CURRENT INSIGHTS INTO HOST IMMUNE RESPONSES TO HUMAN RESPIRATORY SYNCYTIAL VIRUS (RSV) AND CHALLENGES TOWARDS EFFICIENT TREATMENTS AND VACCINES AGAINST RSV

EDITED BY: Alexis M. Kalergis and Steven Varga
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Topic Editors:
Alexis M. Kalergis, Pontificia Universidad Católica de chile, Chile
Steven Varga, The University of Iowa, United States

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OPINION
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Current Insights in the Development of Efficacious Vaccines Against RSV

*Jorge A. Soto[1], Laura M. Stephens[2], Kody A. Waldstein[2], Gisela Canedo-Marroquín[1], Steven M. Varga[2,3,4] and Alexis M. Kalergis[1,5]**

[1] Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Instituto Milenio de Inmunología e Inmunoterapia, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA, United States, [3] Department of Microbiology and Immunology, University of Iowa, Iowa City, IA, United States, [4] Department of Pathology, University of Iowa, Iowa City, IA, United States, [5] Departamento de Endocrinología, Facultad de Medicina, Instituto Milenio de Inmunología e Inmunoterapia, Pontificia Universidad Católica de Chile, Santiago, Chile

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INTRODUCTION

Respiratory viral infections are one of the most important global public health burdens, resulting in millions of hospitalizations worldwide annually (1, 2). Respiratory Syncytial Virus (RSV) is the leading cause of acute lower respiratory tract infections (ALRTI) in children under the age of 2 (3) and adults over 65 (4). RSV-induced disease can range from symptoms similar to the common cold to complex respiratory diseases, such as pneumonia or bronchiolitis, leading to extrapulmonary sequelae in the brain and other tissues (5). During the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine was evaluated in children. Vaccinated individuals exhibited increased disease severity upon subsequent natural RSV infection compared to the controls (6–9). This vaccine-enhanced disease resulted from the failure of the vaccine to elicit either potent neutralizing antibodies or memory $CD8^+$ T cells as well as the induction of a strong inflammatory CD4 T cell response (10–13). Currently, the only treatment option available for RSV is a humanized monoclonal antibody against the RSV F surface protein, known as palivizumab (14). However, its usage is limited to high-risk individuals, such as preterm babies, and infants with congenic diseases (15–17). Due to prolonged concerns about vaccine safety, a better understanding of RSV-induced pathogenesis and the host immune response is needed to aid in the development of safe and effective treatments and vaccines for RSV. This Opinion article examines the various vaccine modalities currently undergoing testing and discusses the advantages and disadvantages of the strategies being employed.

RSV VACCINE MODALITIES AND LESSONS FROM THE HOST IMMUNE RESPONSE

Based on the knowledge gained from the unsuccessful FI-RSV vaccine trial, new vaccine formulations are being developed that promote neutralizing antibodies, induce activated memory and lung-resident $CD8^+$ T cells, and can be administered to different target populations including children, elderly and pregnant women. The most promising vaccine candidates currently being evaluated in humans are live-attenuated, recombinant vector-based, and subunit vaccines.

Live-attenuated vaccines demonstrate favorable benefits including a low risk of causing vaccine-enhanced disease, and they can promote both a humoral and cellular immune response. However, potential drawbacks include conserving the stability of the formulation, and balancing the attenuation of the virus while maintaining replicative activity and immunogenicity in the host (18). Additionally, further studies are needed to assess the safety of live-attenuated vaccines in multiple populations (19). Many live-attenuated vaccines are currently undergoing testing in clinical trials and demonstrate a robust induction of a humoral immune response; however, much

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***Correspondence:**
Alexis M. Kalergis
akalergis@bio.puc.cl

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less information is known about the cellular immune responses induced by the vaccines. Deletion of the M2-2 protein from the RSV strain A2 (LIDΔM2-2) induced robust serum RSV-specific IgG and neutralizing antibody titers that correlated with lower nasal wash viral titers administered intranasally to seronegative children (20). A similar induction of serum neutralizing antibodies was observed with a cold-passage/stabilized RSV containing several attenuating point mutations as well as deletion of the small hydrophobic (SH) protein (RSVcps2) (21). Finally, preclinical studies of a recombinant BCG vaccine expressing the RSV nucleoprotein (N) demonstrated an effective cellular and humoral immune response in mice (22–25).

Recombinant vector-based vaccines allow the presentation of one or more antigens expressed on a viral vector such as parainfluenza virus type 3 (PIV3) or adenovirus. This allows for natural presentation of the antigen of interest to immune cells. A PIV3 vector expressing the RSV F protein (MEDI-534) demonstrated safety in a Phase 1 study when administered intranasally to young children (26). Interestingly, some discordance was observed in the specificity of the immune response. Sequencing of viral samples suggested that modifications were generated post-vaccination in a number of subjects that promoted a reduction in the expression of the F protein correlating with lower neutralizing antibodies in those individuals (27). A recently developed vaccine composed of a chimpanzee adenovirus viral vector expressing the RSV F, N, and M2-1 proteins (ChAd155-RSV) induced robust neutralizing antibody titers and interferon gamma (IFNγ)-secreting T cells compared to placebo controls (28). ReiThera Srl developed a similar vaccine using a chimpanzee adenovirus (PanAd3) viral vector expressing the RSV F, N, and M2-1 proteins in combination with a modified vaccinia virus Ankara (MVA). Intramuscular and intranasal delivery of the vaccine to healthy adults was well tolerated and induced both RSV-specific antibody titers and RSV-specific CD4 and $CD8^+$ T cells (29–31). Interestingly, other clinical trials using adenovirus have been developed to date (NCT03982199, NCT03636906, among others).

Subunit vaccines are a common vaccine modality; however, some disadvantages are associated with these formulations such as the frequent need to use an adjuvant to increase the immunogenicity. A single dose of an RSV F protein subunit vaccine combined with aluminum hydroxide induced RSV F-specific antibodies that persisted for >180 days post-vaccination (32). Similar results were observed following intramuscular administration to women of child-bearing age, suggesting that maternal immunization with this vaccine candidate could generate lasting antibodies to passively transfer to the fetus during the pregnancy (33). Another vaccine utilizing the RSV F protein demonstrated safety and efficacy in Phase 1 and Phase 2 clinicals trials when administered without an adjuvant, suggesting that a subunit vaccine may induce lasting protection without an added adjuvant (34). Interestingly, formulations using the RSV F protein have failed to provide protection against RSV infection in older adult populations, indicating that subunit vaccines may not be the best candidate for this target population (35).

Many vaccine prototypes are focused on viral surface proteins (36, 37). One of the most common viral targets for antibodies is the RSV fusion (F) protein (38, 39). Vaccine formulations containing the N protein also induce long-lasting neutralizing antibodies and could serve as a novel antiviral target (22, 25). The RSV G protein is involved in the initiation of the virus life cycle and has a potent effect on the regulation of the immune response (36). The SH protein can promote a protective immune response in animal models of RSV through Fc receptor-mediated interactions with macrophages and helping the promotion of long-lasting antibodies (40, 41). Furthermore, other protein targets are currently being or have been evaluated in clinical trials, including the nonstructural protein 2 (NS2) (NCT03596801, NCT03473002) and the M2-2 protein (20, 42). However, independent of the antigen evaluated, the key requirement of any RSV vaccine is the ability to promote a safe, but effective and protective immune response.

On the other hand, another type of vaccine strategy that has provided positive and interesting results in human tests is based on intranasal administration of a novel BLP (bacterium like particle) conjugated to the RSV fusion (F) protein eliciting both mucosal IgA responses and elevated IFN-γ production (43). Since BLP prototype is a promising strategy, more assays to evaluate long-lasting immune response are required.

The choice of administration route is an important decision in vaccine development, with most vaccines being delivered via the sublingual, intramuscular, or intranasal route. Sublingual administration of an RSV G protein vaccine induced enhanced cellular infiltration and pro-inflammatory cytokine production compared to intranasal delivery (37). Similar results were observed when a recombinant RSV attachment (G) protein containing the central regions for both RSV A and B serotypes was administrated either intranasally or sublingually (44). Sublingual delivery enhanced pulmonary eosinophil recruitment and body weight loss, while intranasal administration promoted enhanced IgG and IgA antibodies and lower pro-inflammatory cell recruitment into the lung. Mucosal administration may also induce a high titer of IgA in bronchial alveolar lavage (BAL) fluid and IgG antibodies in serum (44, 45). A murine cytomegalovirus vector expressing the RSV matrix (M) protein induced robust lung-resident memory T cell populations when administered intranasally compared to intraperitoneally, where this population was almost undetectable (46, 47). This suggests that intranasal administration of an RSV vaccine would induce an enhanced $CD8^+$ T cell response, a strong secretion of IgG and IgA antibodies, and decrease the inflammatory state of the lung.

One way to aid in the successful development of an RSV vaccine is to gain a better understanding of the host immune response to the virus and the factors required for long-term immunity. Studies examining the host response during acute infection of infants suggest that the virus elicits a pathogenic Th2 dominant response (10–13). Th2-biased T cells, driven by IL-4, IL-5, and IL-13 cytokines, lead to inflammation and hyperreactivity of the airways (48–51). Other T cell populations, including regulatory T cells (Tregs) and Th17 cells, also play an important role during RSV infection (52). Th17 cells can promote a pro-inflammatory state leading to enhanced neutrophil

recruitment and reduced $CD8^+$ T cell activation (53). Tregs are associated with the active recruitment of cytotoxic $CD8^+$ T cells in the lung; however, unbalanced Tregs could promote enhanced lung damage (54, 55). Interestingly, peripheral blood mononuclear cells (PBMCs) from infected children exhibit reduced Tregs compared to age-matched controls (56). Similarly, depletion of Tregs in mice promoted enhanced lung pathology following RSV infection (57, 58). Thus, a successful vaccine should induce a balanced T cell response characterized by Th1-biased T cells as well as Tregs.

The induction of type I IFN are essential for RSV viral clearance. The absence of type I IFN promotes a pro-inflammatory response that helps to induce a lung pathology in both human and murine models of infection (59). The administration of IFN-α in RSV-naïve high-risk infants is associated with a decrease in lung pathology and enhanced viral clearance. However, RSV possesses several evasion mechanisms, and both the NS1 protein and the G protein can suppress the type I IFN response (60). A vaccine that induces a powerful type I IFN secretion within its response could be considered a good candidate against RSV.

$CD8^+$ T cells play a critical role in RSV-clearance (61). Murine studies of RSV demonstrate a protective role for memory $CD8^+$ T cells in promoting viral clearance and providing protection from reinfection (61, 62). Nevertheless, natural RSV infection induces low levels of $CD8^+$ T cells. Thus, it would be advantageous for a vaccine to promote a Th-1 immune response and generate memory $CD8^+$ T cells (23–25). In contrast, $CD4^+$ T cells have a controversial role during RSV infection. Following natural infection, $CD4^+$ T cells can promote a dysbalanced host response that enhances immunopathology. However, adoptive transfer studies in the mouse model also suggest that $CD4^+$ T cells can play a protective role. The induction of a Th-1 polarized immune response that promotes both $CD8^+$ and $CD4^+$ T cells is essential for a vaccine to induce a protective immune response against RSV.

The decline in neutralizing antibodies after the RSV infection is an important factor in the reinfections that occur in children. Several formulations of vaccines seek to induce neutralizing antibodies in high risk populations and maternal antibodies that will be transferred from the mother to the fetus to protect against early RSV infections. Nevertheless, these formulations have been shown to induce antibodies that are short-lived. Interestingly, intranasal vaccines have demonstrated the ability to induce high levels of neutralizing antibodies and also promote the IgA secretion that is directly associated with a protective immune response against RSV (43–45, 59, 63).

DISCUSSION

Severe RSV-induced disease continues to present a major global health burden in high-risk groups such as preterm infants, newborns, elderly populations, and those with many associated comorbidities. There is no licensed vaccine to prevent RSV infections, and the only prophylaxis currently approved by the Food and Drug Administration (FDA) is the monoclonal antibody palivizumab. However, its limited use in high-risk groups (14), as well as the high cost and moderate effectiveness underscore the need for additional options. There remains a critical need to develop safe and effective RSV vaccines and therapeutics to combat RSV disease severity in infants and high-risk populations.

In conclusion a vaccine against RSV that promotes an effective antiviral response must induce a prolonged neutralizing antibody response, Th-1 polarized immunity that promotes both $CD8^+$ and $CD4^+$ T cells, type I IFN secretion and an efficient mucosa immune response.

AUTHOR CONTRIBUTIONS

JS, LS, and KW wrote and revised the manuscript. GC-M contributed to the revision and editing of the manuscript. SV and AK were the lead investigators and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Type I Interferon (IFN) in the Respiratory Syncytial Virus (RSV) Immune Response and Disease Severity

Diego R. Hijano[1†], *Luan D. Vu*[2,3†], *Lawrence M. Kauvar*[4], *Ralph A. Tripp*[5], *Fernando P. Polack*[6] *and Stephania A. Cormier*[2,3*]

[1] Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, TN, United States, [2] Department of Biological Sciences, Louisiana State University and School of Veterinary Medicine, Baton Rouge, LA, United States, [3] Department of Comparative Biomedical Sciences, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA, United States, [4] Trellis Bioscience, LLC, Redwood City, CA, United States, [5] Department of Infectious Disease, University of Georgia, Athens, GA, United States, [6] Fundacion INFANT, Buenos Aires, Argentina

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***Correspondence:**
Stephania A. Cormier
stephaniacormier@lsu.edu

[†]These authors have contributed equally to this work

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Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract disease in children <2 years of age. Increased morbidity and mortality have been reported in high-risk patients, such as premature infants, patients with cardiac disease, and severely immune compromised patients. Severe disease is associated with the virulence of the virus as well as host factors specifically including the innate immune response. The role of type I interferons (IFNs) in the response to RSV infection is important in regulating the rate of virus clearance and in directing the character of the immune response, which is normally associated with protection and less severe disease. Two RSV non-structural proteins, NS1 and NS2, as well as the envelope G glycoprotein are known to suppress type I IFN production and a robust type I IFN response to RSV does not occur in human infants or neonatal mouse models of RSV infection. Additionally, presence of type I IFNs are associated with mild symptoms in infants and administration of IFN-α prior to infection of neonatal mice with RSV reduces immunopathology. This evidence has driven RSV prophylaxis and therapeutic efforts to consider strategies for enhancing type I IFN production.

Keywords: infant immunity, respiratory syncytial virus, type I interferons, human, mouse, vaccine

INTRODUCTION

Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract disease in infants and young children (1–3). Although 30–70% of infants develop bronchiolitis upon primary RSV infection, only 1–3% are hospitalized (4). Despite this heterogeneous course of disease, the global burden of RSV disease is estimated at 64 million cases and 160,000 deaths annually (5, 6). Increased morbidity and mortality have been reported in high-risk patients, such as premature infants, infants with cardiac disease, and severely immuno-compromised patients (7–9). Moreover, the consequences of severe RSV infection are long lasting and constitute a risk factor for childhood asthma and bronchiolitis (10–14). The elderly and immune compromised also suffer from RSV, particularly those with prior pulmonary problems (15). Notwithstanding the advances in our understanding of the immune response to RSV and the recently determined high resolution

structures of the two major immunogenic viral proteins, the RSV F and G proteins, we still lack adequate therapeutics as well as a safe, robust, and effective vaccine (16).

Both viral and host immune factors have been implicated in severe infections (17–20). RSV is an orthopneumovirus in the Paramyxoviridae family (21, 22). The RNA genome contains 10 genes encoding 11 proteins. The envelope of the virus is formed by the matrix (M) protein, the small hydrophobic (SH) protein, and two abundant, glycosylated surface proteins: the fusion (F) and attachment (G) proteins. The G and F proteins control the initial phases of infection (23, 24). The G protein is composed of three epitope regions identified by murine monoclonal antibodies: mostly invariant epitopes in the central conserved domain (CCD); group-specific epitopes (subtype A or B); and strain-specific epitopes in the C-terminal hypervariable region of the G protein ectodomain (25, 26). The two antigenically distinct subtypes, A and B, can co-circulate during the same epidemic season (27–29). The clinical impact of different subtypes likely contributes to different disease severity. While the F protein has historically been the major target for antiviral and vaccine development, both G and F proteins are naturally targeted by neutralizing antibodies induced by infection (23, 24, 30–33). The two non-structural proteins, NS1 and NS2, suppress IFN production (34–36), with NS1 known to bind RIG-I within the cytoplasm of host cells thereby abrogating the signal transmitted via MAVS (2). Further, the G protein also impedes IFN-α expression through the interaction of the CX3C chemokine-like motif in G, which interacts with CX3CR1 and impairs the immune response to RSV. Infection with an RSV strain that lacks the CX3C motif (mimic of the human chemokine called fractalkine or CX3CL1) or treatment with an anti-G monoclonal antibody (MAb) that blocks binding to CX3CR1 result in increased levels of type I/III IFN (37).

The fractalkine receptor, CX3CR1, is expressed on human plasmacytoid dendritic cells (pDCs) and epithelial cells (37–39). The former are specialized immune cells that infiltrate the lung to produce large amounts of type I IFN in response to viral infection (40, 41).

The link between RSV G protein and type I IFN expression is well established (42–44) with details elucidated that include TLR4 signaling and SOCS3 regulation of type I IFN (45–50). For example, the RSV G protein contributes to immune evasion by modifying host cytokine and chemokine responses whose expression is negatively regulated by suppressor of cytokine signaling (SOCS) proteins (48). SOCS1 and SOCS3 are closely related and well characterized members of the family acting through the JAK/STAT pathway to regulate cytokine expression via a kinase inhibitory region (51). SOCS1 and SOCS3 are downstream from toll like receptors (TLR) and can indirectly regulate them (52). Specifically, SOCS3 induction by TLR is dependent on Myd88 (52). SOCS1 and SOCS3 strongly suppress TLR7-mediated type I IFN production by binding IFN regulatory factor 7 (53). In addition, SOCS1 modulates TIRAP which is downstream of TLR1/2, TLR2/6 and TLR4 but not TLR9 (51). It has been shown that SOCS1 and SOCS3 regulate type I IFN in normal fully-differentiated human bronchial epithelial (NHBE) cells, with the pathway including interferon-regulatory factor (IRF)-3 activation and nuclear translocation (48). Further, interferon-stimulated gene (ISG)-15 expression is altered very early after infection and RSV infection has been shown to upregulate SOCS 1 and SOCS 3 in epithelial cells (46). NHBE cells infected with an RSV mutant virus lacking the G gene have distinct responses as compared to wild-type RSV (30). Notably, RSV mutant strains without secreted G induced less CCL2 and CCL5 with no apparent lung disease in mice. Interestingly, mice developed good antibody responses despite the attenuated infection (54). These findings suggest that RSV surface proteins signal through multiple pathways, and this may be an important means of reducing anti-viral type I IFN expression, thereby promoting virus replication.

Of interest, RSV does not induce robust, long term immunity and people may be repeatedly infected with the same and different strains of RSV (55, 56). These finding are particularly relevant to the multiple failed RSV vaccine trails to date, including the original formalin inactivated RSV (FI-RSV) vaccine as well as more recent subunit and live attenuated vaccines. The deficient response to both natural and artificial exposure to RSV antigens in human represents a barrier to the development of novel therapeutic or preventive strategies (57–64). Further, the immune response to both primary and repeat infections with RSV needs further study to better understand short- and long-term immunity. More detailed characterization of the response of healthy adults as compared to the elderly and to infants is also needed. The importance of elucidating the host response to RSV infection is underscored by recent clinical evaluation of prophylaxis with the anti-F protein monoclonal antibody (mAb) palivizumab in healthy preterm infants. In this single-blind, randomized, placebo-controlled trial, suppression of RSV replication did not have a major effect on reducing the RSV-associated asthma incidence at age 6 years, suggesting that other factors besides viral load contribute to the clinical severity (11, 65).

Type I IFNs are a group of related proteins that help regulate the activity of the immune system. The mammalian types are named IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta) (66, 67). IFN-α has 13 different subtypes in humans (α1/13; α2; α4; α5; α6; α7; α8; α10; α14; α16; α17; α21) (68) and is primarily produced by pDCs, while IFN-β is produced largely by fibroblasts; both have antiviral activity that is an important component of the innate immune response. Quantitative and qualitative differences in gene expression have been observed, with type I IFN being notably absent in the RSV infected cells (69). This result is consistent with results from the INFANT study, conducted by Argentine doctors to investigate the causes of respiratory diseases that seriously affect children such as RSV associated asthma and bronchiolitis, and pneumonia and influenza virus infection. In the INFANT study, RSV infection failed to induce a robust type I IFN response in the nasal mucosa of infants even when co-infected with influenza, which normally induces a robust response (70). Intriguingly, neonatal mouse models of RSV infection recapitulate these data from humans. Specifically, neonatal mice infected with RSV fail to induce a type I IFN response to RSV in contrast to adult

mice infected with RSV (71). Furthermore, as compared to non-treated controls, administration of IFN-α during infection of the neonate enhances the immune response to RSV infection 5 weeks later and prevents Th2 biased immune responses (including perivascular inflammation and mucus production) and airway hyperreactivity (71). Notably, studies examining human cord blood-derived pDCs exposed to RSV showed reduced type I IFN production when compared to vehicle control or left unstimulated (40). These recent correlations between type I IFN responses and RSV disease severity in infants merit further investigation. Here, we review the mechanism surrounding RSV and type I IFN production in humans and mouse models and discuss its implications for development of therapeutics and vaccines.

IFN Biology and RSV Disease

Human IFNs are classified as type I (IFN-I), type II (IFN-II), or type III (IFN-III) with each class binding to specific receptors. All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains (72). The ability to produce and respond to IFN-I is distributed in a wide variety of cells. This confers several autocrine and paracrine effects that have been extensively characterized, mainly in viral infections. IFN-I signaling is mediated through a common cell surface receptor, the IFN-I receptor (IFNAR), signaling through the JAK-STAT cascade leading to transcriptional upregulation of the IFN-ISGs. The IFN-II family is represented by a single gene product, IFN-γ, and is mainly produced by T lymphocytes and natural killer (NK) cells. The associated receptor (IFNGR) regulates several cell functions related to host defense to intracellular pathogens. IFN-λ comprises four subtypes: IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4. The members of this IFN-III family interact through a unique receptor, the IFN-λ receptor (IFN-λR). It has been shown that IFN systems differ in terms of tissue distribution of their receptors (73, 74). While IFN-α/β systems are more prominent on endothelial cells, they are expressed on all cells. On the other hand, IFN-λ expression is more restricted occurring predominantly on epithelial cells of the intestines and lungs (73). RSV infection induces high expression levels of IFN-λ 1–3 in the lungs, and these have been associated with more severe disease in children (75).

Type I and III IFNs are induced in virtually all cell types upon recognition of viral proteins by cytoplasmic and endosomal receptors (67, 68). IFN induction by RSV involves the recognition of RSV by TLRs which activate innate and acquired immunity (47, 49, 76–78). Leukocytes express several TLRs, including TLR2, TLR6, TLR3, TLR4, and TLR7 (79). Using knockout mice, TLR2 and TLR6 signaling in leukocytes has been shown to activate innate immunity against RSV by promoting TNF-α (tumor necrosis factor), IL-6 (interleukin-6), CCL2 (monocyte chemoattractant protein 1), and CCL5 (RANTES) (80). TLR4 was shown to also contribute to cytokine activation, and TLR2 and TLR6 activation was shown to be important for controlling viral replication *in vivo* in mice (81). TLR2 interactions with RSV promoted neutrophil migration and dendritic cell activation within the lung. TLR3 has been associated with more severe disease in mice models (82).

TLR4 is upregulated by RSV F protein interaction with TLR4 (76, 77). RSV G protein reduced TLR4 activity to baseline levels even in the presence of LPS (lipopolysaccharide), a strong stimulus, as assayed using a luciferase reporter construct for TLR4 signaling (76). As previously noted, RSV infection of normal human bronchoepithelial cells has been shown to modulate expression of SOCS, an effect mediated by G protein, leading to inhibition of type I IFN and ISG15 expression (48). These findings suggest that RSV surface proteins signal through multiple TLRs, and that enhanced expression and activation of type I IFNs may promote viral replication. Accordingly, IFN-α has been considered as an adjuvant for RSV vaccines as it is known to promote the activation and survival of virus-specific T cells (83).

The role of type I IFN in RSV infection, shedding, and disease severity in humans has been a subject of interest for decades (84, 85). While early studies struggled to identify a role for type I IFN in RSV disease (84–88), novel findings in recent years implicate type I IFN as determinants of RSV pathogenesis and immune responses (40, 41, 89, 90). RSV is a poor inducer of IFN and as a consequence, these IFNs and related cytokines have been speculated to have a limited role in the host defense against viral infection (84, 85, 87, 88). In fact, most hypotheses for RSV disease susceptibility in infants have been based on unique structural respiratory factors such as smaller airway size, lack of interalveolar pores and channels and different innervation patterns, inflammatory responses, and Th2 polarization of the adaptive immune response (78, 91, 92). Reconsideration of this bias is needed. Unlike the case in infants and children infected with influenza virus, IFN levels were undetectable or low in nasal secretions of infants and young children with RSV lower respiratory tract illness and did not correlate with resolution of clinical signs (84, 85). In a more recent study of infants in Argentina, type I IFN was detected more frequently in those infected with influenza A virus than in those infected with RSV or hMPV (93). RSV infected infants hospitalized with bronchiolitis displayed low, intermittent concentrations of IFN-α in respiratory secretions (87). No significant correlation was seen between these low respiratory IFN levels and RSV shedding (88). In human macrophages and peripheral blood mononuclear cells, RSV infection also induced minimal IFN activity and elicited no detectable transcription of IFN-α or IFN-β gene products (86), which is consistent with low IFN-α production in monocyte cultures from young infants (40).

Intriguingly, RSV-induced IFN-α expression by primary pDC collected from older children (from 1 to 5-year-olds) was notably higher than that of healthy full-term infant counterparts suggesting expression may be linked to age of the patient. Likewise, higher IFN-α expression was detected in primary pDCs obtained from healthy adults (40). Age at the time of initial infection is an important predictive factor for disease severity (94, 95). Cohort studies demonstrated that young infants (<6 months of age at initial infection) are at greater risk for severe disease than older infants (96, 97). Furthermore, long-term consequences

of RSV infection, such as development of asthma, are closely associated with severity of infection (10, 13). Extrapolation of response to vaccines or therapeutics in adults to those in young infants is thus highly problematic.

While clear linkage between IFN expression and RSV infection in humans has been elusive, a factor that needs further study is the prolonged incubation period of RSV disease in infants for whom the mean time from infection to symptoms is 4–6 days (87) in sharp contrast to the considerably shorter incubation period for influenza virus (average of 2 days). Type I IFN levels peak early after infection, and therefore sampling of respiratory secretions after symptoms appear may be too late to detect its antiviral effects for infants infected with RSV (84, 85, 93). Support for a function of type I IFNs in RSV pathogenesis is also growing from analysis of developmental innate immune mechanisms associated with poor type I IFN responses in newborn and young infants. For instance, and as mentioned above, RSV-induced IFN-α production appears to be primarily mediated by pDC, (40, 41). Indeed, compared to adult pDC production of type I IFN during RSV infection is substantially impaired in infants when disease is particularly severe (40, 90). Impairment in infants is explained by deficits either in MAVS or RIG-I at the post-translational level or by signaling events downstream of MAVS (40).

Additional evidence supporting a role for type I IFN in RSV infection and illness is the strong inhibition of IFN induction and signaling mediated by the two earliest genes transcribed among the 11 RSV gene products, NS1 and NS2 (89). NS1 and NS2 have been postulated to have various roles in RSV pathogenesis, generally linked to their anti-IFN activity. In addition to antagonizing type I IFN, NS1, and NS2 may negatively modulate dendritic cell maturation, affect Th17 lymphocyte proliferation, and promote Th2 polarization (35, 98–105). Deletion of anti-IFN proteins NS1 and NS2 in RSV live vaccines is responsible for attenuated phenotypes (89).

In the era prior to availability of antibodies against RSV, topical administration of recombinant IFN-α-2a accelerated control of upper respiratory tract symptoms during RSV infection in a randomized, double-blinded trial while not affecting duration or magnitude of viral shedding (106). This early result is of interest in the context of a more recent study of nasal epithelial cells from children with wheeze and/or atopy that showed reduced IFN-β in the nasal swabs in response to RSV infection, which was associated with increased viral shedding (107). However, consistent with other successful immunotherapies, this regimen elicited adverse effects and severity of those effects were dose-dependent (108). Common side effects due to IFN-α include flu-like symptoms, pulmonary toxicity (109), gastrointestinal symptoms (110), and neurotoxicity (111). Lethal toxicities associated with IFN-α regimen are rare and severe toxicities due to IFN-α are manageable if recognized expeditiously (112, 113). Importantly, IFN-α therapy in children (114) and infants with RSV-induced bronchiolitis (115) is generally safe and well tolerated. However, caution is still warranted in use of recombinant IFN-α in the context of an RSV infection, due to the side effects mentioned above.

It is also possible that antiviral agents may benefit from restoring natural type I IFN responses, which may lead to faster clearance of the virus. Two studies using healthy adult volunteers experimentally infected with RSV and treated with antivirals showed that rapid RSV clearance was related to reduced disease (116, 117). Similarly, a higher RSV load was linked to an increased risk for severe bronchiolitis in a large multicenter trial in the United States (28). None of these studies have attempted to define the mechanism by which higher viral load contributes to disease severity. In that regard, a study in infants with RSV bronchiolitis that described an association between viral load and disease severity (length of hospital stay) is of interest since a correlation was also noted with relative expression of ISG-56 (118). Finally, additional evidence for the role of type I IFN in disease severity comes from two studies of rare loss-of-function variants in *IFIH1* (which encodes a RIG-I-like receptor involved in the sensing of viral RNA); the variants result in defective innate recognition of RNA viruses preventing the activation of an efficient antiviral IFN response. These rare but serious immunodeficiencies lead to extreme susceptibility to RSV and other respiratory viruses (119, 120).

Responses in Mice

Mice provide a semi-permissive model for human RSV and while attempts to adapt a strain to this model have repeatedly failed (121) data from numerous laboratories demonstrate similarities in age related immune responses between humans and neonatal mice. Since, our current understanding of the features that contribute to severe RSV disease in infants is tied to our understanding of developmental immunity during the first year of life, the neonatal mouse model of RSV infection is a helpful tool (122–124). Numerous studies utilizing mouse models of RSV infection have revealed a bias toward a T helper type 2 (Th2) cytokine response when mice are initially infected as neonates as compared to adults (71, 125–128). Upon reinfection, mice initially infected as neonates mount significantly greater Th2 responses as compared to mice initially infected as adults (126). This skewed Th2 response upon reinfection is associated with lung dysfunction (lung eosinophilia, increased mucus production, and air hyperresponsiveness) (126, 127, 129). Such responses mirror observations made in infants with severe RSV disease (130–132). Production of type I IFN by pDC during RSV infection of the neonate mouse, as in humans, is considerably impaired. However, both pDC number and production of type I IFN in response to RSV increase with age; adult mice recruit substantially higher numbers of pDCs to the lungs after RSV infection when compared to those of same age that are not infected and to neonatal mice infected with RSV (71). A single dose of IFN-α or adoptive transfer of adult-derived pDCs (capable of mounting a type I IFN response), prior to a primary RSV infection, substantially impedes the Th2-biased immunopathology observed during reinfection (71). A related strategy to revert poor outcomes associated with RSV infection in neonatal mice has been administering Flt3 ligand to neonates before RSV infection (133). Ftl3 ligand is a growth factor that stimulates the proliferation of hematopoietic cells that triggers expansion of cDCs and pDCs in human cord

blood and strongly promotes IFN-α production by pDCs in response to viral exposure (134, 135). This treatment has led to increased lung DC numbers and reconditioning of the type I IFN pathway toward Th1-mediated immunity. In addition, these mice were protected from exacerbated airway disease upon adult re-exposure to RSV (133).

Treating mice with neutralizing mAbs against the RSV G protein reduced G protein-mediated lung inflammation. Specifically, TRL3D3, a human mAb against the G protein CCD, enhanced IFN responses, decreased airway inflammation, and improved lung function upon secondary infection, whereas mice treated with an anti-F mAb (palivizumab) had less IFN than mock infected mice (30, 33). Since RSV infection is inhibited by IFN-induced transmembrane proteins (71, 117), the impact of counteracting the G protein's suppressive effect on IFN production likely also contributes to the antiviral effect of such mAbs. Consistent with these results, intranasal IFN-α administration in neonatal mice prior to RSV infection appreciably reduced RSV viral load in both nasal associated lymphoid tissue and lungs when compared to age-matched controls (136).

Interestingly, while the IFN-α response to RSV progressively increases with age (40, 136); another cytokine IL33, an alarmin cytokine, decreases with age (126). Recent work has demonstrated that IL-33 is significantly greater in neonatal compared to adult mice during RSV infection. IL-33 signaling in the neonatal mouse model of RSV has been shown to induce RSV immunopathogenesis including Th2 bias (126). Elevations in IL-33 are inversely correlated with age at RSV infection (126) and severity of RSV infantile disease has been associated with elevated levels of respiratory IL-33 and polymorphisms within *ST2*, the receptor for IL-33, (137). IL-33 promotes Th2 responses via multiple signaling pathways that are summarized in **Figure 1**. Similarly, intranasal instillation of IL-33 significantly impaired the production of IFN-α/λ in the BALF and reduced the expression of IFN-stimulated genes in the lung following PVM infection (138). **Table 1** summarizes the significant advances in the role of age-dependent differences in various immune and non-immune cells related to the immune pathogenesis of RSV infection in infants. **Figure 1** highlights age-dependent differences in RSV-mediated immune pathogenesis.

Implications for RSV Vaccines and Therapeutic Agents

Current RSV vaccine candidates seek to induce high levels of RSV-specific serum neutralizing antibodies, which are associated with reduced RSV-related hospitalization rates. However, serum neutralizing antibodies may not be sufficient to prevent infection and/or induce protective responses. This feature of RSV biology was exemplified by the antibody responses induced to the FI-RSV vaccine in the 1960's, which elicited lower avidity, non-protective antibodies as compared to those that develop after natural RSV infection (150). Furthermore, mucosal antibodies have been shown to correlate better with RSV protection than serum antibodies in both infants and adults (151–153).

The majority of vaccine efforts to date have focused on the RSV F protein, based on the assumption that reducing RSV load will reduce or eliminate disease. While mAbs against RSV F protein (palivizumab) given to premature infants (at or before 35 weeks) do help to protect children with certain lung or heart conditions who are at high risk for severe RSV disease, such treatment does not fully protect from disease. Further, in a recent study of viral burden in healthy full-term infants (<70 days old), nearly a third experienced a multi-log rebound in viral load at around 2 weeks after onset of symptoms (154). Since viral load had declined by several orders of magnitude by that point, the most likely cause was mutational escape which is a well characterized response to anti-F protein mAbs (155).

In short, the role of RSV viral load as a driver for severity of infection remains controversial. On the one hand, quantitative RT-PCR correlation with disease severity in patients showed that viral load was associated with disease severity in younger patients although not in older patients (63). For patients intubated due to respiratory distress, RSV infection resulted in higher viral load than those not intubated, and higher viral loads were associated with longer hospitalization (156). In the adult human RSV challenge model, virus replication is inversely correlated with the level of nasal secretory neutralizing antibody prior to infection (157). Higher nasal immunoglobulin (Ig) A predicts lower infectivity and lower measures of viral replication (151) and low RSV-specific nasal IgA is an independent significant risk factor for RSV infection (158). On the other hand, several groups have failed to find an association of higher viral load in nasopharyngeal lavage (159) or nasal aspirates with either length of hospitalization, duration of oxygen supplement or severe bronchiolitis in either infants (160) or children (161).

The picture that is emerging is that primary reduction in viral load is useful, but not sufficient, to reduce the clinically relevant pathology. Accordingly, a combination of an anti-viral agent with an agent that reduces the RSV induced alteration in the innate immune response is the most likely route to improved outcomes. Targeting the F protein addresses the first issue. Targeting the G protein addresses the second issue; since anti-G protein mAbs also have potent antiviral activity, targeting the G protein alone may be sufficient to achieve both goals.

The optimal type of RSV vaccine employed, i.e., RSV F and/or G protein, will likely be dependent on the host target population (162, 163), with four groups being of interest: (1) infants and young children, (2) adults, (3) the elderly, and (4) pregnant woman. Immunization schedule (prime/boost) and the specific platform for delivery of the vaccine are also likely to be important (162, 164, 165). Consequently, there are a spectrum of RSV vaccines being tested that include live-attenuated and chimeric virus, purified F protein (including variants engineered to present predominantly the pre-fusion conformation), particle and vector-based presentations of the antigen(s) (165). For example, RSV F protein particle-based (Novovax) (166, 167) and RSV F subunit (GSK; NIH) vaccines are being evaluated for use in pregnant mothers, while RSV F protein particle-based (Novovax; Mucosis) and live-attenuated vaccines such as RSV deletion mutant vaccines, e.g., ΔM2-2 and ΔNS2 constructs (Sanofi; NIH) are being

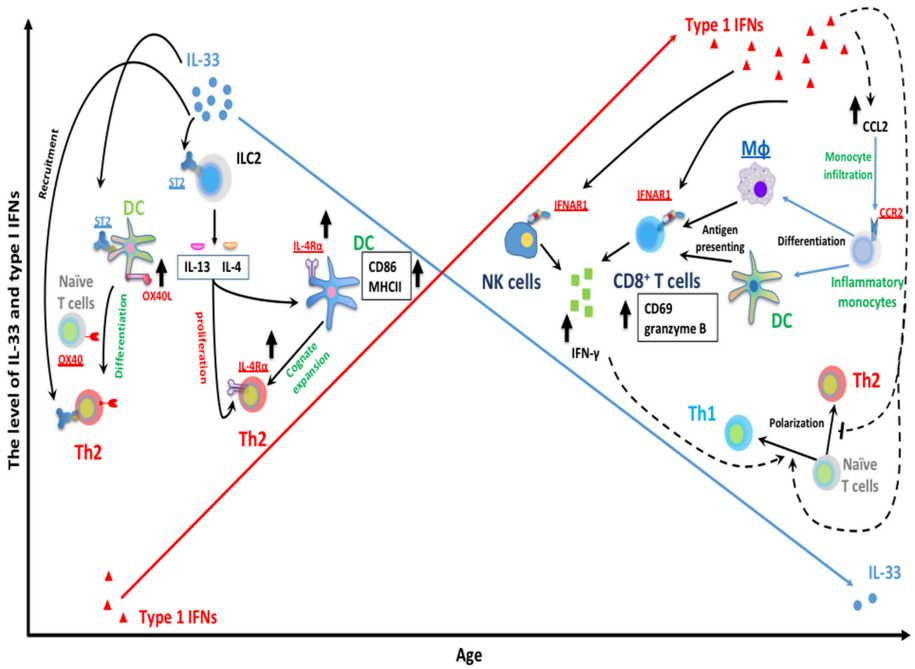

FIGURE 1 | Age-dependent differences RSV-mediated immune pathogenesis. The expression of RSV-induced IFNα is limited during infantile RSV infection and progressively increases with age. Type I IFNs are capable of suppressing Th2 development and promoting type 1 immunity. Type I IFNs have been implicated in the regulation of NK and $CD8^+$ T cells functionality. Type I IFNs can elicit the activation of cytotoxic IFN-γ^+ $CD8^+$ T cells by enhancing the recruitment of inflammatory myeloid cells into infected lungs. These infiltrating myeloid cells then differentiate into macrophage and DC, and acquire antigen presenting ability, subsequently activate $CD8^+$ T cells and trigger $CD8^+$ T cells IFN-γ production. In addition, type I IFNs can also act directly on $CD8^+$ T cells and NK cells by targeting its receptor IFNAR1 on membrane of $CD8^+$ T cells and NK cells. This results in the production of IFNγ, a key mediator for type 1 immunity, which presumably favors Th1 polarization from naïve $CD4^+$ T cells. In contrast with IFN-α, IL-33 has been implicated in the induction of Th2-biased immune pathogenesis during neonatal RSV infection. A large amount of IL-33 is rapidly released following RSV infection in neonatal but not adult mice. IL-33 can elicit ILC2-mediated IL-13/IL-4 production through its cellular receptor ST2 on ILC2. ILC2-derived IL13/IL-4 then can facilitate cognate expansion of Th2 by upregulating the expression of Th2 costimulatory molecules (CD86 and MHCII) on DC. ILC2-derived IL-4 also promotes the proliferation of Th2 cells. It involves the upregulation of IL4Rα on both DC and Th2 cells. Similarly, IL-33 can promote Th2 polarization from naïve $CD4^+$ T cells by targeting DC via ST2 receptors on DC and then enhance the expression of OX40L on DC (ligand for cellular receptor OX40 on naïve T cells).

targeted for the pediatric population with potential extension to older children and young adults (168). An important caveat for using live vaccines is the need to prevent transmission to the immune compromised, or those with reduced or waning immunity. An additional issue for vaccinating infants and young children is that the vaccine needs to balance safety (higher attenuation) and efficacy (lower attenuation). A promising recent study of an RSV vaccine candidate having a deletion of the M2-2 coding sequence showed downregulation of viral replication and upregulation of transcription and antigen synthesis (169). For healthy older adults, several RSV vaccine candidates are being considered, including vector-based platforms such as VXA-RSV F oral (Vaxart) and Ad26.RSV.preF (Janssen) (168). Given the high transmissibility of RSV, even a safe and effective vaccine will likely leave gaps in protection for high-risk, very young infants. Vaccinating pregnant women has become an area of high interest to induce passive protection in the infant by generating high maternal antibody titers.

Antibodies directed to dominant antigenic sites on the F protein have variable neutralization capacities with the most potent neutralization epitopes associated with the pre-F conformation (170–174). Stabilized F protein antigen in both pre- and post-fusion morphology are being explored (31, 172, 175, 176). The typical benchmark is achieving a protective titer at a defined time point, but the time course of increase in antibody titer is also an important parameter, which will likely differ according to vaccine type and composition. The RSV G protein is also an antigenic target for neutralizing antibodies, but despite this fact, the G protein has not usually been considered as a RSV vaccine candidate because of its variability across RSV strains (175–177). However, with the recent discovery of the G protein structure (29, 32), and the known role of the G protein oligomer on the virus surface vs. its monomeric secreted form (54, 178), there has been reinvigorated interest in its potential as a RSV vaccine candidate.

