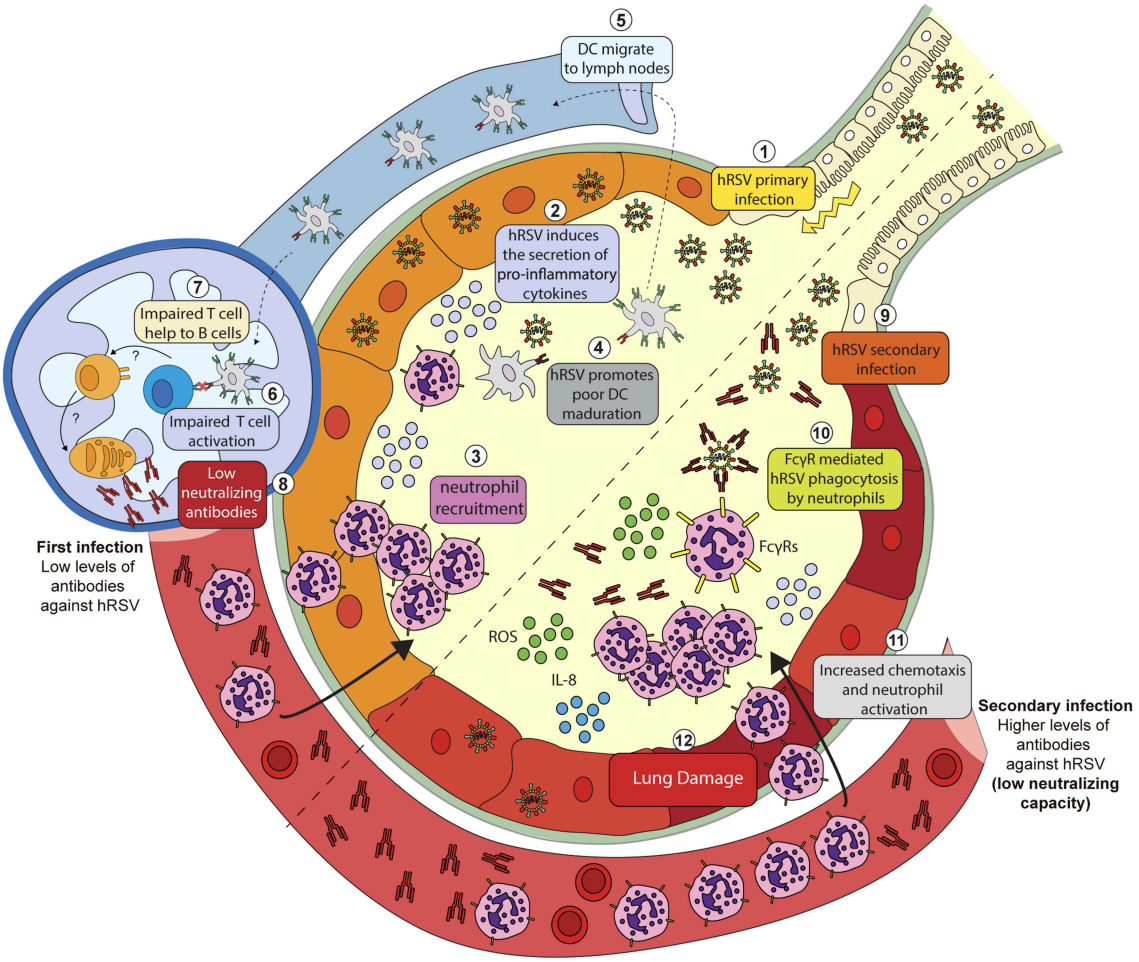

FIGURE 1 | Putative mechanisms explaining hRSV-induced inflammation due to hRSV-IC interaction with Fc-gamma receptors expressed on the surface of neutrophils. During a primary infection (1) hRSV induces the secretion of pro-inflammatory cytokines (2) and chemokines that promote neutrophil recruitment to the lungs and the airways (3). During infection, hRSV is phagocyted by DCs and impair its maturation (4). Infected DCs migrate to lymph nodes (5) but fail to activate T cells (6). By a poorly understood mechanism, T cells fail to help naïve B cells (7) and promote the proliferation of plasma cells that produce anti-hRSV antibodies with a low neutralizing capacity (8). Serum antibodies produced after a primary hRSV infection can opsonize hRSV during secondary infection (9). Opsonized hRSV is then phagocyted by neutrophils through FcγRs (10). The infection of these cells triggers the release of cytokines such as IL-8 that promote the activation and the recruitment of neutrophils (11). Activated neutrophils then release metabolic products, i.e., reactive oxygen species (ROS) that promote lung damage and inflammation (12).

Thus, it is possible that activating FcγRs contribute to prevent re-infection during adulthood, by promoting IFN-γ production by $CD4^+$ T cells through DC-mediated antigen presentation. However, it is necessary to determine whether activating FcγRs on the DC surface also modulate the production of this cytokine by neonatal $CD4^+$ T-cells to prevent re-infection.

Recent studies have shown that another IgG Fc receptor, particularly the neonatal Fc receptor for IgG (FcRn), which is a non-classical Fc receptor that binds IgG at acidic pH (<6,5) (Qiao et al., 2008), does not contribute to the activation of $CD4^+$ T cells when DCs are loaded with hRSV-ICs (Kruijsen et al., 2013). Moreover, Bone Marrow-Derived DCs (BMDCs) from $Fc\gamma Rn^{-/-}$ mice exhibit unaltered capacity to induce the production of IFN-γ by $CD4^+$ T-cells (Kruijsen et al., 2013). These results were validated *in vivo,* as $Fc\gamma Rn^{-/-}$ mice also displayed unaltered IFN-γ production by $CD4^+$ T-cells after being intranasally challenged with hRSV-ICs (Kruijsen et al., 2013).

Results from our group indicate that BMDCs display a reduced capacity to induce IL-2 production by $CD4^+$ T cells after being loaded with hRSV-ICs that had the neutralizing antibody Palivizumab (Synagis^{TM}) (Gomez et al., 2016). In contrast, when the assay was performed with BMDCs derived from either, $Fc\gamma RIII^{-/-}$ or $Fc\gamma RIIb^{-/-}$ mice IL-2 secretion by $CD4^+$ T cells was restored. This results prompts that when present, these receptors impair the capacity of DCs to induce the secretion of IL-2 by $CD4^+$ T cells. It should be noted that, the production of this cytokine is required for the generation of memory regulatory $CD4^+$ T cells (Tregs), which perform anti-inflammatory functions during hRSV infection and protect

against re-infections (Durant et al., 2013). Thus, it is possible that both, FcγRIII and FcγRIIb contribute to hRSV pathogenesis and re-infection by impairing the capacity of DCs to promote the production of IL-2 by $CD4^+$ T cells.

Type II FcγRs Expressed on the Surface of Human DCs Contribute to Immune Responses Against hRSV

In humans, the presence of two types of FcγRs has been recognized (Banegas Banegas et al., 1987). Type-I FcγRs are members of the immunoglobulin superfamily and can be either activating or inhibitory (Nimmerjahn and Ravetch, 2005, 2008). In contrast, Type-II FcγRs are members of the C-type lectin receptor family and comprise two different members: the IgE receptor and the surface protein DC-SIGN (Banegas Banegas et al., 1987; Miettinen, 2004), which is able to recognize the Fc portion of IgG (Kaneko et al., 2006; Svajger et al., 2010), but also the G protein expressed by hRSV (Johnson et al., 2012). Of interest, studies evaluating the role of DC-SIGN in hDCs during hRSV infection, showed that the blockade of this receptor with specific mAbs led to an increase in the expression of maturation markers, such as CD80 and CD86 following hRSV infection (Johnson et al., 2012). This suggests that the interaction between hRSV surface proteins and DC-SIGN can suppress some aspects of DC activation in humans, thus contributing to an impaired protective immunity following hRSV infection. However, further studies are required to study the influence of this receptor during infection *in vivo* and hRSV-induced pathology, as well as its consequences on DC mediated T-cell activation.