Passive transfer of antibodies is protective against severe RSV infection using polyclonal or monoclonal antibodies (mAbs; RSV-IVIG, palivizumab) (179) The ratio of antibody transfer and decay kinetics is considered a principal parameter to measure protection. More recent versions of mAbs have become available with improved antibody transfer and decay kinetics such as

TABLE 1 | Differences in immunological responses toward RSV in the respiratory tract.

Respiratory immune responses	Adult mice	Neonatal mice	Human infants
IFNα UPSTREAM SIGNALING			
Respiratory/pulmonary pDC	+ + + (71, 139)	+ (71, 139)	older (≥4 months) infants had fewer BAL pDCs than younger (<4 months) (140)
IFN-α	+ + + (71, 133, 139)	+ [(71, 139), Remot, 2016 #432]	IFN-α production by primary pDC collected from healthy term infants is lower than older children (from 1-year to 5-year-olds) (40)
IFNα MEDIATED IgA-PRODUCTION OF B CELLS			
Nasal associated lymphoid tissue—B cells	+ + + (136)	++ (136)	
Respiratory IgA	+ + + (136, 141)	+ (136)	IgA levels in nasal aspirates are lower in younger infants (4–8 months) compared to older infants and young children (9–21 months) during RSV infection (142, 143)
OTHER IMMUNE MEDIATORS			
$CD103^+/CD11b^+$ DC	+: $CD103^+/CD11b^+$ ratio (144)	+ + +: $CD103^+/CD11b^+$ ratio (144)	
	+ + +: CD80 and CD86 (144)	+: CD80 and CD86 (144)	
	+: OX40L expression (145)	+ + +: OX40L expression (145)	
$CD4^+$ T cells	Th1 responses >Th2 responses (126, 146)	Th2 responses >Th1 responses (126, 146)	Th2 responses > Th1 responses (131, 132, 147) (Cormier SA, Unpublished Data)
$CD8^+$ T cells	+ + +: IFN-γ producing (148)	+: IFN-γ producing (148)	+ + + (149) and Tc2 responses > Tc1 responses (Cormier SA, Unpublished Data)

(+, low; + + +, high).

MEDI-8897 which is optimized from the human antibody D25 that targets RSV pre-F protein (24, 170, 172, 180, 181). This type of treatment potentially offers novel immunotherapeutic strategies to bridge gaps with RSV vaccine candidates.

Many studies indicate that certain cytokines can mediate strong vaccine responses associated with a good outcome. For example, IFN-α2b is an FDA-approved therapy for adjuvant treatment of patients with certain cancers (182) and hepatitis C (183). Of particular interest is the recent demonstration that administration to neonatal mice of IFN-α prior to RSV infection increased RSV specific IgA production in nasal washes when compared to age matched controls (136). Furthermore, IgA levels became comparable to those of adult mice infected with RSV (136). In addition, IFN-α induced expression of B cell activating factor (BAFF) in nasal associated lymphoid tissue (NALT) (136). BAFF, a B cell survival factor and mediator of B cell activation and class switching, and APRIL, a TNF ligand family member that shares receptors with BAFF, regulate B cell survival, proliferation and differentiation. Gene expression analysis from NALT and lung homogenates further support a role for IFN-α in regulating granulocyte migration and neutrophil-mediated immunity (136).

Comparative studies of genetic background of mice has shown diverse influences on Th cell differentiation by controlling the capacity for IL-2-induced IL-4 production by naive CD4+ T cells. BALB/c mice are Th2-prone, while C57BL/6 mice are Th1-prone (28, 184–186). Notably, type I IFN pathways are reconditioned in neonatal BALB/c mice after RSV infection as lung dendritic cells (DC) numbers increase; the associated shift toward a Th1 response protected the mice from exacerbated airway disease (187). Adult mice produce considerably higher levels of type I IFNs in response to RSV than do neonatal mice. Finally, recent studies have implicated the type III IFN-λ as being significant for mucosal antiviral immune responses to RSV infection (41, 65).

Since SOCS-1 and SOCS-3 negatively regulate the IFN-induced signal cascade, and NS1, NS2, and G protein inhibit the type I IFN response, any of these viral proteins may prove to be useful targets to induce a more effective innate immune response (45, 50). Understanding how these viral proteins modify host immune responses is thus crucial to the development of effective countermeasures. Although no animal model perfectly mimics the human response, the mouse offers a far greater set of tools for analyzing the immune system than other popular models, such as the cotton rat, and the mouse has for that reason become the nearly exclusive model for studies on RSV and the host immune response.

Clinical Implications

Over the past decade, targeting the F protein has repeatedly produced disappointing clinical results. In particular, agents targeting the F protein have not been proven effective post-infection. This is not only problematic for the multiple populations in need of treatment but also for vaccines since healthy full term infants (<70 days old) experienced a significant rebound in viral load at around 2 weeks after onset of symptoms in nearly a third of the study population (154). Moreover, palivizumab is only approved for prophylaxis in premature infants and those at high risk for severe RSV disease. Retrospective analysis of samples from the clinical trials leading to approval of this drug revealed a striking skewing of TLR4

polymorphisms (188). Mutations that interfere with function of this key innate immune system receptor have an incidence in the general population of ∼10%, but 90% of the high-risk premature birth infants had a TLR4 mutation. This striking result has been replicated (78). As described above, the RSV F protein stimulates TLR4, while the G protein suppresses this signaling pathway (48). In the premature birth population, antibody mediated removal of the TLR4 stimulus should not impact the overall response since the pathway is already suppressed genetically. In the broader population, however, removal of that beneficial stimulus may contribute to the lower observed efficacy compared to what was expected.

In light of these empirical failures and the improved understanding of RSV disease mechanisms, interest has increased in the role of the other major viral envelope protein, the RSV G protein, on viral entry, on viral neutralization, and most critically on RSV-mediated pathology (33). In mouse pDCs, mutating the G protein CCD prevented suppression of IFN-α attributable to the G protein; the Fab of a murine mAb against this region of the G protein was nearly as effective as the mutation (39).

Human mAbs targeting the CCD of RSV G protein (189) have recently been compared to anti-F mAbs, as both prophylactic and therapeutic treatment in BALB/c mice. The results showed that targeting the G protein was more effective for reducing viral load, leukocyte infiltration, and pro-inflammatory cytokine expression in cell-free bronchial alveolar lavage (BAL) supernatants (190). These results are consistent with *in vitro* studies on the type I IFN response of normal human bronchial epithelial cells to RSV in conjunction with mAbs to either the F or G protein which showed clear superiority for targeting the G protein (48).

TLR3D3 is a native human mAb that binds the G protein CCD with low pM affinity; it has strong activities as both an antiviral and for immune response normalization (189). It is currently in IND-enabling preclinical development. In light of the accumulated results summarized here on the mechanisms underlying RSV disease, it is appropriate to test this agent as a post-infection treatment. If proven effective, design of a vaccine to induce comparable mAbs will benefit from recently published structural analysis of the binding of TRL3D3 to the G protein CCD (32).

CONCLUSION

RSV infections continue to be a major cause of morbidity and mortality around the world affecting a wide variety of patients. Infants, the elderly, and those with comorbidities are at particularly high risk of hospitalization and death. Mainstream therapy remains restricted to supportive care. Despite successful antigen presentation leading to high titer of neutralizing antibodies by several approaches, we still do not have a licensed vaccine. Although the single licensed monoclonal antibody, palivizumab, is effective, it protects only a minor fraction of the population at high risk. Advances in therapeutic and vaccine development for RSV has mainly been hampered by the lack of understanding of the immune response to the virus both in the setting of primary infection as well as recurrent reinfections. Diverse approaches have converged over the last few years on identification of Type I IFN as a key actor and a readily measured biomarker of the broader innate immune response. Clinical studies in human infants have shown that RSV is a poor inducer of type I IFN responses, and there is accumulating literature reporting an inverse correlation between type I IFN responses and disease severity.

As our understanding improves of how viral proteins modify host immune responses, and the age dependence of those responses, research efforts can focus on development of effective countermeasures to overcome the virus's sophisticated sabotage of the host immune system. Animal models, complemented by studies on human cells *in vitro*, continue to be essential in the discovery and/or confirmation of the key features surrounding the host-virus interaction. Mouse models have proven to be particularly informative, including demonstrations that neonatal mice fail to produce IFN-α in the setting of RSV infection due to poor pDC recruitment, and that administration of IFNα decreases Th2-biased immunopathology and viral load. In addition, and importantly, administration of IFNα enhances mucosal RSV specific IgA production, which is critical given the clinical evidence that suggests that mucosal antibodies correlate better than systemic antibodies with protection. Although the known toxicities of recombinant IFN precludes use in this setting, a variety of approaches to restoring the normal IFN response have been identified, offering new opportunities for both therapeutic and vaccine discovery.

AUTHOR CONTRIBUTIONS

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

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Sublingual Immunization With an RSV G Glycoprotein Fragment Primes IL-17-Mediated Immunopathology Upon Respiratory Syncytial Virus Infection

In Su Cheon[1†], Joo Young Kim[2†], Youngjoo Choi[1†], Byoung-Shik Shim[1], Jung-ah Choi[1], Dae-Im Jung[1], Jae-Ouk Kim[1], Thomas J. Braciale[3], Hyewon Youn[4,5], Man Ki Song[1] and Jun Chang[2*]*

[1] Laboratory Science Division, International Vaccine Institute, Seoul, South Korea, [2] Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea, [3] The Beirne B. Carter Center for Immunology Research and Department of Pathology, The University of Virginia, Charlottesville, VA, United States, [4] Department of Nuclear Medicine, Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea, [5] Cancer Imaging Center, Seoul National University Hospital, Seoul, South Korea

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***Correspondence:**
Man Ki Song
mksong@ivi.int
Jun Chang
tcell@ewha.ac.kr

[†]These authors have contributed equally to this work

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Respiratory syncytial virus (RSV) is the leading cause of serious respiratory tract disease but there is no licensed RSV vaccine. Immunopathological mechanisms have long been suspected as operating in the development of severe RSV disease and have hampered the development of safe and effective vaccines. Here, we show that unlike intranasal immunization, sublingual immunization with RSV glycoprotein fragment containing the central conserved region (Gcf) primes the host for severe disease upon RSV challenge. This increased pathology does not require replication by the challenge virus and is associated with massive infiltration of inflammatory cells, extensive cell death, and excessive mucus production in the airway and lungs. This exacerbated RSV disease primed by sublingual Gcf immunization is distinct from the immunopathology by G-expressing vaccinia virus or formalin-inactivated RSV, and preceded by prominent IL-17 production. IL-17 deficiency abolished the enhanced disease. Our results suggest a novel mechanism of RSV vaccine-induced immunopathology by IL-17, and highlights the importance of vaccination site.

Keywords: RSV, glycoprotein, sublingual, IL-17, immunopathology

INTRODUCTION

Human respiratory syncytial virus (RSV) is a negative sense, single-stranded RNA virus belonging to the Paramyxovirus family. RSV infection is the leading cause of lower respiratory tract disease in infants and young children, and elderly worldwide (1, 2). However, there is no safe and effective vaccine presently licensed for human use. In the 1960s, children that received formalin inactivated RSV (FI-RSV) vaccine experienced severe enhanced disease, characterized by extensive pulmonary inflammation and eosinophilia, upon subsequent natural infection with RSV (3, 4). Similarly, mice immunized with a recombinant vaccinia virus expressing RSV G protein (vvG) also experienced extensive pulmonary inflammation and pulmonary eosinophilia upon RSV infection, mimicking

the enhanced disease in children that received FI-RSV vaccination (5). The vaccine-enhanced disease caused by FI-RSV or vvG vaccination was also typified by extensive secretion of Th2 cytokines, and abrogating the functions of these cytokines reduced disease severity in both models (6, 7). The similar disease characteristics of pulmonary eosinophilia and Th2-biased cytokine response following RSV challenge in FI-RSV- and vvG-vaccinated subjects implicated RSV G protein as the likely cause of disease enhancement (8, 9). Accordingly, subsequent studies have revealed that RSV G protein possesses immuno-modulatory properties capable of altering the immune response in the RSV infected host (10–12). For example, it has been reported that RSV G protein inhibits the development of an innate immune response normally elicited by the virus and endotoxin (12). Johnson et al. also reported that priming with secreted form of G glycoprotein augmented IL-5 production and tissue eosinophilia after RSV challenge (10). Another mechanism of immune modification adopted by RSV G protein includes "chemokine mimicry" utilizing the CX3C chemokine-like motif (aa 182-186) within its central conserved region of G, which contains marked similarity to the receptor binding region of fractalkine, CX3CL1, thereby mimicking fracktalkine and interfering with leukocyte chemotactic activity (13, 14). Also, the immune response to a peptide corresponding to $G_{183-197}$, (a known $CD4^+$ T cell epitope within G protein) has been linked with severe pulmonary eosinophilia suggesting involvement of this T cell epitope in the disease enhancement (15).

In present study, we have identified a novel vaccination route dependent type of RSV vaccine-induced disease, caused by prior exposure to RSV G protein distinct in mechanism and features, from the enhanced disease produced by vaccination with FI-RSV or vvG. We report that sublingual administration of Gcf, a recombinant polypeptide corresponding to the central conserved fragment of the RSV G protein predisposes the immunized animals to enhanced pulmonary disease upon challenge with live RSV. The observed disease enhancement was characterized by prominent IL-17 production, massive infiltration of inflammatory cells, and excessive mucus production in the airway and lungs of the affected animals. Moreover, we show using Gcf vaccinated IL-17 knockout mice that IL-17 is prerequisite for this Gcf-mediated disease enhancement.

MATERIALS AND METHODS

Materials

For cell culture and virus preparation, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY, USA) and Lonza (Basel, Switzerland), respectively. To measure cytokine concentrations, cytometric bead array (CBA) mouse inflammation/Th1/Th2/Th17 cytokine kit, mouse IL-5 and IL-13 flex sets were purchased from BD Biosciences (San Diego, CA, USA). All reagents for flow cytometry including Golgi Plug, Cytofix/Cytoperm solution, anti-mouse CD16/32 (Mouse BD Fc Block^{TM}), CD4-APC-Cy7, CD44-FITC, IFN-γ-APC, IL-17-PE, IL-5-APC, CD11c-FITC, CD45-PerCP, Siglec-F-PE, and Ly6G-PE-Cy7 were purchased from BD Biosciences. For development of ELISA, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from Southern Biotechnology (Birmingham, AL, USA). HRP-conjugated goat anti-mouse IgG1 and IgG2a were purchased from Zymed Laboratories (San Francisco, CA, USA).

Virus Preparation

RSV A2 strain was propagated in HEp-2 cells (ATCC, Manassas, VA, USA) grown in DMEM containing 3% of FBS. RSV replication was confirmed by formation of syncytia. Infected cells were harvested by scraping at day 3 or 4 post infection. Harvested cells were lysed by sonication, and virus particles were isolated via high-speed centrifugation. Virus titer was determined by standard RSV plaque assay.

Preparation of Gcf

The plasmid containing genetic sequence for Gcf derived from RSV A2 G protein spanning from amino acid residues 131–230 was prepared as follows. Gcf plasmid was transformed into *Escherichia coli* BL21 (DE3) strain (Novagen, Madison, WI, USA). Transformed *E. coli* were grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 100 mg/ml of ampicillin for selection. Bacterial culture was transferred into fresh LB medium and cultured at 37°C while shaking at 180 rpm until OD_{600} of 0.6∼0.8. Protein expression was induced by the addition of 0.5 M IPTG. *E. coli* were harvested by centrifugation at 6,000 rpm for 10 min. Bacterial pellets were suspended in binding buffer (20 mM Tris, 0.5 M NaCl, pH 7.9) and disrupted by sonication on ice. Soluble and insoluble fractions were separated by centrifugation for 40 min at 20,000 rpm. Soluble fractions were applied to a Talon metal affinity column, washed with binding buffer containing 20 mM imidazole, and then the proteins were eluted by using an elution buffer (300 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH 7.4). The purified proteins were dialyzed in PBS. The endotoxin in each purified protein was removed by using Triton X-114 as previously described (16). The endotoxin level of each protein was measured by the limulus amebocyte lysate (LAL) assay kit according to the manufacturer's instructions (Lonza). Purified proteins were electrophoresed on 15% SDS-PAGE and the protein bands were visualized by staining with Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA, USA). Protein concentration was determined by Bradford protein assay kit (Bio-Rad Laboratories).

Mice and Immunization

Female BALB/c mice, 6–8 weeks old, were purchased from Orient Bio Inc. (Seoul, Korea). IL-17 knockout mice were provided by Yoon-Keun Kim (POSTECH, Korea). All mice were maintained under specific pathogen-free condition, and all studies were approved by Institutional Animal Care and Use Committee (IACUC) at International Vaccine Institute (Approval No. 2014-001). Mice were immunized with 20 μg of Gcf with 2 μg of CT (List Biological Lab. Inc. Campbell, CA, USA) via i.n. or s.l. route, and a booster immunization was administered 14 days after the primary immunization. For s.l. immunization, 20 μl of prepared antigen was gently placed underneath the tongue of the anesthetized mice. Following antigen delivery, mice were

maintained with heads positioned in anteflexion for at least 30 min. For i.n. immunization, 50 μl of prepared antigen were administered into the left nostril of the anesthetized mice. Three weeks after the booster immunization, mice were challenged with 2–3 × 10^6 PFU of live RSV A2. As control, mice were also immunized with 2 μg of CT sublingually, with 1 × 10^7 PFU of vvG by scarification or with 1 × 10^6 PFU of FI-RSV intramuscularly.

Cytokines

Mice were immunized on days 0 and 14 and challenged with RSV A2 as described above. On expected days (0–9 days) post-challenge, mice were sacrificed, and Bronchoalveolar lavage (BAL) fluids and lungs were harvested. Cytokine levels in BAL fluid and lung homogenate were determined using CBA mouse inflammation/Th1/Th2/Th17 cytokine kit, mouse IL-5 flex set, and mouse IL-13 flex set according to the manufacturer's protocol.

Lung Virus Titration

Lung virus titer was determined from the lungs harvested from mice at day 4 post challenge. Harvested lungs were passed through 70 μm cell strainer (BD Biosciences) in serum-free RPMI-1640. Lung supernatant was collected via centrifugation, and RSV titer in the lung supernatant was determined by plaque assay on HEp-2 cells.

Detection of Eosinophils and Neutrophils in BAL and Lung

On expected days (0–9 days) post-challenge, mice were sacrificed and BAL fluids and lung samples were collected and cells were isolated from the BAL fluid or lung supernatant by centrifugation. Isolated cells were resuspended in FACS staining buffer (PBS with 1% FBS) and stained with CD11c-FITC, CD45-PerCP, Siglec-F-PE, and Ly6G-PE-Cy7 in the presence of anti-mouse CD16/32. Analysis of cell surface marker expression was performed using a BD LSRII flow cytometer (BD biosciences) and FlowJo software (Tree Star, San Carlos, CA, USA). A total of 100,000 events were analyzed per sample. Based on cell surface markers expression two different cell type were identified: $CD45^+$, $CD11c^-$, $SiglecF^+$ cells as eosinophils and $CD45^+$, $CD11c^-$, $Ly6G^+$ cells as neutrophils.

Expression of T-bet, GATA-3 and RORγt in CD4 T Cells

Fourteen days after the booster immunization, immunized mice were sacrificed, spleens were harvested, and single cell suspension was prepared by passage of spleen samples through 70 μm cell strainer (BD Biosciences) in serum-free RPMI-1640. To examine the expression of t-bet (Th1), GATA-3 (Th2), and RORγt (Th17) transcription factors, cells were stimulated with 10 μg/ml of RSV G peptide corresponding to the amino acid (aa) sequence 183–195 (WAICKRIPNKKPG) for 18hr at 37°C. Cells were stained with anti-mouse CD4-APC-Cy7 and CD44-FITC. Cells were then fixed, permeabilized using Cytofix/Cytoperm solution, and further stained with anti-mouse T-bet-BV421, GATA-3-PE, and RORγt-APC and anti-mouse IL-17-PE or anti-mouse IL-5-APC. The cells were analyzed using BD LSRII flow cytometry and FlowJo software.

ELISA for Detection of Antibodies

Levels of Gcf- or RSV A2-specific antibodies in the sera and BAL fluids were detected by ELISA. In brief, 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μl of 2 × 10^4 PFU/ml of purified RSV A2 diluted in 0.05 M carbonate-bicarbonate buffer at 4°C. After blocking with PBS containing 5% dried-skim milk for 1 h at room temperature, serially diluted serum or BAL fluid samples were added into the plate and incubated for 1 h at 37°C. Gcf- or RSV A2-specific antibodies were detected with HRP conjugated antibodies specific for mouse IgG, IgG1, IgG2a, or IgA followed by addition of TMB substrate for development. The absorbance at wavelength 450 nm was measured, and the endpoint titer was determined using O.D. cut-off values of 0.2.

Histology

For histological analysis, mice were sacrificed at day 4 post-challenge and lungs were harvested following perfusion with 10 ml of heparinized PBS. Harvested lungs were fixed in 4% formalin for 48 h, embedded in paraffin, sectioned. Staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) was performed to demonstrate inflammation and mucus production, respectively. Pathological score assigned representing the inflammatory cell infiltration shown in H&E staining and PAS positive cells per millimeter of bronchial basement membrane (mmBM) were measured by MetaMorp 4.6 (Universal imaging, Downingtown, PA, USA). TUNEL assay was performed using *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Fluorescence Imaging

To assess the localization of antigens after their application, the anesthetized mice were injected with 5 μg of Alexa Fluor® 647-streptavidin conjugate (Invitrogen) via i.n. or s.l. route. The distribution of the fluorescence dye was monitored by *in vivo* imaging system (IVIS Lumina III; PerkinElmer Health Sciences, Waltham, MA) with Ex/Em of 640 nm/710 nm at 0.5, 1, and 24 h following injection. The average photon radiance on the certain surface of a mice was expressed as photons *per second* per centimeter squared per steradian (p/s/cm^2/sr).

Statistical Analysis

All data were plotted as a mean ± standard error, and statistical differences were determined using GraphPad Prism version 7 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed for significance using an unpaired two-tailed Student's *t*-test, one-way analysis of variance (ANOVA) or two-way repeated-measures ANOVA with Tukey *post-hoc* test for group comparisons. The difference was considered statistically significant when the $P < 0.05$.

RESULTS

Recombinant polypeptide vaccine corresponding to the structurally conserved central core region of the RSV G protein (Gcf) has previously been developed (17). In an effort to evaluate the effectiveness of mucosal delivery of the polypeptide vaccine, we compared delivery by the intranasal (i.n.) or sublingual (s.l.) route of the Gcf polypeptide in a Cholera Toxin (CT) based adjuvant for protection against RSV infection. Sublingual immunization with CT alone was used as negative control, and FI-RSV- and vvG-immunization were used as controls to compare potential vaccine-enhanced disease which may arise from receiving an RSV G-based vaccine. There was a significant increase in the RSV-specific serum IgG titers detected in animals immunized with Gcf via i.n. and s.l. routes (**Figure 1A** and **Figure S1A**). Upon challenge with live RSV, significant reduction in RSV titer was observed in the lungs of Gcf-, FI-RSV- and vvG-immunized animals indicating that Gcf immunization via both i.n. and s.l. routes effectively reduced lung virus titer (**Figure 1B** and **Figure S1B**). Surprisingly, however, unlike the animals that received intranasal Gcf immunization, animals that received sublingual Gcf immunization suffered severe weight loss following RSV challenge (**Figure 1C** and **Figure S1C**). These observations suggested that prior exposure to this central conserved fragment of RSV G protein delivered via sublingual mucosa primes the host for enhanced morbidity upon subsequent RSV infection with a morbidity profile. Further, these data suggested that enhanced morbidity observed in Gcf s.l. group animals following RSV challenge was not caused by differences in antibody levels or the efficiency of virus clearance at the site of infection.

Next, to determine if enhanced morbidity in Gcf s.l. group, was reflected in the extent of lung injury, lung tissues were harvested at day 4 post RSV challenge and evaluated by light microscopy. Inflammatory cell infiltrations in the peribronchial and perialveolar regions were conspicuous in all RSV-challenged animals. However, the degree of inflammatory cell infiltration was markedly higher in the animals that received sublingual Gcf immunization, FI-RSV immunization and vvG immunization than in the animals that received intranasal Gcf or CT immunization (**Figure 2A** and **Figures S2A,B**). Inflammatory cytokines such as IL-6, TNF-α and MCP-1 were also higher in the animals that received sublingual Gcf immunization and FI-RSV immunization than in the animals that received CT immunization (**Figure 2B**). In addition, detection of cell death by TUNEL assay revealed extensive cell deaths in the airway and lungs of Gcf s.l group animals (**Figure 2C**). Increased cell death was also seen in RSV challenged vvG-immunized animals (**Figure S2C**). Such extensive cell death was not observed following intranasal Gcf or CT immunization (**Figure 2C** and **Figure S2C**). Furthermore, goblet cell hyperplasia and excessive airway mucus secretion which are features of the response to RSV infection in FI-RSV and vvG primed mice was also detected by PAS staining in the airway of animals in sublingual Gcf-immunized animals following RSV challenge (**Figure 2A** and **Figure S2A**). Goblet cell hyperplasia and airway mucus secretion were minimal in challenged animals that received intranasal Gcf- or CT-immunization. Histopathologic scoring of lungs also indicated significant lung pathology exclusively in sublingual Gcf- and vvG-immunized animals undergoing challenge RSV infection (**Figure S2B**). Together, these data demonstrated that the enhanced pathology induced with RSV infection after sublingual Gcf immunization is associated with massive infiltration of inflammatory cells and cytokines, extensive cell death, and excessive mucus production in the airway and lungs upon RSV infection. In addition, the finding that different manifestations of disease were displayed in FI-RSV-, vvG- and sublingual Gcf-primed groups led us to surmise that FI-RSV, vvG and sublingual Gcf immunization may cause disease enhancement by distinctly different mechanisms.

Pulmonary eosinophilia is a well-known disease marker for RSV vaccine-enhanced disease (18, 19). Therefore, we examined eosinophil recruitment to the airway and lungs of Gcf- or FI-RSV-immunized animals following RSV challenge. As expected, we detected significant increase in BAL fluid eosinophil counts in FI-RSV-immunized animals (**Figure 3A**). Unexpectedly, BAL fluid eosinophil counts were also elevated in RSV challenged animals primed by sublingual Gcf immunization and at a level seven-fold higher than the increase detected in FI-RSV-immunized animals. Group-to-group statistical comparison confirmed significance of the difference in eosinophil count in Gcf s.l. group over the Gcf i.n. group (**Figure 3A**). These differences in eosinophil count between sublingual Gcf- and FI-RSV-primed mice undergoing challenge RSV infection were also evident in the infected lung parenchyma (**Figure 3B**).

At the same time, we also examined neutrophil recruitment to the airway and lungs. We detected significant increase in BAL fluid neutrophil counts in animals given sublingual Gcf immunization (**Figure 3C**). BAL fluid neutrophil counts were also elevated but to a lesser extent in challenged mice primed by Gcf via the i.n. route. Again, neutrophil count in the lungs among the immunization groups directly paralleled findings in the BAL fluid i.e., significantly increased neutrophil counts in the lungs of sublingual Gcf-primed animals over intranasal Gcf-primed animals (**Figure 3D**). Of note, we did not detect significant increase in neutrophil recruitment to the lungs in animals that received FI-RSV immunization compared to control CT primed and challenged mice. These results suggest that just as pulmonary eosinophilia characterizes vaccine-enhanced disease generated by prior immunization with FI-RSV, pulmonary neutrophilia appeared to be a prominent feature of enhanced disease produced by prior sensitization to RSV G protein introduced into the sublingual mucosa, and that the enhanced disease produced via sublingual immunization with G protein is distinctly different from the manifestation of disease exacerbation caused by FI-RSV immunization i.e., enhanced disease associated with the recall response to RSV induced by priming with FI-RSV characterized by pulmonary eosinophilia while priming to Gcf via the s.l. route results following RSV challenge in enhanced disease characterized by both pulmonary eosinophilia and neutrophilia.

Next, to further examine the mechanism of enhanced disease caused by sublingual Gcf immunization, we examined cytokine levels in the airway and lungs of all immunized animals at 0∼9 days post-challenge. BAL fluid IFN-γ, IL-4, IL-5, and IL-13

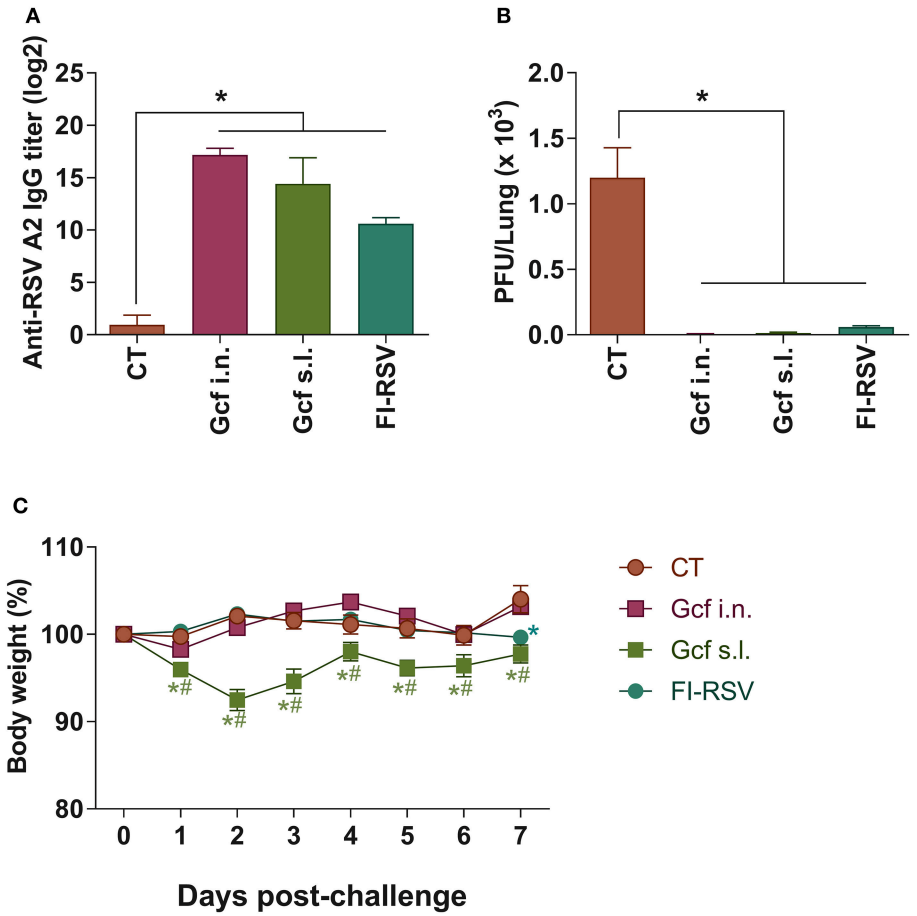

FIGURE 1 | Anti-RSV immunity and enhanced disease in RSV challenged mice following sublingual Gcf immunization. **(A)** RSV-specific serum IgG titers measured at day 14 post-booster immunization by ELISA using 2×10^3 RSV A2 per well as the coating antigen. Titers are indicated in $\log_2$. **(B)** Lung RSV titers at day 4 post-RSV A2 challenge determined by plaque assay. **(C)** Weight loss following RSV A2 challenge. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 5$). *Significant different with CT group ($P < 0.05$). #Significant difference with FI-RSV group ($P < 0.05$).

concentrations were significantly elevated in FI-RSV-immunized animals compared to animal given CT- or Gcf-immunization via either the i.n. or s.l. route. BAL fluid IFN-γ, IL-2, IL-5, and IL-13 concentrations were also significantly increased in Gcf s.l. group animals (**Figure 4**). With the significant increase in IL-17 concentration detected in the BAL fluids of animals in Gcf s.l. group (**Figure 4F**), the IL-17- and/or IFN-γ- producing CD4 T cells were also increased in the lungs and mediastinal lymph node of Gcf s.l. group following RSV challenge (**Figure 5**). Both Th1 and Th2 cytokines represented by IFN-γ and IL-4/5/13, respectively, were notably elevated in the BAL fluid of vvG-immunized mice, as previously described [**Figure S3A**; (20)], and IL-5 producing CD4 T cells were increased in the lung of FI-RSV-immunized mice (**Figure 5B**). However, we observed a significant increase in IL-17 levels in the BAL fluids and IL-17-producing CD4 T cells in the lungs and the draining lymph node of Gcf s.l. group animals, demonstrating the difference in the mechanism of disease exacerbation induced by FI-RSV, vvG and sublingual Gcf immunization. An exaggerated Th17 T cell-dependent cytokine response is most likely responsible for the enhanced RSV disease in animals given sublingual Gcf immunization, whereas, as our results and published findings (21) suggest, excessive Th1 and Th2 cytokine responses contribute to the enhanced RSV disease in animals primed by FI-RSV or vvG immunization.

We next analyzed the expression of canonical Th1, Th2, and Th17 transcription factors by intracellular staining in CD4 T cells in the spleens of immunized mice prior to RSV challenge. In keeping with the above findings, this analysis revealed significant increase in the percentage of RORγt expressing CD4 T cells in animals immunized with Gcf, while the percentages of t-bet (Th1) and GATA-3 (Th2) expressing CD4 T cells were increased in the spleens of vvG-immunized animals (**Figure S3B**). These results indicate that exposure to RSV G protein via the sublingual mucosa primes the host for Th17-biased immune response.

Next, we assessed whether a deficiency in IL-17 would affect the development of enhanced disease during RSV infection by using IL-17 deficient (KO) animals primed with Gcf via

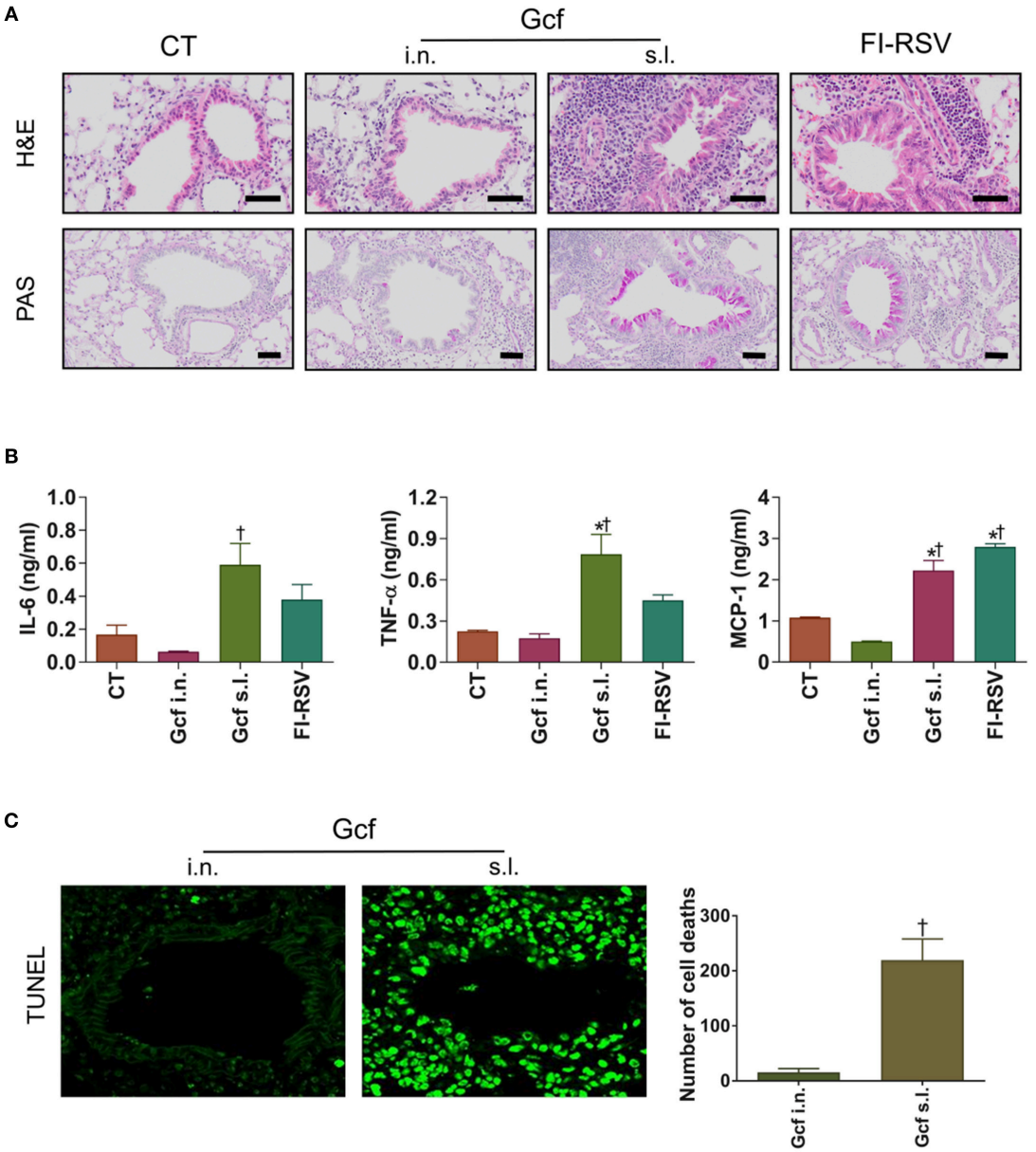

FIGURE 2 | Lung histology and inflammatory cytokines of mice immunized with Gcf through different routes. All samples were collected at day 4 post-RSV challenge and prepared from formalin-fixed, paraffin-embedded lung tissues. H&E staining, TUNEL assay, and PAS staining were performed to determine inflammation, cell death, mucus secretion, respectively. The BAL fluid was collected at day 2 post-RSV challenge and performed CBA assay for the analysis of cytokines. **(A)** H&E staining (1st row, 20X magnification) and PAS staining (2nd row, 20X magnification) of lung samples harvested at day 4 post-challenge. Scale bars indicate 50 micrometer. **(B)** IL-6, TNF-α, and MCP-1 levels in the BAL fluids. **(C)** TUNEL$^+$ cells (40X magnification) and quantification of cell death represented by TUNEL$^+$ cells. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 3$). *Significant difference with CT group ($P < 0.05$). †Significant difference with Gcf i.n. group ($P < 0.05$).

s.l. route. First, we determined Gcf-specific serum IgG2a/IgG1 ratio in order to determine Th1-Th2 bias. In WT animals, the IgG2a/IgG1 ratio was similar between the Gcf i.n. and s.l. groups (**Figure 6A**). However, the difference between serum IgG2a and IgG1 levels was significantly reduced in the FI-RSV group, indicating skewing of the immune response toward Th2. In IL-17 KO animals, the IgG2a/IgG1 ratios did not vary significantly between the immunization groups and there was no significant decrease in the IgG2a/IgG1 ratio in FI-RSV group in contrast to that observed in WT animals (**Figure 6A**).

We also observed a difference following RSV challenge in morbidity between WT and IL-17 KO mice as a function of prior immunization strategy. Among WT mice, animals immunized

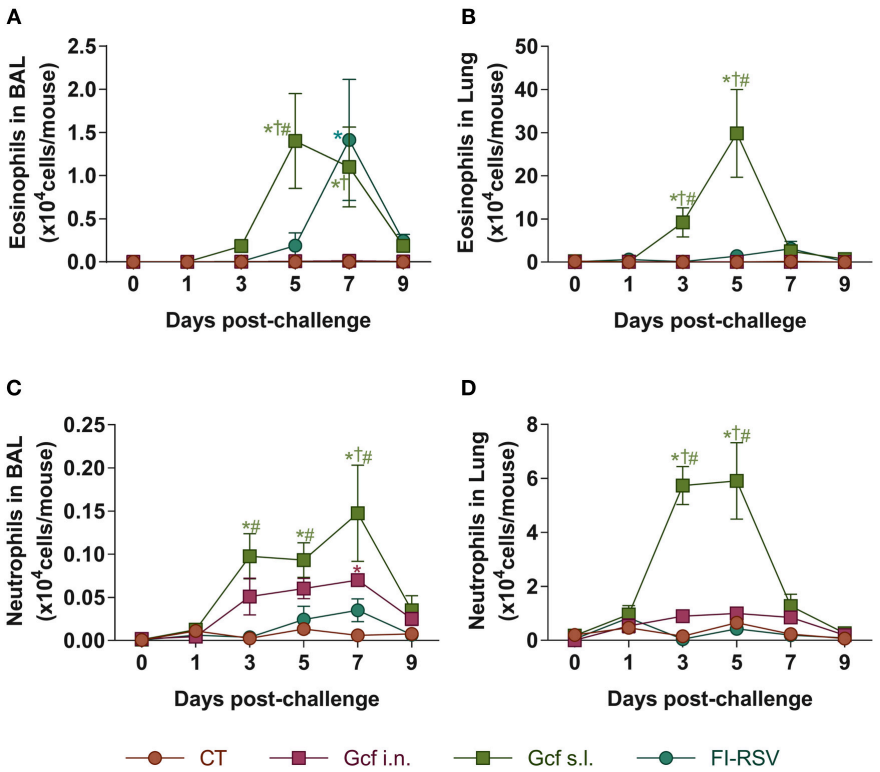

FIGURE 3 | Eosinophil and neutrophil recruitment to the airways and lungs of Gcf-immunized mice following live RSV challenge. Eosinophil ($CD45^+CD11c^-SiglecF^+$) count in **(A)** BAL fluids and **(B)** lung tissues after the RSV challenge. Neutrophil ($CD45^+CD11c^-Ly6G^+$) count in **(C)** BAL fluids and **(D)** lung tissues after the RSV challenge. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 6$). *Significant difference with CT group ($P < 0.05$). †Significant difference with Gcf i.n. group ($P < 0.05$). $^{\#}$Significant difference with FI-RSV group ($P < 0.05$).

with Gcf s.l. or FI-RSV experienced more severe weight loss than animal that received no immunization or intranasal Gcf immunization (**Figure 6B**). The WT Gcf s.l. primed animals experienced prolonged weight loss and delayed weight recovery following RSV challenge, which was more pronounced even than in animals primed with FI-RSV. By contrast, among IL-17 KO mice, the Gcf s.l. primed animals experienced weight loss that was comparable to control unimmunized mice undergoing RSV infection (**Figure 6C**), suggesting a link between IL-17 expression and enhanced morbidity following RSV challenge in mice primed with Gcf via sublingual route. Histological analysis of infected lungs further supported a critical role for IL-17 in enhanced morbidity as there was a significant reduction, compared to WT IL-17 sufficient mice, in inflammatory cell infiltration and mucus secretion in the airway of IL-17 deficient Gcf s.l. primed animals following RSV challenge. Interestingly, severe inflammatory cell infiltration and excessive mucus secretion was still observed in FI-RSV immunized animals, even in the absence of IL-17 (**Figure 7**). Pulmonary eosinophilia was detected in FI-RSV group animals in both WT and IL-17 KO models (**Figure 8**). However, eosinophils as well as neutrophils were significantly reduced in the BAL fluids of IL-17 KO Gcf s.l. primed animals compared to the corresponding primed and challenged WT mice. In sum, these data strongly suggest a critical role of IL-17 as a mediator of enhanced disease following RSV infection in animals sensitized to RSV G via sublingual mucosa.

Finally, to gain insight into the mechanisms leading to severe immunopathology by sublingual administration, we analyzed and compared the antigen trafficking following sublingual or intranasal administration using fluorochrome-conjugated protein. Fluorescence was detected near the tongue and salivary glands of the mice at 0.5 h after sublingual administration of Alexa Fluor 647 conjugated with streptavidin and fluorochrome-conjugated protein was no longer detectable within an hour (**Figure 9**). Draining lymph nodes around the sublingual mucosa, such as submandibular and deep cervical lymph nodes, showed no significant fluorescence during the indicated time period (**Figures 9A,B**). In contrast, in the case of intranasal administration, a large amount of fluorescence was observed in the lung tissue and the mediastinal lymph node at the same time point and even after 24 h (**Figures 9A,C**), suggesting that antigen trafficking and lymphatic drainage is different between sublingual and intranasal routes of immunization.

DISCUSSION

During our evaluation of various mucosal immunization routes to administer our RSV G-based subunit vaccine candidates,

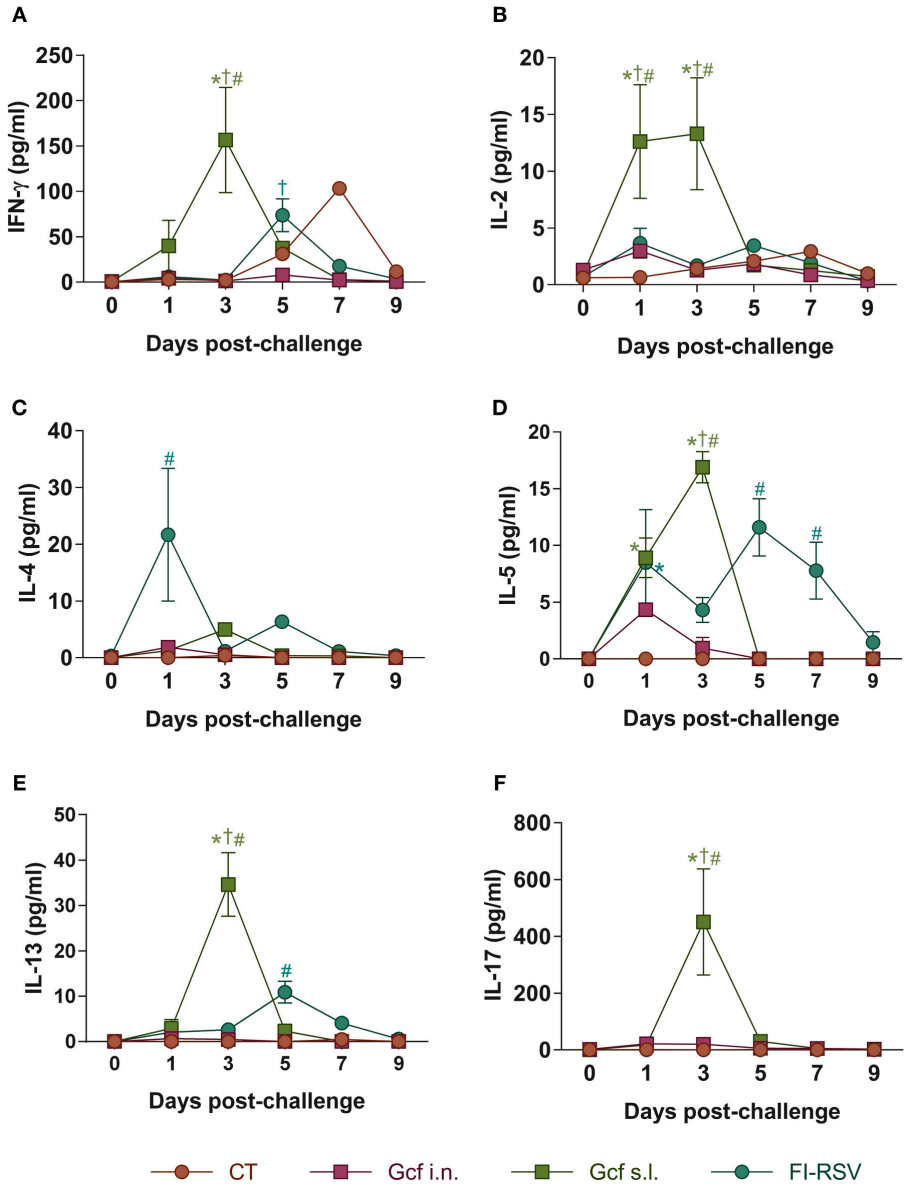

FIGURE 4 | Cytokine profiles in the airway of mice immunized with Gcf. **(A–F)** IFN-γ, IL-2, IL-4, IL-5, IL-13, and IL-17 levels in BAL fluids of mice immunized with Gcf after RSV A2 challenge determined by CBA. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 6$). *Significant difference with CT group ($P < 0.05$). †Significant difference with Gcf i.n. group ($P < 0.05$). #Significant difference with FI-RSV group ($P < 0.05$).

we unexpectedly observed that administration of Gcf via sublingual mucosa exacerbated disease severity upon live RSV challenge. Intranasal administration of the same antigen did not cause disease exacerbation, instead intranasal Gcf delivery conferred protection against RSV infection characterized by significant reduction in morbidity, lung virus titer, lung inflammation, and airway mucus secretion following live RSV challenge. Hence, because of the exacerbated disease severity we further investigated this immunization strategy as it related to the vvG and FI-RSV immunization models, which also have been shown to induce disease exacerbation upon RSV infection (22). First, our study demonstrated no correlation among the severity of disease, viral replication in the lung tissues, and the levels of RSV-specific antibodies distributed either systemically in the serum. The mice in immunization groups that produced similar RSV-specific serum IgG did not experience similar disease exacerbation. Further, our study demonstrated that quantity of live RSV in the lungs was not associated with the disease enhancement given that animals in CT group, even with their significantly higher lung virus titers, did not experience enhanced disease following challenge.

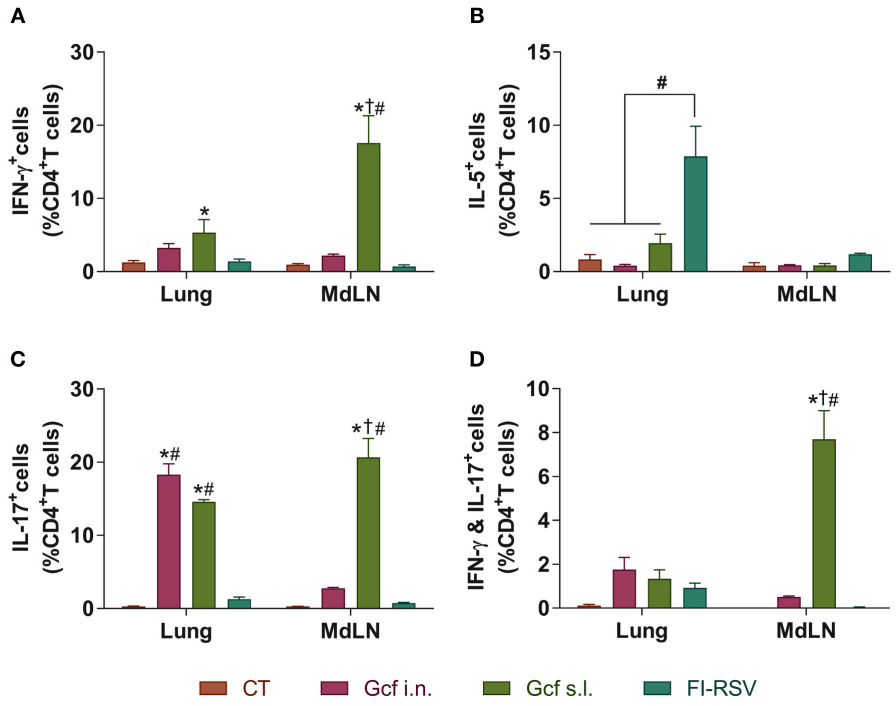

FIGURE 5 | CD4 T cell responses in the mice immunized with Gcf. Percentage of **(A)** IFN-γ-, **(B)** IL-5-, **(C)** IL-17-, and **(D)** both IFN-γ and IL-17-producing CD4 T cells in the lung and mediastinal lymph node after RSV challenge determined by intracellular staining. Representative results of two independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 3$). *Significant difference with CT group ($P < 0.05$). †Significant difference with Gcf i.n. group ($P < 0.05$). #Significant difference with FI-RSV group ($P < 0.05$). *MdLD*, Mediastinal lymph node.

As expected, RSV challenge following FI-RSV or vvG-immunization induced pulmonary eosinophila; enhanced Th2-type responses such as increased airway and lung secretion levels of IL-4, IL-5, and IL-13; eosinophilic airway inflammation; and development of goblet cell hyperplasia and mucus hyperproduction. Elevation in airway secretion of IFN-γ was also simultaneously detected. Interestingly, in a previous study elevated production of IFN-γ was implicated in the increased clinical illness and airway resistance following RSV challenge of animals previously immunized with vvG (18). By contrast while a similar increase in eosinophil recruitment following RSV challenge in animals primed with sublingual Gcf there was also a, significant elevation in neutrophil recruitment and IL-17 secretion in the airway and lungs exclusively in the animals exposed Gcf via sublingual mucosa. Interestingly, disease characteristics in mice undergoing challenge following sublingual Gcf exposure with elevated neutrophil infiltration resemble the disease spectrum in subgroup of severe asthma described as "refractory" asthma. Of note in this regard a previous study demonstrated excessive neutrophilic infiltration to the airway of RORγt-overexpressing mice, with enhanced lung expression of IL-17A following exposure to the sensitizing antigen (23). We observed a remarkably similar disease pattern and cytokine profile in animals that received priming with Gcf by the sublingual route followed by RSV challenge.

It is also interesting to note that there are significant increases in both eosinophil and neutrophil recruitment to the BAL fluids and lung tissues of Gcf s.l. group post RSV challenge. This simultaneous elevation in eosinophil/neutrophil recruitment was unique to animals exposed Gcf via sublingual mucosa. This pattern of mixed granulocyte infiltration into the lungs following RSV challenge, suggests that s.l. priming with Gcf promotes an environment favoring IL-17 production. Consistent with this notion IL-17 has been shown to induce the release of eotaxin from airway smooth muscle cells as well as neutrophil influx (24). However, based on previous findings (18), it is unlikely that eosinophils contribute to the exacerbation of disease, although we observed significant increase in airway and lung eosinophil recruitment in Gcf s.l. group animals experiencing exacerbated disease.

The detrimental role of IL-17 in the development of inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis has been well-documented (25). These evidences strongly suggest that IL-17 might play a prominent role in the pathogenesis of lung inflammation by promoting the recruitment of neutrophils. In our study, severe exacerbation of RSV disease characterized by induction of IL-17-biased immunity and pulmonary neutrophila/eosinophilia in mice that were previously exposed to RSV G protein via sublingual mucosa was observed. The presence of IL-17 in this Gcf-mediated disease exacerbation is critical since the absence of

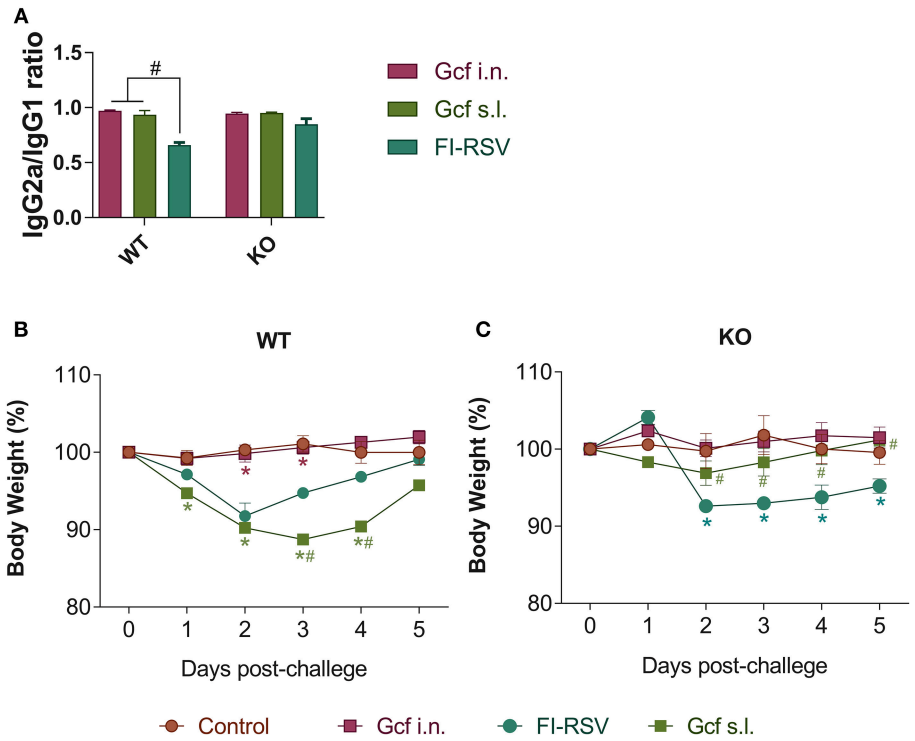

FIGURE 6 | Effect of IL-17 on vaccine-induced immune response and weight loss following RSV challenge in mice immunized with Gcf. **(A)** Ratio of IgG2a/IgG1 in wild type mice and IL-17 KO mice immunized twice with Gcf via sublingual or intranasal route or with FI-RSV via intramuscular route. Sera were collected at 3 weeks after boost immunization. Both G-specific IgG2a and IgG1 were measured by ELISA and the ratio of IgG2a to IgG1 was calculated. #Significant difference with FI-RSV group ($P < 0.05$). Body weight was monitored daily after RSV challenge in **(B)** wild type mice and **(C)** IL-17 KO mice immunized with Gcf or FI-RSV. Representative results of two independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 4$). *Significant difference with Control group ($P < 0.05$). #Significant difference with FI-RSV group ($P < 0.05$). *WT*, wild type Balb/c mice; *KO*, IL-17 knock-out mice.

IL-17, reflected by IL-17 KO model, prevented the phenomenon. These suggest that IL-17 could play a prominent role in the pathogenesis of RSV G-based vaccine-enhanced disease.

The major source of IL-17 produced during the course of RSV infection was most likely Th17 cells because IL-17-producing CD4 T cells were increased in the lungs and mediastinal lymph node of Gcf s.l. group following RSV challenge (**Figure 5**) and the RORγt expression level was increased in splenic CD4 T cells following sublingual Gcf immunization (**Figure S3**). The resident memory CD4 T cells in the lung might produce IL-17 at early times during RSV infection (26). However, we cannot exclude the possibility that other immune cells such as γδ T cells and innate lymphoid cells (ILCs) could produce IL-17 upon RSV challenge (27). Another possibility is that novel IL-17-producing Th2-like cells might contribute to the Gcf s.l. immunization-induced vaccine enhanced diseases. Previously, it has been reported that antigen-specific inflammatory IL-17-producing Th2 cells promote influx of heterogeneous leukocytes including eosinophils and neutrophils and exacerbate allergic asthma (28).

Presently, sublingual mucosa is being targeted for sublingual immunotherapy (SLIT) for treatment of type 1 allergic hypersensitivity (13). Sublingual mucosa is also being extensively evaluated as a delivery route for various vaccines (29). These strategies to target sublingual mucosa for immunotherapy for allergies or as a vaccine delivery route have been broadly gaining momentum. Sublingual antigens might trigger the tolerogenic or immunogenic response depending on the type of antigen and adjuvant through local or systemic pathways (30, 31). Nagai et al. reported that sublingually administered antigens could be transported across epithelial cells in the sublingual ductal system to the ductal antigen-presenting cells within an hour. They also suggested that the sublingual duct, composed of pseudostratified and simple columnar epithelium could spread antigen rapidly through the paracellular and transcellular pathways, although mucosa-associated lymphoid tissues or M cell-like structures were not identified (32). In this study, we observed a rapid disappearance of sublingual antigen within 1 h near the tongue and salivary glands. In particular, it was rarely observed in the draining lymph nodes near the sublingual compartment, which is contrasted with the fact that nasal antigen remained in the mediastinal lymph node for up to 24 h after administration. These results suggest that the initial response to the sublingual antigen may be different from nasal administration.

Our findings represent a cautionary note regarding the use of sublingual route for antigen administration as SLIT or vaccination strategies. Although it is likely that the phenomenon observed in our study is specific to RSV G protein, we cannot

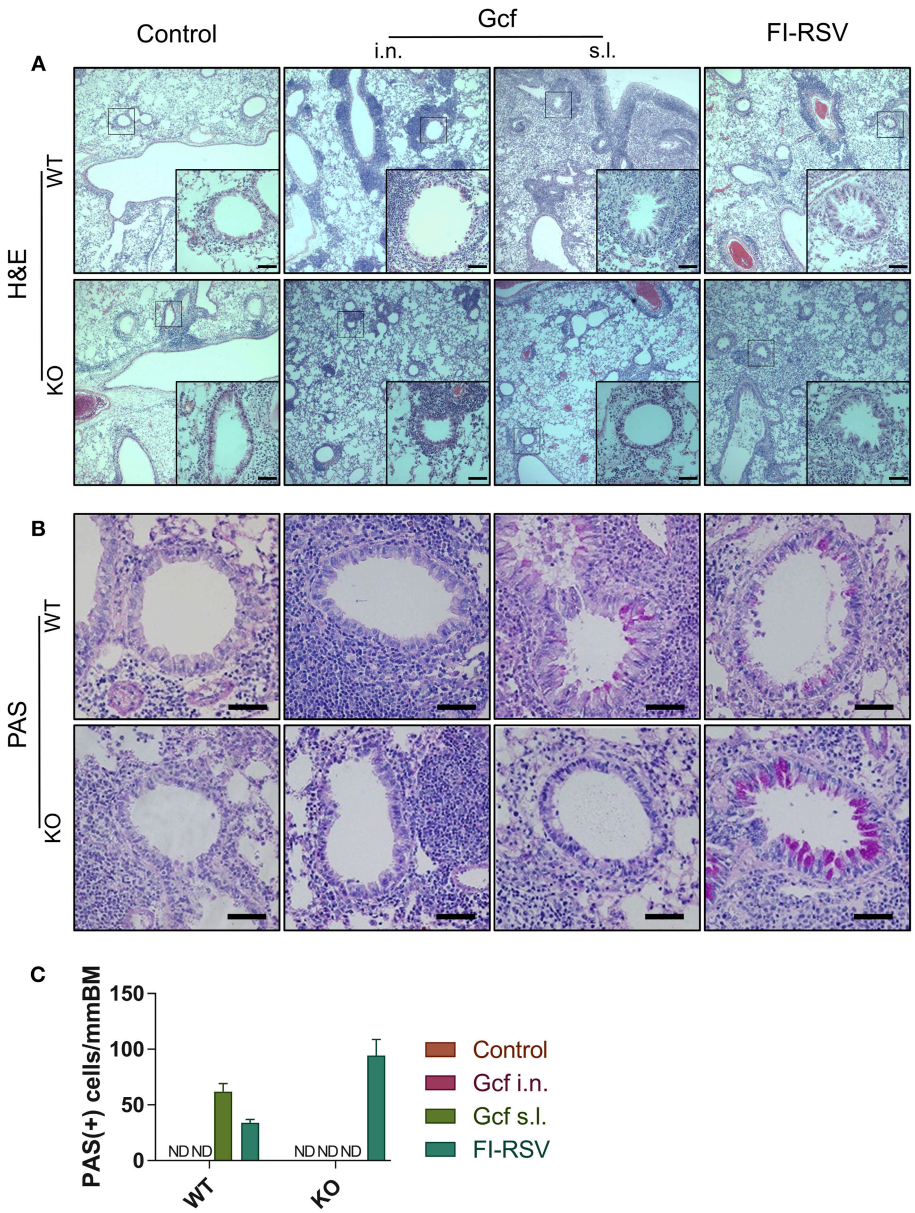

FIGURE 7 | Effect of IL-17 on vaccine-induced histopathologic alterations in lung structure following RSV challenge. Mice were immunized twice with Gcf via sublingual or intranasal route or with FI-RSV via intramuscular route, and then challenged with RSV A2 strain. Histologic samples were prepared from formalin-fixed, paraffin-embedded lung tissues. **(A)** H&E staining and **(B)** PAS staining were performed and examined. **(C)** PAS-positive cells in peri-bronchial regions were measured for quantitative analysis. Representative results of two independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 4$). Original magnification for **(A)** is 10X and the inset shows 40X. Original magnification for **(B)** is 40X. Scale bars indicate 50 micrometer in 40X. *WT*, wild type Balb/c mice; *KO*, IL-17 knock-out mice; *ND*, Not detected.

exclude a possibility that other antigens, when administered via sublingual mucosa, could prime the recipient for an exacerbated disease upon subsequent re-exposure. In this regard, it is worth noting that increase in IL-17 mRNA expression in peripheral blood mononuclear cells (PBMC) was detected in children given SLIT for allergic rhinitis (33). Lastly, exposure to RSV G protein via different delivery routes appears to prime for T helper cell immunity that is differentially biased. For example, intramuscular FI-RSV immunization establishes Th2 bias (34), while vvG immunization via scarification primes for excessive Th1 and Th2 bias. Our findings reveal that sublingual delivery of RSV G institute Th17 bias. It is possible that such capability of inducing selective skewing of T helper immunity may potentially be used beneficially.

In conclusion, we report here a novel class of RSV vaccine-enhanced immunopathology which is primed by sublingual

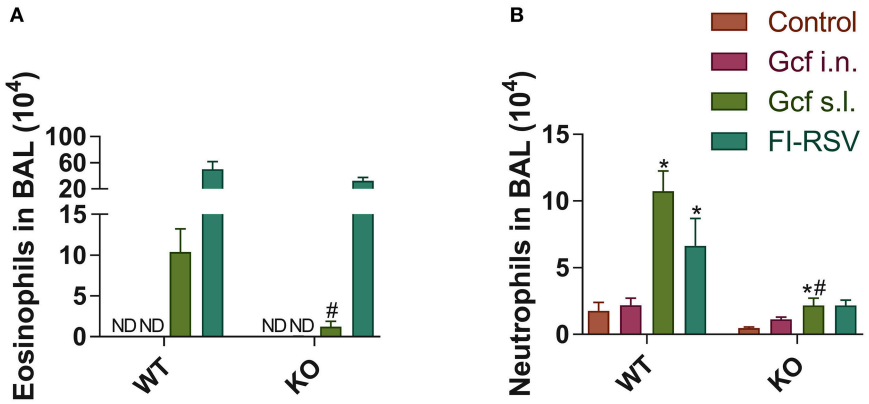

FIGURE 8 | Granulocyte phenotypes in BAL fluid from wild-type and IL-17 KO mice immunized with different routes of Gcf after RSV challenge. BAL fluid cells were collected, counted and then analyzed by flow cytometry at 5 days after RSV challenge. **(A)** Eosinophils and **(B)** neutrophils in BAL fluid from wild type and IL-17 KO mice were measured in accordance with each of the criteria ($CD45^+$ $CD11c^-$ $SiglecF^+$ and $CD45^+$ $CD11c^-$ $Ly6G^+$, respectively). Representative results of two independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 4$). *Statistical significance with "Control" ($p < 0.05$). #Statistical significance with "WT" ($p < 0.05$). *WT*, wild type Balb/c mice; *KO*, IL-17 knock-out mice; *ND*, Not detected.

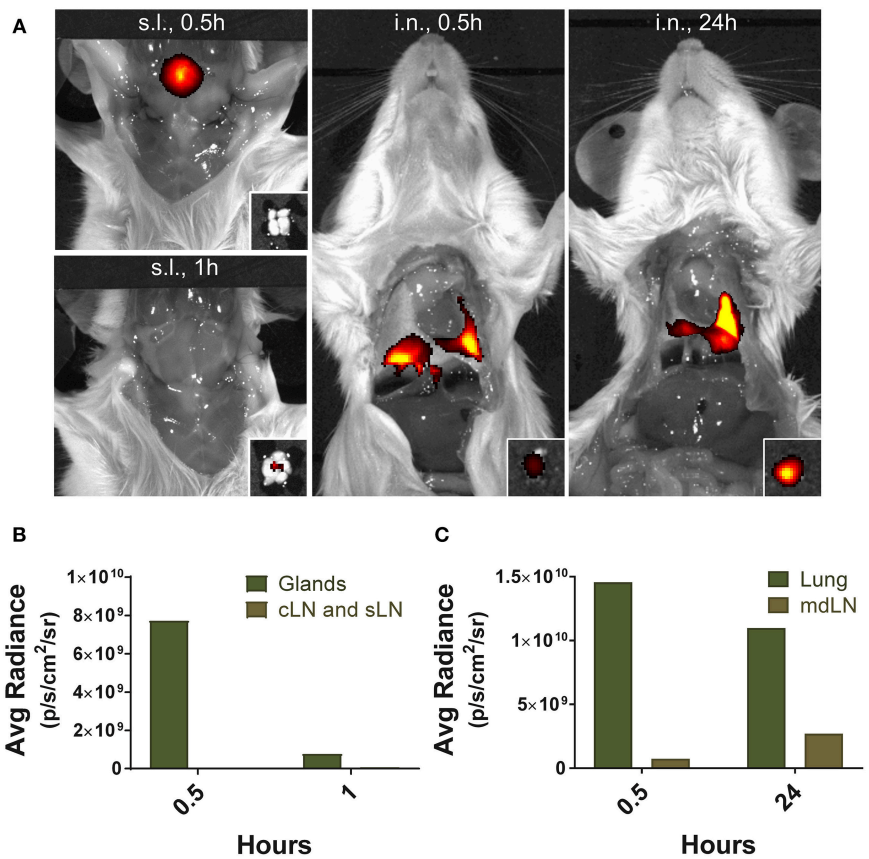

FIGURE 9 | Localization of antigens following sublingual administration. Mice were anesthetized and injected with 5 μg of Alexa Fluor 647-streptavidin conjugate via s.l. or i.n. route. **(A)** Fluorescence images were measured by IVIS Lumina III with Ex/Em of 640 nm/710 nm at 0.5, 1, and 24 h following injection. *Small boxes*, draining lymph nodes from sublingual compartment or lung tissue. Average photon radiance of mice treated with the fluorescence dye via **(B)** s.l. and **(C)** i.n. route. Representative results of three independent experiments are shown. *Avg Radiance*, the average photon radiance on the certain surface of a mice was expressed as photons *per second* per centimeter squared per steradian (p/s/cm^2/sr); *Gland*, Salivary gland; *cLN*, cervical lymph node; *sLN*, submandibular lymph node; *mdLN*, mediastinal lymph node.

immunization and is regulated by IL-17. Understanding the mechanism of vaccine-enhanced disease in RSV infection is essential for the development of safe and effective vaccines, and our results demonstrating IL-17-mediated disease exacerbation provides a new model for evaluating safety of RSV vaccine candidates.

AUTHOR CONTRIBUTIONS

MS and JC designed and conceived the study. IC, JYK, YC, BS, J-aC, DJ, J-OK and HY performed the experiments. IC, JYK, YC, TB, MS, and JC wrote the manuscript. All authors reviewed the manuscript before submission, etc.

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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.2019.00567/full#supplementary-material

Figure S1 | Anti-RSV immunity and enhanced disease in RSV challenged mice following sublingual Gcf immunization**. (A)** RSV-specific serum IgG titers measured at day 14 post-booster immunization by ELISA using 2 x 10^3 RSV A2 per well as the coating antigen. Titers are indicated in $\log_2$. **(B)** Lung RSV titers at day 4 post-RSV A2 challenge determined by plaque assay. **(C)** Weight loss following RSV A2 challenge. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 5$). * indicates significant different with CT group ($P < 0.05$). # indicates significant difference with vvG group ($P < 0.05$). *ND*, Not detected.

Figure S2 | Lung histology of mice immunized with Gcf through different routes. All samples were collected at day 4 post-RSV challenge and prepared from formalin-fixed, paraffin-embedded lung tissues. H&E staining, TUNEL assay, and PAS staining were performed to determine inflammation, cell death, mucus secretion, respectively. **(A)** H&E staining (1st low, 20X magnification), and PAS staining (2nd low, 40X magnification) of lung samples harvested at day 4 post-challenge. **(B)** Lung pathological score assigned representing the inflammatory cell infiltration shown in H&E staining. **(C)** $TUNEL^+$ cells (40X magnification) and quantification of cell death represented by $TUNEL^+$ cells. Representative results of three independent experiments are shown. All data are expressed in mean ± S.E.M ($n = 3$). * indicates significant difference with CT group ($P < 0.05$).

Figure S3 | Cytokine profiles and transcription factor expression levels in the mice immunized with Gcf. **(A)** IFN-γ, IL-2, IL-4, IL-5, IL-13, and IL-17 levels in BAL fluid of mice immunized with Gcf at day 4 post-RSV A2 challenge determined by CBA. **(B)** Percentage of T-bet, GATA-3, or RORγt expressing splenic CD4+ T cells at day 7 post-booster immunization determined by intracellular staining. Representative results of three independent experiments are shown. All data are expressed in mean +/- S.E.M ($n = 6$). * indicates significant difference with CT group ($P < 0.05$). † indicates significant difference with Gcf i.n. group ($P < 0.05$). ND, Not detected.

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Antigenic Site-Specific Competitive Antibody Responses to the Fusion Protein of Respiratory Syncytial Virus Were Associated With Viral Clearance in Hematopoietic Cell Transplantation Adults

Xunyan Ye[1], *Obinna P. Iwuchukwu*[1], *Vasanthi Avadhanula*[1], *Letisha O. Aideyan*[1], *Trevor J. McBride*[1], *Laura L. Ferlic-Stark*[1], *Kirtida D. Patel*[1], *Felipe-Andres Piedra*[1], *Dimpy P. Shah*[2], *Roy F. Chemaly*[3] *and Pedro A. Piedra*[1,4]*

[1] Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States, [2] Department of Epidemiology and Biostatistics, The University of Texas Health Science Center at San Antonio, San Antonio, TX, United States, [3] Departments of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [4] Department of Pediatrics, Baylor College of Medicine, Houston, TX, United States

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***Correspondence:**
Pedro A. Piedra
ppiedra@bcm.edu

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Background: Recent studies of human sera showed that the majority of the respiratory syncytial virus (RSV) neutralizing antibodies are directed against pre-fusion conformation of the fusion (F) protein of RSV and revealed the importance of pre-fusion antigenic site Ø specific antibodies. However, detailed analysis of multiple antigenic site-specific competitive antibody responses to RSV F protein and their contribution to virus clearance in humans are lacking.

Methods: We prospectively enrolled a cohort of RSV infected hematopoietic cell transplantation (HCT) adults ($n = 40$). Serum samples were collected at enrollment (acute, $n = 40$) and 14 to 60 days post-enrollment (convalescent, $n = 40$). Antigenic site-specific F protein antibodies were measured against pre-fusion site Ø, post-fusion site I, and sites II and IV present in both the pre-fusion and post-fusion F protein conformations utilizing four different competitive antibody assays developed with biotinylated monoclonal antibodies (mAb) D25, 131-2A, palivizumab, and 101F, respectively. The lower limit of detection were 7.8 and 1.0 μg/mL for the competitive antibody assays that measured site Ø specific response, as well as sites I, II, and IV specific responses, respectively. Neutralizing antibody titers to RSV A and B subgroups was determined by microneutralization assays.

Results: The overall findings in RSV infected HCT adults revealed: (1) a significant increase in antigenic site-specific competitive antibodies in convalescent sera except for site Ø competitive antibody ($p < 0.01$); (2) comparable concentrations in the acute and convalescent serum samples of antigenic site-specific competitive antibodies between RSV/A and RSV/B infected HCT adults ($p > 0.05$); (3) significantly increased concentrations of the antigenic site-specific competitive antibodies in HCT adults who

had genomic RSV detected in the upper respiratory tract for <14 days compared to those for ≥14 days ($p < 0.01$); and (4) statistically significant correlation between the antigenic site-specific competitive antibody concentrations and neutralizing antibody titers against RSV/A and RSV/B (r ranged from 0.33 to 0.83 for acute sera, and 0.50–0.88 for convalescent sera; $p < 0.05$).

Conclusions: In RSV infected HCT adults, antigenic site-specific antibody responses were induced against multiple antigenic sites found in both the pre-fusion and post-fusion F conformations, and were associated with a more rapid viral clearance and neutralizing antibody activity. However, the association is not necessarily the cause and the consequence.

Keywords: competitive antibody, fusion protein, viral clearance, respiratory syncytial virus, hematopoietic cell transplantation

INTRODUCTION

Respiratory syncytial virus (RSV) is a negative-sense, single-stranded RNA virus of the family *Pneumoviridae*. RSV is transmitted via fomites and large droplet aerosols. RSV, traditionally considered as the most common respiratory pathogen in children <5 years old, has more recently been found to have a high prevalence in immunocompromised individuals (1, 2). RSV is also a leading cause of severe respiratory infection in children and adults with a compromised immune system, with mortality rates as high as 80% (3). The hematopoietic cell transplantation (HCT) recipients are highly vulnerable to the severe consequences of RSV infection with the highest risk for mortality within 100 days after transplantation (4). A licensed vaccine against RSV is not yet available because of problems with the stability, purity, reproducibility, tolerability, and potency of vaccine candidates (5–8). Moreover, immunocompromised patients may not respond adequately to vaccinations because of their relative immunologic suppression. Current treatment strategies of demonstrated efficacy in immunocompromised patients include antiviral therapies such as ribavirin, palivizumab, and immunomodulation with total IVIG (9–11).

RSV fusion (F) surface glycoprotein is a class I protein that mediates viral entry into host cells by transforming from a metastable trimeric pre-fusion conformation (pre-F) to a highly stable post-fusion conformation (post-F). The antigenic topology of RSV F is substantially altered during this transition. Molecules that prevent these structural changes can prevent viral fusion and have potential as therapeutics for treatment of RSV infection. Some groups of epitopes, referred to as antigenic sites, are generally conserved on both the pre-F and post-F conformations, whereas others are found either on the pre-F or post-F conformation (12). Six antigenic sites on the F protein have been described: Ø-V. Antigenic site Ø ("zero") presents at the apex of the pre-F trimer. The pre-F-specific antibodies (MEDI8897 and D25) recognize antigenic site Ø (13). Antigenic site I is found in the post-F conformation. mAb 131-2A binds to antigenic site I on the post-F confirmation (14, 15). Antigenic sites II and IV are found on both pre-F and post-F conformations. Palivizumab (Synagis) is the first characterized monoclonal antibody (mAb) and currently the only mAb licensed for the prevention of severe RSV infection in high-risk infants. It recognizes antigenic site II. mAb 101F recognizes antigenic site IV. Although the secondary structure elements that form site III are present on both pre-F and post-F, they adopt a different spatial arrangement in post-F that results in higher-affinity binding to pre-F (16). The potently neutralizing mAb MPE8 was shown to recognize antigenic site III (16). Antigenic site V is found in the pre-F conformation and located between sites Ø and III. mAb AM14 targets antigenic site V and is a potently neutralizing mAb (17, 18).

RSV is primarily a mucosally restricted virus causing damage to the upper and lower respiratory tracts during its multiple rounds of replication. It can elicit cellular and humoral immune responses at variable levels in HCT recipients, depending upon the degree to which individuals remain immunocompromised post-transplantation. RSV-specific neutralizing antibody play an important role in preventing severe infection while cellular immune responses are thought to play a critical function in destroying RSV infected cells and thus clearing the infection. Previously RSV naïve but immunocompetent infants can shed RSV from their upper respiratory tract (URT) for up to 21 days; by contrast, immunocompromised children can shed RSV for several months (19, 20). In the murine model of RSV infection, CD8+ T cells are capable of clearing RSV infection (14, 21–23). Limited data from studies of infants with primary RSV infection suggest that a cellular immune response with specific cytotoxic T lymphocytes are initiated within 10 days of infection (24, 25). Studies on the humoral immune responses after primary RSV infection in children and re-infection in children and adults have been reported (26–28). Although administration of intravenous immunoglobulin (IVIG) with high neutralizing antibody activity against RSV and palivizumab provides protection against severe RSV infection in high-risk infants, the role of neutralizing antibodies in recovery from an established infection is not clear. We have previously demonstrated that significantly greater levels of humoral palivizumab-competing antibody and neutralizing antibody titers are associated with more rapid RSV clearance in HCT recipients. This cohort of forty RSV infected HCT adults provided us an opportunity to further study other

RSV epitope-specific competitive antibody levels and identify additional immune correlates of viral clearance. Three more RSV epitope-specific competitive antibody assays were developed and standardized to detect competitive antibodies to antigenic sites Ø, I, and IV. In addition, understanding the relationship between neutralization antibody and epitope specificity of the competitive antibodies elicited in response to natural RSV infection will be critical in the selection and design of new monoclonal antibodies and vaccines for prevention of RSV.

METHODS

Study Subjects

HCT recipients with laboratory-confirmed RSV upper respiratory tract infection at enrollment and negative chest radiography findings were enrolled within 72 h of RSV diagnosis and stratified by level of risk for progression to the lower respiratory tract as previously described (29). From January 2012 to April 2015, sera were collected from all patients at enrollment (acute) and 14–60 days after hospitalization (convalescent) for evaluating the humoral immune response. Nasal wash samples were collected at enrollment, day 7 (±1), day 14 (±1), and between day 21 and day 28 (±1) for detecting of viruses in the RSV/A and RSV/B subtypes by real-time, reverse-transcription polymerase chain reaction (rtRT-PCR). At enrollment, an interview was performed to obtain historical information, and medical records were reviewed to extract demographic and clinical data. The institutional review boards of the University of Texas MD Anderson Cancer Center and Baylor College of Medicine approved the study protocol and written informed consent was obtained from all the participants.

Real-Time Reverse-Transcription Polymerase Chain Reaction (rtRT-PCR)

Viral RNA extraction and RSV/A and RSV/B detection in nasal wash samples by rtRT-PCR in a CLIA certified Respiratory Virus Diagnostic laboratory (CLIA ID# 45D0919666) were performed as previously described (30).

Antigenic-Site Competitive Antibody Assays

Four antigenic-site competitive antibody assays were used to measure concentrations of D25-competing antibody (site Ø), 131-2A-competing antibody (site I), palivizumab-competing antibody (site II), and 101F-competing antibody (site IV) in serum that compete with biotinylated mAbs for binding to the respective antigenic site of the RSV fusion protein. D25 and 101F were purchased from Cambridge Biologics, LLC, Brookline, MA, USA. Palivizumab was from MedImmune, LLC, Gaithersburg, MD, USA. 131-2A was from EMD Millipore Corporation, Temecula, CA, USA. mAbs were biotinylated with a Pierce™ Antibody Biotinylation Kit (Pierce, Rockford, IL, USA) per manufacturer's instructions. The source of the fusion protein was from sucrose purified RSV/A/Bernett (spRSV, GA1) for sites I, II and IV. One hundred μL of spRSV (total protein 7 μg/mL) was coated onto the Immulon 2HB 96-well plate (Thermo Scientific, Waltham, MA, USA) for 18 h at 4°C. For site Ø competitive antibody assay, 100 μL of HEp-2 cell suspension (24 x 10^4 cells /mL) in 10% FBS/MEM were seeded on 96-well Falcon tissue culture plates from Corning (Pittston, Pennsylvania, USA). The plates were incubated at 37°C with 5% CO_2 and 85% humidity for 24 h to form monolayers. Cells were inoculated with spRSV (MOI = 0.002) and incubated at the same condition for 2 days. The plates from all four assays were blocked for 1 h with 5% milk (Carnation Instant Nonfat Dry Milk) in 1X phosphate-buffered saline. One hundred μL of D25, 131-2A, palivizumab, and 101F at a concentration of 1.25 μg/mL in 5% milk (for 131-2A, palivizumab, and 101F) and 5% milk in 10% Defined Fetal Bovine Serum in Minimum Essential Medium (for D25) were added in duplicate followed by 2-fold serial dilutions (12,500 to 24.41 ng/mL) for generating a standard curve on each plate. Next 50 μL of 2-fold serial dilutions of serum samples (1:5 to 1:2,560) in duplicate were added to the coated plates, and then immediately 50 μL of 100 ng/mL of biotinylated mAbs (D25, 131-2A, palivizumab, and 101F) generated with a Pierce™ Antibody Biotinylation Kit (Pierce, Rockford, IL) per manufacturer's instructions, were added, followed by 1 h incubation. After washing, HRP-conjugated streptavidin (SeraCare Life Sciences, Gaithersburg, MD) was added for an additional hour. Wells containing biotinylated mAb without sera served as positive controls representing maximum binding. Wells without biotinylated mAbs containing either 5% milk instead of sera or wells containing sera served as negative controls. A four-parameter logistic (4PL) regression model was used to calculate the competitive antibody concentrations (μg/mL) based on the dynamic range of the standard curve by interpolating the concentration of the standards that corresponds to the absorbance value at which the test serum sample resulted in 50% inhibition. The lower limit of detection (LLoD) was 1.0 μg/mL for site I, II, and IV competitive antibody assays, and 7.8 ug/mL for site Ø competitive antibody assay. Samples with concentration below the LLoD were assigned a value of 0.5 and 3.9 μg /mL, respectively.