Contribution of ADE to hRSV Re-infection Episodes

In a recent study, it was shown that young infants (i.e., <3 months old) generate a highly neutralizing antibody response that is biased from the post-fusion to the pre-fusion form of hRSV F protein. However, as children become older (i.e., from <3 months old to >6 months old), this response is re-directed against post-fusion conformation antigens (Goodwin et al., 2018). Thus, the antibodies generated display a weak neutralizing capacity that fail to prevent hRSV infection. Therefore, it is possible that the generation of a pool of low-neutralizing

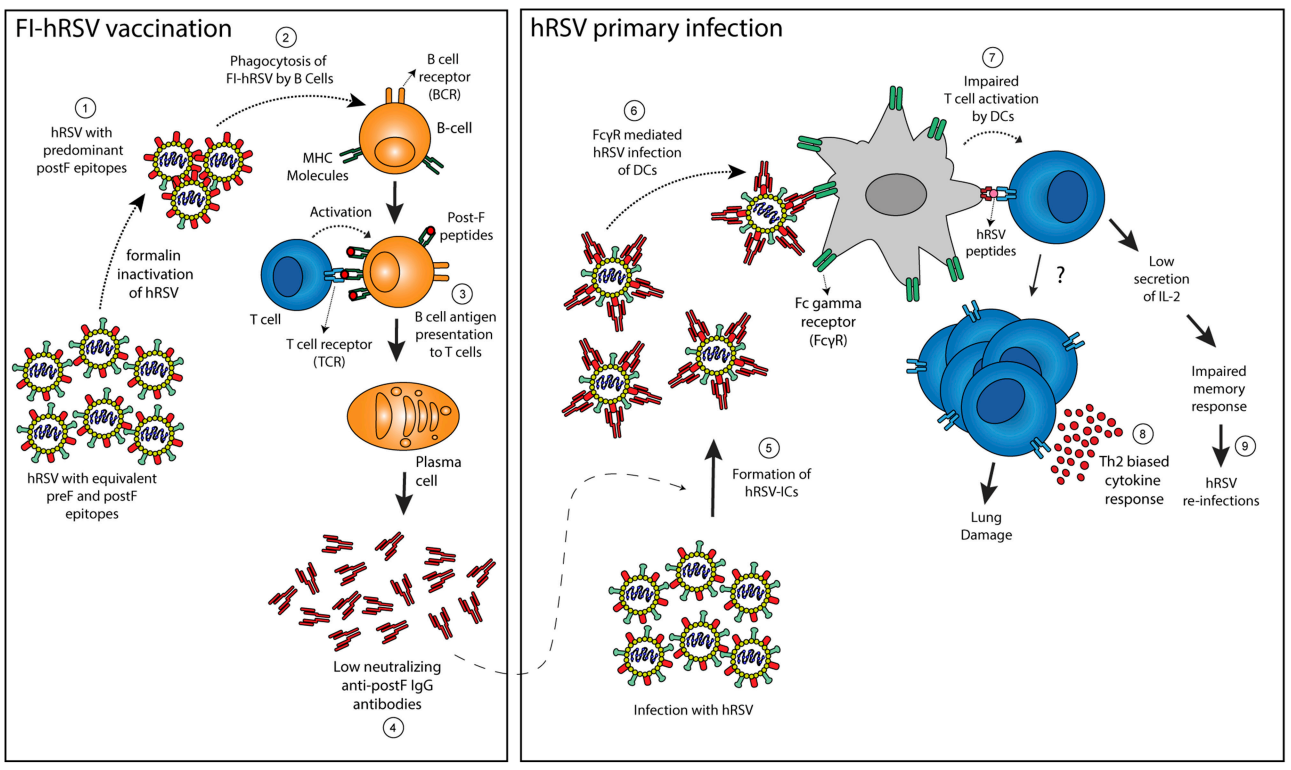

FIGURE 2 | Proposed mechanism to explain enhancement of hRSV-induced disease following FI-hRSV vaccination. Formalin hRSV inactivation produces a non-infectious virus with a high proportion of post-fusion conformation epitopes in the F protein (Post-F) (1). The inactivated virus is then phagocyted by B cells (2) that can present hRSV antigens to T cells in the context of MHC molecules (3). The interaction between B and T cells allows the differentiation of B cells into plasma cells that generate antibodies against the post-fusion conformation of the hRSV F protein (4). Such antibodies failed to neutralize hRSV infection but also may enhance the infection of FcγR bearing cells such as DCs. When infection by hRSV occurs, the low neutralizing antibodies induced by the FI-hRSV vaccine can form immune-complexes (ICs) with hRSV (5) that leads to the activation of Fc-gamma receptors expressed on the surface of DCs (6). Subsequently, an impaired DC-mediated T cell activation (7) can induced $CD4^+$ T cells with a Th2-biased phenotype that promotes lung damage (8). Furthermore, low secretion of IL-2 by $CD4^+$ cells activated by hRSV-IC-loaded DCs can lead to a poor memory response that contributes to hRSV re-infection (9).

antibodies during infancy can facilitate infection of immune cells that express FcγRs, a phenomenon called ADE of infection that has been observed for other viruses such as dengue virus (Flipse et al., 2016), acute respiratory syndrome coronavirus (Yip et al., 2014) and porcine reproductive and respiratory syndrome virus infection (Gu et al., 2015). In this context, antibodies might exert different effector functions through their Fc regions and for hRSV, ADE during infection is an effect that has been reported in *in vitro* studies (Gimenez et al., 1989; Krilov et al., 1989; Osiowy et al., 1994). However, a role of ADE during hRSV pathogenesis *in vivo* has been proposed, but remains to be confirmed during re-infection.

To date, *in vitro* enhancement of infection of monocyte-derived cell lines due by FcγR binding by mAbs and patient sera has been reported (Gimenez et al., 1989; Krilov et al., 1989; Osiowy et al., 1994), demonstrating that non-neutralizing mAbs can enhance the infection of phagocytic cell lines expressing these receptors (Gimenez et al., 1996). Further, when neutralizing antibodies were applied at sub-neutralizing concentrations (i.e., diluted), they induced ADE in phagocytic cells bearing FcγRs. This was also observed using human sera and purified human immunoglobulin (IVIg) (van Erp et al., 2017). Together, these results suggest that the interaction of hRSV-ICs generated with low neutralizing antibodies can promote the infection of immune cells *in vitro,* therefore contributing to hRSV pathogenesis under physiological conditions.

Contribution of ICs Containing hRSV to Enhanced Disease Elicited by Vaccination With Formalin-Inactivated hRSV

The administration of a formalin-inactivated hRSV vaccine to children nearly 50 years ago, which was aimed at preventing severe respiratory disease elicited by hRSV infection was unable to produce protective immunity against hRSV. Contrarily to what was expected, its administration resulted in increased morbidity and mortality in vaccinated infants when they were later infected by the virus (Kim et al., 1969). Although the mechanisms underlying the pathological effects of FI-RSV vaccine have not been totally elucidated, this episode revealed complexities associated to vaccine development, which has been hampered, and raised hypothesis about the pathologic roles of hRSV-ICs (Kim et al., 1969; Polack et al., 2002; Delgado et al., 2009). Enhanced hRSV disease (ERD) after FI-RSV immunization of BALB/c mice has been associated with alveolar deposition of ICs, which was observed 7 dpi of hRSV by means of co-localization of IgG with the complement component 3 (C3 protein). The role of complement fixing ICs in ERD was supported by experiments in $C3^{-/-}$ mice, which showed significantly less airway hyper-responsiveness (AHR) in comparison to WT counterparts, after FI-hRSV vaccination and hRSV challenge, arguing for a role of complement in bronchoconstriction observed in ERD (Polack et al., 2002). These experimental studies were supported by histological analysis of lung sections from two infants that suffered fatal ERD, in which IC-mediated complement activation was observed through extensive peribronchiolar complement component 4d (C4d) deposition in the airway tissue (Polack et al., 2002). Furthermore, a sub-optimal, non-protective antibody response in mice, characterized by high levels of non-neutralizing anti-F and anti-G IgG antibodies, was observed after immunization with FI-hRSV, but not infectious hRSV (Polack et al., 2002). The lack of affinity maturation in Abs elicited by FI-hRSV was associated with enhanced lung histopathology and AHR, whereas the supplementation of Toll-like receptor (TLR) agonists, performed during immunization promoted proper affinity maturation that prevented ERD after hRSV challenge, showing that a deficient TLR stimulation in B cells is likely responsible for the lack of Ab affinity maturation after FI-hRSV vaccination (Delgado et al., 2009). Furthermore, cotton rats vaccinated with FI-RSV elicited high levels of hRSV-specific antibodies, which displayed low neutralizing titers in Vero cells (Piedra et al., 1993). These antibodies were also able to cause ADE in *in vitro* assays. These studies suggest that sub-optimal antibody production and the generation of ICs play a role in ERD development (**Figure 2**). Furthermore, recent studies suggest that $CD4^+$ subsets and a Th2-biased immune response are key for AHR and ERD (Knudson et al., 2015). In this context, TAM (Tyro3, Axl, and Mertk) receptors, which are expressed in various cells and tissues, and their ligand Growth arrest-specific 6 (Gas6) could be involved in the production of a Th2-biased immune responses that reduce the production of type IgG2a subclass antibodies (Shibata and Ato, 2017). These antibodies could have an effective neutralizing capacity against hRSV and therefore prevent hRSV induced disease, but their production is lowered as a consequence of FI-hRSV immunization followed by hRSV infection. Therefore, it is possible that the TAM/Gas6 signaling axis can contribute to the generation of low neutralizing antibodies that failed to neutralize hRSV infection and instead contributes to the pathology caused by hRSV infection through the engagement of FcγRs.