RSV F Protein Specific Microneutralization (MN) Assay

Serum samples and the four mAbs (D25, 131-2A, palivizumab, and 101F) were analyzed for neutralizing antibodies (Nt Ab) against RSV/A/Tracy and RSV/B/18537 in HEp-2 cells using a qualified microneutralization assay as previously described (31–33). Serum samples and mAbs (final concentration was 40 μg/mL) were diluted 1:8 initially, followed by 2-fold serially dilution. An equal volume of RSV/A Tracy or RSV/B 18537 was added to each dilution and incubated at 36°C with 5% CO_2 for 90 minutes. One hundred μL of HEp 2 cells was added to each well in the 96-well plate and incubated for 6–7 or 7–8 days for RSV/A and RSV/B microneutralization assays, respectively. Then the 96-well plates were fixed and stained with 10% formalin/0.01% crystal violet solution for ∼24 h. After the 96-well plates were air-dried, they were read. Neutralizing antibody titers were defined as the final dilution at which there was a 50% reduction in viral cytopathic effect (CPE). Any serum sample resulting in a titer <LLoD (2.5 log2) was assigned a value of 2 log2. Any mAbs resulting in a titer <LLoD was retested using the stock

TABLE 1 | RSV Antibody Levels in Acute and Convalescent Sera from RSV infected HCT Adults.

RSV antibody	Ab target	Acute ($n = 40$)	Convalescent ($n = 40$)	Fold increase	P-value[b]
Competitive antibody	Antigenic site Ø	21.1 [14.9, 30.6][a]	55.1 [37.5, 78.0]	2.6	<0.001
	Antigenic site I	2.0 [1.4, 3.0]	8.2 [4.8, 14.7]	4.1	<0.001
	Antigenic site II	2.9 [1.9, 4.4]	16.3 [8.9, 29.8]	5.6	<0.001
	Antigenic site IV	7.0 [5.0, 10.4]	28.2 [18.0, 47.3]	4.0	<0.001
Neutralizing antibody	RSV/A	6.9 [6.3, 7.5]	9.6 [8.7, 10.4]	6.5	<0.001
	RSV/B	7.2 [6.4, 8.1]	9.9 [8.8, 10.9]	6.5	<0.001

[a] *Geometric mean conc., μg/mL for competitive antibody and log2 for neutralizing antibody titer [95% Confidence Interval].*
[b] *Paired t-test for difference in means of competitive antibody conc. (log2 μg/mL) or neutralizing antibody titer (log2) between acute and convalescent.*

(1.0 mg/mL) and assigned a value of 0.2 log2 if the titer was negative again.

Statistical Analysis

For demographic characteristics and clinical outcomes, continuous variables and categorical variables were analyzed. A paired t-test was used to determine whether the geometric means of neutralizing antibody titer (GMT) (log2) or geometric mean of log transformed competitive antibody concentrations (GMC) differed significantly between acute and convalescent samples. A two-sample t-test was used to determine whether the neutralizing antibody (log2) or GMC of log transformed competitive antibody concentrations differed significantly between RSV/A and RSV/B infected patients, as well as between HCT recipients who shed virus for <14 and $\geq$ 14 days. Statistical significance was indicated for p-values <0.05. Pearson's correlation coefficients were calculated between neutralizing antibody titers and site-specific competitive antibody concentrations. Statistical analyses were performed using the SPSS Statistic 22 (IBM, Armonk, NY).

RESULTS

Demographic and Clinical Variables of RSV Infected HCT Adults Were Comparable Between Study Groups

Clinical characteristics at enrollment are summarized for all 40 HCT adults in **Table 1** from previous publication (29), stratified by duration of RSV shedding (<14 or ≥ 14 days) or by RSV infection subtype (RSV/A or RSV/B). Briefly, all listed variables were comparable between the groups. The only significant difference observed was recipients who shed RSV for ≥ 14 days were more likely to have received an allogenic stem cell transplant compared to recipients with a shorter duration of viral shedding (18/20 vs. 11/20, $p < 0.025$). The absolute neutrophil count and the absolute lymphocyte count are within the normal ranges for these cohorts. The median time from transplantation to RSV infection is 169 and 100 days for adults shedding RSV from the URT for <14 and ≥ 14 days, respectively.

The Site-Specific Competitive Antibody Assays Were Specific to the Corresponding Competitive Antibodies

Specificity of the four competitive antibody assays were confirmed using a panel of monoclonal antibody (12.5 μg/mL) to compete with biotinylated monoclonal antibody specific to their respective antigenic sites (**Figure 1**). Using a 125-fold higher concentration, the panel of monoclonal antibodies that bound antigenic sites on the F protein other than the site targeted by the biotinylated monoclonal antibody were not able to inhibit the biotinylated monoclonal antibody from binding to its antigenic site. For example, D25 inhibited or competed with the biotinylated D25 in the site Ø competitive antibody assay, while the rest of the monoclonal antibodies in the panel did not inhibit the biotinylated D25 from binding to site Ø.

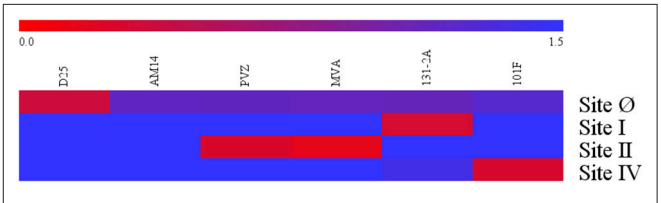

FIGURE 1 | Specificity of RSV antigenic site-specific competitive antibody assays. Blue indicates there was the mAbs had no inhibition to the biotinylated mAbs specific to the RSV antigenic site. Red indicates there were strong inhibition to the RSV antigenic site. The 0.0–1.5 scale represents the level of mAb inhibition from the strongest to the weakest. PVZ, palivizumab; MVA, motavizumab. Data represented are from 3 independent duplicate competitive antibody assays.

Monoclonal Antibodies Showed Different Levels of Neutralizing Potency

Neutralizing activity of the monoclonal antibodies at a concentration of 40 μg/mL was evaluated in the RSV/A and RSV/B microneutralization assays (**Figure 2**). The mAb D25, that binds antigenic site Ø, is the most potent of the mAbs used in the competitive antibody assays. Palivizumab and 101F mAbs that bind site II and site IV, respectively, have moderate neutralizing antibody activity, and 131-2A, a site I mAb, did not have measurable neutralizing activity, which are consistent with the results previously reported (34).

Convalescent Sera Had Higher Concentration of Site-Specific Competing or Neutralizing Antibody Than Acute Sera

A significant increase in RSV antibody levels (competitive and neutralizing antibodies) in the convalescent sera compared to the acute sera was measured by all four competitive antibody

assays and both microneutralization assays (**Table 1**). The GMC of D25-competing antibody (**Table 1**) was the highest in both acute and convalescent sera, followed by the 101F-competing antibody (site IV), palivizumab-competing antibody (site II), and 131-2A-competing (site I) antibodies. The RSV/A and RSV/B neutralizing antibody GMTs were comparable. However, the fold-increase in the competitive antibody concentration was greatest for palivizumab-competing antibody (5.6), and lowest for D25-competing competitive antibodies (2.6). The fold-increase was comparable between RSV/A and RSV/B neutralizing antibody titers (6.5).

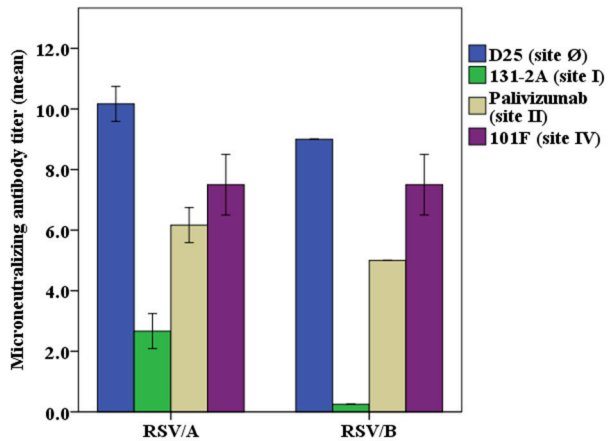

FIGURE 2 | Neutralizing potency of RSV antigenic site-specific monoclonal antibody. RSV/A Tracy and RSV/B 18537 were used in the experiments. The concentration of the mAbs were 40 μg/mL for D25, palivizumab, 131-2A, and 101F in both RSV/A and RSV/B microneutralization assays, except that mAb 131-2A was used at 1.0 mg/mL in the RSV/B microneutralization assay. The Y axis is the RSV/A Tracy or RSV/B 18537 neutralizing antibody titers (log2) for different mAbs used in the microneutralization assay. Data represented are from 3 independent duplicate microneutralization assays ± standard deviation.

Higher Antibody Levels Were Detected in HCT Adults Shedding RSV <14 Days

The competitive antibody in GMC (μg/mL) and neutralizing antibody in GMT (log2) were compared between HCT adults shedding virus from their URT for <14 and ≥14 days (**Table 2**). For the acute sera, there was no significant difference between the two groups for either site-specific competitive antibodies or RSV-specific neutralizing antibodies. However, for the convalescent sera, significantly higher levels (<0.01) were detected of both site-specific competitive antibodies and RSV-specific neutralizing antibodies in HCT adults shedding RSV <14 days, except for the site Ø competitive antibody. In addition, all the convalescent sera had higher antibody levels than acute sera for both site-specific competitive antibody and RSV-specific neutralizing antibodies. The total GMC for the four competitive antibodies in the convalescent sera increased about 5-folds for HCT adults shedding virus <14 days and about 2.5 folds for those shedding virus ≥14 days. For HCT adults shedding virus <14 days, the total GMC of the four site-specific antibodies increased from 47.4 μg/mL in the acute sera to 228.6 μg/mL in the convalescent sera. For HCT adults shedding virus ≥14 days, the total GMC increased from 34.1 μg/mL in the acute sera to 91.8 μg/mL in the convalescent sera.

The proportion of the contribution for each site-specific competitive antibody GMC to the total GMC for acute and convalescent sera from HCT adults shedding virus < 14 days and ≥14 days are illustrated in **Figure 3**. For HCT adults shedding virus <14 days, ∼60% of the total GMC was composed by D25-competing antibody, and decreased to ∼30% in the convalescent sera; while the percentages of 131-2A, palivizumab-competing antibody, and 101F-competing antibodies increased. For HCT adults shedding virus ≥14 days, ∼70% of the total GMC was also composed by D25-competing antibody in the acute sera, and the percent contribution by the four competitive antibodies did not increase even though the total GMC increased

TABLE 2 | RSV Antibody Levels between RSV Infected HCT Adults Who Shed RSV <14 days and ≥14 Days.

RSV antibody	Ab target	Serum	<14 days (n = 20)	≥14 days (n = 20)	P-value[b]
Competitive antibody	Ø	Acute	22.6 [13.6, 37.2][a]	26.6 [19.2, 38]	0.712
		Convalescent	59.7 [33.9, 103.8]	50.8 [33.0, 79.1]	0.672
	I	Acute	2.4 [1.3, 5.2]	1.6 [1.0, 2.6]	0.307
		Convalescent	18.2 [7.8, 41.1]	3.7 [2.3, 6.5]	**0.001**
	II	Acute	3.9 [1.9, 8.1]	2.2 [1.4, 3.3]	0.139
		Convalescent	44.4 [18.1, 108.9]	5.9 [3.3, 10.8]	**<0.001**
	IV	Acute	9.0 [5.7, 16.2]	5.5 [3.5, 8.1]	0.156
		Convalescent	60.2 [28.6, 134.0]	13.2 [8.5, 20.6]	**<0.001**
	Ø, I, II, IV	Total Acute	47.4 [29.9, 79.8]	34.1 [22.9, 48.9]	0.306
		Total Convalescent	228.6 [116.8, 400.3]	91.8 [66.6, 123.1]	**0.013**
Neutralizing antibody	RSV/A	Acute	6.97 [6.14, 7.89]	6.22 [5.28, 7.20]	0.281
		Convalescent	10.21 [9.09, 11.37]	8.24 [7.30, 9.29]	**0.016**
	RSV/B	Acute	7.32 [6.30, 8.48]	6.35 [5.35, 7.32]	0.170
		Convalescent	10.78 [9.31, 12.44]	8.01 [6.82, 9.28]	**0.006**

[a] *Geometric mean conc., μg/mL for competitive antibody and log2 for neutralizing antibody titer [95% Confidence Interval].*

[b] *Two-sample t-test for difference in means of antigenic site competitive antibody conc. (log2 μg/mL) or neutralizing antibody titer (log2) between <14 and ≥14 days. The bold values significant means that the p-values are statistically significant at 0.05 level.*

from 34.1 μg/mL in the acute sera to 91.8 μg/mL in the convalescent sera.

No Significant Differences Were Observed in Antibody Levels in the Acute and Convalescent Sera Between HCT Adults Infected With RSV/A vs. RSV/B

The site-specific competitive antibody GMC and neutralizing antibody GMT were compared between RSV/A and RSV/B infected HCT adults (**Table 3**). No significant differences were observed in both the neutralizing antibody level and site-specific competitive antibody concentrations in the acute and convalescent sera between HCT adults infected with RSV/A vs. RSV/B. An increase in antibody activity were detected in the convalescent sera of RSV/A and RSV/B infected HCT adults by all 6 assays. In addition, the total GMC of the 4 competitive antibodies were comparable (133.6 vs. 159.9 μg/mL) in convalescent sera between RSV/A and RSV/B infected HCT adults. This observation is consistent with antigenic site II and IV being well conserved among RSV isolates in the RSV/A and RSV/B subgroups.

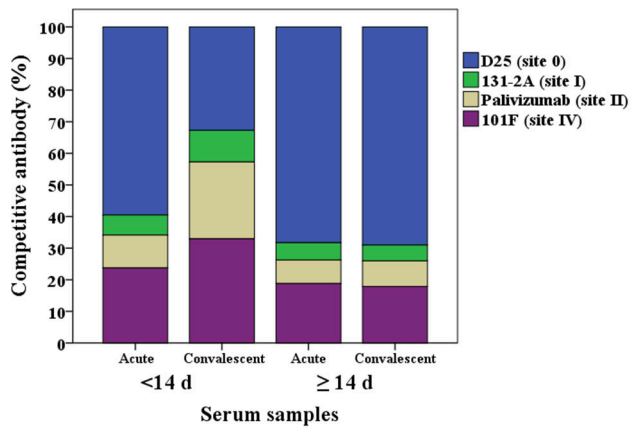

FIGURE 3 | Percentage of each RSV antigenic site-specific competitive antibody GMC (μg/mL) to the total GMC (μg/mL) for acute and convalescent sera from HCT adults shedding virus <14 days and ≥14 days. The Y axis is the percentage of each competitive antibody GMC to the total GMC. The X axis is the serum types: acute or convalescent sera from HCT adults shedding virus <14 days or ≥14 days. The total GMC was the sum of 4 competitive antibody GMC. The percentage of each competitive antibody was that the competitive antibody GMC divided by the total GMC. GMC, geometric mean concentration. $N = 40$ in each competitive antibody assay.

The proportion of the contribution for each site-specific competitive antibody GMC to the total GMC for RSV/A and B infected HCT adults is illustrated in **Figure 4**. D25-competing antibody constitutes ∼60% of the total GMC in the acute sera for both RSVA and B infected HCT adults, and decreased to ∼50% in the convalescent sera; while the percentages of 131-2A, palivizumab-competing antibody, and 101F-competing antibodies increased in the convalescent sera for both A and B infected HCT adults.

Site-Specific Competing Antibodies Correlate to RSV-Specific Neutralizing Antibodies

Pearson's correlation coefficient was calculated to measure the strength of the linear association between site-specific competitive antibodies and RSV-specific neutralizing antibodies. We observed a significant positive correlation between site-specific competitive antibody measured by the four site-specific competitive antibody assays to neutralizing antibody measured by the RSV/A and RSV/B microneutralization assays. Correlation coefficients ranged from 0.33 to 0.83 for acute sera, and 0.50 to 0.88 for convalescent sera with all correlations being significant ($p < 0.05$) (**Figure 5**). The highest correlation was observed between the site IV 101F-competing antibody concentration and

TABLE 3 | RSV Antibody Levels between RSV/A and RSV/B Infected HCT Adults.

RSV antibody	RSV antibody target	Serum	RSV/A infected adults ($n = 22$)	RSV/B infected adults ($n = 18$)	*P*-value[b]
Competitive antibody	Ø	Acute	23.0 [16.1, 42.5][a]	20.3 [12.9, 31.8]	0.423
		Convalescent	50.0 [27.6, 86.5]	62.1 [39.0, 92.1]	0.570
	I	Acute	2.5 [1.4, 5.1]	1.5 [0.9, 2.4]	0.213
		Convalescent	8.7 [3.5, 21.7]	7.6 [3.5, 15.9]	0.814
	II	Acute	3.7 [1.9, 7.5]	2.1 [1.4, 3.2]	0.176
		Convalescent	14.9 [6.0, 36.7]	18.1 [7.5, 43.7]	0.754
	IV	Acute	8.2 [5.2, 14.4]	5.8 [3.9, 8.8]	0.339
		Convalescent	25.7 [12.2, 60.8]	31.5 [16.0, 62.4]	0.678
	Ø, I, II, IV	Total Acute	45.0 [28.8, 75.1]	35.1 [25.0, 49.5]	0.442
		Total Convalescent	133.6 [72.7, 234.1]	159.9 [104.4, 256.1]	0.640
Neutralizing antibody	RSV/A	Acute	6.7 [5.6, 7.8]	6.9 [5.7, 8.1]	0.510
		Convalescent	9.1 [8.1, 10.3]	8.9 [7.5, 10.4]	0.807
	RSV/B	Acute	6.5 [5.7, 7.2]	6.7 [6.0, 7.7]	0.593
		Convalescent	9.3 [8.0, 10.7]	9.8 [8.3, 11.6]	0.395

[a] *Geometric mean conc., μg/mL for competitive antibody and log2 for neutralizing antibody titer [95% Confidence Interval].*

[b] *Two-sample t-test for difference in means of antigenic site competitive Ab conc. (log2 μg/mL) or neutralizing antibody titer (log2) between RSV/A and RSV/B.*

RSV neutralizing antibody titers, and the lowest correlation was observed between site Ø D25-competing antibody concentration and RSV neutralizing antibody titers in RSV infected HCT adults.

DISCUSSION

An in-depth understanding of the human antibody response to RSV infection will aid the development and evaluation of vaccines and therapeutics against RSV disease. Previous studies have reported on the epitopes targeted by RSV-specific neutralizing antibodies in human sera (35, 36), as well as reported the specificities and functional properties of antibodies elicited by natural RSV infection (17). To our knowledge, the present study is the first to describe site-specific competitive antibody responses to the pre-F and post-F conformations and to correlate these site-specific competitive antibody responses to viral clearance and neutralizing antibody responses in RSV naturally infected HCT adults.

In this prospective cohort study, pre-F and post-F competitive antibodies were elicited after RSV infection in HCT adults. The highest site-specific competitive antibody concentration in acute sera was against site Ø on the pre-F conformation, which is consistent with prior published results showing that prefusion F-specific antibodies are prevalent in polyclonal neutralizing antisera (36). However, the high levels of site Ø specific competitive antibodies present in the ≥14 days group were ineffective in viral clearance once virus infection was established. Ngwuta et al. (36) used methods that relied on absorption to define the neutralizing activity associated with pre-F and post F conformations as well as site Ø and site II. Our site-specific competitive antibody assays measured site-specific competitive antibody concentrations but did not directly demonstrate the neutralizing activity of the antigenic site-specific competing-antibodies. In general, RSV-specific antibodies play an important role in preventing infection and a lesser role in

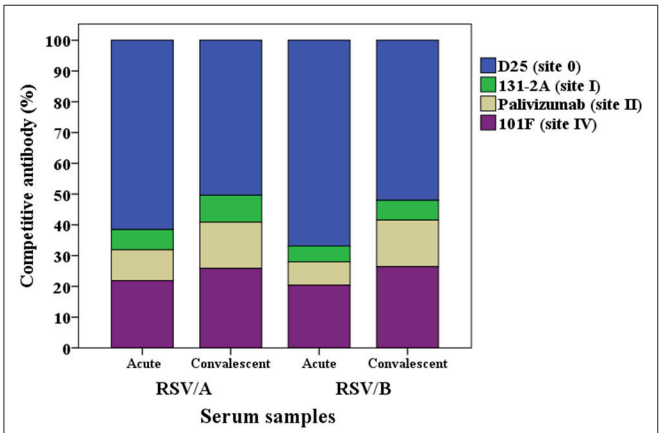

FIGURE 4 | Percentage of each RSV site-specific competitive antibody GMC (μg/mL) to the total GMC (μg/mL) for RSV/A and B infected HCT adults. GMC, geometric mean concentration. $n = 40$ in each competitive antibody assay.

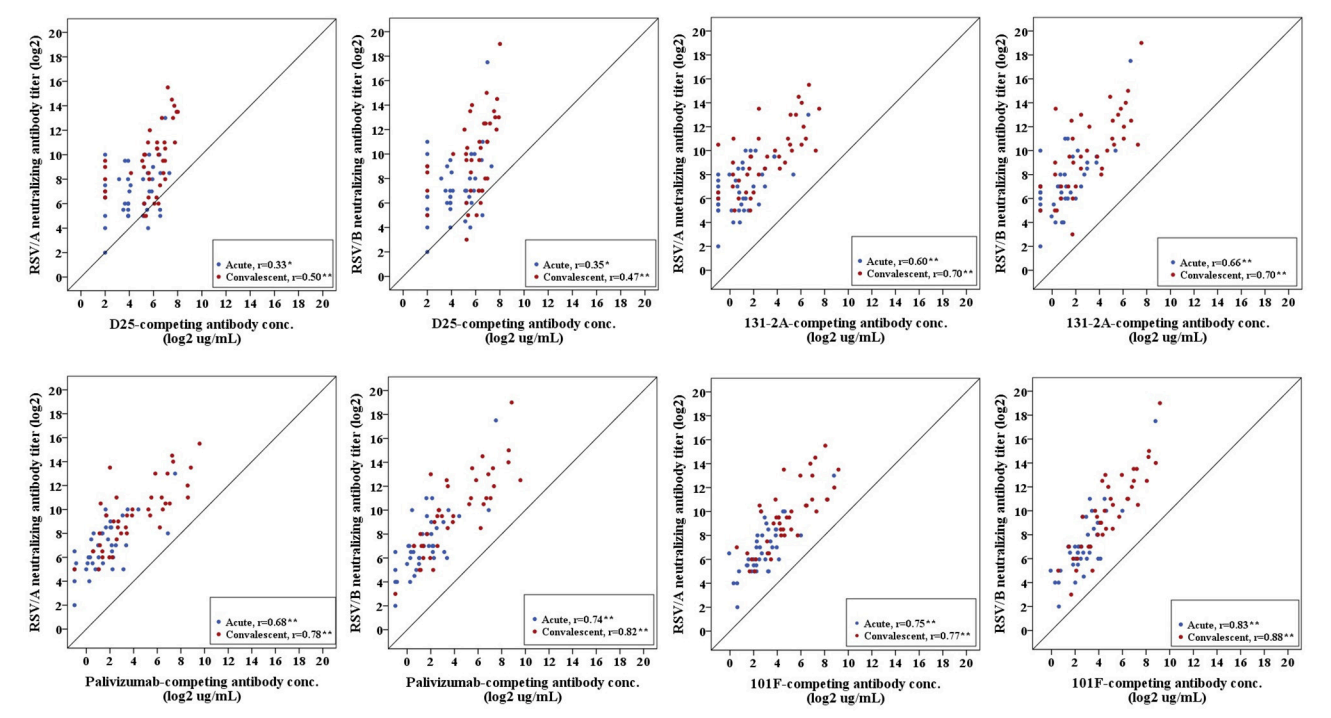

FIGURE 5 | Correlation of RSV antigenic site-specific competitive antibody and RSV-specific neutralizing antibody. Pearson's correlation coefficient was calculated to measure the strength of the linear association. Correlation coefficients ranged from 0.33 to 0.83 for acute sera, and 0.50–0.88 for convalescent sera. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). $n = 40$ in each competitive antibody assay and microneutralization assay.

viral clearance. The group that cleared the virus in <14 days were able to generate a more vigorous humoral response likely reflecting greater immunologic reconstitution after stem cell transplantation. Although we did not measured cell mediated immunity, it is highly likely that this group also had a more robust cellular immune response that contributed to improved viral clearance.

Site I had the lowest competitive antibody concentration, which is also consistent with the results published by Gilman and others (17, 37). The highest fold increase was detected against site II, suggesting that following RSV infection the immune response recognizes other antigenic sites that are not unique to the pre-F form. This was demonstrated by the increase in the proportion of site II, site IV and site I competitive antibody GMC but not site Ø to the total GMC in the convalescent sera of HCT adults who rapidly cleared their viral infection. It is not clear why antibodies that target sites unique to the pre-F form appear to persist for longer duration compared to sites shared by the pre-F and post-F forms. It is important to point out that most of the competitive antibodies were directed to site Ø in the acute serum samples. If similar concentration increases occurred to the four antigenic sites in the convalescent serum samples, this would have resulted in higher proportion increase of site-specific competitive antibodies that had lower concentration in the acute serum samples such as sites II and IV. The change in proportion in site-specific competitive antibody was compounded by a higher LLoD (7.8 μg/mL) for the cell based site Ø competitive antibody assay that was appreciably higher compared to the LLoD (1.0 μg/mL) for the other three competitive antibody assays.

We used Pearson's correlation to determine the associations between the four site-specific competitive antibodies and between site-specific antibody concentrations to neutralizing antibody titers. The highest correlations to RSV-specific neutralizing antibody levels were observed with sites II and IV competitive antibodies. These results suggest that antibodies against palivizumab and 101F epitopes are responsible for a significant proportion of the virus neutralizing capacity of sera. It also suggest that the site-specific competitive antibody assays measures changes that directly relates to changes in neutralizing antibody levels. The lower correlation detected between antigenic-site Ø competitive antibody concentrations and neutralizing antibody titers might be related to antigenic site Ø being the least conserved region compared to other antigenic sites in the F protein (13, 38, 39), although this was not analyzed in the present study. The low correlation also might be due to the relative high LLoD of the site Ø antibody competitive assay compared to the lower LLoD for the other three site-specific competitive antibody assays. Additionally, small changes in antigenic site Ø competitive antibody concentration might be associated with greater changes in neutralizing antibody activity, thereby making it harder to detect a strong correlation due to assay variability. Of the four mAbs we tested for neutralizing antibody activity, D25 mAb that targets site Ø was the most potent of the mAbs, followed by 101F and palivizumab; 131-2A shows no neutralizing potency, which were consistent with what Phung and others reported (40). These different neutralizing antibody activities suggest that sites Ø, II, and IV are major target of the human neutralizing antibody response, and antigenic site I is not a major target. Interesting, although there was a strong correlation between site I competitive antibody concentration and RSV-specific neutralizing antibody titers, the site I mAb (131-2A) did not have detectable neutralizing antibody activity. The direct correlation observed likely reflects the HCT adults' ability to mount an effective humoral response to antigenic sites on the F protein rather than a mechanistic correlation between site I competitive antibody concentration and neutralizing antibody activity.

The effect of RSV infection by viral subgroups (RSV/A and RSV/B) on the humoral immune responses to the four antigenic sites was evaluated in this prospective cohort study. No significant differences were observed in both the neutralizing antibody level and site-specific competitive antibody concentrations measured between HCT recipients infected with RSV/A vs. RSV/B. In addition, the proportion and the total GMC of the four competitive antibodies were comparable in convalescent sera between RSV/A and RSV/B infected HCT adults. This observation is consistent with antigenic site II and site IV being well conserved between RSV/A and RSV/B subgroups, and suggests a monovalent RSV-F vaccine against infection with RSV/A and RSV/B is likely sufficient for protection against severe RSV disease.

Our study has some limitations. The small number of RSV infected HCT adults ($n = 40$) is not representative of adults in the general population. Higher rises in competitive antibody concentration and neutralizing antibody titer after an RSV infection might occur in healthy adults compared to the HCT adults. In addition, we did not measure competitive antibody concentration to site III and site V, the remaining pre-F antigenic sites. Thus, their contribution to the neutralizing antibody activity post-RSV infection was not determined in this cohort of HCT adults infected with RSV.

In summary, the study revealed significantly higher concentrations in RSV F site-specific competitive antibodies (except for site Ø) in HCT adults who shed RSV <14 days compared to ≥14 days, comparable concentrations of competitive antibodies in the convalescent sera between RSV/A and RSV/B infected HCT adults, and a significant positive correlation between site-specific competitive antibodies and RSV-specific neutralizing antibody activity. In conclusion, in RSV naturally infected HCT adults, site-specific competitive antibody responses occurred to antigenic sites found in both the pre-fusion and post-fusion F conformations and were associated with viral clearance. The data might suggest but does not demonstrate that these antibody responses are contributing to the observed more rapid viral clearance. The faster clearance in the individuals with higher antibody responses might just reflect the level of immune competence of these individuals. Therefore, the observed relation between higher antibody responses and more rapid clearance is not necessarily the cause and the consequence. In addition, further evaluation of how the T cell–mediated responses assist in RSV viral clearance is needed. Lastly, HCT recipients would likely benefit from monoclonal antibody immunoprophylaxis or RSV-F vaccine when licensed

for prevention of severe RSV infection during their period of vulnerability post-transplantation.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional review boards of the University of Texas MD Anderson Cancer Center and Baylor College of Medicine with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Texas MD Anderson Cancer Center and Baylor College of Medicine.

AUTHOR CONTRIBUTIONS

XY, RC, and PP designed the research. XY, OI, VA, LA, TM, and PP performed the research. XY, LF-S, and PP analyzed the data. DS and RC provided the samples. KP processed the samples. F-AP, VA, LA, TM, LF-S, KP, DS, RC, and PP edited the text. XY wrote the first draft of the manuscript.

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REVIEW
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Immune-Modulation by the Human Respiratory Syncytial Virus: Focus on Dendritic Cells

*Eduardo I. Tognarelli, Susan M. Bueno and Pablo A. González**

Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

The human respiratory syncytial virus (hRSV) is the leading cause of pneumonia in infants and produces a significant burden in the elderly. It can also infect and produce disease in otherwise healthy adults and recurrently infect those previously exposed to the virus. Importantly, recurrent infections are not necessarily a consequence of antigenic variability, as described for other respiratory viruses, but most likely due to the capacity of this virus to interfere with the host's immune response and the establishment of a protective and long-lasting immunity. Although some genes encoded by hRSV are known to have a direct participation in immune evasion, it seems that repeated infection is mainly given by its capacity to modulate immune components in such a way to promote non-optimal antiviral responses in the host. Importantly, hRSV is known to interfere with dendritic cell (DC) function, which are key cells involved in establishing and regulating protective virus-specific immunity. Notably, hRSV infects DCs, alters their maturation, migration to lymph nodes and their capacity to activate virus-specific T cells, which likely impacts the host antiviral response against this virus. Here, we review and discuss the most important and recent findings related to DC modulation by hRSV, which might be at the basis of recurrent infections in previously infected individuals and hRSV-induced disease. A focus on the interaction between DCs and hRSV will likely contribute to the development of effective prophylactic and antiviral strategies against this virus.

Keywords: dendritic cells (DCs), DC maturation, antigen presentation, T cell activation, inflammation, recurrent infection, immune evasion, immunity

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University of Edinburgh, United Kingdom
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San Raffaele Scientific Institute (IRCCS), Italy

****Correspondence:***
Pablo A. González
pagonzalez@bio.puc.cl

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INTRODUCTION

The human respiratory syncytial virus (hRSV) is the leading cause of infant pneumonia worldwide and also elicits significant morbidity in the elderly and children (1–3). Importantly, infants with partial airway development due to premature birth, airway hyperreactivity, pulmonary hypertension, cystic fibrosis, Down syndrome, neurologic conditions, congenital heart disease, and those that are immunosuppressed are at increased risk of developing severe complications due to hRSV infection, which may even lead to death (4, 5). Nevertheless, individuals that are otherwise healthy, such as infants 2 months old or older can also be infected with hRSV and suffer respiratory illness leading to significant morbidity and eventually life-threatening disease, mainly because of complicated pneumonia (3, 6, 7). Noteworthy, at present there are no vaccines available against hRSV, yet many are under development and being assessed clinically, although few would be suitable for direct application on to newborns (8–11).

An important feature of hRSV is that it is capable of re-infecting healthy children and adults that have been previously infected with this virus (12, 13). hRSV can be classified into two groups (A or B) that mainly differentiate from each other based on nucleotide variability in the attachment glycoprotein G (14). Thanks to affordable sequencing costs and high-throughput sequencing techniques, at present circulating hRSV isolates, can be further sub-classified into at least 14 A genotype groups and 23 B genotypes groups, based on similarities of the G protein gene (14). Furthermore, improved access to whole genome sequencing has opened the possibility for molecular classification and molecular epidemiology studies on hRSV (15). However, despite nucleotide and antigenic variability in the attachment G protein of hRSV, recurrent infections with the same virus can occur at a high frequency within healthy individuals. For instance, adults that had a previous natural infection with hRSV and were then exposed to a virus belonging to the same strain group subsequently manifested several reinfections. At 26 months, 73% of individuals were shown to have two or more re-infections, and 47% had three or more infections (16). Thus, other immune-evasion mechanisms distinct from antigenic variation are likely at the base of host reinfections with hRSV. Because relatively few hRSV-encoded genes are known to directly interfere with the host's antiviral response in a somewhat direct manner, one could suggest that the capacity of hRSV to repeatedly infect the host may derive from its ability to elicit an adaptive antiviral immune response that is non-optimal for the host. Indeed, hRSV has been exhaustively described to skew the host's antiviral immune response toward phenotypes that promote exacerbated lung inflammation in response to lung infection which favor the virus (17–20). Importantly, hRSV lung infection can induce macrophages and monocyte-derived macrophages in this tissue to upregulate the surface expression of PD-L1, which will likely have adverse effects over the function of T cells (21). Furthermore, hRSV can elicit human neonatal regulatory B cells to secrete IL-10, which may also result in non-optimal antiviral T cell responses (22). However, an immune cell of choice targeted by hRSV seems to be dendritic cells (DCs), critical immune cells that initiate and regulate antigen-specific adaptive antiviral immunes responses. Indeed, the phenotype and function of these cells have been broadly reported to be modulated during hRSV infection, both *in vitro* and *in vivo*. Here, we review the latest studies that describe the interaction between hRSV and DCs and how the outcome affects relevant functions of these cells, which will ultimately impact the establishment of an effective antiviral response against hRSV in the host.

HRSV GENES AND THE VIRION STRUCTURE

hRSV is an enveloped, negative-sense, and single-stranded RNA virus that encodes 10 genes that are translated into 11 proteins (**Figure 1**) (23). Its replication and gene transcription occur in the cytoplasm, thanks to the aid of an RNA-dependent RNA-polymerase that is encoded within the viral genome by the L gene (24, 25). For its adequate function, the L protein requires the viral phosphoprotein P, which associates to this RNA polymerase (26, 27). Importantly, the replication of the viral genome and the transcription of its genes are modulated by the hRSV-encoded factors M2-1 and M2-2, which are generated from a common mRNA transcript by a ribosome shift that occurs on the mRNA after producing the M2-1 protein; initiation of M2-2 takes place at a start codon that overlaps with the M2-1 open reading frame (ORF) (23, 28). Noteworthy, in the virion and infected cells, the viral genome is covered by the nucleoprotein N, which is highly expressed within infected cells (29–31). Covering the nucleocapsid, yet beneath the envelope is the matrix protein M, which has been reported to travel to the nucleus of infected cells during the replication cycle of hRSV to inhibit the transcription of host genes and was recently described to interact with actin within infected cells, likely contributing to the transport of virion components into budding virions (30, 32, 33). Importantly, the virion envelope is covered on its surface by the attachment glycoprotein G, which may be dispensable for infection in some cells (34–36), the fusion F glycoprotein which is a type-I integral membrane protein that binds nucleolin for cell infection (37, 38), and the transmembrane protein SH, which forms a viroporin that transports cationic ions (39, 40). Importantly, to date, there is accumulating data that describes the molecular interactions between hRSV structural components, which has allowed establishing an overall comprehensive scenario of how the virus' components are coordinately assembled within infected cells to favor its replication and exit (30). Finally, the non-structural (NS) genes NS1 and NS2 that are at the foremost 3′ of the viral genome, are solely expressed within infected cells (not contained within the virion), and are known to negatively modulate the cellular antiviral interferon type-I response early after infection (**Figure 1**) (41, 42). Importantly, several host factors that modulate the replication cycle of hRSV, such as factors involved in the regulation of host transcription, innate immune responses, regulation of the cytoskeleton, membrane remodeling, and cellular trafficking have been identified and confirmed. These factors could eventually be overexpressed or silenced in host cells to reduce infection or hamper virus replication during infection to avoid pathology (43–45). Although many of the abovementioned hRSV proteins have been studied individually *in vitro*, only few of them have been assessed within immune cells or more specifically DCs, which if performed could eventually reveal relevant immune-evasion or immune-modulation properties for hRSV-encoded viral factors and help identify key viral and host factors that modulate the virus' replication cycle in these cells.

HRSV INFECTS DENDRITIC CELLS

Dendritic cells (DCs) are immune cells that play vital roles in initiating and regulating antigen-specific immune responses against foreign and self-antigens in the organism (46–48). DCs are strategically located both, at peripheral sites and internal

Abbreviations: hRSV, Human respiratory syncytial virus; DCs, dendritic cells; IL, interleukin, LNs, lymph nodes.

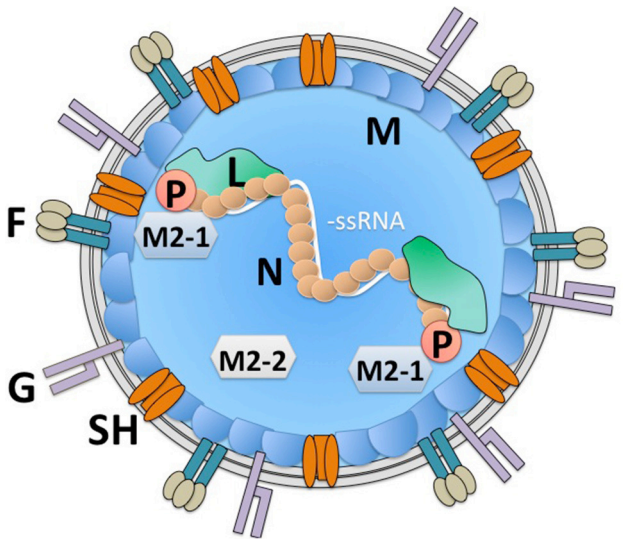

FIGURE 1 | hRSV virion structure. The hRSV genome is a negative-sense single-stranded RNA virus. Its genome is wrapped by the nucleoprotein (N). An RNA-dependent RNA polymerase (L) is associated to the viral genome in the virion. Also, within the virion is the viral phosphoprotein (P), which is required by the L polymerase for its function. Additionally, within the virion are the viral proteins M2-1 and M2-2, derived from a single mRNA, which modulate transcription of viral genes and genome replication by the L polymerase. Beneath the virus envelope is the matrix protein (M), which has been described to inhibit host gene translation in the nucleus of infected cells. Three proteins are immersed in the virus envelope: the small hydrophobic (SH) protein, which forms a viroporin, that transports cations, the attachment glycoprotein (G) and the fusion protein, which arranges as a trimer on the virion surface (F). The viral proteins NS1 and NS2 are non-structural and hence, are not found within the virion.

organs in such a way to sense and capture both, foreign and self-proteins. If the captured protein is immunogenic or associated with activating molecules, DCs undergo phenotypic transformations, and migrate to lymph nodes (LNs) to present protein-derived peptides to antigen-specific $CD8^+$ and $CD4^+$ T cells in MHC-I and MHC-II molecules, respectively (49–52). Importantly, DCs express a battery of molecular sensors that detect pathogen-associated molecular patterns (PAMPs), which leads in most cases to transcriptional and phenotypical changes in these cells in a process known as DC maturation (46, 53, 54). In turn, this process will lead to the activation and modulation of other immune cells that can help resolve infection (49, 50, 55, 56). If DCs capture virus components DCs, these cells will ideally encounter, activate and differentiate virus-specific $CD4^+$ T cells into helper cells (Th) that support the production of antiviral antibodies by B cells, as well as promote the generation of cytotoxic T cells (CTLs) that eliminate virus-infected cells (57–59). Given the crucial role of DCs in initiating antigen-specific adaptive antiviral immune responses, mainly through the activation and differentiation of T cells, such as $CD4^+$ T helper cells, numerous viruses and other pathogens have evolved molecular determinants and mechanisms to interfere with the function of DCs, in such a way to impair the establishment of an effective antiviral immune response (60–67).

Noteworthy, hRSV infects DCs *in vitro* and is known to interfere with their functions, even though DCs seem not to be an optimal viral substrate for this virus. Indeed, many *in vitro* studies report relatively low virus yields from hRSV-infected DCs, even at multiplicity of infection (MOI) values that generally lead to complete infection of epithelial cell cultures (MOI >3) (66, 68–71). This phenomenon is suggestive of abortive hRSV infection in a significant proportion of DCs (66, 68, 69, 71, 72). Thus, it seems that hRSV likely infects DCs as a strategy to target a pivotal immune component to indirectly favor its infectious process in the host, namely the infection of epithelial lung cells that yield high amounts of infective virions, which will expand the magnitude of the infection within the individual and promote its dissemination onto others. Interestingly, hRSV may reach other tissues besides the airways during infection, such as the central nervous system (CNS) (73, 74).