CONCLUDING REMARKS

The hRSV is a leading cause of respiratory illness in infants and a major health burden worldwide. Re-infections with this virus are common and can contribute to additional clinical manifestations, such as asthma and allergies. For this reason, several studies have focused on understanding the mechanisms that can contribute to hRSV induced pathology, but also to elucidate the factors that contribute to re-infection episodes throughout life. In this context, some studies suggested that low number of memory hRSV-specific $CD8^+$ T cells could be associated with re-infection episodes and that the levels of such cells could be regulated by virus-specific antibodies, by modulating the function of antigen presenting cells, such as DCs. Furthermore, recent studies suggest that the generation of regulatory memory T cells could be impaired by the interaction of hRSV-ICs with DCs, pointing out these phenomena as an interesting research topic that deserves analysis. In this review, and based on several studies, we discussed the role of FcγRs during hRSV infection and their immune-modulatory properties that can account for recurrent hRSV infection episodes and the enhancement of the disease caused

by FI-hRSV vaccination. However, further research is needed to understand how hRSV induces the production of antibodies that fail to prevent re-infections. Knowledge of such mechanisms would certainly be appreciated for vaccine and therapy development against hRSV, which represents a major global health problem.

AUTHOR CONTRIBUTIONS

OA and FD are responsible for the writing of this review article. TB, FB, JE-V, JS, and PG 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.

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Immune response during hantavirus diseases: implications for immunotherapies and vaccine design

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Summary

Orthohantaviruses, previously named hantaviruses, cause two emerging zoonotic diseases: haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Overall, over 200 000 cases are registered every year worldwide, with a fatality rate ranging between 0·1% and 15% for HFRS and between 20% and 40% for HCPS. No specific treatment or vaccines have been approved by the U.S. Food and Drug Administration (FDA) to treat or prevent hantavirus-caused syndromes. Currently, little is known about the mechanisms at the basis of hantavirus-induced disease. However, it has been hypothesized that an excessive inflammatory response plays an essential role in the course of the disease. Furthermore, the contributions of the cellular immune response to either viral clearance or pathology have not been fully elucidated. This article discusses recent findings relative to the immune responses elicited to hantaviruses in subjects suffering HFRS or HCPS, highlighting the similarities and differences between these two clinical diseases. Also, we summarize the most recent data about the cellular immune response that could be important for designing new vaccines to prevent this global public health problem.

Keywords: haemorrhagic fever with renal syndrome; hantavirus; hantavirus cardiopulmonary syndrome; immune response; vaccines.

Introduction

Orthohantaviruses (hantaviruses from now on in this review) are a genus of viruses belonging to the *Bunyavirales* order and *Hantaviridae* family.[1] Hantaviruses contain a negative-sense single-stranded tri-segmented RNA genome. The large (L) viral genomic segment encodes an RNA-dependent RNA polymerase, the medium (M) segment encodes a glycoprotein precursor (GPC, which generates Gn and Gc glycoproteins in the host cell), and the small (S) segment encodes a nucleoprotein (NP) and, in some hantaviruses, a non-structural protein (NSs).[2] The

Abbreviations: ANDV, Andes orthohantavirus; CFR, case fatality rate; CRP, C-reactive protein; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DOBV, Dobrava–Belgrade orthohantavirus; dsRNA, double-stranded RNA; EC, endothelial cell; FDA, Food and Drug Administration; GPC, glycoprotein precursor; HCPS, hantavirus cardiopulmonary syndrome; HFRS, haemorrhagic fever with renal syndrome; HTNV, Hantaan orthohantavirus; IL, interleukin; ISG, interferon-stimulated gene; mAbs, monoclonal antibodies; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation-associated protein 5; MMP-9, matrix metalloproteinase 9; moDC, monocyte-derived dendritic cell; nAb, neutralizing antibody; NE, nephropathia epidemica; NK, natural killer; NP, nucleoprotein; NSs, non-structural proteins; PB, plasmablast; PBMC, peripheral blood mononuclear cell; PHV, Prospect Hill orthohantavirus; PKR, protein kinase R; PUUV, Puumala orthohantavirus; RIG-I, retinoic acid-inducible gene I; SEOV, Seoul orthohantavirus; SNV, Sin Nombre orthohantavirus; Th, T helper; TLR, Toll-like receptor; TNF, tumour necrosis factor; Treg, regulatory T cell; TULV, Tula orthohantavirus; VEGF, vascular endothelial growth factor; VLP, virus-like particle; VSV, vesicular stomatitis virus

pleomorphic virion is enveloped, with a size between 70 and 160 nm.[3,4] A tetrameric assembly of the Gn and Gc glycoproteins on the virus surface constitutes the spike complex that mediates cell entry and virus assembly.[3]

The main animal reservoirs of hantaviruses are rodents, showing a strong degree of host-virus specificity.[5] Hantavirus outbreaks are reported after dynamic changes in the host population, which are influenced by several factors, including environmental forces and anthropogenic disturbances.[6–8] Transmission to humans takes place mostly after inhalation of contaminated droplets from rodent excreta.[9] Humans are usually dead-end hosts for these viruses[10]; however, person-to-person transmission has been described with the highly pathogenic Andes orthohantavirus (ANDV).[11–13]

The hantavirus species are the aetiologic agents of two different diseases in humans: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Nearly 200 000 cases per year of hantavirus infection are reported worldwide,[14] and the number of hantavirus species is still on the rise, with 50 species described to date, of which over 24 are recognized as pathogenic.[15] HFRS-related hantavirus species are distributed in the Eurasia region, causing case fatality rates (CFR) between 0·1% and 15%, mainly by Hantaan orthohantavirus (HTNV), Dobrava–Belgrade orthohantavirus (DOBV) and Puumala orthohantavirus (PUUV) infection.[16] On the other hand, Sin Nombre orthohantavirus (SNV) and ANDV are the leading agents of HCPS in the Americas, with case fatality rates of up to 20–40%.[17–19] To date, no specific treatment has been approved for hantavirus-caused diseases, despite the constant efforts to develop effective therapies and vaccines against these viruses.[14] Early intravenous ribavirin treatment has been described as useful for HTNV-infected patients with HFRS in Asia,[20,21] but not for PUUV[22] or HCPS-related viruses.[23] Due to the lack of effective antiviral treatments or vaccines, clinical interventions rely mostly on treating symptoms and on supportive care to provide haemodynamic and oxygen support in patients suffering more severe clinical forms of these diseases.[24]

An early neutralizing antibody (nAb) response has been associated with a favourable clinical outcome in HCPS individuals.[25] Despite the importance of T cells in controlling viral infections, little is known about their contribution to hantavirus infection and immunity. Both HFRS and HCPS are characterized by a robust cytotoxic T lymphocyte (CTLs) response,[26] and clearance of ANDV-RNA mediated by cytotoxic $CD8^+$ T cells without an increase in nAbs titres has also been reported.[27] These data suggest that these cells might be necessary for protective immunity against ANDV infection.[27] However, no additional observations have been published to support this hypothesis. It is noteworthy that hantavirus-infected patients exhibit an elevated proinflammatory cytokine profile in the serum, organs and tissues.[28–30] It has been proposed that hantavirus-specific $CD4^+$ T cells and $CD8^+$ T cells contribute to the cytokine storm observed and capillary leak, with the consequent pulmonary oedema and cardiogenic shock during HCPS.[2,31]

Several features of hantavirus pathogenesis and immunity,[32–34] as well as advances in vaccines and therapeutics,[35,36] have been recently reviewed and discussed elsewhere. Here, we summarize the main findings relative to the immune response in humans and animal models for both HFRS and HCPS, emphasizing the similarities and differences among both syndromes, and discussing the immunological basis for the rational design of vaccines and immunotherapeutic interventions. A more comprehensive understanding of the protective response is needed to develop efficacious vaccines to protect against each syndrome.

Hantavirus-caused diseases: hantavirus cardiopulmonary syndrome and haemorrhagic fever with renal syndrome

Hantavirus infection causes two different zoonotic human diseases: HFRS, caused by Old World hantaviruses, and HCPS, produced by New World hantaviruses.[9] Exposure to hantaviruses is associated with domestic, recreational and occupational activities in areas with wild rodents.[37] Environmental factors, such as precipitations, temperature and landscape disturbances, might also impact hantavirus dynamics in rodents and contribute to human disease outbreaks.[8]

Human infection occurs after inhalation of aerosolized contaminated faeces, urine or saliva. Microvascular endothelial cells (ECs) are the main target for hantaviruses,[38] and although infections are systemic, viral dissemination within the body in humans has not been conclusively demonstrated.[18] The incubation period ranges from 7 to 39 days in HCPS[39] and 14 to 28 days in HFRS cases.[2] These syndromes share initial symptoms, such as headache, myalgia, vomiting and abdominal pain. In HCPS, following a short prodromal phase with flu-like symptoms, patients develop a severe acute cardiopulmonary stage, with dyspnoea, pulmonary oedema, hypotension and shock.[40,41] It is noteworthy that cardiogenic shock and severe respiratory failure are frequent in lethal outcomes by HCPS.[42] Furthermore, haemorrhagic and renal manifestations such as petechiae, haemorrhages and higher creatinine levels in the blood might also be present during ANDV infections.[43] Respiratory sequelae, such as dyspnoea, have been registered even after 3 years in HCPS survivors infected by Choclo orthohantavirus and SNV.[44]

On the other hand, the clinical manifestations of HFRS might involve five phases: febrile, hypotensive, oliguric, polyuric and convalescent.[45] However, the absence or

overlapping of clinical stages is frequent.[46] Severe cases develop renal dysfunction, with proteinuria and haematuria.[46] Renal involvement is the most frequent condition in patients with HFRS, but pulmonary dysfunction might also present in the severe cases of HFRS,[47] being life-threatening conditions.[48] Nevertheless, most of the individuals present a full recovery in the convalescent stage.[49]

The pathophysiological mechanisms of these syndromes remain unclear, and probably multiple pathways contribute to the development of these two diseases. Viral loads have not been associated with disease severity in PUUV, SNV or ANDV infections.[50–52] However, due to the high viral RNA levels in patients with HCPS, it has been suggested that viraemia might trigger the immunopathology.[50] Along these lines, viral replication elicits EC dysfunction in the vascular niche, with an increase in the secretion of vascular endothelial growth factor A (VEGF-A) and an alteration of vascular permeability that ultimately leads to pulmonary oedema.[17,53] Changes in plasma VEGF levels might not be evident in patients; however, the presence of VEGF in pulmonary oedema fluid has also been associated with disease and severity in patients with HCPS.[53] Along these lines, studies in DOBV-affected patients indicate that plasma levels of VEGF remain normal during the disease.[54] On the other hand, another study reported that severe cases of DOBV had higher and prolonged VEGF levels as compared to moderate illness, suggesting that VEGF could be more associated with tissue repair than tissue dysfunction.[55] Additional studies are required to elucidate the specific roles of VEGF in the pathogenesis or endothelial repair in the hantavirus disease context.