Although cell surface receptors that lead to hRSV cell infection have been identified, such as cellular heparan sulfate glycosaminoglycans that act as attachment factors for the hRSV G glycoprotein (75, 76), as well as nucleolin (37) and ICAM1 (77) as ligands for the F fusion protein, the exact mechanism by which hRSV enters DCs has not been corroborated and could eventually be different compared to that observed in other cells, such as epithelial cells (78). Noteworthy, opsonized hRSV particles (hRSV covered with virus-specific antibodies), which is known to hamper virus-infection of epithelial cells, were recently shown to be nevertheless capable of infecting DCs and interfere with their function, such as activating T cells (**Figure 2**). Importantly, this process was shown to be mediated by Fcγ receptors (FcγRs) expressed on the surface of DCs (79). Because opsonized hRSV particles retained the same ability as free hRSV to interfere with DC activation of T cells, this process would favor impaired DC function in time despite the individual having anti-hRSV antibodies. Thus, hindered DC function by hRSV would ensue during each exposure to the virus, likely hampering the capacity of the host to mount an effective response against this virus.

HRSV-DENDRITIC CELL INTERACTION

Growing amounts of studies have focused on the relationship between hRSV and DCs increasing our knowledge on the outcome of this interaction. While some reports indicate that DCs infected with hRSV can sense viral components, which can lead to somewhat activating signaling pathways within these cells, other reports indicate that hRSV determinants interfere with antiviral signaling pathways within DCs, such as those related to interferon type-I responses, which is mediated by STAT-1 and STAT-2 (80). Importantly, the activation or inhibition of distinct intracellular signaling pathways within DCs by hRSV generally leads to DC outcomes that are overall poorly activating for T cells, with hRSV-infected DCs displaying poor- or only partial-maturation phenotypes both, in human and murine DCs (58, 59, 66, 68, 69, 72, 81).

NS1 and NS2 have been reported as two hRSV factors that are directly involved in inhibiting the maturation of human DCs and impairing the secretion of type-I IFNs by

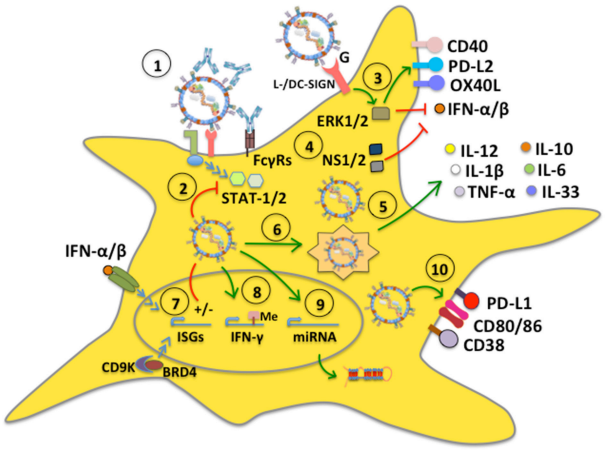

FIGURE 2 | hRSV modulates dendritic cell function. (1) DC infection with hRSV can occur even in the presence of antibodies bound to the virus (opsonized virus), which enter DCs through Fcγ receptors (FcγRs). (2) hRSV is capable of inhibiting antiviral signaling pathways mediated by STAT-1 and STAT-2, likely through its NS proteins. (3) The G glycoprotein signals through L-/DC-SIGN and phosphorylates ERK1/2, which translates into the upregulation of surface expression of CD40, OX40L, and PD-L2, whereas it downregulates IFN-α secretion. (4) The hRSV NS1 and NS2 proteins interfere with type-I interferon secretion. (5) hRSV induces the secretion of proinflammatory cytokines by DCs. Some mDC subsets (BDCA-1$^+$ and BDCA-3$^+$) secrete IL-10. (6) hRSV induces autophagy and is processed by the autophagosome leading to cytokine release and lung inflammation. (7) hRSV differentially modulates the expression of interferon-stimulated genes (ISGs), through IFN-dependent and independent pathways. (8) hRSV induces the activity of demethylases to modulate gene expression, such as IFN-γ, preventing an antiviral response. (9) hRSV upregulates the expression of specific host microRNAs. (10) hRSV stimulates the expression of CD80 and CD86. Additionally, the virus upregulates PD-L1 and CD38 expression on the DC surface to modulate inflammation in the lungs.

myeloid DCs (mDCs), which can enhance the differentiation of CD4$^+$ T cells into Th2-phenotypes and promote the generation of Th2-polarized anti-hRSV immune responses in the host. Consequently, these immune responses can be detrimental to the host, as they promote exacerbated inflammation in the lungs (80, 82). Another hRSV-encoded determinant that has been reported to alter the maturation of DCs directly is the surface glycoprotein G, which was described to trigger ERK1 and ERK2 phosphorylation within these cells, mainly through DC- and L-SIGN molecules on the DC surface. Neutralization of DC- and L-SIGN induced significant secretion of IFN-α, MIP-1α, and MIP-1β in plasmacytoid DCs (pDCs) inoculated with hRSV, suggesting that this virus alters DC maturation through this signaling pathway thanks to this glycoprotein (**Figure 2**) (83). Such intracellular signaling events induced by the hRSV G glycoprotein in these cells may explain why mice immunized with a recombinant vaccinia virus (rVV) expressing hRSV G and subsequently challenged with hRSV displayed lung inflammatory DCs that expressed increased levels of the programmed cell death 1 ligand 2 (PD-L2), as well as low CD40 and OX40 ligand (OX40L), when compared to mice inoculated with a rVV expressing the hRSV F fusion protein, which were also challenged with hRSV. Noteworthy, the expression or not of these co-stimulatory molecules on the DC surface was shown to have profound effects over T cell activation, suggesting that the hRSV G glycoprotein has some important immune-modulatory properties, possibly mediated through DCs (84).

Other studies have found that hRSV infection promotes DC maturation and the secretion of pro-inflammatory cytokines by these cells, either directly or through the infection of other cells. For instance, primary human DCs characterized as mDC1, mDC2 or pDC were found to upregulate phenotypic markers associated to maturation after hRSV inoculation, which was dependent on divalent cations suggesting the participation of C-type lectin receptors in this process (71). Other human DC subsets studied with hRSV have been BDCA-1$^+$ and BDCA-3$^+$ mDCs obtained from peripheral blood. Similar to other DC subsets, these cells were susceptible to infection with hRSV, and while they expressed increased amounts of CD80 and CD86 in response to this virus as compared to non-infected cells, they also expressed the inhibitory costimulatory receptor PD-L1 and secreted IL-10. Furthermore, hRSV-infected BDCA-1$^+$ mDCs produced pro-inflammatory cytokines and chemokines, namely IL-1β, IL-6, IL-12, MIP-1α, and TNF-α and displayed a reduced capacity to stimulate T cells (85). Hence, hRSV can produce significant changes in DCs once infected, namely by modulating the expression of T cell-activating molecules on their surface. etSuch modulation was accompanied by the expression of inhibitory receptors and the secretion of numerous immune-modulatory cytokines, mostly inflammatory.

Another study reported that depending on the hRSV strain used; human DCs can respond differentially to this virus by secreting different kinds of type-I and type-III IFNs, and transcribe distinct interferon-stimulated genes (ISGs). Although both serotypes of hRSV A and B induced the expressing of IFN-β, IFN-α1, IFN-α8, and IFN-λ1-3, only the serotype A2 induced IFN-α2, -α14, and -α21 (86). Type-I IFN-dependent activation of ISGs during an hRSV infection was shown to be modulated by the virus' ability to downregulate suppressor of cytokine signaling (SOCS1 and SOCS3) through its RSV G protein, in turn affecting IFN-β and ISG15 expression (87). Moreover, during hRSV infection, airway epithelial cells activate cyclin-dependent kinase 9 (CDK9) and associates with bromodomain 4 (BRD4) to activate IRF3-dependent IFN-stimulated genes, independent of IFN-signaling. Altogether, these processes contribute to increased RSV-induced airway inflammation and disease (88, 89). Although these findings may have important implications over disease severity and the outcome of the host's immune response, as well as the modulation of immunity, the implications of different hRSV serotypes in clinical infections and *in vitro* studies are somewhat seldom assessed.

IL-33 is a key cytokine involved in Th2 immune responses and inflammatory airway diseases and is usually secreted in high amounts by epithelial cells in this tissue (90, 91). Interestingly, hRSV-infected DCs within the lungs of hRSV-infected animals have been reported to have elevated levels of IL-33 mRNA and were suggested to be a relevant source of IL-33 in the lungs of hRSV-infected mice (92). Noteworthy, blocking TLR3 or TLR7 signaling with antagonists significantly reduced the levels of IL-33 mRNA produced by DCs, suggesting that IL-33 expression

in these cells upon hRSV infection is TLR-dependent (92). Interestingly, a study on the identification of enzymes that alter the methylation status of the host DNA suggests that the profile of cytokines secreted by DCs in response to hRSV may be driven by specific demethylases induced by infection with this virus (**Figure 2**). In a study by Ptaschinski and colleagues, it was shown that hRSV upregulates the expression of Kdm5b/Jarid1b H3K4 demethylase in response to *in vitro* hRSV infection of DCs and that inhibiting this factor with siRNA led to a 10-fold increase in IFN-β production, as well as other cytokines. Furthermore, mice that had Kdm5b specifically deleted in DCs showed higher production of IFN-γ and reduced IL-4 and IL-5 secretion after hRSV infection, as well as lesser lung inflammatory mucus production in this tissue. Some of these findings were mirrored in human DCs treated with an inhibitor of KDM5B suggesting that this factor, which is induced by hRSV can directly inhibit the expression of type-I IFN and other cytokines within infected DCs, likely favoring hRSV replication and virus-induced lung disease (93). This finding calls for further studies assessing the roles of such DNA-modification enzymes in host cells and how they are modulated by hRSV infection, potentially unveiling novel antiviral strategies.

Studies that have assessed the role of autophagy in hRSV-infected DCs have found that this process is involved in driving the production of cytokines that lead to lung inflammation (**Figure 2**). Indeed, inhibition of autophagy with inhibitors such as siRNA, or experiments with Beclin$^{+/-}$ mouse-derived DCs, or exposing Beclin$^{+/-}$ mice to hRSV significantly reduced the production of cytokines by CD4^{+} T cells. In these cases, hRSV-infected lungs displayed increased amounts of mucus secretion, and cellular infiltrates, unveiling important roles for autophagy in DCs in response to hRSV infection (94, 95). Additionally, Beclin-1$^{+/-}$ DCs were shown to express reduced amounts of MHC class II molecules on their surface and were less effective at stimulating the production of IFN-γ and IL-17 in co-cultures with CD4^{+} T cells, as compared to controls; furthermore, they promoted the secretion of Th2-cytokines by these T cells. On the other hand, transfer of hRSV-infected Beclin-1$^{+/-}$ DCs into the airways of wild-type mice elicited lung disease accompanied with the production of significant amounts of Th2 cytokines upon later challenge with hRSV (94, 95). Notably, a recent study found that hRSV induces Sirtuin-1 (SIRT1) expression in DCs, which is a NAD(+)-dependent deacetylase that is associated with the induction of autophagy. In this study, it was found that SIRT1 exerts antiviral effects against hRSV *in vitro* and that using an inhibitor of this enzyme, siRNA o analyzing the specific effect of SIRT1 knockout in DCs not only attenuated autophagy in these cells, but these animals manifested exacerbated hRSV-pathology (96).

When searching for particular markers induced by hRSV infection in DCs or cytokines elicited by hRSV-infected DCs, a recent study found that this virus induces CD38 expression in these cells, which is an ectoenzyme that catalyzes the synthesis of cyclic ADPR (cADPR). The expression of this enzyme was found to be dependent on hRSV-induced type-I IFN and inhibitors of CD38 significantly reduced the expression of type-I/III IFNs, suggesting that CD38 is regulated by- and influences IFNs in DCs and thus, modulating this enzyme may be an intriguing target for improving the host's response to hRSV infection and pathology (97).

Despite poor, or relatively low expression of surface markers associated with the potential capacity of DCs to activate or promote the activation of T cells, a common feature that has been repeatedly observed in hRSV-infected DCs is the secretion of cytokines that may promote the differentiation of T cells into phenotypes that are not favorable for the effective resolution of infection, such as IL-6 and IL-10, which lead to Th2 CD4^{+} T cell responses (66, 81). Concomitantly, cytokines such as IL-12 that tend to elicit T cells with phenotypes that are commonly associated with efficient viral clearance, such as Th1 are usually not secreted by hRSV-infected DCs, (66, 68, 69, 98–101). Interestingly, a study reported that the secretion of different cytokine profiles by hRSV-infected human DCs depends on whether these cells originate from neonates or adults. For instance, DCs derived from blood cord samples secrete more TGF-β1 than DCs obtained from adult blood in response to hRSV, suggesting the existence of age-related phenotypes in DCs that may translate into differential responses to hRSV (further discussed below) (102).

Interestingly, a somewhat novel approach that is being undertaken to study the relationship between hRSV and DCs is analyzing the profile of miRNA expression in these cells (**Figure 2**). A recent study found that DC infection with hRSV elicited the upregulation of a specific miRNA, namely let-7b (103). This study is complemented by another report that found that hRSV infection induces significant expression of three miRNAs, namely hsa-miR-4448, hsa-miR-30a-5p, and hsa-miR-4634 in human DCs (104). Interestingly, this latter study also performed comparative analyses of miRNA profiles between DCs infected with hRSV and a related virus, namely the human metapneumovirus, and found that both viruses induced the expression of elevated levels of hsa-miR-4634. Elucidating the contribution of these miRNAs in DCs in response to hRSV remains to be determined.

DENDRITIC CELL PHENOTYPE AND MIGRATION UPON HRSV INFECTION *IN VIVO*

Although the study of DC infection with hRSV *in vitro* has provided valuable insights on the consequences that hRSV infection has over these cells, studying the effects of hRSV over DCs at the site of infection is likely key for understanding the contribution of this interaction to airway disease. They are also important as they help determine if the results obtained *in vitro* mirror what occurs in the respiratory tissue. Interestingly, several studies have addressed the question of how DCs respond to lung infection with hRSV, yet only a few have directly assessed whether the analyzed DCs are actually infected with hRSV, or if the observed effects are driven by viral antigen or other factors in the virus-infected environment. Evidence for the participation of hRSV-infected DCs in the exacerbated inflammatory response to hRSV has been reported by the instillation of hRSV-infected DCs

directly into the airways, which produced a pathological Th2-type response in mice (105). Regarding how DCs are infected by hRSV *in vivo,* a study by Ugonna et al. explored in an *in vitro* setting whether cells present in the respiratory tissues may contribute to hRSV access to DCs. Interestingly, by analyzing the interrelationship between DCs and epithelial cells, and their reciprocal infection in co-culture transwell assays they found that macrophages on the apical surface of differentiated epithelia helped hRSV infect DCs in the basal chamber, suggesting that lung macrophages may have a potentially relevant, and previously unknown role in DC infection with hRSV (106). However, this remains to be assessed and demonstrated in *in vivo* settings. Furthermore, other reports have analyzed whether cells that are usually adjacent to DCs in the infected tissues may influence the outcome of DCs. Interestingly, one study found that hRSV-infected rat airway epithelial cells elicited DC activation, increasing MHC-II and CD86 surface expression, as well as enhancing T cell proliferation in mixed lymphocyte reactions. Noteworthy, this activation was dependent on thymic stromal lymphopoietin (TSLP), a pleiotropic cytokine implicated in inflammatory diseases, which was secreted by hRSV-infected airway epithelial cells (**Figure 3**) (107, 108). On the other hand, airway DCs incubated with inflammatory mediators secreted by hRSV-infected lung epithelial cells was shown to induce their differentiation into functional DCs capable of activating T cells characterized by a type-I IFN antiviral response. Nevertheless, these DCs only had a partial mature phenotype, as they were unable to up-regulate CD80, CD83, CD86, and CCR7, and were unresponsive to TLR triggering, suggesting that the airway epithelium elicits DCs with a somewhat suppressive phenotype, even under inflammatory conditions induced in the lungs after infection with hRSV (109).

Given the existence of diverse types of DCs in the lung tissue, different studies have focused on analyzing the effects of hRSV over distinct subtypes of DCs in the respiratory tissue and their contribution to hRSV-associated lung pathology (110). Early studies on the dynamics of DCs *in vivo* in the lungs of hRSV-infected animals showed that pDCs accumulate in this tissue and secrete type-I IFNs, thus contributing to limit viral replication and the extension of pathology induced by hRSV infection (111, 112). Interestingly, other subsets besides pDCs, such as conventional DCs (cDCs) also accumulate in the lungs of hRSV-infected animals (113, 114). Noteworthy, together with increased accumulation of DCs in the lungs, an increase in the amount of these cells in the associated LNs also occurs, with DCs exhibiting varying phenotypes at this site (114–118). However, in most cases, it is unclear whether the analyzed DCs are infected by hRSV, or if their migration is influenced by other factors within the infected tissue, such as hRSV antigens or cytokines elicited in the infected tissue.

Importantly, differences in the phenotype of DCs obtained from the lungs of hRSV-infected animals have also been assessed based on the age of the individual, by analyzing these cells in the lungs of neonates and adults. One such study found that while CD103+ DCs dominated the response to hRSV in neonates, $CD11b^+$ DCs were underrepresented in this group both, in number and function as compared to adult animals. For instance, pDCs from neonate animals display limited type-I IFN responses during hRSV infection, as compared to adult pDCs (119). Noteworthy, the transfer of adult pDCs into neonate animals reduced the Th2-biased immunopathology produced by hRSV which was elicited after a subsequent challenge with hRSV, further evidencing significant differences between DCs obtained from these different age-groups in response to hRSV infection (120). In line, with this notion, another study found that neonatal $CD11b^+$ mDCs expressed increased levels of the IL-4 receptor IL-4Rα, as compared to adult DCs and that specifically deleting this cytokine receptor from $CD11b^+$ mDCs significantly decreased hRSV-induced immunopathophysiology. Concomitantly, overexpression of IL-4Rα on the surface of $CD11b^+$ DCs of adult animals and transferring them into adult mice elicited hRSV-induced immunopathology. Finally, an important finding in this study was also the fact that increased IL-4Rα expression in DCs was associated with reduced maturation of DCs during hRSV infection (121). Interestingly, another study found that age-dependent DC responses against hRSV could be modified through the use of TLR agonists, such as agonists for TLR4 or TLR9 at the time of infection. By using such agonists, a significant change in the response of hRSV-specific $CD8^+$ T cells could be observed, evidenced as a shift in the immunodominance of the antigens to which these T cells responded when activated by neonate DCs. The shifted response found resembled more that was observed in adults, which is associated with less severe disease (122). Overall, the findings outlined above suggest particular and distinctive features between lung DCs from neonates and adults after hRSV infection, at least in the mouse model, and could be considered in the future for potential therapeutic and prophylaxis approaches in neonates and adults.

Another area of intense research regarding the interaction between hRSV and DCs is the migration of these cells, as hRSV lung infection may result in alterations on the of migration pattern of different subsets of DCs from the lungs to LNs. Interestingly, an *in vitro* study found that human monocyte-derived DCs infected with hRSV failed to downregulate CCR1, CCR2, and CCR5 from their surface, which is required for DCs to effectively migration to LNs. Indeed, these infected DCs migrated significantly less in chemokine gradients in *in vitro* assays. Furthermore, hRSV-infected DCs failed to upregulate CCR7 on their surface, which is known to promote the migration of antigen-exposed DCs to LNs for presenting antigens to T cells (123). Nevertheless, these findings need to be corroborated in hRSV-infected individuals. Even though cDCs accumulate in the lungs of hRSV-infected animals as mentioned above (113, 114), it has been reported that monocyte DC precursors are depleted during infection from this tissue. Importantly, this phenomenon has been suggested to favor opportunistic lung infections by pathogens, such as bacteria (116). Notably, two major subsets of lung tissue cDCs have been shown to transport hRSV RNA to the LNs, namely $CD103^+/CD11b^{low}/CD11c^+$ and $CD103^-/CD11b^{high}/CD11c^+$ cDCs and present hRSV antigens to T cells on MHC-I and MHC-II molecules (118). Interestingly, a study that was mentioned in the section above which analyzed the effects of TLR agonists over DCs infected with hRSV showed

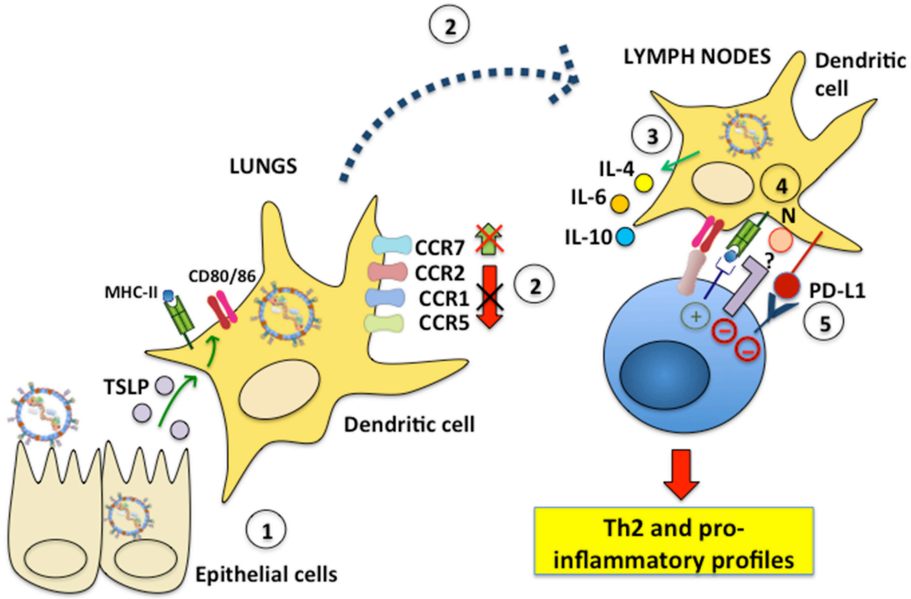

FIGURE 3 | hRSV modulates dendritic cell migration and induces inflammatory profiles in T cells. (1) Epithelial cells infected with hRSV produce TSLP, which elicits MHC-II, CD80, and CD86 expression in lung DCs. (2) hRSV-infected DCs fail to downmodulate the surface expression of the chemokine receptors CCR1, CCR2 and CCR5, which is needed for effective DC migration to lymph nodes. Additionally, CCR7, which favors DC migration to the LNs, is not upregulated on the surface of hRSV-infected DCs. (3) Nevertheless, during infection hRSV-infected DCs migrate to the LNs to interact with T cells. hRSV-infected DCs secrete IL-4, IL-6, and IL-10 and promote the differentiation of $CD4^+$ helper T cells toward a Th2 phenotype. (4) The hRSV N protein expressed on the DC surface mediates impaired immunological synapse assembly. The signaling events led by host proteins that interact with N are unknown. (5) hRSV induces PD-L1 expression on the DC surface which signals negatively within inflammatory T cells.

that the treatment with these molecules increased the numbers of $CD11b^+$ and $CD103^+$ DCs migrating from the lungs to draining LNs in neonates, likely supporting an improved antiviral response thanks to DCs with adult-like phenotypes migrating to this site for optimal T cell activation (122).

Although some studies suggest a positive role for lung cDCs during hRSV infection, other reports indicate that these cells may play detrimental functions for the host during hRSV infection (111, 113, 115). These effects have been evidenced, for example by blocking the chemokine CCL20 in hRSV-infected animals or knocking-out its associated receptor (CCR6), which significantly reduced the presence of cDCs in the airway tissue without affecting pDCs. These scenarios overall translated into improved outcomes of hRSV infection, suggesting that a balance between pDCs and cDCs in the lungs is likely associated with hRSV-induced pathology (114, 124). The finding supports this notion that depletion of pDCs from the lungs of animals significantly increases pulmonary disease after a challenge with hRSV (112). Concomitantly, activation of pDCs in the lungs of hRSV-infected animals was shown to limit the replication of hRSV in the airways and decrease hRSV-associated pathology (114). Thus, pDCs, as well as cDCs in the airways, are considered to interplay limiting hRSV replication and regulating inflammation (111, 112, 114). However, whether the findings described above in the mouse model hold in humans remains to be determined. Interestingly, some observations performed in animal models have been mirrored in patients, such as individuals experiencing hRSV bronchiolitis having significantly higher numbers of cDCs than pDCs in the blood, suggesting an imbalance in the proportion of DC subtypes in children with bronchiolitis, as compared to healthy individuals after hRSV infection (125).

MOLECULAR MECHANISMS INVOLVED IN THE REDUCED CAPACITY OF HRSV-INFECTED DCS TO ACTIVATE T CELLS

A substantial effect of hRSV over DC function is its ability to reduce the capacity of hRSV-infected DCs to effectively activate $CD4^+$ and $CD8^+$ T cells (**Figure 3**). Although this phenomenon has been reported *in vitro* and is not necessarily echoed *in vivo* in humans or animal models, a relationship between the *in vitro* observations and potential *in vivo* effects likely exists in terms of non-optimal T cell activation taking place, as a result of DC infection with hRSV (66, 68, 72, 126). Given that the reported phenotype of hRSV-infected DCs is generally associated to weak or incomplete maturation, it is somewhat expected that the activation of T cells by infected DCs will not be optimal and may lead to less potent, or inadequately differentiated or polarized hRSV-specific T cells. Interestingly, some studies have identified particular hRSV factors that are involved in hampering the capacity of DCs to activate T cells. For instance, a report published in 2008 that studied the interaction

between DCs and hRSV suggested that hRSV factors driving poor T cell activation by hRSV-infected DCs were membrane-bound and interfered with the establishment of the immunological synapse (IS) between T cells and hRSV-infected DCs *in vitro*, which is essential for productive T cell activation (49, 66). This notion was reinforced by the fact that supernatants from hRSV-infected DCs enhanced the activation of T cells in the presence of plate-bound activating anti-CD3 and anti-CD28 antibodies (66). However, another study suggested that soluble factors secreted by hRSV-infected DCs were involved in impaired T cell activation by hRSV-infected DCs (68, 99). It is possible thus that both, membrane-bound and soluble factors secreted on to the extracellular media, or at the DC-T cell immunological synapse negatively modulate the activation of T cells, given that the is highly susceptible to modulation by factors of both natures (49).

A membrane-bound hRSV factor that has been reported to mediate negative effects over T cells is the hRSV F fusion protein, which expressed on the surface of epithelial cells was shown to inhibit T cell activation *in vitro* (127). However, the effect of the hRSV F protein has not been assessed in the context of DC-T cell interactions. Still, a study that assessed the role of the hRSV N nucleoprotein in mediating detrimental effects over the DC-T cell interaction found that this protein was present on the surface of hRSV-infected DCs and could directly mediate the inhibition of T cell activation (128). An interesting finding in this study was the fact that the hRSV N protein was shown to be able to interfere with the establishment of productive immunological synapses between T cells and cognate ligand mounted on lipid bilayers (128). Importantly, the identification of the hRSV N protein on the surface of infected cells had not been previously reported for this virus. Another hRSV factor known to hamper the capacity of DCs to activate T cells is NS1, which has been found to negatively modulate the capacity of human DCs to activate both, $CD4^+$ and $CD8^+$ T cells (129). Additionally, NS1 has also been reported to favor the differentiation of DCs toward phenotypes that promote the activation of $CD4^+$ T cells that secrete IL-4, yet by a mechanism that is independent of its capacity to modulate IFN-I signaling (129).

Again, depending on whether DCs inoculated with hRSV originate from neonate or adult animals, a study by Thornburg et al. reported differences in the capacity of such hRSV-infected DCs to activate autologous T cells, with DCs from adult mice eliciting IFN-γ, TNF-α, and IL-12 secretion in co-cultures and neonate DCs (from blood cords) eliciting IL-1β, IL-4, IL-6, and IL-17 release (102). Furthermore, neonatal $CD103^+$ DCs have been shown to promote the proliferation of T cells differently, as compared to adult $CD103^+$ DCs, namely by eliciting the expansion of T cells against distinct antigens, defining different hRSV antigenic hierarchal profiles. The differences observed with DCs from animals of different ages in this and other studies, suggest that neonatal DCs overall display limited T cell co-stimulatory properties when compared to adult DCs, which could eventually relate to infants being more susceptible to severe disease than adults after hRSV infection (119). Another report found two phenotypically and functionally distinct populations of $CD103^+$ DCs in the lungs of neonatal mice following hRSV infection, and that those that were $CD103^{lo}$ were functionally limited at activating hRSV-specific T cells, while those that were $CD103^{hi}$ were capable of potently activating T cells (130). Whether such differences mirror the adaptive immune responses to hRSV *in vivo* in both adults and infant humans, remains to be determined.

Although several studies have reported impaired T cell activation by hRSV-infected DCs *in vitro*, *in vivo* studies reveal that hRSV-specific T cells are expanded in the organism after infection, yet they generally display pro-inflammatory phenotypes that likely contribute, or are the root of exacerbated lung damage during hRSV infection (131–133). Thus, reduced activation of T cells *in vitro* seems to translate *in vivo* as the activation of virus-specific T cells with detrimental phenotypes that respond to an hRSV lung infection. Yet, a role for hRSV-infected DCs has also been described in regulating or controlling pathogenic T cells during infection. Analyses of human and murine lung DCs report that these cells express PD-L1 and that this molecule is critical for suppressing the activity of inflammatory T cells (**Figure 3**). This finding suggests a vital role for the PD-L1/PD-1 axis in DC-T cell interactions for limiting the inflammatory response of T cells to hRSV (134). Nevertheless, the results of this study contrast with those of another report that found that hRSV inhibited the capacity of pDCs to produce a regulatory T cell response to inhaled antigens, eliciting an alteration in their immunotolerogenic potential (135).

Because of the key role of DCs in mounting and regulating immune responses to viruses such as hRSV, novel vaccines are needed to strategically seek and target these cells in a specific manner. A recently described approach that directly involves DCs consists on a DNA vaccine encoding the ectodomain of the hRSV F protein fused to a single-chain variable fragment F (scFv) that directly targets the viral antigen to DEC205 on the DC surface. This viral protein is then phagocytosis by DCs through this receptor and processed for antigen presentation to T cells. Interestingly, this strategy has been reported to elicit high levels of anti-hRSV antibodies with neutralizing capacity and induce F-specific $CD8^+$ T cells that elicit a Th1 response in mice (136).

CONCLUDING REMARKS

Over the last years, new studies have revealed novel features of the DC-hRSV interaction, providing unanticipated outcomes in DCs after infection with this virus and helping identify different host and viral factors that participate in these processes. Because DCs play pivotal roles in initiating and regulating antigen-specific immune responses to infections, it seems relevant that particular focus should be given to these cells both, before and after interacting with hRSV. Indeed, these cells are needed for establishing an effective antiviral immune response in the lungs to promote viral clearance, while altogether avoid exacerbated inflammation of the airways. The fact that hRSV can repeatedly reinfect the host without the need of varying its antigens calls for special attention to the steps that determine the founding events of the host antiviral response, in such a way to train the immune system to withstand the negative immune-modulatory properties of this virus or counteract its potent Th-skewing effects. In both

cases, a protective immune response elicited against hRSV, such as one that could be induced by a vaccine, should be strong enough to bear subsequent viral reinfections that will push to revert this outcome and elicit scenarios that are favorable for the virus. Overall, significant efforts should be invested in identifying viral and host factors that hamper hRSV-infected DCs, or bystander DCs in the infected tissue from promoting effective antiviral immune responses against this virus. Importantly, promoting positive hRSV-DC interactions during re-infections, after virus-specific immune components have already been polarized toward detrimental phenotypes by hRSV may be more complicated than promoting an effective immune response before primary infection. Indeed, shifting a pre-existing antigen-specific immune profile has proven somewhat challenging in the context of other diseases, such as cancer and autoimmunity, although the antigens involved in these pathologies are seldom known, which is not the case for hRSV.

Finally, some questions that remain open regarding the roles of DCs in hRSV infection are: How can we enable hRSV-infected DCs to elicit effective antiviral immune responses during primary infection and re-infections against this virus? Are there hRSV factors, or hRSV-induced factors elicited during reinfections that revert, through their effects over DCs, otherwise effective primary anti-hRSV immune responses? Do the different circulating hRSV A and B genotypes affect the outcomes of hRSV-infected DCs equally? What are the roles of hRSV-infected and non-infected DCs in the lungs of hRSV-infected individuals? Do the findings reported in the mouse model hold in humans? Hopefully, answers to these and many other questions regarding DCs and their interaction with hRSV will provide novel insights that will help limit the burden and mortality associated with the epidemiology of this important respiratory virus.

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PG and SB wrote, revised, and edited the article and figures. ET wrote and revised the article and drew the figures.

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LAIR-1 Limits Neutrophilic Airway Inflammation

Kuldeep Kumawat[1,2†], Ruben J. Geerdink[1,2†], Marije P. Hennus[3], Mojtaba Abdul Roda[4], Ingrid van Ark[4], Thea Leusink-Muis[4], Gert Folkerts[4], Anita van Oort-Jansen[1], Alexandra Mazharian[5], Steve P. Watson[5,6], Frank E. Coenjaerts[7], Louis Bont[1,8] and Linde Meyaard[1,2]**

[1] Laboratory for Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, Netherlands, [2] Oncode Institute, University Medical Center Utrecht, Utrecht, Netherlands, [3] Department of Pediatric Intensive Care, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, Netherlands, [4] Division of Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, Netherlands, [5] Centre for Cardiovascular Sciences, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [6] Centre of Membrane Proteins and Receptors, Universities of Birmingham and Nottingham, Birmingham, United Kingdom, [7] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, Netherlands, [8] Department of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, Netherlands

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Edited by:
Steven Varga,
The University of Iowa, United States

Reviewed by:
Kartika Padhan,
National Institutes of Health (NIH), United States
Giulia Fabozzi,
National Institute of Allergy and Infectious Diseases (NIAID), United States

***Correspondence:**
Louis Bont
l.bont@umcutrecht.nl
Linde Meyaard
l.meyaard@umcutrecht.nl

[†]These authors have contributed equally to this work

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Neutrophils are crucial to antimicrobial defense, but excessive neutrophilic inflammation induces immune pathology. The mechanisms by which neutrophils are regulated to prevent injury and preserve tissue homeostasis are not completely understood. We recently identified the collagen receptor leukocyte-associated immunoglobulin-like receptor (LAIR)-1 as a functional inhibitory receptor on airway-infiltrated neutrophils in viral bronchiolitis patients. In the current study, we sought to examine the role of LAIR-1 in regulating airway neutrophil responses *in vivo*. LAIR-1-deficient (*Lair1*$^{-/-}$) and wild-type mice were infected with respiratory syncytial virus (RSV) or exposed to cigarette smoke as commonly accepted models of neutrophil-driven lung inflammation. Mice were monitored for cellular airway influx, weight loss, cytokine production, and viral loads. After RSV infection, *Lair1*$^{-/-}$ mice show enhanced airway inflammation accompanied by increased neutrophil and lymphocyte recruitment to the airways, without effects on viral loads or cytokine production. LAIR-1-Fc administration in wild type mice, which blocks ligand induced LAIR-1 activation, augmented airway inflammation recapitulating the observations in *Lair1*$^{-/-}$ mice. Likewise, in the smoke-exposure model, LAIR-1 deficiency enhanced neutrophil recruitment to the airways and worsened disease severity. Intranasal CXCL1–mediated neutrophil recruitment to the airways was enhanced in mice lacking LAIR-1, supporting an intrinsic function of LAIR-1 on neutrophils. In conclusion, the immune inhibitory receptor LAIR-1 suppresses neutrophil tissue migration and acts as a negative regulator of neutrophil-driven airway inflammation during lung diseases. Following our recent observations in humans, this study provides crucial *in-vivo* evidence that LAIR-1 is a promising target for pharmacological intervention in such pathologies.

Keywords: LAIR-1, neutrophils, RSV, airway, inflammation, bronchiolitis, cigarette smoke

INTRODUCTION

The lungs are constantly exposed to potential pathogens and other harmful agents. To protect against sudden incursions, neutrophils patrol the lung capillaries. A rapid and robust neutrophil response is crucial to antimicrobial defense (1, 2). Neutrophilic inflammation is a common trait of some respiratory diseases. We have recently reviewed literature showing that due to the promiscuous cytotoxicity of neutrophils, excessive neutrophilic inflammation induces immune injury in viral infection (3). Therefore, balancing pathogen eradication with neutrophil-induced tissue injury is of the utmost importance to preserve tissue homeostasis. However, the mechanisms that regulate neutrophilic inflammation in the airways are still unclear.

Leukocyte-associated Ig-like receptor (LAIR)-1, also known as CD305, is an ITIM-bearing inhibitory receptor expressed on majority of immune cells (4). Mouse and human LAIR-1 share ∼40% homology, potent inhibitory capacity and bind to collagen and collagen-like molecules (5–8). Circulating neutrophils do not express LAIR-1 on the cell surface, but surface expression can be induced by *in vitro* stimulation, suggesting that LAIR-1 is involved in the regulation of activated, tissue-infiltrated neutrophils (9). We recently identified the collagen receptor LAIR-1 as functional inhibitory receptor on airway neutrophils obtained from RSV bronchiolitis patients (10). Activated airway-infiltrated neutrophils, but not their resting circulating counterparts, express LAIR-1 at the cell surface. Resting neutrophils store LAIR-1 intracellularly in granules, which allows for rapid surface upregulation upon activation. Agonistic antibody-mediated ligation of LAIR-1 on patient airway neutrophils suppresses neutrophil extracellular traps (NET) formation *ex-vivo*. Ligands for LAIR-1 are abundant in the lungs, including collagen in the extracellular matrix and surfactant protein-D (SP-D, which contains a collagen-like domains, in the airway lumen (11, 12). We, therefore, hypothesized that LAIR-1 regulates neutrophilic inflammation in lung diseases to minimize tissue injury. However, patient studies do not allow for experimental settings required to investigate the *in-vivo* role of LAIR-1. To test this hypothesis, we used mouse models and examined two distinct lung diseases in which neutrophilic inflammation plays a key role, namely, acute viral bronchiolitis caused by RSV infection, and lung inflammation induced by short-term smoke-exposure.

MATERIALS AND METHODS

Animals

Lair1$^{-/-}$ mice were generated on the C57BL/6 background by Taconic Artemis as described (13). BALB/c mice were procured from Harlan (Horst, the Netherlands). All animal studies were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the national and institutional guidelines.

Mouse RSV Infection

Eight to Twelve-week-old female C57BL/6 *Lair1*$^{-/-}$ mice or their wild-type littermates were intranasally infected with 1×10^7 PFU of RSV-A2 in 50 μl PBS. RSV-A2 preparation, quantitative assay for RSV-A2 titration and RSV-A2 infection of mice, including, intranasal inoculation, termination, and sample collection, was performed as described previously (14, 15). Mice were sacrificed on day 2 or 5 post-infection.

LAIR-Fc Administration

For LAIR-1 blocking experiments, recombinant mouse LAIR-1 fused with the Fc portion of mouse IgG2a was produced in-house. The Fc tail was mutated to prevent binding to Fc receptors and complement as described previously (16).

Eight to twelve-week-old female BALB/c wild-type mice were injected intraperitoneally with 200 or 400 μg of mouse LAIR-1-Fc chimeric protein in 100 μl PBS or PBS alone as control, 1 day before and 2 days after RSV infection. Intranasal RSV infection was performed as described above.

Bronchoalveolar Lavage Collection and Processing

Bronchoalveolar lavage (BAL) fluid collection was performed by flushing the lungs 2 times with 1.0 ml of ice-cold PBS. BAL fluid was centrifuged; supernatants were stored at −80°C for further analyses. Total cell counts in BAL were determined using a Bürker-Türk hemocytometer. BAL cells were analyzed by flow cytometry or examined by light microscopy.

For differential cell analysis by light microscopy, at least 200 cells were counted to assign relative quantities of macrophages, lymphocytes, and neutrophils based on morphology after May–Grünwald–Giemsa stain.

RSV-A2 concentrations in BAL fluid supernatants were analyzed by real-time PCR as described previously (15). Mouse CXCL1 (KC) and IL-6 levels in BAL fluid supernatants were measured by ELISA (Peprotech, London, UK) according to the manufacturer's instructions.

Cigarette-Smoke Exposure

Male, 8–12 weeks old, wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were randomly assigned to undergo cigarette smoke (CS) or control air exposure. Mice were exposed to whole body mainstream CS generated from standard research cigarettes (3R4F; 9.4 mg tar/0.726 mg nicotine, University of Kentucky) using a Watson-Marlow roller pump (323 E series, speed 35 RPM; Watson Marlow, Rotterdam, The Netherlands) that directed the CS into the exposure chamber (25 L of air volume). Carbon monoxide and oxygen levels in the exposure chamber were measured using a gas analyzer from Bacharach (PCA-3 series; Bacharach, New Kensington, PA, USA) carbon monoxide concentrations were held at 150–300 ppm and oxygen levels were kept at 20%. Mice were exposed to CS twice daily for a maximum of 30 min with a 5-h smoke-free interval, for 10 consecutive days. On the first day of CS exposure, mice were exposed to CS from 2 pairs of cigarettes, followed by CS of two times 3 cigarettes on the second day, CS of 4 and 5 cigarettes on the third day, CS of 5 and 6 cigarettes on the fourth day, and CS of two times 6 cigarettes on fifth and remaining days. Mice were weighed daily. On the final day, mice were sacrificed and BAL fluid was collected.

Intranasal Instillation of CXCL1

Eight to Sixteen week-old male and female *Lair1*$^{-/-}$ mice (on a C57BL/6 background) or their wild-type littermates were randomly assigned to be instilled with 0.1 μg or 0.5 μg CXCL1 (R&D Systems, Minneapolis, MN, USA) in 50 μl PBS or PBS alone. After 4 h, mice were sacrificed and BAL fluid was collected. Cells were counted and analyzed by flow cytometry as described below.