Thrombocytopenia, leucocyte activation and release of proinflammatory cytokines and chemokines are inherent changes that might be related to pathogenesis.[53,56] Several reports have demonstrated an upregulation of VEGF-A and proinflammatory markers, such as nitric oxide, C-reactive protein (CRP) and proinflammatory cytokines, including IL-1β, IL-2, IL-6, IL-8, TNF and IFN-γ, among others.[30,53,57–61] This 'cytokine storm' contributes to the illness associated with the viral infection. A study in cases of HCPS or nephropathia epidemica (NE, a mild form of HFRS caused by PUUV) revealed that HCPS is characterized by a massive upregulation of proinflammatory cytokines and chemokines, such as IL-6, IL-18, CXCL9, CXCL10 and MIF.[62] Instead, NE cases display an increase in IL-6 and IL-12p40, and downregulation of IL-18 compared with healthy controls.[62] Further, a more robust innate immune response and an earlier secretion of cytokines related to a T-helper (Th) 1 response are observed during HCPS as compared to NE patients.[62] Besides, DOBV-infected cases are related to higher serum cytokine expression in comparison with cases of PUUV,[58] suggesting that the elevated cytokine expression correlates with disease severity. Consistent with this notion is the observation that high IL-6 levels have been associated with more severe forms of HFRS and HCPS[63–65] and with fatal outcomes of HCPS.[64] The upregulation of these chemo-attractant cytokines might be responsible for the recruitment of hantavirus-specific immune cells and extensive bystander activation of innate and cytotoxic cells that destroy ECs.[34] This scenario, combined with the cell-to-cell junction disruption, could promote platelet migration at the injury site, with the consequent thrombocytopenia commonly found in hantavirus disease.[56,66] Additionally, alterations between platelets and their ligands are reported during hantavirus infection, with high levels of fibrinogen and von Willebrand factor (VWF) in the acute stage of NE,[67] both directly involved in the initial steps of clot formation. Furthermore, β3 integrin, the receptor of pathogenic hantaviruses, is expressed in platelets and is involved in vascular permeability and platelet function.[68] Thus, although hantavirus infection does not produce cytopathic effects, current studies suggest that several pathogenic effects can be induced by hantavirus infection, ultimately leading to vascular leakage (Fig. 1).

Innate immunity elicited by hantavirus infection

Hantavirus recognition by innate immunity

Hantaviruses replicate predominantly in ECs, macrophages and dendritic cells (DCs).[9] Upon viral infection, pathogen-associated molecular patterns are recognized by host cell receptors at extra- and intracellular levels as Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and protein kinases (PKR).[69–76]

TLR3 recognizes the double-stranded RNA (dsRNA) viral replication intermediate, promoting the synthesis of alpha/beta interferon (IFN-α/β) and interferon-stimulated genes (ISGs) to fight the viral infection.[69] Nevertheless, pathogenic hantaviruses apparently alter the activation of TLR3 and its downstream effectors. *In vitro* studies have shown that HTNV infection delays the secretion of antiviral effectors, which may contribute to the innate evasion by delaying the inflammatory response, subsequently allowing a higher viral replication.[69]

After recognizing viral RNA motifs, RIG-I and MDA5 activate mitochondrial antiviral signalling protein (MAVS), inducing the expression of type I IFN and proinflammatory cytokines at the early stages of infection.[77] In this context, according to studies on human ECs, RIG-I-like receptors are fundamental to elicit an antiviral response against HTNV infection through the IFN production and ISG expression to limit the viral replication *in vitro*.[70] It is noteworthy that ANDV presents various ways to inhibit both RIG-I and MDA-5 pathways. NP-ANDV interferes with interferon regulatory

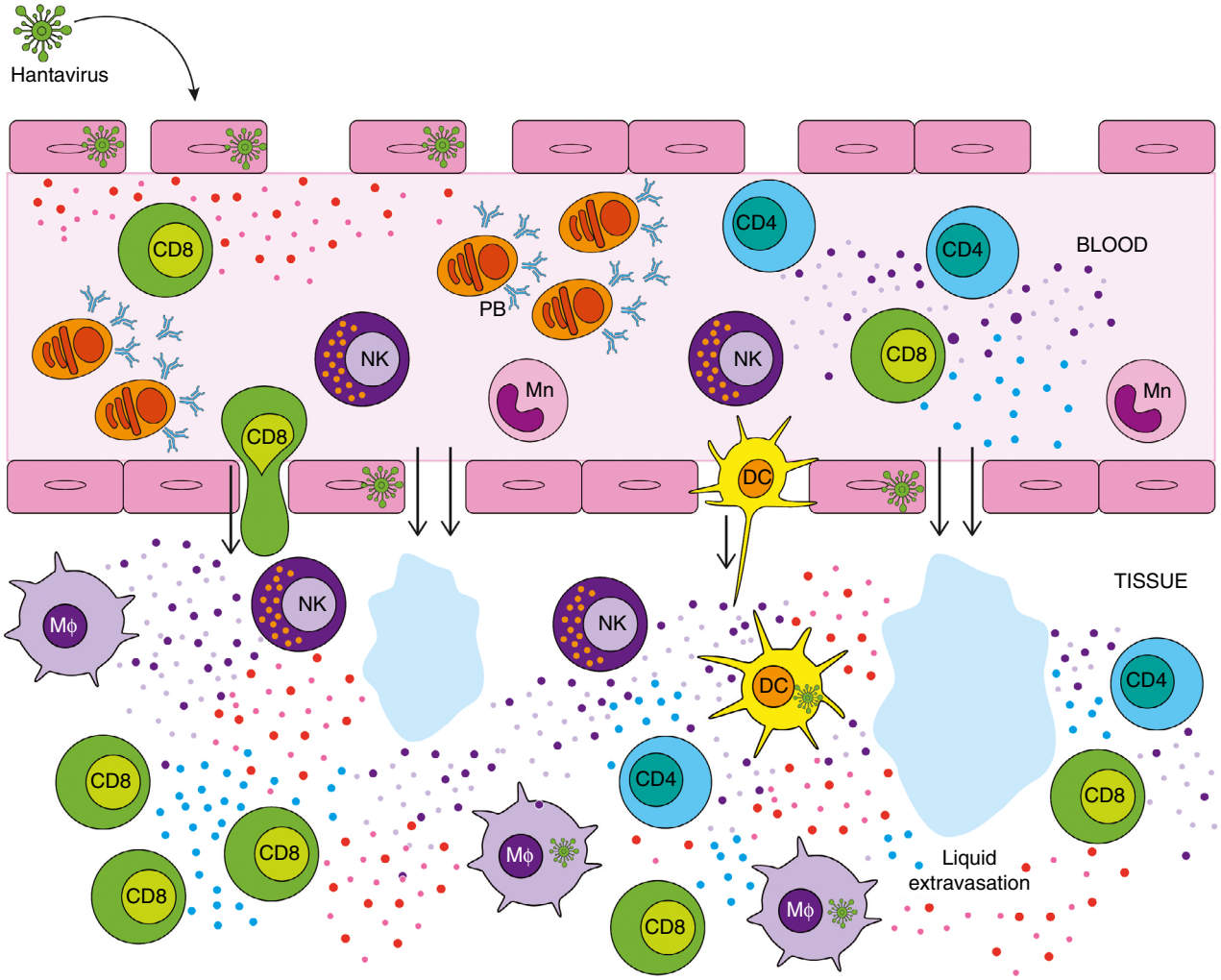

Figure 1. Cellular responses associated with hantavirus diseases. Hantaviruses infect endothelial cells, monocytes and macrophages without cytopathic effect but producing an endothelial disruption. The detection of virions activates the immune response, with the primary production of cytokines from innate immune cells such as types I and III IFNs, IL-1β, TNF, IL-6 and IL-15, and chemokines such as CCL5 and CXCL10, among others. Later, in addition to NK cells and monocytes, $CD8^+$ and $CD4^+$ T cells are recruited to infected tissue, releasing granules as perforins, granzyme and specific Th1/Th2 cytokines inducing a 'cytokine storm'. Due to the disruption of the endothelial layer, massive oedema is present in severe cases. Furthermore, a significant increase in plasmablasts (PBs) is present early in the disease with an early IgM/IgG production.

factor 3 phosphorylation and TANK-binding kinase 1 autophosphorylation,[74] and NSs-ANDV interacts with MAVS decreasing its ubiquitination and therefore downregulating the innate immune response.[71] Additionally, the pathogenic hantaviruses ANDV, Tula orthohantavirus (TULV) and New York 1 (NY-1V) can modulate the IFN pathway through the cytoplasmic tail of the Gn protein, while, on the other hand, non-pathogenic Prospect Hill orthohantavirus (PHV) does not inhibit the IFN pathway but induces a strong IFN- β response in human ECs.[75,76]

On the other hand, PKR senses dsRNA leading to the attenuation of mRNA translation and inhibition of the protein synthesis and assembly of RNA stress granules (SGs).[72] PUUV and ANDV actively inhibit PKR-dependent SG formation despite causing a transient, limited SG formation in the early stages of infection.[72] Interestingly, when NP expression increases in the late stages of infection, these proteins inhibit PKR autophosphorylation, keeping the translation process active.[73]

Apoptosis is another pathway triggered by infected cells against viral infection; nevertheless, hantaviruses can prevent cell death by the overexpression of NP in the late stages of the infectious cycle.[78] NP motifs are recognized by caspase-3, which leads to their degradation over caspase-3 targets,[78] thus preventing the activation of intrinsic apoptosis effectors. Moreover, HTNV has also been shown to inhibit the extrinsic pathway of apoptosis through the downregulation of dead receptor 5, in a TRAIL-mediated mechanism activated by cytotoxic granules.[79]

Despite the viral strategies to avoid the innate immune system, in the late stages of infection type I IFN

production has been reported in pathogenic hantavirus species.[80,81] The activation of immune cells and secretion of proinflammatory cytokines (such as IL-1β, TNF and IL-6) and chemokines (such as CCL5 and CXCL10)[28,41] are likely responsible for the recruitment of further mononuclear cells at the injury site, promoting a sustained proinflammatory state where cytokines and cytolytic activity may perpetuate endothelial damage.[28,81] All these components may contribute to the development of the disease.

Natural killer cells

Natural killer cells are innate lymphoid cells with the ability to rapidly respond during viral infections by releasing cytotoxic granules that contain granzymes and perforin and the secretion of cytokines, such as IFN-γ, TNF, IL-12, IL-15, IL-18 and IL-21.[59] The dynamics of NK cells during hantavirus infection are poorly understood. Particularly, studies have focused on PUUV infection, where peripheral NK cell counts change along the infection time. During the febrile stage, NK cell counts from NE patients are lower than in healthy donors,[82] probably caused by extravasation. One week after this stage, NK cells increase and remain high for 2 months after NE onset, and then return to normal levels after a year.[83] $CD56^{dim}$ NK cells are increased in infected patients, showing elevated expression of activation markers, such as CD69, NKG2D, 2B4 and cytotoxicity receptors that include NKp30 and NKp46, with granzyme B and perforin secretion during the acute phase of HFRS.[84] This active and proliferative phenotype is associated with an elevated IL-15 secretion by infected epithelial cells.[84] Similarly, elevated levels of IL-15 in serum were reported in SNV-infected rhesus macaques,[85] and increased levels of IL-15 are associated with high severity course and fatal outcome in ANDV-infected patients with HCPS,[65] but not in patients with HCPS from North America.[60]

It is noteworthy that *in vitro* studies have shown that HTNV-infected ECs, but not uninfected ECs, are protected from NK cell cytotoxicity.[78,84] Despite significant TRAIL upregulation in infected ECs and NK cells, HTNV-infected cells are protected by TRAIL-mediated killing, as previously discussed.[79] Moreover, inhibition of cell-mediated apoptosis has been observed in pathogenic and non-pathogenic hantaviruses, associated with NP-driven inhibition of granzyme B and caspase-3.[86] Those events would explain why infected ECs are not damaged despite strong cytotoxic lymphocyte activation in patients, according to the authors.[78,86]

Dendritic cells and macrophages

Upon HTNV infection, DCs mature and upregulate the expression of class I and class II major histocompatibility complex molecules, as well as costimulatory and adhesion molecules.[87] DC activation also induces a proinflammatory response with TNF and IFN-γ secretion.[87] Moreover, HTNV infection of human monocyte-derived DCs (moDCs) causes their maturation and promotes their antigen cross-presentation capacity,[88] an essential process for the activation of the antiviral activity mediated by $CD8^+$ T cells.[89] According to these findings, *in vitro* ANDV infection of moDCs promotes their maturation and a proinflammatory cytokine secretion profile, with high levels of TNF, and diminished production of IL-10 and TGF-β. Additionally, these cells increase the secretion of matrix metalloproteinase 9 (MMP-9), whose activity is associated with higher endothelial permeability, a characteristic finding in HCPS cases.[90]

Interestingly, the ability of hantaviruses to replicate in mononuclear phagocyte system cells has been suggested to be related to their pathogenicity. Opposite to HNTV, the low-pathogenic Tula orthohantavirus (TULV) does not replicate in human monocytes when inoculated *in vitro*.[81] Similarly, non-pathogenic PHV was unable to replicate in inflammatory DCs.[81] Although mononuclear phagocytic system cells express both β1 and β3 integrins, the expression of CD86 is less sustained in TULV than in HTNV infection.[81] Additionally, experiments using immature DCs with defective signalling capacity of integrins prove that the capability of pathogenic hantavirus to replicate is dependent on the integrin signalling pathway.[81] Analyses of peripheral blood cells from HFRS patients with respiratory problems showed a significant decrease in mononuclear cells with an increase in CCR7 expression, suggesting a concomitant migration of these cells to the airways.[47] These findings indicate that the activation of the immune response mediated by infected DCs could contribute to the pathogenesis during viral infection.[34]

Moreover, analyses of human tissue samples from ANDV fatal cases and infected wild *Oligoryzomys longicaudatus* rodents identified ANDV in alveolar macrophages and submandibular glands.[91] It has been proposed that replication in human salivary glands and expectoration of alveolar macrophages could contribute to person-to-person transmission.[91] Therefore, it is of great importance to understand the role of macrophages during hantavirus infection, not only as part of the immune response against the virus but also their potential role in viral transmission.[91]

Adaptive immunity to hantavirus infection

$CD8^+$ T-cell response

The specific role of T cells during hantavirus disease has not been elucidated. Hantavirus infection does not produce a cytopathic effect on the vascular endothelium.

Therefore, it has been suggested that strong immune activation of CTLs is involved in capillary leakage during a severe clinical presentation.[92] Additionally, NP-specific $CD8^+$ T cells could persist for more than 15 years after PUUV infection, suggesting strong, long-lasting $CD8^+$ T-cell responses.[93] Furthermore, there is an early increase in circulating $CD8^+$ T cells, specifically in the effector population ($Ki67^+$ $CD38^+$ $HLA\text{-}DR^+$).[94]

A vigorous virus-specific $CD8^+$ T-cell response has been observed with the onset of symptoms, with high levels of granzyme B and perforin accompanied by elevated expression of the inhibitory receptor CTLA-4.[95] Besides, 10 days after symptoms had started, a concomitant decline in the $CD8^+$ T-cell effector population and viral load is observed, suggesting an essential role for the CTLs in PUUV clearance and patient recovery.[95] In contrast, a lack of correlation between $CD8^+$ T-cell effector population response and clinical severity has been described.[96] Discrepancies between results studying PUUV could be due to differences among the immunological panel analysed and the incorporation of clinical and biodemographic variables.

$CD8^+$ T cells might also be involved in the clinical HCPS outcome; however, current evidence is inconclusive. Immunoblasts are present in peripheral blood, and $CD3^+$ infiltrates are found in lung necropsies from patients with HCPS.[97] Furthermore, patients with severe HCPS by SNV infection might have higher specific circulating $CD8^+$ T cells than those with a mild course of the disease.[26] Although a case report showed a late viral clearance mediated by $CD8^+$ T cells secreting granzyme B and IFN-γ, ANDV-RNA remained in blood cells even 67 days after viral infection.[27] Later, a strong memory $CD8^+$ T-cell response against Gn-ANDV with an effector phenotype ($CD27^-$ $CD28^-$ $CCR7^-$ $CD127^-$) that lasted up to 13 years after HCPS onset was described.[98] Along these lines, it was observed that $CD8^+$ T-cell responses are long-lasting during hantavirus infections.[93,98]

The Syrian golden hamster model of fatal ANDV infection also exhibited a $CD8^+$ T-cell increase in peripheral blood and lungs. However, no differences in severity or outcome were observed when the disease was evaluated in T-cell-depleted hamsters as compared to control animals, suggesting that T cells play neither pathological nor protective roles in the hamster model of fatal ANDV infection.[99] A study in rhesus macaques infected with SNV showed a high proportion of activated cytotoxic T cells during early infection and the development of a memory effector phenotype with granzyme B expression after disease,[85] similar to the findings in humans.[98] Despite evidence for the implication of $CD8^+$ T cells during and after hantaviruses infections, their participation in pathology or clearance remains unclear.