Single-Cell Suspension Preparation

Spleen and lymph nodes were mechanically dissociated and filtered sequentially through 100 and 70 μm cell strainers (BD Biosciences, San Jose, CA, USA). Bone marrow was flushed from femurs and tibiae and filtered through 70 μm cell strainers. Lungs were mechanically dissociated using the gentleMACS Dissociator (Miltenyi Biotec, Leiden, the Netherlands) as recommended by the manufacturer, and were enzymatically digested for 30 min at 37°C with 0.13 Wünch U/ml of LiberaseTM (Roche, Basel, Switzerland) and 200 μg/ml DNAse I (Roche, Basel, Switzerland) in RPMI-1640. Subsequently, the digested lung cell suspension was passed through 70 μm cell strainers. Red blood cells were lysed by 10 min incubation at 4°C in ammonium chloride carbonate buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Single-cell suspensions were analyzed by flow cytometry.

Flow Cytometry

Single-cell suspensions from tissues and cells from BAL fluid were stained for surface markers for 30 min at 4°C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) BSA. Propidium iodide (0.3 μg/mL, Sigma, St. Louis, MO, USA) in PBS was used to distinguish vital cells. Samples were treated with rat anti-mouse CD16/CD32 antibody (clone 2.4G2, BD Biosciences, Basel, Switzerland) to block FcR-mediated non-specific antibody binding prior to incubation with fluorochrome-conjugated antibodies. Acquisitions were made with a BD Canto II or LSR Fortessa and analyzed using FlowJo software (version 10.0.7, Treestar).

Statistics

The statistical significance of differences between two groups was calculated with the unpaired Mann-Whitney or Student's *t* test where appropriate and more than two groups were compared with the 2-way ANOVA or Kruskal-Wallis test as mentioned in the legends. Statistical analyses were performed using Prism 6 (GraphPad) software. *P*-values < 0.05 were considered significant and are marked in the graphs where applicable. All unmarked differences are non-significant.

RESULTS

LAIR-1 Negatively Regulates Neutrophil Recruitment During RSV Infection

We recently demonstrated that LAIR-1 is a functional inhibitory receptor on airway-infiltrated neutrophils of RSV infection-induced bronchiolitis patients in an *ex-vivo* setting (10). We therefore hypothesized that LAIR-1 regulates the neutrophil response *in vivo* during viral bronchiolitis. To test this hypothesis, wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were intranasally inoculated with the RSV-A2 strain. Mice were sacrificed on day 2 or 5 post-infection and BAL was performed to assess the cellular airway infiltrate, cytokine levels, and viral load.

Total leukocyte influx into the airways was notably increased in LAIR-1-deficient compared with wild-type mice at both the time points (**Figure 1A**). Early in infection, the increase in cell numbers was mostly contributed by enhanced neutrophil recruitment in LAIR-1-deficient mice, while at day 5 post-infection lymphocytes mainly constituted the infiltrating population (**Figures 1B,C**). There were no differences in macrophage recruitment between genotypes (**Figure 1D**). Thus, LAIR-1 limits neutrophil and lymphocyte recruitment during RSV infection *in vivo*.

Despite the enhanced leukocyte recruitment, concentrations of the major neutrophil chemoattractant CXCL1 and the inflammatory cytokine IL-6 were not increased in the BAL fluid of RSV-infected *Lair1*$^{-/-}$ mice compared with wild-type mice (**Figures 1E,F**), nor were there differences in the viral load (**Figure 1G**). Thus, the data suggest that LAIR-1 negatively regulates neutrophil and lymphocyte recruitment during RSV infection without directly affecting the local inflammatory milieu or viral replication.

Tissue-Infiltrated Neutrophils Express LAIR-1

To rule out basal differences, we performed immunophenotyping of unchallenged *Lair1*$^{-/-}$ C57BL/6 mice and confirmed that there were little to no differences in the composition of immune cell populations (**Supplemental Figures S1**, **S2**) as described before (13, 17). Moreover, we demonstrate that unchallenged wild-type and *Lair1*$^{-/-}$ mice did not differ in neutrophil numbers and activation state—indicated by the CD11b, CD62L, and CD182 markers—in either blood or bone marrow (**Figure 2A**). In unchallenged wild-type mice, circulating neutrophils did not express LAIR-1, whereas tissue-infiltrated neutrophils did (**Figure 2B**). However, upon infection with RSV, circulating neutrophils started to express LAIR-1 (**Figure 2C**). In line with the observation regarding tissue infiltrated neutrophils in unchallenged mice, airway-infiltrated neutrophils also expressed LAIR-1 and were highly activated—indicated by upregulation of CD11b and shedding of CD62L (**Figure 2C**). Thus, RSV infection induced LAIR-1 expression on circulating and airway-infiltrated neutrophils which may directly regulate the function/adhesion of neutrophils.

Blocking LAIR-1-Ligand Interaction Enhanced Neutrophil Recruitment During RSV Infection

To further rule out developmental differences in *Lair1*$^{-/-}$ mice as cause of the observed phenotype, the interaction between endogenous LAIR-1 and its ligands was blocked during RSV infection by injecting wild-type mice with LAIR-1-Fc chimeric protein. Here, BALB/c mice rather than C57BL/6 mice were used as the former are more sensitive to RSV

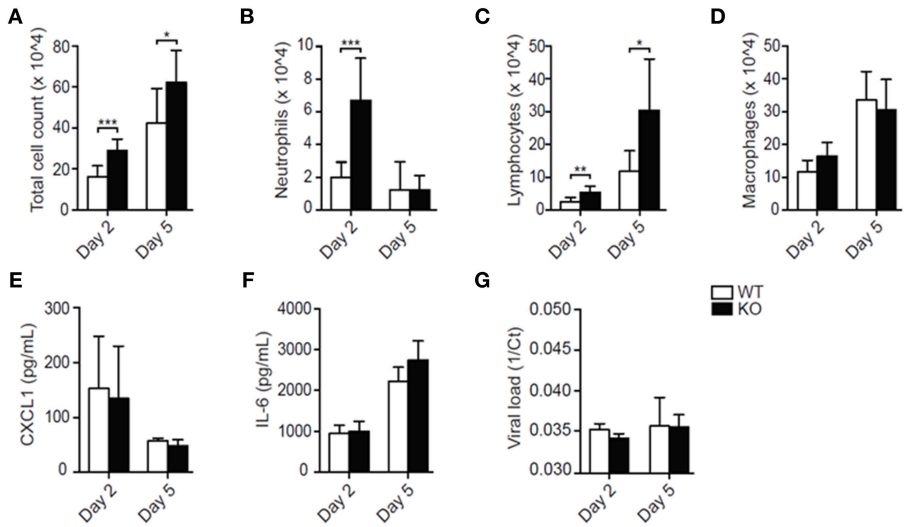

FIGURE 1 | LAIR-1 regulates neutrophil and lymphocyte recruitment during RSV-A2 infection in mice. **(A–G)** Mice were inoculated with RSV-A2 and sacrificed on day 2 or 5. Total **(A)** and differential **(B–D)** BAL cell counts, BAL CXCL1 **(E)** and IL-6 **(F)** concentrations, and viral loads **(G)** were determined. Data are presented as means ± SD and represent 8 mice per group in 2 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; unpaired Student's *t-test* with Welch's correction. WT, *wild-type* and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

infection (18, 19). The results obtained with RSV-infected BALB/c mice in which endogenous LAIR-1-collagen interactions were blocked, mimic those of the RSV-infected *Lair1*$^{-/-}$ C57BL/6 mice, when compared to their respective vehicle-treated or wild-type mice. The total cell count in the BAL of RSV-infected LAIR-1-Fc chimeric protein-treated mice was increased (**Figure 3A**). This was due to enhanced neutrophil and lymphocyte recruitment, whereas macrophage numbers remained unaffected (**Figures 3B–D**). Concentrations of CXCL1 and IL-6 as well as viral load were comparable between vehicle- and LAIR-1-Fc-treated mice (**Figures 3E–G**). The data further confirm that LAIR-1 negatively controls neutrophil and lymphocyte recruitment during RSV infection with no direct effect on the local inflammatory milieu or viral replication.

LAIR-1 Limits Disease Severity and Neutrophil Airway Recruitment in Response to Cigarette-Smoke Exposure

Despite the enhanced neutrophil recruitment during RSV infection in mice that lack LAIR-1 signaling, there was no effect on disease severity as measured by weight loss (data not shown). However, RSV pathophysiology in humans and mice is notably different. While neutrophils dominate the cellular inflammatory response in RSV bronchiolitis patients, with neutrophils comprising ≥80% of infiltrating leukocytes (20–22), lymphocytes are more prominent in the airways of mice during experimental RSV infection (**Figures 1A–D**, **3A–D**). We hypothesized that in a genuine neutrophil-driven disease model the loss of LAIR-1-mediated immune regulation and the corresponding increase in neutrophil infiltration would exacerbate disease severity. Therefore, we employed another model of neutrophilic airway inflammation and exposed wild-type and *Lair1*$^{-/-}$ C57BL/6 mice to cigarette smoke. Smoke-exposed *Lair1*$^{-/-}$ mice lost more body weight and showed delayed and significantly attenuated recovery as compared to their wild-type counterparts (**Figure 4A**). In line with this, BAL fluid analysis revealed that *Lair1*$^{-/-}$ mice had significantly higher lung immune infiltration in response to smoke-exposure as compared to the wild-type (**Figure 4B**). This increase in total cell influx was contributed by increased recruitment of neutrophils, macrophages and lymphocytes to *Lair1*$^{-/-}$ lungs (**Figures 4C–E**). The increase in BAL cell counts in *Lair1*$^{-/-}$ mice was highest for neutrophils (∼3 fold) followed by macrophages (∼2 fold), while the increase in lymphocytes was modest and failed to reach statistical significance. We confirmed that airway-infiltrated neutrophils of smoke-exposed mice expressed LAIR-1 and were highly activated (**Supplemental Figure S2**). Thus, LAIR-1 limits neutrophil recruitment to the airways during cigarette smoke-induced lung inflammation and regulates disease severity.

LAIR-1 Directly Suppresses Neutrophil Migration

The local inflammatory milieu in the lung, as reflected by CXCL1 and IL-6 production, was not directly regulated by LAIR-1 (**Figures 1E,F**, **3E,F**). Since the lung is enriched in LAIR-1 ligands such as collagen, SP-D and airway-infiltrated neutrophils in both the RSV infection and the smoke exposure model express LAIR-1 (**Figure 2C**; **Supplemental Figure S3**), we hypothesized that LAIR-1 can directly limit the migratory capacity of neutrophils. To examine this hypothesis, the lungs of wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were instilled with the neutrophil chemoattractant CXCL1 by intranasal administration and analyzed for the BAL fluid cellular infiltrates. We observed significantly higher total BAL cell influx in *Lair1*$^{-/-}$ mice as compared to wild-type mice

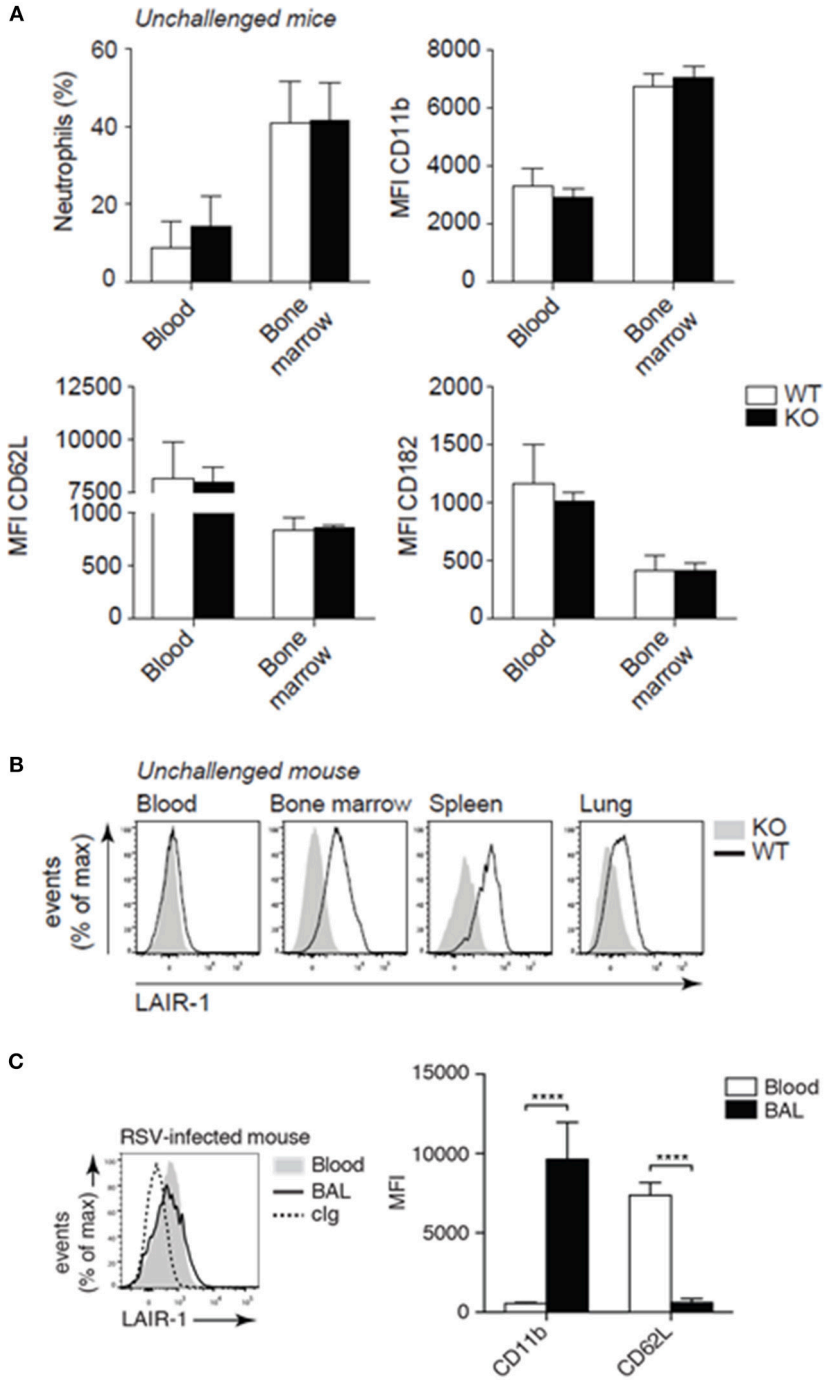

FIGURE 2 | LAIR-1 expression and deficiency in mice. **(A–C)** Mouse leukocytes were examined for lineage and activation markers and LAIR-1 expression by flow cytometry. Neutrophils were identified based on characteristic forward- and side-light scatter properties and the expression of Ly-6G. **(A)** The percentage neutrophils (Ly-6G+) among total live leukocytes in blood and bone marrow as well as the expression of activation markers (CD11b, CD62L, and CD182) were compared by flow cytometry. **(B)** Flow cytometric analysis of LAIR-1 expression on blood and tissue (bone marrow, spleen, and lung) neutrophils. **(C)** Wild-type mice were inoculated with RSV and sacrificed 2 days post-infection. Expression of LAIR-1 and activation markers (CD11b and CD62L) was measured on BAL and blood neutrophils. $^{****}p < 0.0001$; 2-way ANOVA with Holm-Šídák multiple comparison correction. Data are representative of eight mice **(A, B)** or three independent experiments with at least three mice **(C)**. Error bars in **(A)** and **(C)** represent mean ± SD. WT, *wild-type* and KO, *Lair1*$^{-/-}$ on C57BL/6 background; clg, *isotype-matched control antibody*; MFI, *mean fluorescence intensity*.

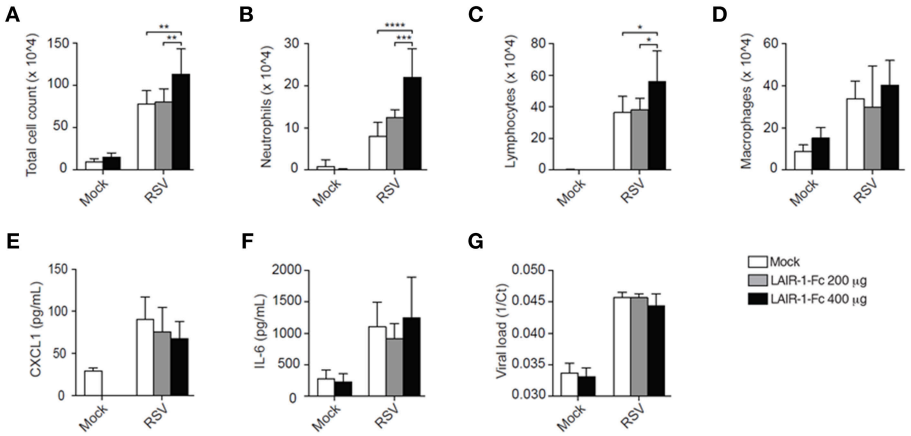

FIGURE 3 | LAIR-1-Fc chimeric protein administration effects pulmonary neutrophil recruitment during RSV infection. **(A–G)** Wild-type BALB/c mice were inoculated intranasally with RSV and sacrificed on day 5. One day before RSV inoculation and 2 days after inoculation mice were treated intraperitoneally LAIR-1-Fc fusion protein or PBS (vehicle). Total **(A)** and differential **(B–D)** BAL cell counts, BAL CXCL1 **(F)** and IL-6 concentrations **(G)**, and viral load **(G)** were determined. Data are presented as means ± SD and represent 6 mice per group for panels **(A–G)** in 2 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. 2-way ANOVA with Holm-Šídák multiple comparison correction.

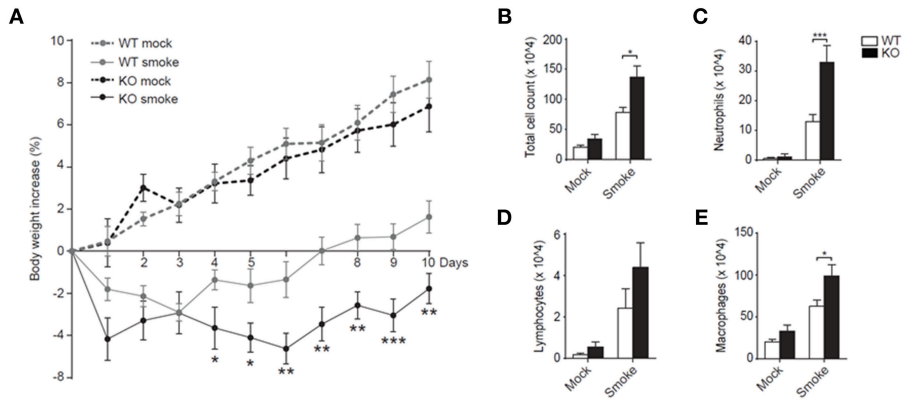

FIGURE 4 | LAIR-1 regulates neutrophilic airway inflammation during cigarette-smoke exposure. **(A–E)** Mice were exposed to cigarette smoke or mock (air) in whole-body chambers twice daily for 10 consecutive days. **(A)** Body weight of mice was measured daily and percentage change relative to day 0 was calculated. Total **(B)** and differential counts **(C–E)** in BAL were determined after 10 days of cigarette-smoke exposure. Data are representative of at least 10 mice per group in 2 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The Mann-Whitney U test was used to calculate the statistical significance of differences between WT and KO mice **(A)** and a 2-way ANOVA with Holm-Šídák multiple comparison correction was used for the cell counts **(B,C)**. WT, *wild-type* and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

in response to 0.5 µg of CXCL1 (**Figure 5A**). Flow cytometry confirmed that CXCL1 specifically attracted neutrophils, as they constitute ∼88% of total cell population in BAL fluid (**Figure 5B**), which were highly activated and expressed LAIR-1 (in wild-type mice) (**Figure 5C**). These data demonstrate that LAIR-1 intrinsically limits neutrophil infiltration of the airways, thereby controlling neutrophilic airway inflammation.

DISCUSSION

The pulmonary immune response must protect against the ever-present threat of pathogens, while limiting immune-induced tissue damage to allow for gas exchange. Neutrophils are crucial for antimicrobial defense but such responses must be tightly regulated to prevent bystander damage. The mechanisms underlying the regulation of neutrophil activation and responses are incompletely understood. We have recently identified LAIR-1 as an inhibitory receptor on activated airway neutrophils which limits NET formation during RSV bronchiolitis (10). In the current study, we investigated the role of LAIR-1 in regulating airway inflammation using two different models of neutrophil pre-dominant lung diseases. Using a mouse model of RSV bronchiolitis, we demonstrate that LAIR-1 functions as a negative regulator of airway inflammation as LAIR-1 deficiency or administration of Lair1-Fc chimeric protein led to enhanced recruitment of neutrophils and lymphocytes. Similar results were

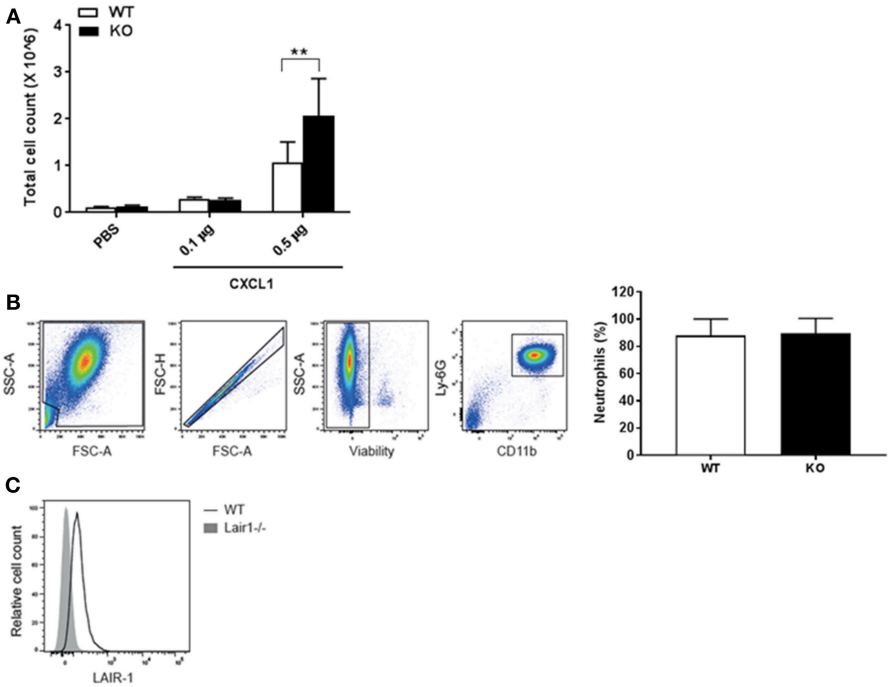

FIGURE 5 | LAIR-1 directly controls neutrophil migration. **(A–C)** Mice were intranasally instilled with CXCL1 or vehicle (PBS). After 4 h mice were sacrificed and BAL was performed. **(A)** Total cell counts in BAL fluid were determined and represented as mean ± SD ($n = 4$–8 mice per group per genotype; 2 independent experiments). Differences between WT and KO in cell influx at CXCL1 (0.5 μg) were consistent in both experiments. $^{**}p < 0.01$; 2-way ANOVA with Tukey's multiple comparison test. **(B,C)** Cells in BAL fluid were analyzed by flow cytometry. **(B)** Neutrophils were identified by concurrent Ly-6G and CD11b expression; graph represents neutrophils as percent of total live leukocytes in BAL fluid after 0.5 μg CXCL1 administration in WT and KO mice, mean ± SD and, **(C)** a representative histogram of LAIR-1 expression on BAL fluid neutrophils. WT, *wild-type* and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

obtained in the cigarette smoke exposure model where LAIR-1 deficient mice show marked increased neutrophilia. Our study, thus, underlines a key regulatory role of LAIR-1 in limiting neutrophilic inflammation in lung diseases.

Ligands for LAIR-1 are abundant in the lungs. En route to the airways, for instance in response to RSV infection or irritants in smoke, neutrophils will traverse the extracellular matrix, which contains collagen. In the airway lumen, neutrophils will encounter pulmonary surfactant-associated protein-D (SP-D) and C1q, which possess a collagen-like domain (11, 12). The interaction of LAIR-1 with the ligands present in the extracellular matrix may limit the recruitment of neutrophils to the airways. In support hereof, we observed an increased neutrophil recruitment to airways of RSV-infected and smoke-exposed mice that lack functional LAIR-1. Interestingly, this enhanced neutrophilia in *Lair1*$^{-/-}$ mice was not associated with augmented chemokine production—for example, by LAIR-1-expressing alveolar macrophages—that attracts more neutrophils. There were no changes in the abundance of CXCL1, a potent neutrophil chemoattractant (23) and IL-6, a major pro-inflammatory cytokine, among wild-type and *Lair1*$^{-/-}$ mice. This is suggestive for an enhanced intrinsic cellular migratory capacity in the absence of an inhibitory interaction of LAIR-1 with extracellular matrix. Indeed, in response to an intrapulmonary challenge with the neutrophil chemoattractant CXCL1 (23), neutrophil recruitment was strongly enhanced in *Lair1*$^{-/-}$ mice compared with wild-type mice, thereby demonstrating a neutrophil-intrinsic role for LAIR-1 in migration to the airways. Thus, during an active inflammation and in response to chemoattractant stimuli, LAIR-1 interaction with its ligands impedes neutrophil migration in wild-type mice but not in *Lair1*$^{-/-}$ mice.

A prior study of *Lair1*$^{-/-}$ mice did not reveal an overt clinical phenotype in multiple lymphocyte-driven disease models (13, 17). Similar to our study (**Supplemental Figure S3**), LAIR-1 deficiency had little effect on the composition of immune cell populations or neutrophil activation state in unchallenged mice. However, in the previous studies neutrophils were not extensively studied. In contrast, we examined two different models of lung diseases where neutrophils are a dominant contributor, namely RSV bronchiolitis and smoke-induced inflammation. In both cases, LAIR-1 regulated neutrophilic lung inflammation. We observed no differences in disease severity between RSV-infected *Lair1*$^{-/-}$ and wild-type mice. A possible explanation for this is that while in RSV bronchiolitis patients, the immune response to RSV is characterized by massive infiltration of neutrophils into the airways—≥80% of infiltrating leukocytes are neutrophils (20–22)—the contribution of airway infiltrating neutrophils on disease severity is notably less pronounced in mice (24). However, in response to cigarette smoke exposure, a bona

fide neutrophil-driven lung inflammation model in mice, the enhanced neutrophilic inflammation in LAIR-1-deficient mice was accompanied by worsened weight loss and retarded recovery underlining the critical role of LAIR-1.

Neutrophils are crucial to antimicrobial defense, but cytotoxic effector mechanisms such as protease secretion, reactive oxygen species production, and NET formation cause bystander tissue damage (1–3, 25). Therefore, excessive neutrophilic inflammation is harmful. Major lung diseases, including RSV bronchiolitis and chronic obstructive pulmonary disease (COPD), are characterized by a massive neutrophilic inflammation (26). However, the regulatory mechanisms hereof are not yet fully elucidated. A better understanding of how neutrophilic inflammation is regulated could reveal potential targets for pharmaceutical intervention.

In human neutrophils, LAIR-1 is stored in intracellular granules and is rapidly recruited to the surface upon activation (10), presenting a plausible mechanism that would ensure a proper balance between neutrophil function and tissue injury. Under steady state, a low or lack of expression of LAIR-1 would ensure neutrophil activation against invading pathogens whereas rapid recruitment of LAIR-1 from intracellular stores would promptly impede the neutrophil influx and limit the tissue injury during neutrophilic inflammation. Whereas, our study shows that LAIR-1, indeed, impedes neutrophil influx during active inflammation in mouse models, the mechanisms of LAIR-1 surface expression on mouse neutrophils remains to be investigated.

Our study sheds light on a novel regulatory mechanism involved in neutrophilic inflammation but has several limitations. First, we cannot rule out the contribution of other LAIR-1-expressing immune cell populations, such as lymphocytes and macrophages, to the observed phenotypes. In addition to increased neutrophil infiltration, we also see increased recruitment of lymphocytes after RSV infection and increased number of macrophages after smoke-exposure in the airways of LAIR-1-deficient mice. Possibly, the augmented neutrophil response directly contributes to the subsequent increase in lymphocyte and macrophage influx. Indeed, neutrophils, by depositing CXCL12-containing vesicular trails during migration, are critical to the recruitment of T cells to the airways of influenza virus-infected mice (27). Whereas, both lymphocytes and macrophages could possibly contribute to increased disease severity, increased airway infiltration of neutrophils remains a consistent observation in LAIR-1-deficient mice in both RSV and smoke-exposure models.

Second, we measured a limited number of cytokines. Also, we did not investigate a direct effect of LAIR-1 on mouse neutrophil functions such as NET formation and migration *in-vitro*. Which aspect of the observed increased airway infiltration of neutrophils aggravates disease severity, remains unresolved. These questions require further investigation. The strength of our study lies in being the first to discern the role of LAIR-1 in neutrophil-predominant airway diseases *in vivo*. We show that LAIR-1 acts as a crucial regulator of neutrophils and is therefore a potential target for pharmacological intervention in neutrophil-driven lung diseases.

ETHICS STATEMENT

The study was carried out in accordance with the national and international guidelines. The protocols were approved by the Institutional Animal Care and Use Committee of Utrecht University/University Medical Center Utrecht, the Netherlands.

AUTHOR CONTRIBUTIONS

KK and RG acquired, analyzed and interpreted data and wrote the manuscript. MH, MR, AvO-J, IvA, and TL-M performed mouse experiments. GF, AM, SW, and FC provided essential tools and technical insights. LB and LM designed the study, interpreted the data, and edited the manuscript. All authors reviewed and approved the manuscript.

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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.2019.00842/full#supplementary-material

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Conflict of Interest Statement: LB and LM have regular interaction with pharmaceutical and other industrial partners. They have not received personal fees or other personal benefits. LB's institute has received major funding (>€100,000 per industrial partner) for investigator-initiated studies from AbbVie, MedImmune, Janssen, the Bill and Melinda Gates Foundation and MeMed Diagnostics. LB's institute has received minor funding participation in trials by Regeneron and Janssen since 2015 (total annual estimate <€20,000). LB received minor funding for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, Novavax, Janssen (total annual estimate <€20,000). LM's institute has received funding for investigator-initiated studies from Nextcure, Boehringer Ingelheim, Ono Pharmaceuticals, Ablynx and Janssen. LM received minor funding for consultation from Novo Nordisk, Biogen and Boehringer Ingelheim.

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

frontiers in Immunology

REVIEW
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Prevention of Pediatric Respiratory Syncytial Virus Lower Respiratory Tract Illness: Perspectives for the Next Decade

*Sofia S. Aranda and Fernando P. Polack**

Fundacion Infant, Buenos Aires, Argentina

The landscape of infant bronchiolitis and viral pneumonia may be altered by preventive interventions against respiratory syncytial virus under evaluation today. Pediatric wards in 2018 in developing countries may differ from those attended by future generation pediatricians who may not witness the packed emergency rooms, lack of available beds, or emergency situations that all physicians caring for children with RSV experience every year. In this review, we describe and discuss different prevention strategies under evaluation to protect pediatric patients. Then, we outline a number of potential challenges, benefits, and concerns that may result from successful interventions after licensure.

Keywords: RSV, RSV vaccines, monoclonal antibodies, maternal immunization, live attenuated vaccines

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Alexis M. Kalergis, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Juan Pablo Jaworski, National Council for Scientific and Technical Research (CONICET), Argentina
Bernhard Resch, Medical University of Graz, Austria

***Correspondence:**
Fernando P. Polack
fpolack@infant.org.ar

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In the next decade, we may witness a change in the burden of office and emergency room visits, and hospitalizations in infant wards during the winter. Swamped emergency rooms and pediatricians and nurses overwhelmed with work, caring for an endless number of young infants in respiratory distress may start to become a scene of the past. Vaccines, monoclonal antibodies of extended half-life and antivirals against respiratory syncytial virus (RSV) are being evaluated in clinical trials (1). It is reasonable to expect that several of these candidates will be partly or completely successful in the next 10 years and become part of the preventive and therapeutic tools for pediatric public health.

RSV is the main cause of hospitalization in infants and young children worldwide. Millions of children are hospitalized every year and the vast majority of them live in the developing world. While infants born prematurely, those with congenital heart disease and other specific subgroups are at increased risk for hospitalization, the majority of severe cases affect previously healthy infants and children. The only available intervention licensed to prevent severe disease today is the administration of a neutralizing anti-RSV humanized monoclonal antibody, palivizumab®. Palivizumab® is recommended for high risk populations in high and middle-income countries (2). But its cost is prohibitive for low income nations, where most of the fatal cases of RSV disease occur.

Evidently, a safe and effective, affordable preventive strategy against RSV is necessary. One of these strategies under evaluation is to immunize pregnant women against the virus to transfer high titers of protective antibody to infants before birth (1). Alternatively, long-lived monoclonal antibodies against neutralizing epitopes in the RSV F protein could be administered to newborns and young infants to prevent disease in the first months of life (1). Other attractive approaches under study include immunization of infants with recombinant live attenuated RSV vaccines or using a variety of live vectors carrying genes encoding RSV proteins (1). Even though natural infection with RSV does not induce lifelong protective immunity, antibodies against the RSV F glycoprotein can prevent severe disease in humans. RSV F is a target for neutralizing antibodies, as

evidenced by the effectiveness of palivizumab for nearly two decades. Hence, F alone or in combination with other viral proteins is the preferred antigen in RSV candidate vaccines (3).

ELICITING PROTECTIVE IMMUNITY AGAINST RSV LRTI

Humans are the only natural host for RSV. The virus is spread from person to person via respiratory droplets, and spreads into the respiratory tract, where it preferentially targets apical ciliated epithelial cells (4). The incubation period for RSV from time of infection to onset of illness is between 3 and 5 days (5). Natural immunity includes innate responses by polymorphonuclear (PMN) and mononuclear cells, activation of numerous pattern recognition receptors (e.g., TLR3, TLR2/6, TLR7/8, NOD-like, and RIG-I-like receptors), and type I and III interferon responses (6–9). These responses are important, as PMNs and macrophages have been postulated to enhance and prevent severe disease, and PRRs have been reported to modulate numerous responses during RSV infection (5, 10). Moreover, although often underappreciated because their levels peak before RSV symptoms become evident to pediatricians, IFNs are increasingly recognized as relevant actors in RSV immunity (9, 11). In phase I trials with intranasal live attenuated RSV vaccines in seronegative infants, a small but significant number of subjects is not infected despite direct inoculation, suggesting that innate immune responses may be an important barrier against infection.

Adaptive immunity is critical for protection against RSV disease. The humanized monoclonal antibody palivizumab® and polyclonal sera enriched for antibodies against the RSV fusion (F) protein, Respigam®, demonstrate that antibodies against RSV F can prevent severe RSV LRTI. RSV infection elicits polyclonal, high avidity, neutralizing antibody responses against RSV F (12), that cross-react between RSV subgroups A and B. The F protein is highly conserved between RSV subgroups, with amino acid sequence identities of >90% (5, 13). This protein mediates entry into host cells by converting from a metastable trimeric pre-fusion conformation (pre-F) to a highly stable post-fusion conformation (post-F) (14). There are two pre-F-specific antigenic sites Ø and V, target of the most potent neutralizing antibodies, and two sites that are present on both conformations II and IV (12, 14). Although present on both pre-F and post-F, antibodies against site III bind tighter to pre-F (14). Instead, site I antibodies bind tighter to post-F (14). A newly recognized antigenic site in F, designated antigenic site VIII, occupies an intermediate position between the previously defined sites II and Ø (15).

The RSV attachment (G) protein also elicits polyclonal neutralizing responses. RSV G is more variable than RSV F, with ∼50% sequence homology between subgroups A and B (5). Both RSV subgroups co-circulate during yearly epidemics, although one typically predominates every season (16–18). While IgA responses are probably important in protecting the respiratory tract, their exact role in RSV immunity is only now becoming clearer and requires further study (11).

T cell responses are also important for protection against RSV. $CD4^+$ T lymphocytes contribute to T-cell dependent antibody responses and indirectly to viral clearance (as evidenced by persistent infections in HIV-infected individuals) and $CD8^+$ T lymphocytes clear the virus from infected cells (5, 19).

The aforementioned innate and adaptive immune responses are mimicked to different degrees by live attenuated vaccines against RSV, discussed below. But in specific situations, immune responses against RSV -particularly those elicited by non-replicating vaccines in naive individuals- can also potentiate disease severity.

The first time an enhanced form of RSV disease (ERD) was observed in children during a vaccine trial, its manifestations were not recognized by investigators. Between 1962 and 1963, a formalin-inactivated vaccine against RSV (FIRSV) was evaluated in 54 children in the United States. Unfortunately, none of them received placebo. Twenty-one of fifty-four (39%) vaccine recipients were infected with RSV, and 10 (18%) experienced severe disease. But only 5 years had passed since the isolation and initial characterization of the chimpanzee coryza agent (now RSV), and its burden of illness was still unclear (20). As a consequence, researchers attributed the observed severity to an unusually bad season (20).

In 1966, a similar formalin-inactivated vaccine against RSV was administered to infants and toddlers in four trials in the United States. Immunized subjects had measurable non-neutralizing, low avidity anti-F IgG responses, no RSV-specific cytotoxic T lymphocytes and a $CD4^+$T lymphocyte response primed to respond to wild type infection with an exuberant production of Th2 cytokines (10, 21–23). Indeed, upon infection with wild type RSV during the winter of 1966-1967, non-protective, low avidity IgG coupled with the virus to activate the complement cascade and in synergy with a strong Th2 polarization of the immune response led to increased hospitalization rates and two deaths in toddlers due to this enhanced form of RSV disease presenting with wheezing and bronchopneumonia (20, 24–26). Consequently, generation of an IgG response dominated by low avidity, non-neutralizing antibodies against RSV F (**Figure 1**), and priming for a Th2 bias upon RSV infection are considered undesirable features for RSV vaccine candidates.

IMMUNIZING MOTHERS TO PROTECT YOUNG INFANTS

Maternal immunization, a strategy well-accepted to prevent infant influenza and pertussis, aims to provide passive immunity to infants by transfer of maternal antibodies across the placenta. During the third trimester of pregnancy, IgG antibodies are actively transferred via the FcRn receptor in the placenta from the maternal to the fetal circulation. The first candidate in clinical trials to complete phase III evaluation in eleven countries enrolling approximately 4,600 women of childbearing age was the Novavax prefusogenic RSV F nanoparticle adjuvanted with alum (NCT02624947) (27). The Novavax vaccine elicited responses against epitopes displayed by both pre-fusion and post-fusion

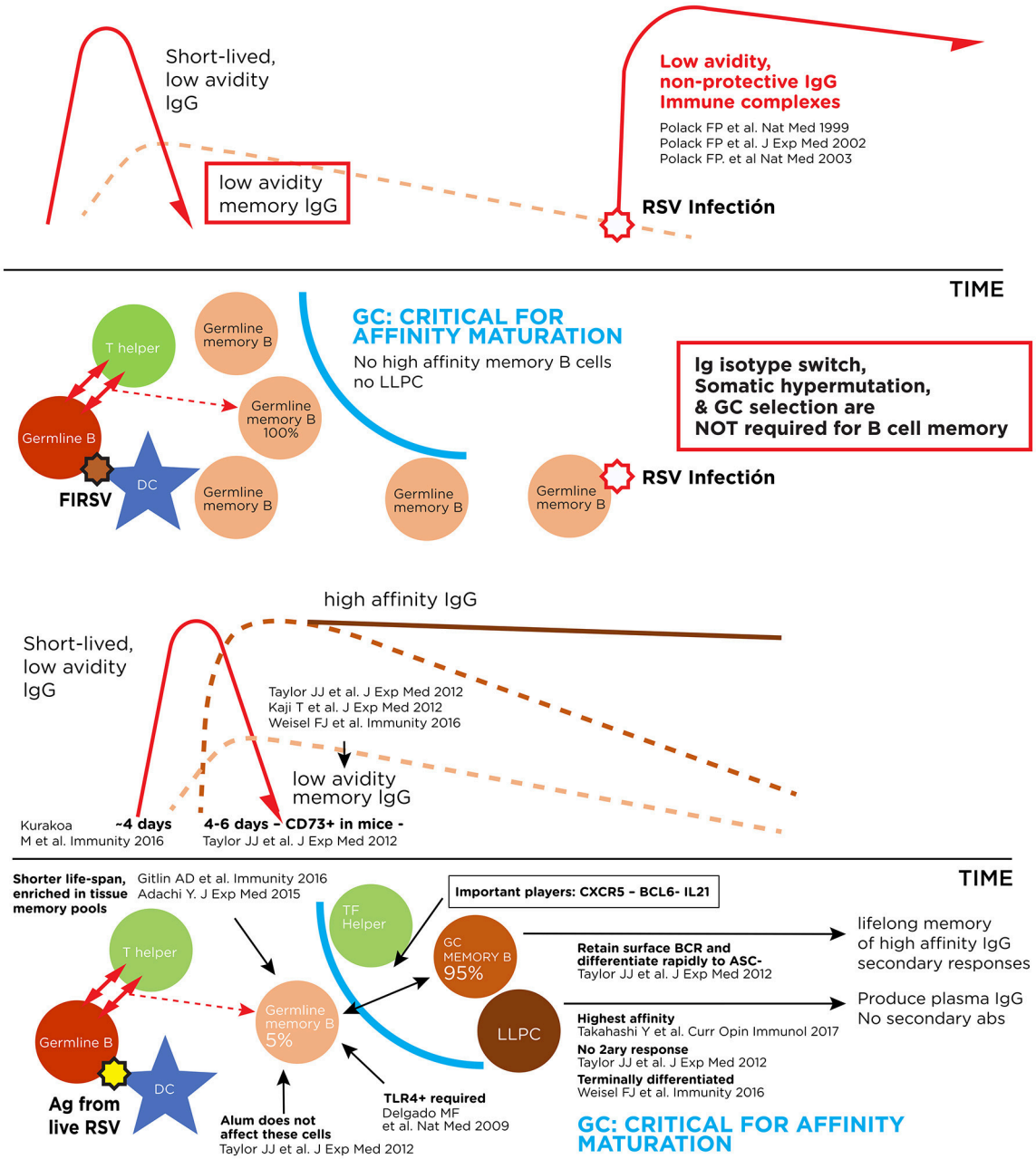

FIGURE 1 | Pathogenic and protective antibody responses against RSV. Upper panel: Immune complex formation with antibodies of low affinity for the virus. Lower panel: Antibody response to live RSV infection with production of high affinity long-lived memory B cells and long-lived plasma cells (LLPC).

conformations of the F protein (28). The trial aimed to reduce the rate of medically significant RSV LRTI in infants through 90 days of life (27), but failed its primary endpoint despite an overall efficacy of 39.4% (95% CI, 5.3, 61.2) (29). Interestingly, the vaccine protected against RSV hospitalizations (40%) and severe disease (44%) worldwide (29), but its efficacy was radically different in the U.S. compared to developing countries: results in South Africa were considerably better than in the American population at 57% (95% CI, 32.7, 72.5) vs.−32.7% (95% CI,−238.9, 48.1). Unfortunately, the study lacked sufficient power to confidently ascertain whether these differences simply represented variations within the confidence interval.