$CD4^+$ T-cell response

$CD4^+$ T-cell responses in natural infections have been less addressed in studies than $CD8^+$ T-cell responses. Nevertheless, in hantavirus infection, $CD4^+$ T cells may also be a part of the complex immune response in the affected organ in humans. During early PUUV infection, a decrease in $CD4^+/CD8^+$ ratios has been reported, which returned to baseline levels during the convalescent stage.[94] Moreover, $CD4^+$ T-cell responses against hantaviruses are described as mixed Th1/Th2 responses based on sera cytokine profiles[30] without a correlation between effector $CD4^+$ T cells and clinical parameters.[96] Analyses performed in DOBV-affected patients show a negative correlation between total Th cells expressing CD69 and urea, creatinine and CRP levels, suggesting that greater activation of T-cell subsets might contribute to ameliorate disease.[100] Nevertheless, the roles of each Th subtype during hantavirus diseases remain unclear.

Regulatory T cells (Tregs) are the most studied subpopulation of $CD4^+$ T cells, due to the notion of an exacerbated immune response involved in hantavirus pathogenesis. It has been suggested that Tregs are essential for establishing persistent hantavirus infection in rodent hosts and are involved in modulating the immunopathology.[101–103] Increased levels of Tregs are observed in lungs of rats during Seoul orthohantavirus (SEOV) infection.[101] Interestingly, inactivation of Tregs not only led to a reduction in viral RNA in lungs and virus shedding in rats, but also led to reduced amounts of acute multifocal lesions in lungs.[101] Besides, FOXP3 mRNA levels remain static in infected hamsters during HCPS.[104]

The contribution of Tregs during human infection and clinical disease has not been elucidated yet, and studies addressing this T-cell subset differ in methodological approaches. In the course of HFRS, it has been observed that FOXP3 expression levels are upregulated during PUUV infection and are directly correlated with hospitalization days, suggesting that Tregs play a role in disease severity.[96] On the contrary, suppressive cytokines, such as TGF-β1 and TGF-β2, are decreased in severe PUUV cases as compared to mild clinical courses.[105] Despite no differences in Treg frequency in PUUV cases in comparison with healthy donors, two main immune checkpoints involved in T-cell regulation, $PD1^+$ and $CTLA4^+$, are elevated in $CD4^+$ T cells.[95] Also, an increased frequency of Tregs is observed early in PUUV cases as compared to healthy controls and DOBV-infected individuals,[100] suggesting that regulatory mechanisms might be promoting a more balanced and less harmful immune response. This observation is in line with the recent findings in an HCPS survivor cohort after ANDV infection. In this group, the frequency of PD-1 expression in Tregs remains elevated years after the infection, suggesting that immune-

suppressive mechanisms might be involved in regulating HCPS disease, leaving an immunological signature.[106] Furthermore, the dissection of regulatory subtypes indicates a downregulation of a Th1-like Treg response, with a consequent increase in the Th2-like Treg population, possibly mediated by CXCR3 downregulation through GP-ANDV.[106]

B-cell response

A robust IgM response emerges early after hantavirus infection, with a subsequent increase in IgG antibodies.[107–109] Although surface glycoproteins first engage cellular receptors, the initial antibody response seems to target NP protein predominantly, being NP-specific antibodies detectable shortly after symptom onset.[110,111] A low specific IgG response is associated with severe disease in PUUV-infected patients,[51] and higher nAbs titres have been detected in survivors when compared to deceased individuals, suggesting that nAbs production is directly associated with the chance of survival.[25] Furthermore, nAbs have been detected years after PUUV, SNV and ANDV infections, implying a long-lasting immune response.[112,113] Surprisingly, ANDV survivors also show an increase in nAbs titres during time, even years after disease, suggesting that viral antigens might be present at later times after infection.[98]

Moreover, studies have suggested differences between New and Old World hantavirus species regarding the IgG subclass produced by the infected individual during the disease. IgG1 is the most prevalent subclass in patients infected by PUUV,[114] while IgG3 is the main subclass in HCPS by SNV.[115] However, no association between IgG subclass and clinical severity has been reported. An in-depth characterization of IgG responses against different hantaviruses is required to better understand the relevance of antibody-mediated responses for patient survival.

Despite the importance of B cells in antibody production, little is known about the dynamic changes in B-cell populations after hantavirus infection. Recently, a 100-fold increase in the plasmablast (PB) population during acute ANDV infection was described.[116] Remarkably, they found a virus-specific population of specific antibody-secreting cells and a significant rise in reactivity against virus-unrelated antigens.[116] Nevertheless, none of these observations were associated with disease severity. It is noteworthy that this is not the first report that detects the presence of unrelated antibodies during hantavirus disease. This finding is consistent with a previous article describing autoantibodies due to PUUV infection.[117] Moreover, this finding is consistent with research that showed a significant risk of Hodgkin's lymphoma after HFRS by PUUV, similar to other viral infections, such as with the Epstein–Barr virus and the human immunodeficiency virus type 1.[118] However, additional research is required to elucidate whether those autoreactive antibodies have any role in hantavirus disease or are associated with a higher risk of autoimmune illness.

The current knowledge about the immune responses during hantaviruses disease is summarized in Table 1. Furthermore, according to published data, immune mechanisms associated with a positive or negative prognosis for hantavirus diseases are proposed in Fig. 2.

Current therapeutic approaches for treating hantavirus diseases

At the clinical level, current therapies targeting hantavirus diseases are based mostly on supportive care, with extracorporeal membrane oxygen therapy as a unique option in HCPS life-threatening conditions.[119] As high nAbs titres are related to a favourable outcome, fresh frozen plasma from recovered individuals has been used as a therapy in patients with HCPS.[120] This treatment decreased CFR from 32% to 14% in a non-randomized multicenter trial[121] and is currently being administered to patients with HCPS, even though standardization of this intervention is still required.

Recombinant antibodies are a promising treatment for hantavirus diseases. At the practical level, the use of purified monoclonal and polyclonal nAbs has also been explored with promising results in pre-clinical stages. Mapping and isolation of two monoclonal antibodies (mAbs) from an HCPS-recovered individual with high nAbs titres showed high neutralizing activity *in vitro* and protected against lethal HCPS in Syrian hamsters when administered as a post-infection treatment.[122] More recently, neutralizing mAbs targeting both Gn and Gc ANDV proteins were developed through murine hybridomas, after vaccinating mice with a VSV-DNA vaccine expressing ANDV glycoproteins. Besides the neutralizing functions of these mAbs, they displayed antibody-dependent cellular cytotoxicity and were also protective in the lethal ANDV Syrian hamster model.[123]

Additionally, purified polyclonal human IgG has been produced after DNA vaccination of transchromosomal bovines against ANDV, SNV, and, more recently, HTNV and PUUV.[124,125] All these purified polyclonal human IgG displayed high neutralizing activity and were protective against lethal HCPS. As was the case for mAbs, rationally designed human polyclonal IgGs require further testing and should be considered promising prophylaxis therapy.

Nevertheless, nAbs are not the only promising immune treatments, as some strategies employed in cancer therapies could successfully be applied to treat hantavirus diseases. IL-6, as described above, is elevated in HCPS and HFRS, being associated with a severe outcome in HCPS.[62–65] Moreover, the increase in other cytokines is

Table 1. Main findings associated with cellular immune response during hantavirus diseases

HCPS	References	HFRS	References
Natural killer cells			
No data published	-	1 Lower peripheral blood levels in comparison with healthy donors during febrile stage. 2 Peak during renal phase. 3 Elevated for at least 2 months after infection 4 High expression of activation markers	82–84
Dendritic cells/monocytes	90		47,81,87,88
1 Release of active MMP-9 2 Proinflammatory cytokine profile: elevated TNF, and decreased IL-10 and TGF-β secretion (*in vitro*).		1 Decreased antigen uptake 2 HLA-I upregulation after HTNV challenge 3 Increase in CCR7 in peripheral monocytes 4 Differential expression of CD86 after the infection of low and high pathogenic viruses	
$CD8^+$ T cells	26,98,99		93–95
1 Higher frequency in mild HCPS course 2 Memory effector phenotype with Gn predominance over 4 years 3 Not involved in pathogenesis according to the Syrian hamster model		1 Effector population increase during acute stage. 2 High levels of perforin and granzyme. 3 NP-specific response persists more than 15 years	
$CD4^+$ T cells	30,104,106		83,96,100
1 Th1/Th2 profile 2 Static mRNA levels of FOXP3. 3 High frequency of PD-1 in Tregs from survivors 4 Treg Th1-like downregulation		1 Higher expression of FOXP3 in severe course. 2 Suppressor phenotype 3 Increase in Treg population	
B cells	25,113,115,116		112,114
1 Exacerbated PB response 2 Higher titres of nAbs in mild disease 3 Long-lasting immune response 4 IgG3 as the main subclass		1 Long-lasting immune response 2 IgG1 as the main subclass	

Summary of cellular immune responses with emphasis in correlation with mild and severe course of each hantavirus cardiopulmonary syndrome (HCPS) and haemorrhagic fever with renal syndrome (HFRS).

also involved in hantavirus diseases. The cytokine storm phenomena have also been observed in other viral illness, as coronavirus disease 19 (COVID-19). Like hantavirus diseases, severe cases of COVID-19 are related to pneumonia with cellular infiltration and cytokine storm.[126] Due to the global emergency and impact of COVID-19, many therapeutic approaches have been tested simultaneously. Among them, the use of commercial mAbs to block the cytokine release syndrome is currently a strategy being tested under clinical trials to treat critical cases of COVID-19, and successful results have been reported when a cocktail of antibodies is administered to COVID-19-infected patients.[127] Along these lines, data obtained from these clinical trials could help dampen the hyperreactive response after SARS-CoV-2 infection and provide baseline information on whether the decrease in proinflammatory cytokines could be considered as a successful therapeutic strategy for severe hantavirus cases.