Another vaccine designed for maternal immunization is a recombinant RSV protein F vaccine, engineered to preferentially maintain prefusion conformation by GSK. The vaccine progressed through early phase clinical trials in healthy adults and non-pregnant women (NCT02753413) (30–32). In addition, a subunit vaccine encoding a stabilized prefusion

molecule developed by Pfizer also targets pregnant women (and the elderly) and is entering phase 2 trials (NCT03529773) (33–35). A fourth attractive investigational candidate developed by NIAID and entering clinical evaluation is RSV F DS-Cav1 (NCT03049488) (36), a formulation of engineered soluble site Ø–stabilized RSV F trimers (site Ø is only present on pre-fusion F) adjuvanted with alum (37, 38).

LONG-LIVED MONOCLONAL ANTIBODIES

The concept of administering an optimized mAb with extended half-life and strong neutralizing activity to protect infants after birth is appealing. MEDI8897 from Medimmune is a highly potent, extended half-life antibody that completed phase 2 trials and is expected to enter phase 3 in 2019 (NCT02878330) (39). Recently, MEDI8897 was granted breakthrough therapy designation by the FDA. MEDI8897 is a recombinant human RSV monoclonal antibody with a modified Fc region. The antibody has been optimized from antibody D25, which targets site Ø in the pre-F conformation of the RSV F protein. MEDI8897, with a mean half-life 85 to 117 days, is intended to prevent RSV disease in all infants for the duration of the RSV season with a single dose (40). A triple-amino-acid (M252Y/S254T/T256E [YTE]) substitution within its Fc region extends its half-life by increasing binding to the major histocompatibility complex class I-related neonatal Fc receptor (FcRn) at acidic conditions and preventing degradation (40–42). A second long-lived monoclonal antibody candidate in clinical development is MK-1654 from Merck (NCT03524118) (43), undergoing early phase evaluations and targeting site IV in RSV F.

Despite the intuitive attraction of using a targeted mAb to neutralize RSV and prevent severe illness, this virus has proven once and again to be an annoying creature. A recent phase 3 study evaluating suptavumab (REGN2222), an antibody developed by Regeneron against site V in RSV F, did not meet its primary efficacy endpoint in healthy preterm infants (NCT02325791) (44). REGN2222 had a non-significant protective trend against medically attended infections in preterm infants up to day 150 of life caused by RSV subgroup A. But due to an unexpected mutation in site V of RSV F in circulating RSV B viruses, failed to prevent disease against this subgroup (44).

LIVE-ATTENUATED VACCINES AGAINST RSV

Several live-attenuated RSV vaccines candidates to protect infants and young children are in clinical development (1). One of their strengths as infant vaccines is that decades of studies in infants have shown that these vaccines do not appear to prime for enhanced RSV disease in RSV-naïve infants (45). In addition, these vaccines are administrated intranasally, needle-free and, given that they replicate in the upper respiratory tract, can generate an immune response even in the presence of passively acquired maternal antibodies (46). Their greatest challenge is to attain the adequate balance between attenuation and immunogenicity. A clever deletion of the coding sequence for the RSV M2-2 protein in one of these candidates attenuates viral replication while upregulating gene transcription and antigen expression (NCT02237209, NCT02040831) (47, 48). The NS2 non-structural protein was deleted in a second candidate(NCT03422237, NCT03099291) (49, 50) reducing viral suppression of type interferon responses in the host (51). Both candidates were developed by the Laboratory of Infectious Diseases at NIAID and were safe in early studies in infants and young children and may soon progress to larger trials for evaluation. While no head-to-head comparison was described, the similarities in trial design suggest that the M2-2 vaccine has more restricted virus shedding but was more immunogenic than the live vaccine with the NS2 deletion (52).

VECTORS ENCODING RSV GENES

Vectored-based vaccines for children are now in clinical trials, using adenoviruses. Adenoviruses are highly immunogenic and induce both innate and adaptive immune responses (53). Moreover, adenovirus-based vaccines have been and are currently being investigated as vectors targeting viral, bacterial, and protozoan pathogens (53). The candidate Ad26.RSV.preF uses a human adenovirus 26 expressing pre-F RSV protein. Ad26.RSV.preF is now in phase 2 trials in adults and 12-24-month-old RSV seropositive toddlers (NCT03303625) (54). A second adenovirus-based candidate in phase 2 trials in seropositive children is ChAd155-RSV (NCT02927873) (55), using a replication-incompetent chimpanzee adenovirus 155. This vector encodes the F, N, and M2-1 RSV proteins (51, 55).

Finally, a chimeric candidate in clinical development is the rBCG-N-hRSV vaccine (NCT03213405) (56). A recombinant BCG expressing RSV N protein is targeted for use in newborns. BCG induces Th1 immunity, skewing responses away from undesirable Th2 priming (57). Vaccination with rBCG-N-hRSV is expected to elicit cellular immunity in addition to a humoral response against the virus (58).

THE FUTURE

Whether one or many of these vaccines and/or mAbs prove effective, several questions, concerns, and speculations remain to be answered through the trials and subsequent studies post-licensure. Some of these issues are discussed below.

CONCERNS OF ENHANCED RSV DISEASE

The mechanism of illness of ERD has not been completely elucidated, in part because every significant scientific advancement in structural virology and immunology continues to uncover new angles of a complex problem. For several years, two immune correlates described above have been accepted as indicators of candidate RSV vaccines that may prime for enhancement: the presence of low avidity, non-protective antibodies after immunization (**Figure 1**) (10, 21, 59), and a Th2 polarization of the immune response in the respiratory tract

after RSV infection (23, 60). Now, novel observations in the field suggest a potential relationship between RSV F conformations in the vaccine and disease enhancement (14, 61); experience with other immunogens question whether route of vaccination may be an important determinant of RSV vaccine responses (62); improved understanding of B cell memory, class switching and affinity maturation may allow a clearer identification of primed B cell memory populations associated with undesirable outcomes (63–65); and detection of subpopulations with genetic mutations in molecules essential to B and T cell maturation interrogate whether enhancement could ever be possible in seropositive adults (66–68).

Importantly, given that memory B and T helper cells play a critical role in ERD pathogenesis, concerns for this problem are lower in several leading approaches to RSV prophylaxis that rely on passive acquisition of antibody: immunization of pregnant women to protect infants through transplacental transfer of antibody and administration of virus-specific monoclonal antibodies (mAb) of extended half-life (69, 70). Moreover, a series of maternal immunization studies and years of clinical experience with mAb in vulnerable infants never identified a case of ERD (2, 71, 72). Finally, infant intranasal immunization with live attenuated RSV vaccines (LAV) mimics natural infection, and after extensive testing in early phase trials were never found to associate with ERD in seronegative subjects (45).

Perhaps the greatest challenge will come from novel platforms that defy our traditional vaccine testing paradigms and may also alter our criteria for discriminating ERD in the future. For example, vaccine replication may not be necessary to prevent ERD priming in PAMP-adjuvanted vaccines (10, 73, 74), or a stabilized pre-fusion RSV F may elicit protective antibodies of high affinity.

ENDOTYPES IN RSV LRTI

The diversity in clinical presentations, combination of signs and symptoms, and variety in long-term consequences strongly suggest that RSV LRTI is not a single disease. In fact, it is probably a collective noun used to describe a set of clinical signs, which may obey different pathophysiological mechanisms. While subtypes of LRTI sharing similar observable characteristics are often designed as phenotypes, endotypes identify discrete subtypes based on specific mechanisms of illness. For example, middle-class urban and suburban infants with loss-of-function single nucleotide polymorphisms in Asp299Gly and/or Thr399Ile ($TLR4^{+/-}$) are severely ill when infected with RSV due to an exaggerated Th2 responses in the respiratory tract. Moreover, these infants are not protected by the administration of RSV-specific mAb when premature (75). Children in Navajo and Apache reservations are also particularly susceptible to RSV (76). And a high-affinity mAb against RSV failed to prevent long-term recurrent wheezing in them, despite reducing the rate of severe acute RSV disease (77). Alaskan native children are equally susceptible to acute infection with the virus. Therefore, efficacy and subsequent effectiveness of preventive strategies against RSV may differ in certain RSV LRTI endotypes, and be affected by environmental exposures, population genotype, and/or circulating viruses.

VIRAL REPLACEMENT OR EXTENDED PROTECTION

An interesting dilemma is whether a successful vaccine or mAb against RSV will lead to replacement of the virus by a different pathogen as a cause of pediatric illness or vaccination will impact other pulmonary ailments currently not known to be triggered by RSV.

In principle, vaccines and mAbs are not expected to prevent RSV infection in the upper respiratory tract, and therefore other agents would still compete for the niche with RSV in vaccinated subjects (78). However, recent data suggest that, upon prevention of RSV LRTI with palivizumab, other respiratory viruses may indeed replace a portion of the LRTI burden (78). A recent study in 429 premature infants, described a reduction in RSV LRTI, but reported no differences in the absolute number of respiratory episodes (78). These observations paralleled an increased rate of rhinovirus infections in recipients of palivizumab (79). A possible explanation may be that infants intermittently experience "*windows of susceptibility*" that allow pathogens to cause disease. RSV may outcompete other viruses for the nasopharyngeal niche in early life, but following immunization (or administration of mAb), infectivity of RSV may be "weakened". Then, other viruses may outcompete this "weakened" version, becoming more frequent agents of LRTI. Therefore, it is conceivable that some of the burden caused today by RSV will be elicited in the future by other viral pathogens.

Interestingly, the maternal Novavax RSV prefusogenic vaccine evaluated worldwide conferred significant protection against all-cause LRTI (25.3%) and all-cause LRTI with significant hypoxemia (<92% O_2 sat; 39.1%). Therefore, while it is conceivable that RSV may be replaced in certain situations, recent data suggest that significant, partly unexpected benefits may follow transplacental acquisition of maternal antibody in infants in developing nations.

REDUCING BURDEN OF RECURRENT WHEEZING AND ASTHMA

RSV LRTI in infants and young children associates with 25–80% greater subsequent rates of recurrent wheezing and asthma when compared to children not experiencing severe RSV LRTI (80, 81). And while some of these RSV lung ailments affect infants genetically predisposed to develop asthma at an older age (82), severe RSV LRTI may also contribute to the inception of recurrent wheezing and asthma in children (80, 82). In fact, enough evidence exists today to prompt long term follow up of vaccinated subjects in clinical trials to ascertain a potential role for RSV vaccines in decreasing the burden of recurrent wheezing (77, 78, 83, 84).

A variety of studies in premature infants examined the efficacy or effectiveness of palivizumab® in preventing long term wheezing and asthma at ages 1 and 6 years (78, 83–86). Most

of them described a protective role for RSV prevention against subsequent episodes of wheezing in infancy (78, 83, 84). The long-term effect elicited by RSV appears to be specific, and not triggered by infections with other viruses (78). However, a similar study with a virus-specific mAb in term, healthy Native Americans in Arizona prevented severe acute RSV LRTI but had no effect on rates of medically attended wheezing in children aged 1-3 years highlighting the potential importance of "endotypes" in future results (77).

At age 6 years in the randomized clinical trial in premature infants in The Netherlands, treatment significantly reduced parent-reported current asthma. The observation was based on different rates in infrequent wheezing (1-3 episodes/ year) (85), while physician diagnosed asthma and lung function were not different in drug and placebo recipients (85). In Japan, a second study revealed that palivizumab® prophylaxis administered to preterm infants did not suppress atopic asthma but lowered the incidence of recurrent wheezing (86).

Ongoing RCTs constitute a unique opportunity—perhaps the only ever- to settle these questions and determine the role of RSV in asthma inception. In recent years, a group of investigators reported guidelines for the evaluation of recurrent wheezing and asthma in upcoming studies (87). Yet, we must remain mindful of the fact that asthma is also a set of heterogeneous diseases sharing common symptoms. Therefore, it is highly likely that preventing severe RSV LRTI may affect one or few of these asthma endotypes but not others. And only a more sophisticated discrimination of the diseases under the "RSV LRTI and asthma umbrellas" will allow a definitive understanding of the mechanistic associations between both syndromes.

DECREASING INFANT MORTALITY

In 2015, more than three million children were hospitalized with RSV LRTI and up to 118,000 died at hospitals and in the community. Ninety-nine percent of deaths occurred in developing countries (88). Deaths attributable to RSV in industrialized countries are not frequent, affecting children with congenital heart disease, chronic lung illness, neuromuscular disorders or genetic syndromes (89). In contrast, healthy term infants from socially vulnerable environments in the developing world die at the hospital with bacterial sepsis and/or affected by clinically significant pneumothoraxes (90). Importantly, approximately 50% of deaths due to RSV in the developing world are known to occur at home. These deaths affect infants and young children from families affected by serious socioeconomic challenges (91).

The overall impact of an effective RSV vaccine in the developing world remains to be determined. Shi et al. estimated that a vaccine against RSV with 80% efficacy would prevent 22,000 in-hospital deaths every year (88). But studies of RSV mortality show sequential seasonal peaks of RSV and pneumococcal disease suggesting a potential synergic association between both pathogens (92), and a third of community deaths during the winter associated with RSV in the only study with information about viral etiologies so far (91). In fact, the recently observed protection conferred by ResVax® against all-cause LRTI and against all-cause LRTI with significant hypoxemia suggest our estimations may require revision in the near future. In fact, detecting RSV using RT-PCR is challenging in underserved settings where deaths typically occur, and testing for it in critically ill patients is infrequent given the absence of specific therapies (93). Moreover, randomized clinical trials and formal studies enhance surveillance and standardize care, perhaps providing an overly optimistic perspective on this problem.

BREAST MILK AND RSV VACCINES

Breastfeeding is a critical asset to protect infants against respiratory viruses in developing countries (94). But the potential benefit conferred by antibodies in human milk elicited by maternal immunization is uncertain.

Numerous molecules in breast milk, passively transferred to the baby during lactation, have been postulated to confer protection. Among them, sIgA has been the most widely accepted example of this passive mechanism (95). However, this hypothesis has potential inconsistencies that require further consideration. First, protecting the lung with passively acquired defenses would require frequent "mini" gastric aspirations to coat the nasopharynx, as the predicted persistence of molecules in the upper respiratory tract is short lived (96). Such a mechanism would be highly inefficient, notwithstanding the repetitive risk for a more severe aspiration. Second, babies are often exposed to RSV simultaneously with their mothers (97). This timing is problematic, as Ig protection of the respiratory tract requires high levels of local antibody. Therefore, the situation would demand instantaneous boosting of maternal immunity to timely prevent infection in infants. Because it is not entirely logical to postulate that mothers carry protection in adequate concentrations against all pathogens at all times. Third, studies on RSV and nasal IgA neither exhibited a strong correlation between baseline IgA levels and ARI (98), nor were able to correlate neutralizing activity and anti-RSV IgA levels (99).

But there is another argument that supports the hypothesis that the main mechanism of protection against RSV in human milk is not through passive transfer of sIgA. At least five studies in different populations from different regions and risk condition, described sex differences in breast milk-mediated prevention of severe viral LRTI in children. In all these studies, milk protects female infants better than males (100–104). Therefore, human milk appears to activate a process in the baby. And this process, in line with evolution and preservation of the species, is better suited for females (100–104). It will be interesting to learn whether such benefits can be boosted through RSV immunization.

BENEFITS FOR PREGNANT WOMEN FROM MATERNAL IMMUNIZATION

Influenza virus and *B. pertussis* maternal vaccines prevent severe disease in infants, but differ on their ability to affect maternal illness. Influenza virus is known to cause severe disease in pregnant women and its effects extend to influence both timing

of delivery and fetal growth (105). Conversely, pertussis in adults is relatively mild (106). Post-vaccine licensure studies will define whether RSV vaccines belong to the first or the second group.

Defining RSV disease burden during pregnancy is challenging. Proper ascertainment of the problem would require very large prospective studies, intense outpatient follow-up, and consequently considerable funding. Therefore, most information today about RSV in pregnant women derives from *post-hoc* analyses of trials evaluating maternal immunogens against flu (107). This strategy is not perfect, particularly due to two important limitations: unlike in flu, fever is not a frequent clinical sign associated with RSV infection in pregnant women (108); and often RSV and influenza seasons do not overlap exactly (109). Then, studies using criteria tailored for influenza-like illness in flu studies to prompt sick visits or collect samples from mothers to study RSV probably underestimates the burden of illness (110). In absence of RSV-targeted studies, we cannot convincingly state today that RSV elicits milder disease than influenza during pregnancy. While RSV in pregnancy elicits mild illness, it can occasionally cause acute respiratory failure (111).

STRATEGY-SPECIFIC CHALLENGES

Very specific logistical challenges may impact each immunization strategy. Maternal immunization may be most effective with different windows for immunization in pregnant women, should preparations differ. These decisions should be influenced by the levels of pre-existent antibodies in women and will also depend on the quality of prenatal care to maximize immunization rates. In the ResVax® RCT, immunization >30 days before delivery enhanced infant protection. In addition, acceptance of maternal immunization may differ in different populations due to cultural idiosyncrasies. Passive prophylaxis using mAb of extended half-life will require precise characterization of the local RSV season in different regions. This may prove challenging in tropical climates, where seasonality can oscillate by weeks in different years (112). And, importantly, cost of the drug in developing countries may become a critical factor for universal administration. These interventions are most needed in LMIC, where most morbidity and mortality occur. Finally, strategies like intranasal live attenuated RSV vaccine administration may demand a fine balance between optimal immunogenicity and no pathogenicity, require temperature stability for tropical climates, and inaugurate inoculation of RSV vaccines in older infants through a novel route of immunization, that for flu intranasal vaccines is not entirely comfortable.

In summary, the landscape of bronchiolitis and viral pneumonia may be altered directly and indirectly by the interventions under evaluation today. A long list of candidate vaccines (i.e., FIRSV, PFP-2) and mAb (i.e.: motavizumab®, suptavumab®) have failed over the years for different reason to control this feisty virus in young children. But both palivizumab® and the results from ResVax® in LMIC demonstrate that protection against RSV is possible. Just like many physicians in the industrialized world have rarely ever seen a case of measles, pediatricians in 2030 may be entirely unfamiliar with the packed emergency rooms, lack of available beds, or emergency situations that we experience every year due to RSV. Human metapneumovirus, rhinoviruses and human parainfluenza viruses may concentrate a lot more attention. A brighter future may be near.

AUTHOR CONTRIBUTIONS

SSA and FPP reviewed the literature, conceived and wrote the manuscript.

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REVIEW
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Cytokines in the Respiratory Airway as Biomarkers of Severity and Prognosis for Respiratory Syncytial Virus Infection: An Update

*Yaneisi Vázquez[1], Liliana González[1], Loreani Noguera[1], Pablo A. González[1], Claudia A. Riedel[2], Pablo Bertrand[3] and Susan M. Bueno[1]**

[1] *Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile,* [2] *Millennium Institute on Immunology and Immunotherapy, Departamento de Ciencias Biológicas, Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago, Chile,* [3] *División de Pediatría, Unidad de Enfermedades Respiratorias Pediátricas, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile*

The human respiratory syncytial virus (hRSV) is one of the most important causes of upper and lower respiratory tract infections in children and the main cause of bronchiolitis worldwide. Disease manifestations caused by hRSV may vary from mild to severe, occasionally requiring admission and hospitalization in intensive care units. Despite the high morbidity rates associated to bronchiolitis, treatment options against hRSV are limited and there are no current vaccination strategies to prevent infection. Importantly, the early identification of high-risk patients can help improve disease management and prevent complications associated with hRSV infection. Recently, the characterization of pro- and anti-inflammatory cytokine patterns produced during hRSV-related inflammatory processes has allowed the identification of potential prognosis biomarkers. A suitable biomarker should allow predicting the severity of the infection in a simple and opportune manner and should ideally be obtained from non-invasive samples. Among the cytokines associated with hRSV disease severity, IL-8, interferon-alpha (IFN-alpha), and IL-6, as well as the Th2-type cytokines thymic stromal lymphopoietin (TSLP), IL-3, and IL-33 have been highlighted as molecules with prognostic value in hRSV infections. In this review, we discuss current studies that describe molecules produced by patients during hRSV infection and their potential as biomarkers to anticipate the severity of the disease caused by this virus.

Keywords: biomarker, cytokines, LRTI, hRSV, severity, prognosis

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South Korea

****Correspondence:***
Susan M. Bueno
sbueno@bio.puc.cl

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INTRODUCTION

The human respiratory syncytial virus (hRSV) is a viral agent predominantly involved in acute lower respiratory tract infections (LRTIs), frequently associated to bronchiolitis and pneumonia in children and infants (1, 2). HRSV is responsible for approimately 60% of all LRTIs in children under 5 years old and causes more than 80% of the reported cases in infants (3, 4). At the age of 2 years, almost all children have been infected with hRSV at least once, and disease severity among these children may vary from mild to severe manifestations, sometimes requiring hospitalization with oxygen administration or admission into intensive care units (5, 6). Moreover, hRSV infection may cause exacerbated airway

diseases and has been associated with recurrent wheezing and asthma development (7, 8).

Several attempts to reduce the impact of hRSV-LRTI in health-care have been made. The first vaccine trial for hRSV was based on a formalin-inactivated hRSV formulation (FI-hRSV) in the 1960's, but this formulation was unable to generate an effective immune response and conversely produced an exacerbated disease in children after hRSV infection (9). Since this first failed attempt, several other vaccination strategies have been addressed, ranging from live-attenuated viral approaches to recombinant proteins, as well as recombinant organisms using both, viral and bacterial vectors as immunoadjuvants (10). It is important to highlight a growing number of clinical vaccine trials in the last decades aiming to identify a protective approach (phase I and II, ClinicalTrials.gov 2017: Identifier: NCT03213405 and 2018 Identifier: NCT03636906) (2, 11). However, despite the significant progress achieved in this field, until now there are no commercially available vaccines against hRSV (12).

Regarding hRSV disease management in high-risk groups, prophylaxis based on neutralizing monoclonal antibodies has been implemented to prevent severe manifestations associated to hRSV-LRTI (13–15). Palivizumab and Motavizumab are two humanized monoclonal antibodies generated against the hRSV fusion protein F that have shown efficacy in preventing hRSV infection and the capacity to decrease the rate of hospitalization of hRSV-infected infants (16). However, only Palivizumab has been licensed to be used as a therapy against hRSV severe infections associated with bronchiolitis and pneumonia. Yet, it is unable to induce long-lasting protection in those treated and the costs associated to its use make difficult the implementation of this strategy as a first treatment option (14). Despite the existence of the neutralizing antibodies described above as prophylactic and therapeutic strategies, these approaches do not work as vaccines. Hence, to date there is no successful and affordable strategy available to control hRSV outbreaks, which represent an important public health problem worldwide (17, 18).

Therefore, strategies to prevent complications derived from hRSV infection and improve disease management are needed. Based on this premise, early diagnosis, and prediction of disease severity has raised considerable interest in researchers and the search for biological biomarkers to predict disease severity during hRSV infection. In this review we discuss the latest studies available in PubMed on potential prognosis biomarkers and revise the feasibility of including them during routine hRSV diagnosis.

CHARACTERISTICS AND PATHOGENESIS OF HRSV

HRSV is an enveloped, negative, single-stranded RNA virus belonging to the *Pneumoviridae* family (19, 20). The genome of hRSV has 10 genes encoding 11 proteins required for the replicative cycle of hRSV in infected cells (21, 22), as well as for the modulation of the host immune response (23). Two hRSV subtypes have been identified, A and B, with the subtype A mostly associated to outbreaks during winter in countries with temperate climates (24, 25). hRSV is transmitted by direct contact or aerosol particles and once in the airways it replicates in mucosal epithelial cells, starting in the upper respiratory tract and then continuing to the lower respiratory tract (26). When hRSV arrives to the lower respiratory tract, viral antigen recognition by innate immune cells induce an inflammatory response, a process that is the result of complex interactions between the pathogen and host factors (27, 28). Lung inflammation is likely the result of a non-effective activation of the innate immunity by hRSV infection, mainly leading to Th2 and/or Th17 immune responses that generate mucus overproduction in the airways and enhance the inflammatory immune response in this tissue, leading to lung immunopathology (29, 30). After airway epithelial cells (AECs) recognize hRSV components (e.g. F protein and virus-related nucleic acids) through Toll-like receptors (i.e., TLR3 or 4) (**Figure 1A**) and retinoic-acid inducible gene I (RIG-I) receptors, signaling pathways activate transcription factors, such as interferon-regulatory factor 3 (IRF-3), and nuclear factor κB (NF-κB) (**Figure 1A**). In turn, these proteins promote the transcription of several anti-viral genes and soluble molecules (30, 31). In response to hRSV infection, AECs produce proinflammatory molecules such as type-I and type-III interferons (IFN) (31, 32). IFNs bind to IFN receptors (e.g., IFNAR) located on the surface of target cells and activate signaling pathways via Signal Transducer and Activator of Transcription 1 (STAT-1) and STAT-2 transcription factors. Ideally, STAT will bind to IFN-regulatory factors for a complete promotion in the transcription of interferon-stimulated genes (ISGs). Concomitantly, pro-inflammatory cytokines such as IL-6, tumor necrosis factor alpha (TNF-α) and chemokines (e.g., CXCL8, CCL3, CCL2, and CCL5) are induced and secreted to the extracellular medium. Importantly, some of these molecules (i.e., CCL2 and CCL5) will promote the recruitment of leukocytes (i.e., monocytes and neutrophils), dendritic cells, macrophages, natural killer cells, and $CD4^+$ T cells to the site of infection (31, 32).

Effective clearance of the hRSV requires a balanced Th1 and Th2 adaptive immune response, which promotes IFN-γ production by cytotoxic $CD8^+$ T cells (27, 33). However, during hRSV infection a weak type-I IFN response is elicited in the host, whereby viral replication is effective in infected cells and a pro-inflammatory Th2-response is generated (34) (**Figures 1A,B**). Because hRSV infection does not produce an effective memory response that confers protective immunity to subsequent viral exposure, re-infections are very frequent which lead to hyperreactivity, recurrent wheezing and an increased susceptibility of developing asthma (35).

CLINICAL MANIFESTATIONS OF HRSV INFECTION

Clinical manifestations of LRTI caused by hRSV might vary depending on the individual's co-morbidity, age or sex, air pollution exposure, parental asthma history or previous

Abbreviations: hRSV, Human respiratory syncytial virus; LRTI, Lower Respiratory Tract Infection; TSLP, Thymic Stromal Lymphopoietin; IL, Interleukin; IFN, Interferon; BALF, Bronchoalveolar lavage fluid; NPA, Nasopharyngeal aspirate; TLR, Toll-like receptors; AECs, Airway epithelial cells; NF-κB, Nuclear factor κB.

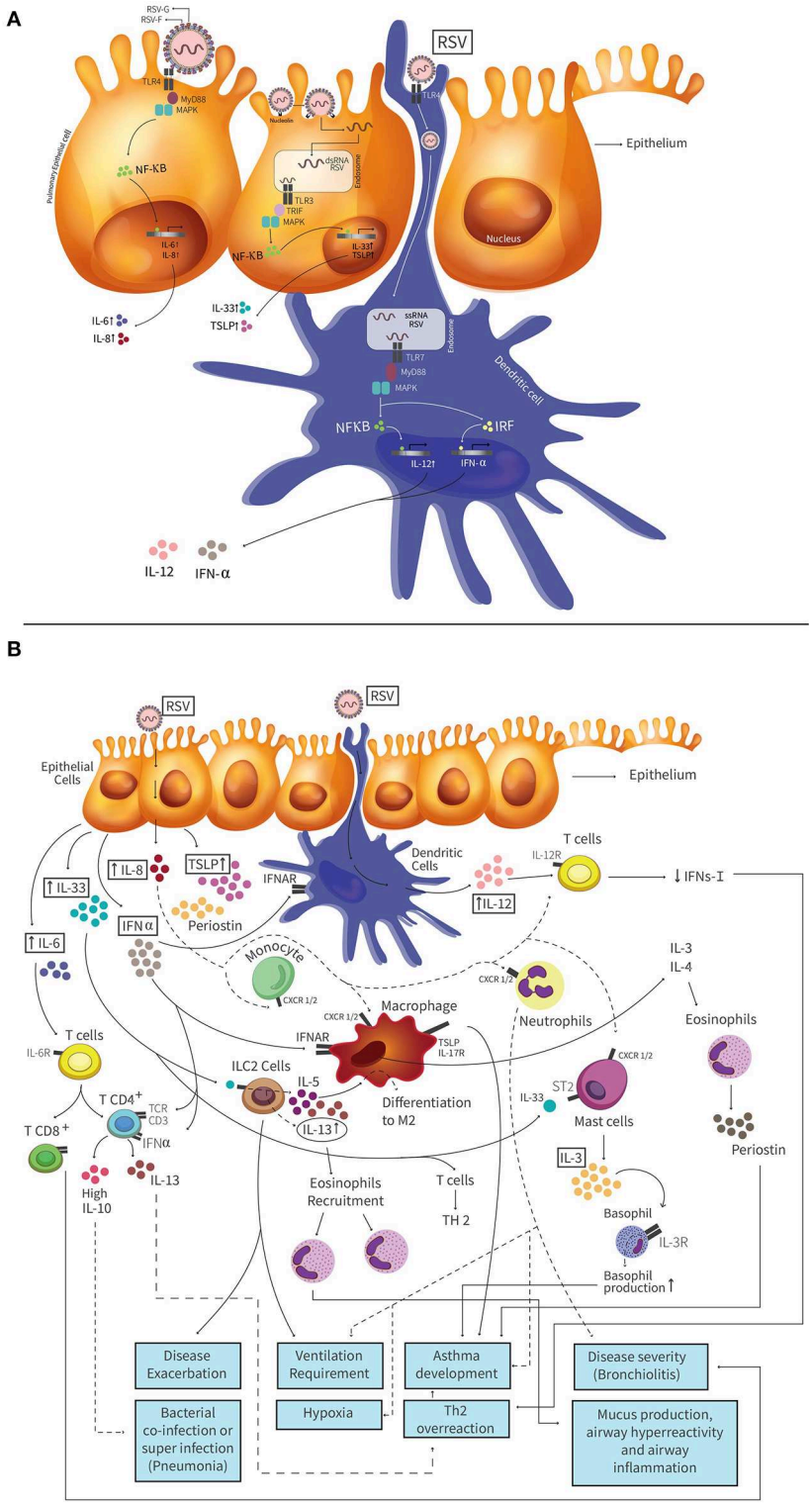

FIGURE 1 | Pathogenesis of hRSV and molecules with a biomarker potential induced in the airways during hRSV infection. **(A)** HRSV attaches to airway epithelial cells and this binding is mediated by the interaction between the fusion (F) or glycoprotein (G) protein of hRSV. Toll-like receptor 4 **(TLR4)** is expressed on AECs and it is involved in the hRSV entry. When hRSV F protein binds to TLR4, this triggers a cascade of signaling, where the protein myeloid differentiation primary response 88 (MyD88) is activated. The activation of MyD88 leads to activation of mitogen-activated protein kinase (MAPK), and the NF-kB transcription factor. Activated NF-kB translocates to the nucleus and promotes the production of Th1 cytokines (like as TNF-α, IL-6, and IL-8). **Nucleolin** is a protein located on the cell surface that is also

(Continued)

FIGURE 1 | involved in the entry process of hRSV, which generates a fusion between host cell membrane and the virus. This fusion allows the entry of the viral genetic material to the cell, and the binding of dsRNA to TLR3. TLR3 triggers a cascade of signaling by the TIR-domain-containing adapter-inducing interferon-β (TRIF), MAPKs and NF-kB transcription factor. This signaling pathway promotes the IL-33 and TSLP production. HRSV also can infect Dendritic Cells **(DCs)** and the virus mediates its entry by TLR4 receptor, present on the surface of the DC. DCs are then infected and the genetic material of the virus enters the cell. dsRNA binds TLR7 receptor, present in the endosome produced by the fusion, which one TLR3 triggers a cascade of signaling by the MyD88 protein, MAPKs and NF-kB transcription factor or interferon-regulatory factor (IRF). Those signaling pathways promote the IL-12 and IFN-α production, respectively. **(B)** Infected AECs secrete several cytokines and chemokines that have been described as potential biomarkers. High **IL-33** levels are produced by AECs and cells expressing ST2 receptor, such as ILC2s, respond to IL-33 through the production of IL-5 and IL-13, which promote the recruitment of eosinophils that generate disease exacerbation and is associated to ventilation requirement. The mast cells also express the ST2 receptor and when IL-33 binds to these receptors the production of IL-3 is promoted. AECs produce high levels of **IL-8**, promoting the recruitment neutrophils to the infection site, that could generate a degree of hypoxia, ventilation requirement and asthma development. **TSLP** production is mediate by AECs. This cytokine is recognized by the receptor TSLPR, which is expressed by macrophages, generating an exacerbation of the disease and asthma. **Periostin** is produced by AECs or eosinophils. This protein increases the expression of inflammatory mediators. Deposits of periostin in the lung is associated with increased severity of asthma. **IL-6** is produced by AECs and promotes a Th2 response. This cytokine is involved in the promotion of naïve differentiation to $CD4^+$ and $CD8^+$ T cell. $CD4^+$ T cells trigger the IL-13 production and Th2 overreaction response. $CD8^+$ T cells increase the disease severity. **IFN-α** is produced by pDCs and AECs. At late times of infection, high levels of this cytokine produce high IL-10 levels by T cells. **IL-12** is produced by pDCs and promotes the differentiation of naive T cells into Th1 cells and induces weak IFN-γ-production by T cells. This low IFN-γ-production generate a Th2 overreaction response. **IL-3** promotes basophil and eosinophil production, triggering inflammatory and allergic diseases as asthma. **IL-13** is produced by ILC2 cells and $CD4^+$ T cells, among other. High IL-13 levels result in a Th2 overreaction response and the recruitment of eosinophils that generate exacerbated mucus production, airway hyperreactivity and inflammation. Different lines (dotted and solid) were used to facilitate understanding of the figure and the different signaling pathways involved.

infections, among others (2, 36). HRSV-LRTI might be accompanied by nasal congestion, rhinorrhea, cough, wheezing and shortness of breath (36, 37), with an increased risk of subsequent wheezing episodes that can last for several years after acute infection. Indeed, pathology induced in the airways by respiratory viruses is characterized by alterations in the respiratory epithelium, which stimulates the production of pro-inflammatory cytokines and chemokines that promote the infiltration of immune cells into the lungs (38, 39). In some cases, this response might become exacerbated and bring temporary or lifetime changes in the lungs, leading to the recurrent wheezing episodes and asthma (3, 40). Although most viral infections induce a transient airway hyperresponsiveness (41, 42), those with a history of atopy or asthma might display enhanced virus-related inflammation with significant airway obstruction leading to a more severe disease (43, 44). Therefore, the identification of hRSV-infected patients susceptible to develop more severe diseases would be important for performing better clinical decisions.

DIAGNOSIS OF HRSV INFECTION

Early clinical diagnosis of hRSV infection could help to improve the care management of patients with respiratory infections and anticipate severe outcomes, according to the clinical predisposing factors, such as age. Currently, the available methods for hRSV diagnosis include tests that are based on molecular, virologic, or immunologic diagnostic.

Nowadays, the most used methods for hRSV diagnosis are based on direct immunofluorescence (DIF), reverse transcriptase-PCR (RT-PCR), immunochromatographic assay (CIA) and enzyme immune-assay (EIA). Other more complex methodologies that have been used more frequently in the last years are based in the detection of multiple analytes in high-complexity multiplex assays (such as Luminex or Affimetrix), as these approaches are faster than viral culture (11, 45, 46). Some molecular assays, such as RT-PCR and Luminex have high diagnostic sensitivity as compared to cell culture technique, but only RT-PCR is used as reference technique (47). Although RT-PCR is the fastest, its implementation is expensive as compared to DIF, EIA, or CIA assays. However, while the latter are low cost and fast, their sensitivity is lower than that of RT-PCR or Luminex and, in some cases (i.e., DIF), the interpretation of the results is somewhat subjective and requires technical skills, time, and expertise (47).

Immunologic diagnosis of hRSV is based on the characterization of cellular and cytokine/chemokine profiles (48, 49). In this case, flow cytometry is the main technique used to identify the cell types present in the bronchoalveolar lavage fluid (BALF) and peripheric blood samples of patients with hRSV infection. Cells recruited to the lungs include neutrophils, dendritic cells, T and B cells, alveolar macrophages and monocytes (10, 35). Clinical studies with hRSV infected children have shown an increased amount of neutrophils ($CD11b^+$, $CD18^+$, and $CD54^+$) (50), alveolar macrophages (expressing TNF-α) (51), monocytes ($CD69^+$) (52) and B cells (53) infiltrating the infected airways. Contrarily, the presence of T cells ($CD4^+$ and $CD8^+$) and plasmacytoid dendritic cells (DCs, HLA-DR^+, $CD123^+/CD11c^-$) significantly decrease in peripheral blood of infected children, as compared to healthy children control groups (54, 55). Besides the characterization of the cells infiltrating the airways, the cytokines/chemokine profile observed in the infected tissue is also informative. The main cytokines evaluated in the BALFs of hRSV infected individuals are mainly IL-2, IL-12, IFN-γ, IL-8, IL-6, and TNF-α (35). Importantly, all these cytokines can be evaluated by flow cytometry, ELISA, RT-PCR, or Luminex (56–58).

The types of samples used to detect hRSV or immune-related markers can be nasal washes, nasopharyngeal aspirates (NPA), nasopharyngeal swabs, BALFs, serum and peripheral blood (11, 45, 46). However, cytokines as biomarkers should be assessed at the site of infection (upper and lower respiratory tract) and to a lower extent in peripheric blood. The role of the above-mentioned cytokines during infection is discussed below in the following sections.

SEVERITY PROGNOSIS IN HRSV INFECTION

Among the patients diagnosed with LRTI, a significant number of hRSV-infected children treated as outpatients will require additional medical attention due to respiratory complications. Furthermore, a significant percentage of diagnosed patients will display recurring wheezing episodes and other complications in the following months after the first LRTI episode (59, 60). It is worth mentioning that these patients can not be identified early after infection due to a lack of accurate tools for predicting disease severity. Furthermore, at present there is no consensus on predicting the outcome of patients with LRTI caused by hRSV, which represents a problem for disease management due to the rapid evolution of the disease in which mechanical ventilation might be unexpectedly required in the course of 24 hours or less (61). Currently, methods that are based on clinical parameters used by physicians are widely accepted to support clinical decisions (62). However, these parameters may be somewhat subjective and are not accurate enough to perform a precise categorization or prognosis of disease severity (63, 64). To address this problem, biomarkers within samples of patients might contribute to a better diagnosis and could help physicians take more accurate decisions, increasing the possibility of obtaining better outcomes (4, 65). In line with this notion, in the last years several research groups have focused on identifying an accurate method for determining the severity and progression of LRTI by hRSV (62, 66–68). Below, we describe diverse parameters and soluble molecules currently used to assess disease severity in hRSV-infected patients (**Table 1**).

Clinical Score as a Biomarker Related to Disease Severity

The use of prediction models to calculate the risk of severe outcome in LRTI in children has been previously implemented based on the clinical characteristics of patients, radiological findings, and laboratory results (77). In the last 10 years, remarkable progress in diagnostics has been achieved thanks to the availability of transcriptional profiles that have allowed establishing fingerprints related to disease progression and severity caused by hRSV infection (78). Among the available methods based on transcriptomic approaches, the "molecular distance to health" (MDTH) has shown to be a promising diagnostic tool for respiratory tract infections (68, 79). The MDTH is a tool designed to measure alterations in the transcriptional profile of immune cells (i.e., neutrophils, cytotoxic cells, and T-cells) obtained from patients (80). Data is obtained from the test as a single score that is compared with a basal score from healthy controls. Importantly, MDTH scores performed during the first days of hRSV infection have been able to predict disease severity in terms of hospitalization days and intensive care requirements (78).

Microbial Factors as Severity Biomarkers

It is well known that the higher microbial load at the site of infection, the greater the possibility to cause tissue damage, which is related to worse prognosis. Based on this premise, several research groups have tried to demonstrate a direct relationship between viral loads and the severity of the disease (81), but the conclusions are somewhat controversial. Different studies have shown a direct correlation between the increase of viral loads with more severe clinical manifestations (81–83). In fact, these studies showed that high hRSV viral loads at day 3 are significantly associated with requirement for intensive care and respiratory failure (84). In contrast, studies, such as (69, 85) and Piedra et al. have reported the opposite, where high hRSV loads at the beginning of the infection correlate with protective immune response and less severe disease progression (86). These findings raise the discussion about the role of viral loads in disease progression and the possibility of considering this factor as a potential biomarker to determine disease severity in hRSV-LRTI, as viral loads could be leading the host immune response to the virus.

Soluble Proteins as Biomarkers for Disease Severity

In the last few years, the analysis of protein expression patterns has become one of the most explored fields in diagnosis. The samples used to obtain the protein expression patterns range from blood to nasopharyngeal samples, with both suggesting helpful insights into the identification of molecules related to the severity of the infection. For example, increased levels of serum transaminases, aminotransferases and antidiuretic hormones have been related to severe cases of hRSV bronchiolitis (70, 71). Furthermore, increased levels of lactate dehydrogenase (LDH) in nasopharyngeal samples has also shown to have a predictive value of 88% in determining the severity of the disease in young children with bronchiolitis (72, 73). Another molecule proposed as a disease severity biomarker is mucin 5AC (MUC5AC), a highly glycosylated protein present in the airway mucus (74). This protein has been reported to be detected in nasal aspirates obtained from hRSV-infected children and its presence and concentration is correlated to disease severity caused by hRSV (75). Taken together, several soluble molecules show a correlation with the severity of hRSV-related disease and can be easily detected in samples that are simple to obtain, and thus may be used as biomarkers of disease severity related to infections caused by hRSV.