Impact of the current knowledge for the vaccine development

Up to date, there are no FDA-approved vaccines for HFRS and HCPS. Considering the worldwide impact and the high fatality rate of both diseases, the development of protective vaccines for these syndromes is imperative. The high eco-epidemiological complexity of these emerging infectious diseases makes it difficult to rely only on control measures to prevent human–reservoir contacts. However, this approach should be considered as a required

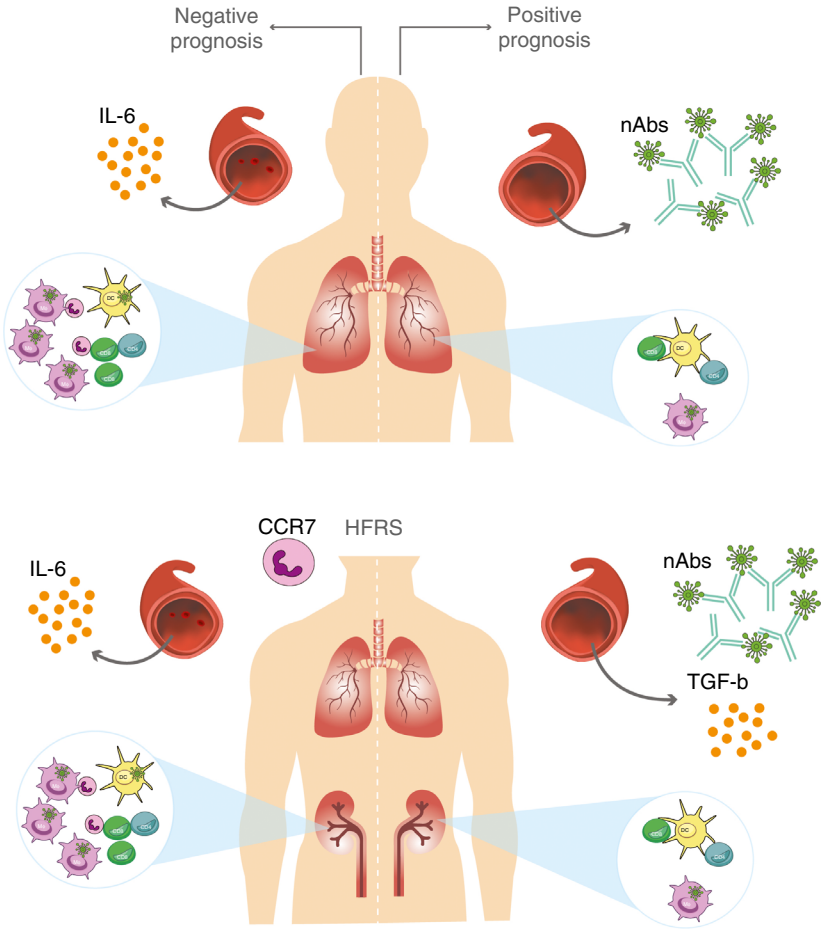

Figure 2. Immune mechanisms involvement in hantavirus prognosis disease. Early nAb production is broadly associated with a positive prognosis in both HCPS and HFRS cases. Further, an increase in serum TGF-β levels has been related to mild HFRS presentation. Higher levels of the proinflammatory IL-6 cytokine have been found in deceased patients with HCPS in comparison with survivors. Similarly, it has been associated with a more severe course of HFRS. Moreover, lung compromise could be associated with a higher expression of CCR7 during HFRS disease.

complement to achieve a successful immunization scheme.[128] Vaccine development for hantaviruses has been hindered by several factors, such as a lack of animal models that recap critical aspects of the illness, the limited knowledge on the immunopathology and protective immunity mechanisms, and other obstacles inherent to clinical studies.[129] However, during the past years, notorious advances have been made with several types of vaccines at the experimental stage and, most notably, with promising DNA vaccine prototypes being tested in clinical studies.[35] This section discusses vaccine approaches under development, as well as some critical missing aspects of immunity, and implications of humoral and cellular immunity for the rational design of vaccines.

Hantavirus vaccines at experimental and clinical stages

A formalin-inactivated HTNV vaccine (Hantavax) has been used since 1990 in parts of Asia.[36] Also, bivalent vaccines based on inactivated HTNV and SEOV have been used for more than 10 years in China, supported by the extended immunization programme applied in that country.[130,131] These vaccines elicit nAbs titres that might last 2–3 years when used in three-dose schedules.[132] Although these vaccines are considered safe,[132] studies assessing their efficacy are scarce, with a 10-year retrospective cohort study failing to demonstrate the protective effect of Hantavax in Korea.[133]

Several strategies have been tested to develop a vaccine against one or more hantaviruses species, including purified recombinant proteins,[134,135] virus-vectored antigens,[136–138] virus-like particle (VLPs)[139,140] and nucleic acid-based vaccines.[141–145]

Studies using recombinant proteins indicate that purified antigens are enough to elicit immunogenicity and provide partial or total protection against hantavirus infection, including cross-protective responses.[146] Immunization of hamsters with recombinant viruses expressing both Gn and Gc provided protection from infection after

an HTNV challenge inducing nAbs. It is noteworthy that immunization with baculovirus encoding for the NP of HTNV also protected hamsters in this model.[147] Other groups showed that vaccination with recombinant PUUV NP expressed in yeast and *E. coli* provides protection in the bank vole model, and cross-protective responses could be achieved using NP from other hantaviruses, including ANDV. Interestingly, the degree of protection was neither related to the amino acid sequence identity nor the cross-reactive humoral responses of the different NPs.[146] In mouse models, administration of DOBV-NP in a three-dose immunization scheme elicited robust and long-lasting NP-specific IgG antibodies with a cross-reactive response against HNTV and PUUV.[148] Similarly, a three-dose immunization of C57BL/6 mice with DOBV-NP provided protection from infection in 75% of challenged animals when administered with Freund's complete adjuvant but only of 12·5% when the antigen was administered with alum. When evaluating cytokine secretion of PBMCs in ELISpot assays, the immune response towards the NP antigen was associated with a strong IL-4 secretion, suggesting that a Th2 response would not be optimal to confer protection to infection.[149]

Regarding virus-vectored antigens, there are promising cross-protective vaccine candidates aimed to protect from HCPS. Initial studies using a non-replicating adenovirus (Ad), a single dose of Ad vaccine encoding Gn, Gc, Gn + Gc or NP individually or in combination, confer protection in the Syrian hamster model for ANDV infection.[136] Vaccines carrying Gn or Gc were able to provide sterile immunity despite a lack of nAbs; similarly, Ad-expressing NP also protected in the absence of nAbs.[136] Vesicular stomatitis virus (VSV) pseudotypes encoding hantavirus glycoproteins have been explored as a vaccine approach against HFRS and HCPS. Regarding HCPS, a single dose of a VSV that expressed GPC-ANDV (rVSVΔG/SNVGPC) provided sterilizing immunity when administered 28 days before a lethal dose of ANDV in the Syrian hamster model, with a robust nAb response.[137]

Furthermore, the cross-reactive antibody response was observed after testing VSV expressing ANDV or SNV GPC in both lethal ANDV and non-lethal hamster-adapted SNV infection models.[138] According to *in vitro*-neutralizing assays on primary human ECs, the protective response elicited by these VSV-based vaccines is associated with a humoral response that blocks the interaction between GPC and protocadherin-1, a membrane protein that has been recently recognized as a critical host factor for ANDV entry and infection,[138,150] supporting the importance of that molecule for ANDV infection, and as an intervention target. Long-term protection studies with the rVSVΔG/ANDVGPC vaccine suggest that protective immunity can last up to 6 months, but not for a year.[137] Interestingly, this vaccine also provided protection when administered 1 week before lethal ANDV challenge, in the absence of specific nAbs, suggesting other mechanisms of immunogenicity.[151] Moreover, oral and intramuscular (IM) vaccination with a single dose of rVSVΔG/SNVGPC reduced viral loads in the lungs and blood of *Peromyscus maniculatus*.[152] Remarkably, partial protection was achieved without inducing significant nAbs, but was enough to prevent SNV infection of uninfected/vaccinated animals when directly exposed to infected deer mice.[152] These data suggest the relevance of cellular immune mechanisms involved in the protection against hantavirus infection.

Some of the most promising delivery approaches to date are nucleic acid-based vaccines, with several candidates currently advancing to clinical trials, as recently reviewed.[36] Nucleic acid vaccines have advantages over viral vector-based vaccines, such as not interfering with pre-existing immunity and lacking irrelevant viral antigens. Initial studies in Syrian hamsters showing increased protection after delivering SEOV-M segment compared with SEOV-S segment[153] led to the development of HNTV-M segment DNA vaccines. These candidate vaccines were tested in rodent and non-human primate (NHP) models for hantavirus infection with promising results on disease protection and nAb responses.[141] Up to date, and after successful pre-clinical studies, several DNA vaccines targeting PUUV, HTNV and ANDV are being tested in clinical trials.[35] Clinical studies using a 4-week interval with three-dose scheme indicate that PUUV and HNTV DNA vaccines delivered through IM electroporation increase immunogenicity as compared to gene gun delivery, rising detectable nAbs titres in near of 78% of volunteers.[24] Current clinical trials focus on vaccine dosage and different delivery methods to optimize immunogenicity with a successful safety profile.[35] DNA vaccines have also been experimentally tested as a pan-hantavirus vaccine candidate, showing that rabbits immunized with a plasmid mix targeting SNV, ANDV, PUUV and HNTV develop nAbs.[154] Further research is required to validate this approach as up to date, no DNA vaccines have been approved for use in humans.

Cellular immune response induced by hantavirus vaccines

A common approach for determining the efficacy of some vaccines is quantifying the presence of nAbs.[155] As stated before, because nAbs are enough to confer protection against hantavirus-related diseases, the evaluation of immunogenicity of hantavirus vaccine candidates has relied mainly on this determination. However, evidence from diverse models and prototypes suggests that cellular immunity may be necessary for clearance and protective immunity against these viruses (summarized in Table 2). During the development of a PUUV DNA vaccine, cross-protection against DOBV and ANDV was observed

Table 2. Multiple strategies for the development of a hantavirus vaccine

Hantavirus vaccine candidates				
Vaccine type	Vaccine/antigens	Animal model	Immunogenicity evaluation	References
Recombinant proteins	Nucleoprotein from ANDV, TOPV, DOBV or PUUV	Bank voles	1 Specific $CD8^+$ T-cell production 2 Cross-reactive response against PUUV	146
	Yeast-expressed DOBV nucleoprotein	Mice	1 NP-specific IgG response, with IgG1, IgG2a, IgG2b and IgG3 subclass production 2 Th1/Th2 response 3 Cross-reactivity with HTNV and PUUV	134
	Truncated recombinant PUUV nucleoprotein linked to bacterial membrane protein	Mice	1 NP IgG response 2 $CD8^+$ T-cell response	135
DNA vaccines	HTNV and ANDV M gene segments	Rhesus macaques	1 Neutralizing antibodies	142
	HTNV M segment	Rhesus macaques	1 Neutralizing antibodies 2 Cross-reactivity with SEOV and DOBV	141
	SNV M gene segment	Syrian hamsters	1 Neutralizing antibodies	154
	HTNV/PUUV/SNV/ANDV M gene segment mix	Rabbits	1 Neutralizing antibodies	154
	PUUV M gene segment	Syrian hamsters	1 Neutralizing antibodies 2 Protection against lethal ANDV infection, without nAbs	144
Virus-vectored	Replication-competent VSV-vectored ANDV glycoproteins	Syrian hamsters	1 Neutralizing antibodies	137
	Replication-competent VSV-vectored ANDV or SNV glycoproteins	Syrian hamsters	1 Cross-reactive IgG response 2 Neutralizing antibodies	138
	Non-replicating Ad vector expressing N, Gn, Gc or Gn/Gc	Syrian hamsters (protection studies) Mice (cytotoxicity assays)	1 Neutralizing antibodies in SHs after challenge 2 Specific T $CD8^+$ cell response	136
Virus-like particles (VLPs)	HTNV-VLP with CD40L or GM-CSF incorporation	Mice	1 Neutralizing antibodies 2 Antigen-specific IFN-γ production 3 CTL response	140
Inactivated virus	Hantavax (formalin-inactivated HNTV)	Humans	1 Neutralizing antibodies 2 B-cell response 3 Th1 response 4 Cytotoxic response	156

Published approaches for vaccine development against haemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome.

without the detection of nAbs, suggesting that protection could be mediated directly by immune cells.[144] As discussed above, T cells, but not the neutralizing activity of antibodies, have been associated with homologous and heterologous protection against hantavirus infection in mice.[146] In addition, $CD8^+$ T-cell cytotoxic activity, as well as IFN-γ and TNF secretion, seems to be required for the clearance of HTNV.[146] More recently, an HTNV-VLP vaccine (VLP-CD40L; VLP-GM-CSF) was developed, which elicited high titres of nAbs with a specific cytotoxic response mediated by T $CD8^+$ cells in mice.[140] Regarding HCPS, protection in the absence of neutralizing activity of antibodies has been reported after immunization with adenoviral vectors encoding for Gn, Gc or NP.[136] Moreover, a pan-hantavirus DNA vaccine against HTNV/PUUV/SNV/ANDV was reported to protect against ANDV infection despite low nAbs titres.[154]

These observations suggest that T-cell immunity, particularly cytotoxic T-cell activity, can be important for generating cross-protection against hantavirus species. However, mechanistic studies should be carried out in hantavirus animal models to support these conclusions.

In humans, preliminary results of a phase 3 study with the Hantavax vaccine (NCT02553837) revealed that high responders (described as volunteers with higher nAb titres) present a differential expression gene profile related to T and NK responses, upregulating the activation markers CD69 and CD83, and the CXCR4 chemokine after the second and third boost. Additionally, after the fourth boost, Khan *et al.* observed a later immunoglobulin-related signature, including the upregulation of IGLV1-40, IGLV2-11, IGKV3D-20, IGLC2 and IGHG1. However, this correlation was not observed in low responders, suggesting that this differential expression gene profile might have ultimately led to an enhanced neutralizing response.[156] This study indicates that several interrelated pathways of the immune response, such as phagocytosis, B cells, T cells and NK responses, are involved in the protective mechanisms induced by Hantavax.

Discussion

Despite the worldwide distribution of pathogenic hantaviruses and the constant efforts invested in vaccine development, to date there are no approved vaccines against these viruses. The only vaccine in use, which is based on an inactivated virus (Hantavax), does not provide an effective, long-lasting immune response.[157] Vaccine availability is a necessary complement to currently applied preventive actions to avoid incidences, such as the recent HCPS outbreaks in South American countries, with fatality rates above 30%.[158]

Many investigations have demonstrated differences between hantavirus species in many aspects, such as the biological receptors used, the immune responses induced, the clinical disease course, and fatality rates. The clinical course of HCPS could evolve faster in comparison with HFRS, and therefore, samples for longitudinal studies are not always available. A common finding in hantavirus survivors is the early nAb response.[25] Furthermore, it has been demonstrated that nAbs can persist years after infection,[113] and their presence is enough to protect against lethal challenge in several animal models. For this reason, most of the therapeutic or prophylactic strategies are currently based on nAb production to provide protection. However, other humoral immune mechanisms are, in concert with the cellular immune response, an essential arm of the immune system and therefore need to be considered in evaluating immunogenicity and protection against these viruses.[159]

The study of hantavirus involves many challenges, starting from the critical requirement of biosafety and biosecurity standards that limit animal studies to laboratories with the highest biosafety levels, which are frequently absent in areas with elevated HCPS endemicity cases. In this line, animal models that accurately recapitulate clinical disease are insufficient for comprehensive mechanistic studies of the many pathogenic species of hantavirus. Additionally, immune response analyses in hamsters are limited due to a lack of reagents to measure specific immune populations. Thus, the diversification of animal models and the development of immunological tools for studying cellular immunogenicity are key aspects to be reached to advance towards efficient, protective vaccines against hantaviruses.

Studies on the hamster model of HCPS have suggested that the immunopathogenesis of HCPS might not be related to T cells[99]; however, analysis of human cases indicate that T $CD8^+$ cells could be involved in the viral clearance without nAb participation,[27] and similar results are observed in the cross-protection against different hantavirus species. $CD8^+$ memory cells maintain an effector phenotype years after infection,[93,98] and thus, vaccine studies should consider the response of effector memory profiles in the design of future vaccine candidates. Remarkably, cross-reactivity between different species of hantaviruses has been observed in immunization studies indicating that a pan-hantavirus vaccine could be a feasible, successful approach.

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Disclosures

The authors declare no conflict of interest.

Author contributions

Farides Saavedra and Alexis M. Kalergis performed *conceptualization*. Farides Saavedra, Fabián E. Díaz, Angello Retamal-Díaz and Camila Covián wrote the original draft. Camila Covián, Angello Retamal-Díaz, Farides Saavedra and Fabián E. Díaz prepared figures and tables. Farides Saavedra, Fabián E. Díaz, Camila Covián, Angello Retamal-Díaz, Pablo A. González and Alexis M. Kalergis wrote, reviewed and edited the manuscript.

Data availability statement

No new data sets were generated for this manuscript.

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