Pro-inflammatory Cytokines as Biomarkers for Disease Severity in hRSV Infections

During hRSV infection, the host innate immune response generated against the virus can be unbalanced and ultimately detrimental to the host. Non-optimal responses against the virus are Th2-like responses with the generation of cytokines, which in turn can recruit numerous pro-inflammatory immune cells (35, 56). Furthermore, several studies have reported an increase in the levels of Th2-like cytokines in different types of samples (BALF, serum, blood, plasma, nasopharyngeal, or aspirate washes), which can be correlated with disease severity in children. Such cytokines, which could be used as prognosis biomarkers are IL-33, IL-8, TSLP, IL-6, periostin, and IFN-α. Those biomarkers could predict hRSV disease severity in children

TABLE 1 | Molecules and cells as severity markers in respiratory diseases.

Marker	Sample type	Market for	References
Viral loads	Nasal washes	Disease progression in hRSV-LRTI.	(69)
Transaminases, aminotransferases and antidiuretic hormones	Serum and nasopharyngeal	Bronchiolitis caused by hRSV	(70, 71)
Lactate dehydrogenase	Nasopharyngeal	Bronchiolitis caused by hRSV in children.	(72, 73)
MUC5AC	Mucus	Severity disease caused by hRSV infection	(74, 75)
Neutotrophins (BDNF and NGF)	BALF	Severity disease caused by hRSV infection Developed asthma later hRSV infection	(76)

BDNF, Brain-derived neurotrophic factor; NGF, nerve growth factor.

(**Table 2**). Other cytokines, such as IL-12, IL-3, and IL-13 could also be potential biomarkers, although more clinical studies are required (**Table 3**). Next, we will explain further how some of these cytokines could be useful to predict the severity of hRSV infection.

Interleukin-33 (IL-33)

IL-33 is constitutively expressed by endothelial and epithelial cells. The main function of this cytokine is the initiation and development of the innate and adaptive Th2 type immune response (103). Cells expressing the ST2 receptor respond to IL-33, including mast cells, eosinophils, and basophils, among others (104). Type-2 innate lymphoid cells (ILC2s) are also targeted by IL-33 to produce Th2-type cytokines (IL-6, IL-8, IL-5, IL-13), which in turn promote a Th2 response with eosinophil recruitment, generating an exacerbated disease (105) (**Figure 1B**). Recent studies with mice in which IL-33 was neutralized during hRSV infection, showed that severe pathology was not induced and that mice treated with IL-33 during hRSV infection quickly developed the disease, resulting in more severe clinical outcome (35, 88). Interestingly, Saravia et al. measured IL-33 levels in NPA and showed a link with ventilation requirement in infants hospitalized by bronchiolitis caused by hRSV (87). In 2015, Bertrand et al. performed a study in children with bronchiolitis caused by hRSV and detected high levels of IL-33 expression levels in NPA in patients with a family history of atopy (66). García-García et al. measured IL-33 levels from NPA in children infected with hRSV, associating bronchiolitis with high levels of this cytokine. Furthermore, both studies describe that IL-33 cytokine is elevated when coinfection occurred (88). Taken together, these results indicate that IL-33 could be a good biomarker to determine the severity and prognosis during bronchiolitis caused by hRSV.

Interleukin-8 (IL-8)

IL-8 has a mayor chemotactic role, and is mainly produced by monocytes, endothelial cells, macrophages, and T cells (106, 107). IL-8 binds to G protein-coupled receptors CXCR1 and CXCR2 expressed by cells that include monocytes, neutrophils, endothelial cells, macrophages, and T cells, among others (108, 109) (**Figure 1B**). During an infection with hRSV, McNamara et al. found that the concentration of IL-8 remains elevated during the disease, even when the number and recruitment of neutrophils ultimately decreased (110). Elevated IL-8 levels (in nasopharyngeal samples) have been widely correlated with disease severity caused by hRSV infection, including the risk of mechanical ventilation (4, 90). In 2013, Díaz et al. found high IL-8 levels in NPA in children with severe hRSV bronchiolitis as compared to controls and patients with mild disease manifestations. More specifically, they observed an increase in IL-8 in a group of patients with severe disease (111), which may suggest that higher levels of this cytokine relate to higher severity of hRSV infection. Tabarani et al. identified in nasopharyngeal washes increased levels of IL-6, IL-8, and TNF-α associated to hRSV disease severity in young children (89). In this study, the authors associated the severity of disease with the age of the individuals, chronic diseases and elevated concentrations of IL-8, as well as other molecules (89). In another study, which was performed in children with severe hRSV infection, Brand et al. assessed the levels of IL-8 in plasma and NPA and found an increase in IL-8 in the plasma of children with severe disease, as compared to children with mild or moderate disease (48). In 2015, Díaz et al. performed another study in children with bronchiolitis caused by hRSV and Rhinovirus (RV). This study showed higher IL-8 levels in NPA of children infected with both, hRSV and RV than children infected with RV alone, which was associated with more days requiring O_2 treatment (92). Based on this study, it can be suggested that high IL-8 levels in children infected hRSV will act as a good predictor for determining the days that requiring O_2 treatment. In 2016, Huang et al. performed a clinical study that included 96 patients with asthma-chronic obstructive pulmonary disease (COPD) and 35 healthy controls. Their results showed an increment of IL-8 and other cytokine levels that were related to the severity of airway diseases. The researchers suggest that IL-8 could be a potential marker for the evaluation of asthma and COPD (91). There are not new clinical studies that correlate high levels of this cytokine with the disease severity.

Thymic Stromal Lymphopoietin (TSLP)

TSLP is expressed by several cell types, but mainly by epithelial cells and keratinocytes (112, 113). Two isoforms have been described for this cytokine: a long and a short form of TSLP (114). The short isoform is constitutively expressed in several tissues, particularly in those that are highly sensitive to inflammation. Importantly, the long isoform of TSLP has been widely correlated with exacerbated immune responses and the establishment of allergic and asthma in patients with atopic

TABLE 2 | Pro-inflammatory cytokines as prognosis biomarkers in respiratory diseases.

Cytokine	Sample type	Biomarker for	References
IL-33	Nasal aspirates	Risk for asthma or severe hRSV disease in children after reinfection.	(87)
		Ventilation requirement in infants hospitalized by bronchiolitis caused by hRSV	
	NPA	Bronchiolitis, asthma, and allergic diseases.	(88)
		Allergic inflammation.	(66)
IL-8	Plasma	Predictors of mechanical ventilator requirement during hRSV infection and bronchiolitis.	(4, 48)
	Nasopharyngeal wash	Severity during hRSV infection.	(89)
	Plasma and nasal secretion	Prognosis for children evolving to bronchiolitis by hRSV.	(90)
	Plasma	Severity of airway diseases, asthma and COPD.	(91)
	NPA	Predictive value for the number of days with need of supplemental oxygen.	(92)
TSLP	NPA	Severe bronchiolitis by hRSV.	(93)
	NPA	Increased infant hospitalization and disease severity.	(88)
	BALF	Asthma development by hRSV.	(94, 95)
Periostin	NPA	Severe bronchiolitis by hRSV.	(93)
	NPA	Increased infant hospitalization	(88)
	Bronchial and nasal cells	Persistent or uncontrolled asthma in children.	(96)
	Serum	Persistent or uncontrolled asthma in children.	(97, 98)
	Tracheal aspirates and nasal wash	Pulmonary hypertension and prognosis during hRSV bronchiolitis.	(99)
IL-6	Blood, plasma and serum	Increased infant hospitalization and severe hRSV bronchiolitis.	(4)
	Nasopharyngeal wash	Severity during hRSV infection.	(89)
	NPA	High hRSV disease severity.	(100)
	NPA	Predictive value for the number of days with need of supplemental oxygen.	(35, 92)
IFN-α	Blood	Severity of the disease in children under 2 years infected by hRSV.	(101)
	Blood and nasopharyngeal swabs	More severe illness and recurrent wheezing in in hRSV bronchiolitis.	(89, 102)

BALF, Bronchoalveolar lavage fluid; NPA, Nasopharyngeal aspirate.

dermatitis (95) (**Figure 1B**). Asthma may result as a consequence of different factors in children. However, a possible association with viral infections has gained increased attention of researchers in the last decade (88). At present, there is increasing evidence suggesting an association between TSLP elicited upon infection with hRSV or RV and the development of asthma (88, 94). However, it still remains to be elucidated whether asthma favors severe viral disease or if asthma is the result of severe disease elicited during respiratory infection. Lee et al. reported that viral antigen recognition triggers a signaling cascade involving the NF-κB nuclear factor and retinoic acid induced gene 1 (RIG-1) (115). The activation of this cascade resulted in TSLP production and a strong Th2 response, contributing to the pathophysiology observed in severe bronchiolitis, which eventually in some cases progressed to asthma (115). Later, García-García et al. showed an association between TSLP, together with periostin and IL-33, with disease severity in the infection of the respiratory tract of children. This study showed a correlation between increased levels of TSLP with hRSV bronchiolitis and coinfections with rhinovirus, as well as with severe disease and intensive care unit (ICU) admission (88).

Interleukin-6 (IL-6)

IL-6 is a soluble mediator that can be produced by macrophages and epithelial cells (116). After its synthesis, IL-6 moves to the liver through the bloodstream and generates a pleiotropic effect over immunity and inflammation (117). This cytokine is involved in the promotion of the differentiation of naïve $CD4^+$ and $CD8^+$ T cells and is an important link between innate and acquired immunity (117) (**Figure 1B**). In 2013, Tabarani et al. evaluated the levels of IL-6 in nasopharyngeal wash samples from children with LRTI and hRSV. Interestingly, they found a correlation between the magnitude of the clinical manifestations elicited by hRSV infection and high levels of IL-6 amongst other inflammatory mediators (CCL2, TNF-α, CXCL8, IL-10) (89). On the other hand, Brown et al. have suggested that high levels of IL-6 in the plasma could indicate a higher probability of infant hospitalization and severe bronchiolitis caused by hRSV (4). In 2016, Lu et al. also detected high levels of IL-6 in NPA of patients with hRSV and this was correlated with higher hRSV disease severity (100). Increased levels of IL-6 and other cytokines have also been found in nasal lavage fluids of children with LRTI, particularly those which needed O_2 treatment (35, 118). Other studies performed in children with bronchiolitis caused by hRSV infection showed that high IL-6 levels in nasal samples and BALF correlated with the need for ventilation and with a higher degree of hypoxia (35, 92). In this study, the authors suggested that IL-6 and other cytokines assessed could be reliable biomarkers to determine the severity of hRSV infection.

Periostin

Periostin is a protein that is expressed at basal levels in almost all human tissues (119). Its expression is also found in the respiratory epithelium and is elevated levels in asthmatic children

TABLE 3 | Pro-inflammatory cytokines as potential prognosis biomarkers in respiratory diseases.

Cytokine	Sample type	Potential Biomarker for	References
IL-12	BALF	Recurrent wheezing due to hRSV infection.	(66)
		Developed asthma later hRSV infection.	
		Developed asthma in infants with bronchiolitis caused hRSV infection.	(35)
IL-3	BALF	Recurrent wheezing due to hRSV infection.	(66)
		Developed asthma later hRSV infection.	
	NPA	Severe bronchiolitis by hRSV.	(100)
IL-13	Nasal aspirates	Ventilation requirement in infants hospitalized by bronchiolitis caused by hRSV.	(87)
	Blood	Asthma diagnosis.	(100)
	Nasal washes	High IL-13 levels are elevated in children with hRSV LRTI.	(35)

BALF, Bronchoalveolar lavage fluid; NPA, Nasopharyngeal aspirate.

(120). This protein is produced by eosinophils in response to IL-4 an IL-13 signaling (121) (**Figure 1B**). The role of periostin is related to the generation of allergic inflammation and the development of a Th2 phenotype, among others (120) (**Figure 1B**). Periostin has been associated with asthma severity and increased levels of periostin have been found in the serum of children with exacerbated manifestations of asthma (122). Lopez-Guisa and colleagues evaluated periostin levels in bronchial and nasal cells from asthmatic, non-asthmatic, atopic, and healthy children and found a significant increase in periostin levels in asthmatic children (3.7 times), as compared to the other groups (96). These results were confirmed in studies that showed a correlation between high levels of periostin in the serum with persistent or uncontrolled asthma in children (97, 98). In fact, clinical manifestations of asthma are considered to be very similar to bronchiolitis symptoms (123). These findings suggest that asthma could be a sequel of severe bronchiolitis in children (123). García-García et al. showed in NPAs that increased concentrations of periostin were associated with more severe hRSV infection, as compared to healthy children (88, 93). More recently, periostin levels were associated with the severity of viral bronchiolitis, as children with severe pulmonary hypertension had high levels of this protein as compared to children with mild pulmonary hypertension (8,887 ± 1,582 pg/ml vs. 5,016 ± 1,017 pg/ml) (99). These results indicate that periostin could be another good biomarker for the prognosis of hRSV infection and particularly bronchiolitis.

Interferon Alpha (IFN-α)

IFNs are a large family of pleiotropic cytokines. Particularly, IFN-α and IFN-β are type-I interferon family members produced by epithelial cells and most of immune cells (124). To exert its biological action, type-I IFNs binds to the type-I IFN receptor (IFNAR1/2) (125), which triggers the expression of pro-inflammatory molecules and antiviral genes, such as those involved in the degradation of viral RNA (126). Importantly, the recognition of the hRSV non-structural protein 1 (NS1) has been correlated with impaired IFN-α function, particularly through the induction of the miRNA miR-29a, which inhibits the expression of the IFN-α receptor in infected cells (101). These studies suggest that low levels of IFN-α could be related to the severity of hRSV infection and hence could be used as a biomarker. However, other studies based on the transcriptional profile of blood samples and nasopharyngeal swabs, report contrasting results, indicating that type-I interferons, particularly IFN-α/β are increased in hRSV bronchiolitis and correlate with severe illness and recurrent wheezing (89, 102). These studies suggest that interferon signaling pathways may serve as important biomarkers associated to hRSV loads and severity (102). Resolving the discrepancies found among different studies analyzing the role of IFN-α in hRSV disease severity will require further investigations that ideally relate transcriptional findings with protein levels in blood and nasopharyngeal samples.

OTHER POTENCIAL PRO-INFLAMMATORY CYTOKINES AS BIOMARKERS FOR SEVERITY CAUSED BY HRSV

Besides the cytokines described above as potential biomarkers for hRSV severity (4), recent studies have preliminarily pointed out other pro-inflammatory cytokines that show positive correlations with hRSV severity and are potential prognosis biomarkers for respiratory diseases (**Table 3**). Some of these cytokines are described below.

Interleukin-12 (IL-12)

IL-12 is produced in response to viral or bacterial infections by DCs and other antigen-presenting cells and is involved in promoting naïve T cell differentiation into Th1 T cells (127) (**Figure 1B**). Bertrand et al. have shown that nasal and lung samples display increased levels of IL-12 in LRTI patients. Furthermore, they showed for first time that high levels of IL-12p40 (in BALF) and other cytokine could be correlated with recurrent wheezing and the development of asthma in infants with bronchiolitis caused by hRSV infection (35, 66).

Interleukin-3 (IL-3)

IL-3 is mainly expressed by mast cells and activated T cells located in the airways (128). This cytokine induces an increase in basophil and eosinophil production (129) (**Figure 1B**) and is involved in the pathogenesis of asthma (128). In 2015, Bertrand et al. described for the first-time the presence of high levels of IL-3 in BALF and NPA obtained from children <9 months with acute bronchiolitis caused by hRSV. Furthermore, the authors found a correlation between high levels of IL-3 with episodes of recurring wheezing and the development of asthma in the future (66). Lu et al. also found high levels of IL-3 in NPA in children with bronchiolitis caused by hRSV and an increased risk of asthma, which was associated with higher disease severity

(100). The results of this study suggest that IL-3 could be involved in the development of chronic airway inflammatory diseases and that it could be used to predict clinical outcomes in hRSV-LRTI. Consistently, the authors suggested that IL-3 could be eventually used to predict the clinical outcome of patients.

Interleukin-13 (IL-13)

In the lungs, IL-13 is the mediator of eosinophilic inflammation, mucosal secretion, and bronchial hyper reactivity (130). It has been observed that IL-13 is elevated in COPD, as well as in asthma and other lung diseases (131). Importantly, IL-13 is produced in response to IL-33 signaling and is released from various cells, including alveolar macrophages, basophils, mast cells, eosinophils, ILC2 and $CD4^+$ T cells (132) (**Figure 1B**). In 2015, Saravia et al. linked high levels of IL-13 and IL-33 with the requirement for ventilation in infants hospitalized with bronchiolitis caused by hRSV (87). Consistently, in an animal model of hRSV (BALB/c mice), an up-regulation of IL-13 has been reported, which results in the recruitment of eosinophils to the airways that generates exacerbated mucus production, lung hyperreactivity and airway inflammation (132). A more recent study performed in 2016 evaluated IL-13 levels in the blood of children being treated for respiratory symptoms following severe hRSV bronchiolitis and found that IL-13 could be used as a clinical asthma diagnosis marker (100).

CONCLUDING REMARKS

Biomarkers for classifying the severity of respiratory tract infections have become a global need due to the lack of effective strategies to decrease the impact of such diseases and the need for improving the management of patients and their potential outcomes. Most efforts point to the development of highly sensitive, rapid, and low-cost techniques that allow predicting in an accurate way the prognosis of patients with respiratory infections. Nowadays, an important number of molecules have been identified which could help asses disease severity, however their specificity and sensitivity remain challenging and are not strong enough yet to accurately predict disease outcome and become a canonic biomarker for predicting LRTI severity associated to hRSV. Hence, more studies are needed to establish the pro-inflammatory cytokine and cytokine expression patterns that are related to disease development during the different stages of hRSV infection. Ideally, particular pro-inflammatory cytokine profiles will ultimately allow determining early on during infection the severity of disease caused by hRSV.

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YV, LG, and LN are responsible for the writing of this review article. PG, CR, and PB are responsible for reviewing the article and SB is the leading investigator and assisted in the organization and revision of the manuscript.

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Biology of Infection and Disease Pathogenesis to Guide RSV Vaccine Development

Seyhan Boyoglu-Barnum[1], *Tatiana Chirkova*[2] *and Larry J. Anderson*[2]*

[1] Vaccine Research Center, National Institutes of Health, Bethesda, MD, United States, [2] Department of Pediatrics, Emory University and Children's Healthcare of Atlanta, Atlanta, GA, United States

Respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory tract disease in young children and a substantial contributor to respiratory tract disease throughout life and as such a high priority for vaccine development. However, after nearly 60 years of research no vaccine is yet available. The challenges to developing an RSV vaccine include the young age, 2-4 months of age, for the peak of disease, the enhanced RSV disease associated with the first RSV vaccine, formalin-inactivated RSV with an alum adjuvant (FI-RSV), and difficulty achieving protection as illustrated by repeat infections with disease that occur throughout life. Understanding the biology of infection and disease pathogenesis has and will continue to guide vaccine development. In this paper, we review the roles that RSV proteins play in the biology of infection and disease pathogenesis and the corresponding contribution to live attenuated and subunit RSV vaccines. Each of RSV's 11 proteins are in the design of one or more vaccines. The G protein's contribution to disease pathogenesis through altering host immune responses as well as its role in the biology of infection suggest it can make a unique contribution to an RSV vaccine, both live attenuated and subunit vaccines. One of G's potential unique contributions to a vaccine is the potential for anti-G immunity to have an anti-inflammatory effect independent of virus replication. Though an anti-viral effect is essential to an effective RSV vaccine, it is important to remember that the goal of a vaccine is to prevent disease. Thus, other effects of the infection, such as G's alteration of the host immune response may provide opportunities to induce responses that block this effect and improve an RSV vaccine. Keeping in mind the goal of a vaccine is to prevent disease and not virus replication may help identify new strategies for other vaccine challenges, such as improving influenza vaccines and developing HIV vaccines.

Keywords: pathogenesis, RSV (respiratory syncytial virus), vaccine development, biology of infection, protective immunity

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***Correspondence:**
Larry J. Anderson
larry.anderson@emory.edu

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BACKGROUND

Respiratory syncytial virus (RSV) is estimated to cause 3.4 million hospitalizations and 95,000–150,000 deaths globally and up to 175,000 hospitalizations in the United States in children <5 years of age each year (1, 2). It is also estimated to cause 14,000 deaths each year in adults in the United States (3). Its disease burden has made RSV a priority for vaccine development for over 50 years but no vaccine is yet available for any of groups targeted for an RSV vaccine including young

children (∼ <6 months of age), older children (∼ 6 months to 24 months of age), pregnant women, and elderly adults (∼ >65 years of age) (4, 5). The challenges to developing an RSV vaccine include: concern that a non-live virus vaccine in young children may predispose to enhanced RSV disease (ERD) in RSV-infected young children who earlier received a formalin-inactivated RSV plus alum vaccine; difficulty in inducing and assessing protective immunity; cost of clinical vaccine trials; and the young age, 2–4 months of age, for peak of disease. The first RSV vaccine, formalin-inactivated RSV with alum adjuvant (FI-RSV), given to young, likely RSV naïve, but not older, RSV primed children, led to enhanced RSV disease (ERD) with later infection, i.e., a high rate of hospitalization and two deaths (6–9). This experience raised concern that any non-live virus vaccine may induce an aberrant immune response that predisposes to ERD in young children and a focus on live attenuated RSV or virus vector vaccines for this target population. Since ERD is not a concern for RSV-primed older children and adults and live attenuated RSV replicates poorly in primed persons, subunit vaccines are under development for older children and adults. The difficulty in inducing protective immunity is highlighted by repeat infections and disease throughout life (3, 10).

The fact that prior infection and high titers of neutralizing antibodies, e.g., maternally derived antibodies or from an earlier infection, are associated with some protection suggest that a vaccine should be achievable (11–17). In addition, immune globulin with a high RSV neutralizing antibody titer and a neutralizing monoclonal antibody are effective in preventing serious disease in high-risk young infants (18, 19).

The past failures, however, suggest that novel vaccines may be required for success. In considering novel vaccines, it is useful to remember that the goal of a vaccine is to prevent disease caused by the infection. Though obviously important to an effective vaccine, a singular focus on induction of neutralizing antibodies or preventing virus replication, may lead to missing other, important effects of a vaccine. For example, if a vaccine does not induce sterilizing immunity, as is likely for RSV, other effects such as virus-induced inflammation become relevant. The pathogenesis of RSV disease, reviewed elsewhere (20, 21), is the foundation for designing a vaccine that addresses disease pathogenesis. The prominence of wheezing as a manifestation of infection (10) with its similarity to asthma and the association between mucus production and disease severity (22) suggest a prominent role of host inflammatory responses in disease pathogenesis. Blocking such effects could be important to a successful vaccine.

The role of RSV's proteins in biology of infection and disease pathogenesis provides clues to their potential contribution to a vaccine. RSV has 10 genes that encode for 11 proteins (23). RSV has two major antigenic groups of strains, A and B, and multiple genotypes within the two groups (24–27). Though only two RSV proteins induce *in vitro* neutralizing antibodies, F and G (28), as illustrated in **Table 1**, all RSV proteins have played a role in design of one or more vaccines. The type of vaccine under development varies among the target populations. Live attenuated or virus-vector subunit vaccines are under development for infants and young children and non-live or virus-vector subunit vaccines for older children and adults.

LIVE VIRUS VACCINES

A live attenuated RSV vaccine needs to both have mutations that attenuate virus replication for safety while maintaining sufficient replication to maintain immunogenicity. The first attenuated vaccines were generated by chemical mutagenesis and low temperature passage. Subsequently, reverse genetics has identified specific mutations associated with temperature sensitivity and attenuation (30, 31). A set of five mutations, one in the N, two in the F, and two in the L protein genes, are associated with attenuation in primates and designated "cp" for cold passage. Six additional mutations, 5 in L and 1 in the gene-start transcription signal for M2, contribute independently to temperature sensitivity and attenuation. Five RSV genes, i.e., NS1, NS2, SH, G, and M2-2, can be deleted and virus recovered. All viruses are attenuated in animals. Live attenuated RSV candidate vaccines with deletions of NS2, G, or M2-2 are in clinical trials (32). A live attenuated RSV candidate vaccine with the 5 cp mutations, two other attenuating mutations, and deletion of the SH gene was also in a clinical trial (33, 34).

A virus vector vaccine's safety is likely not dependent on the RSV antigen present but the vector. Since the virus vectors present antigen to the immune system similar to the way that live RSV does, they are likely safe from ERD risk. A parainfluenza virus that expressed the RSV F protein did not led to ERD in RSV naïve children (35).

Codon pair de-optimization is another way to attenuate RSV and different combinations of RSV proteins including NS1 and NS2; NS1, NS2, N, P, M, and SH; G and F; L; or all proteins except M2-1 and M2-2 have been codon de-optimized to attenuate the virus (36, 37). With codon pair de-optimization, the level of attenuation can be fine-tuned by varying levels of protein production and makes it possible to attenuate through changes to any protein without relying on specific attenuating mutations or gene deletion.

Several live attenuated RSV vaccines show promise in early clinical trials (38). It is yet uncertain if they will achieve the balance between safety and immunogenicity needed for the young child. Maternal vaccination, or longer lasting immune prophylaxis, followed by vaccination at 4–6 months of age should make safety easier to achieve. A safe virus vector is another possible way to protect young children.

SUBUNIT VACCINES

With the exception of virus-vector subunit vaccines, subunit vaccines are under development for RSV-primed older children and adults. Virus vector vaccines are under development for both. The goal for a subunit vaccine is to safely, induce a more effective immune response than natural infection. One or both of RSV proteins that induce neutralizing antibodies (F and G) are likely required for an effective subunit vaccine. Proteins

TABLE 1 | RSV proteins in live attenuated or subunit vaccines.

Protein	Size aa	Functions related to vaccine design	Role in a live virus vaccine	Role in a subunit vaccine
NS1	139 aa	Inhibits type 1 interferon production to block host response to control infection	Attenuation when deleted or codon de-optimized	None
NS2	124 aa	Inhibits type 1 interferon production to block host response to control infection	Attenuation when deleted or codon de-optimized	None
Nucleoprotein (N)	391 aa	Nucleocapsid formation and T cell epitopes	Attenuation or temperature sensitivity when mutated	Induce T cell immunity
Phosphoprotein (P)	241 aa	Nucleocapsid formation, replication	Attenuation when codon pair de-optimized	Platform for RSV VLPs
Matrix protein (M)	256 aa	Envelop, virion assembly	Attenuation and temperature sensitivity when the gene start signal mutated	Induce T cell immunity and platform for RSV VLPs
Small hydrophobic (SH)	64 aa	Ion channel	Attenuation when deleted or codon pair de-optimized	Induce ADCC antibodies to decrease virus replication
G protein	292-319 aa	Attachment and immune modulation	Attenuation when deleted and improved safety and immunogenicity when mutated	Induce antibodies to inhibit virus replication by blocking binding to the cell surface receptors CX3CR1 and glycosaminoglycans and/or ADCC and to block virus-induced inflammation
F protein	574 aa	Attachment, entry, fusion	Attenuation when mutated or codon pair de-optimized and improved protective immunity and virus stability when mutated	Induce antibodies to inhibit virus replication by blocking fusion and possibly by ADCC
M2-1 protein	194 aa	Anti-termination factor during transcription	Attenuation when mutated	Induce T cell immunity, platform for RSV VLPs
M2-2 protein	90 aa	Switch from transcription to replication	Attenuation and enhanced immunity when deleted	None
L protein	2,165 aa	Viral polymerase	Attenuation when mutated or codon pair de-optimized	None

Adapted with permission from Anderson (29).

that induce T cell immunity (N, M2-1, and other proteins) or antibody dependent cellular cytotoxic antibodies (ADCC) including the F, G, and SH proteins are incorporated into subunit vaccines (**Table 1**). Co-expression of the M protein and P proteins produces RSV virus-like-particle (VLPs) vaccine platform. A number of subunit vaccines, some in a virus vectors, including F protein; G protein; SH protein; F plus G; or F, G, and other RSV proteins are under study in clinical trials (4, 32).

Several pre-fusion F subunit vaccines are in early clinical trials and expected to induce higher titers of neutralizing antibodies and be more effective than previous F protein vaccines. Recently, two non-prefusion stabilized F protein vaccines were ineffective in elderly adults in phase II or III clinical trials (4). In a phase III maternal vaccination trial, one of these F protein vaccines did not significantly decrease medically significant RSV lower respiratory tract illness in infants (its primary endpoint) but did significantly decrease hospitalization in the infant (38), a result that suggests an F protein can be an effective maternal vaccine. An extended half-life, anti-F neutralizing monoclonal antibody is in phase II or III clinical trials and a promising alternative to vaccination to protect infants (39).

FUNCTION AND ROLE OF RSV PROTEINS IN VACCINE DESIGN

As noted above and outlined in **Table 1**, all RSV proteins are included in design of one or more vaccines. Understanding the role of RSV proteins in the biology of infection and disease pathogenesis helps determine if, and how, individual proteins might contribute to a vaccine.

Below we discuss each proteins function relative to vaccine design with an emphasis on the F and G proteins. F and G are most effective at inducing protective immunity and one or both likely needs to be included in a RSV vaccine. Though G is often not included in candidate vaccines, its role in disease pathogenesis suggest it might make important contributions to a vaccine.

NS1 and NS2 Proteins

NS1 is a 139 aa and NS2 is a 124 aa non-structural proteins, i.e., not incorporated into the virus but produced during transcription and replication. They both participate in virus replication and antagonize host innate responses designed to control infection (40–49). Deleting or codon de-optimizing

he gene ability to alter host cell responses that control the infection that reduces virus replication and attenuates the virus (36, 37, 50–55).

N Protein

The 391-amino acid N protein binds to and encapsidates the viral RNA generating an RNAse resistant nucleocapsid that is the template for transcription and replication of RSV genome (56, 57). N also inhibits host cell down regulation of cellular and viral protein production (58) and may impair dendritic cell and T cell interactions (59). It does not induce neutralizing antibodies but does induce T cell responses that protect animals at 4 weeks post vaccination (28, 60, 61). Given its role in virus replication, de-optimizing N gene codons should attenuate a live virus vaccine and its induction of T cell responses might contribute to efficacy of subunit vaccines.

P Protein

The P protein is a 214 aa protein that is part of the ribonucleoprotein complex (RNP) (56, 57). The P protein interacts with both the N and L proteins and is an essential co-factor for L function. P also interacts with the M2-1 protein (62). Since co-transfection of P and M proteins produces RSV VLPs (63), it could be used in a subunit RSV VLP vaccine. P's role in virus replication suggest that de-optimizing P gene codons should attenuate the virus (36).

M Protein

The M protein is 256 aa and guides assembly, budding, and virion formation (64). It lines the inner surface of the viral envelop, helps determine the shape of virus particles, and, with P, forms VLPs (63, 65–69). Since M induces T cell responses in vaccinated animals and memory T cells in humans after natural infection (70, 71), it might improve a subunit vaccines efficacy.

SH Protein

The SH, small hydrophobic protein is a 64–65 amino acid type II protein located on the surface of the virus. It forms a pentameric cation-selective ion channel, or a viroporin, and can activate NLRP3 inflammasome leading to IL-1b expression (72, 73). Deletion of the SH gene is often used to attenuate live RSV candidate vaccine strains (74). Codon pair de-optimization (CPD) (36, 75) might also attenuate the virus. Though SH does not induce neutralizing (76), an SH vaccine induces antibody-dependent cell-mediated cytotoxicity (ADCC) antibodies and protection in animals (77, 78) and being studied in clinical trials (4, 32).

G Protein

The G protein is a class II protein of 292-319 amino acids (AA) long. The extracellular domain contains a variable, highly glycosylated domain and a central conserved domain (CCD-G) followed by a second variable, highly glycosylated domain. Within the CCD-G are 13 aa conserved among all strains (aa 164-176) and a CX3C chemokine motif (aa 182-186). Through the CX3C motif, G, like the one CX3C chemokine, fractalkine, binds to the chemokine receptor CX3CR1 (79). G, as does F, also binds to cell surface glycosaminoglycans (GAGs) through its heparin binding domains and GAGs are one receptor for RSV infection. In primary human airway epithelial cells, RSV also uses CX3CR1, through the CX3C motif in G, as a receptor for infection (80–82). G binding to CX3CR1 can also induce fractalkine-like responses (79). CX3CR1 is expressed on the surface of many cell types, including neurons and microglia (83), smooth muscle (84), and various immune cells including monocytes, dendritic, NK, T, and B cells (85–87) and binding to it can induce a variety of downstream responses. In mice, the G protein/CX3CR1 interaction is associated with depressed respiratory rates (88), inhibition of migration of CX3CR1+ T cells to RSV-infected lungs (89), induction of aberrant pulmonary inflammation with RSV challenge after FI-RSV vaccination (90), increased pulmonary inflammation and mucous production and airway resistance during infection, and induction of Th2-type immune responses in the lung with infection (91). In *In vitro* studies, the G protein through its interaction with CX3CR1 dampens Type I IFN production by innate immune cells and Type 1 cytokine responses of memory T cells (92). Recently, the G-CX3CR1 interaction has been shown to induce IL-10 in neonatal regulatory B cells (nBreg) resulting in downregulation of Th1 cell responses (93).

The ability of the anti-G monoclonal antibody, 131-2G, to block these effects of G (91, 94–97) suggests a role for G in vaccine design. As illustrated in **Figure 1**, immunity designed to block infection, if successful, will prevent disease. However, if only partially successful, as occurs with naturally acquired immunity, RSV will replicate and produce G leading to G induced host immune/inflammatory responses that cause disease. Vaccine-induced anti-G antibodies can block G-induced disease and essentially have an anti-inflammatory effect that decreases disease. Interestingly, the anti-inflammatory effect of 131-2G is independent of its anti-viral effect, i.e., intact 131-2G has both an anti-viral effect and anti-inflammatory effect while 131-2G F(ab')2 has no anti-viral effect but a similar anti-inflammatory effect (95, 96). Since CX3CR1 is an important receptor in primary human airway epithelial cells, likely in natural human infection, antibodies that block G's interaction with CX3CR1 should neutralize virus in humans by a mechanism different from F. Finally, studies in mice suggest that anti-G immunity, through passively administered 131-2G before RSV challenge or actively induced by a CCD-G peptide vaccine given with FI-RSV, can block ERD in RSV-challenge of FI-RSV vaccinated mice (98, 99). These data suggest that including G, or a CCD-G containing peptide, in an RSV vaccine might decrease the risk of ERD in infants and young children.

Thus, G in a subunit vaccine can induce antibodies that block binding to CX3CR1 that should enhance the antiviral activity of an F protein subunit vaccine and uniquely add an anti-inflammatory effect not present in an F only vaccine (**Figure 1**). In a live attenuated vaccine, mutating G to block binding to CX3CR1, from studies in mice, should markedly decreased disease and maintain, or enhance, the vaccine-induced immunity (100).This mutation by blocking binding to CX3CR1 would also attenuate virus replication in humans.

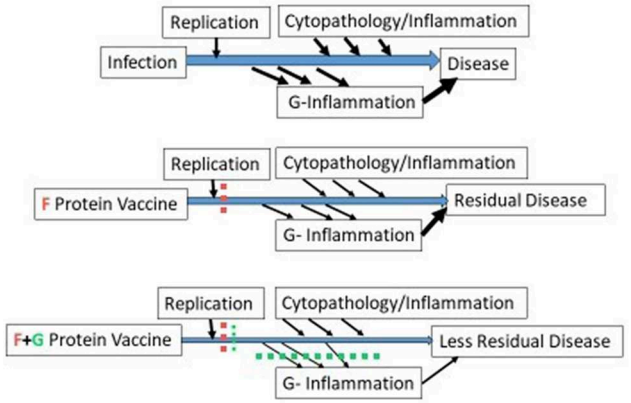

FIGURE 1 | Enhanced disease prevention with the addition of G to an F protein vaccine. The three schematics represent disease pathogenesis associated with no vaccine (1st schematic), an F protein vaccine (2nd schematic), and an F + G protein vaccine (3rd schematic). For all three, two types of disease pathogenesis are represented, one associated with virus replication and cytopathology (above the line) and the other induced by the RSV G protein (below the line). In mice, G induced disease includes increased inflammatory cells and mucus in the lungs and increased signs of obstructive airway disease and is not dependent on level of virus replication (95–97). In the second schematic, an F protein vaccine prevents much but not all virus replication and much of the disease pathogenesis represented above the line. In the third schematic, addition of G to an F protein also prevents disease pathogenesis represented below the line. The width of the arrows indicate level of virus replication, cytopathology/inflammation, G-inflammation, or residual disease.

Thus, including G in RSV vaccine design could improve a vaccine through multiple mechanisms. A number of G, or G peptides that include CCD-G, vaccines have been effective in preventing disease in animal studies (101–109). A G construct based on CCD-G will likely need to account for antigenic differences between groups A and B and not within the two groups.

F Protein

The F protein is a class I fusion protein of 574 amino acids (AA) long. It has two furin cleavages sites, at aa position 109 and the other at aa 136. Cleavage at these sites gives the 50 kDa carboxy-terminal F1, the 20 kDa N-terminal F2, and a 27 aa fragment. F1 and F2 form dimers and the F1-F2 dimers form trimmers (110, 111). The F protein is highly conserved among RSV strains with 25 AA differences between RSV subtypes A and B and induces neutralizing antibodies and protection in animals across the two groups (110, 112). F binds to glycosoaminoglycans (113), nucleolin (114), and EGFR (115) on the cell surface with GAGs and nucleolin presumed to be receptors for infection of cells. F binding to EGFR is associated with induction of IL-13 and mucin production.

The F protein mediates fusion of RSV with cellular membranes which is essential to infection and requires F to go from the metastable pre-fusion (pre-F) structure to a stable post-fusion (post-F) structure (116, 117). Many neutralizing epitopes on F are on the pre- and not post-fusion structure and most of the neutralizing antibodies in humans react against the pre- and not post-fusion form of F (118, 119). Pre-fusion stabilized F protein constructs have been developed and these F constructs, e.g., Ds-Cav1 and SC-TM, are highly effective at inducing neutralizing antibodies (120). Anti-F antibodies can also mediate antibody dependent cell-mediated cytotoxicity (ADCC) (121, 122) though it is unknown what role ADCC antibodies play in controlling natural infection. The initial two neutralizing antigenic sites identified on F have been expanded to at least five and more will likely be identified in the future (24, 123). Anti-antigenic site Ø antibodies have high levels of neutralizing activity and are a high proportion of neutralizing antibodies in human serum specimens (118). Interesting, F proteins in some circulating strains have been shown to have increased stability of pre-fusion F, increased virus temperature stability, induce mucus and airway resistance in mice, and bind to EGFR (115, 124, 125).

Stabilization of pre-fusion F in subunit vaccines substantially increases the neutralizing antibody response in animals and is a promising development in design of RSV subunit vaccines. In a live virus vaccine, mutations in F that increase pre-fusion stability and temperature stability should be advantageous. Mutations at other sites in F have been associated with virus attenuation. It is possible that mutations that block F binding to EGFR will attenuate disease and improve a live attenuated RSV vaccine.

The F protein's essential role in infection through fusion suggest it is key to protection for both subunit and live virus vaccines.

M2-1 and M2-2 Proteins

The internal viral matrix protein M2 is unique to the family Pneumoviridae, plays a significant role in virus assembly (66), and contains two overlapping translational open reading frames, one for M2-1, a 194 aa protein, and one for M2-2, a 83-90aa protein (126).

The *M2-1* protein functions as an intragenic transcription anti-termination factor allowing the synthesis of complete RNA (127–129) and link the RNA/nucleocapsid with the M protein just inside the virus surface (67, 130). M2-1 can induce short term, T cell based RSV immunity (28, 70, 131) and could be included in a subunit vaccine to enhance induction of T cell immunity.

The *M2-2* protein facilitates the shift from gene transcription to production of viral RNA and infectious virus (126, 132). Deletion of M2-2 results in a decrease genome replication and increase in gene transcription and protein production resulting in both attenuation and increased immunogenicity. M2-2 deletion viruses are being evaluated in a phase 1 clinical trials (34, 133).

L Protein

The L protein is a large, 2,165-amino acid, protein that mediates transcription and replication of RSV RNA and capping and methylation of mRNA (56, 57, 134). The active form of L is a

heterodimer of the L and P proteins with P essential to L's catalytic activity. Given its central role in transcription and replication it is not surprising that attenuating mutations, and likely codon pair de-optimization of L, attenuate live RSV (30, 31).

COMMENT

Though a number of candidate RSV vaccines are under development and some promising candidate vaccines have moved into clinical trials, past failures suggest that we should continue look for better candidate vaccines. Though the composition of a successful RSV vaccine remains uncertain, it likely will need to induce both antibody and Th1 biased T cell memory responses. It is, also, useful to remember that the goal is to prevent disease and not just to control infection. For example, tetanus and diphtheria toxoid vaccines prevent the disease and not pathogen growth. The RSV G protein has the potential to enhance a vaccine by not only helping to control infection but independently decreasing disease by controlling virus-induced inflammation. Virus protein-specific contributions to biology of infection and disease pathogenesis might also suggest ways to decrease disease for other vaccine challenges such as improving influenza vaccines and developing HIV vaccines.

AUTHOR'S NOTE

Respiratory syncytial virus is a high priority for vaccine development but, despite nearly 60 years of research no vaccine is yet available. Understanding the biology of infection and pathogenesis of disease has and will continue to be key to developing new vaccine strategies to finally achieve a successful vaccine. New vaccines are being developed and their safety and efficacy will ultimately be determined by clinical trials in the target population. Given past failures it is important to continue to pursue better candidate vaccines. In developing new vaccines, it is useful to remember that the goal of a vaccine is to prevent disease and not, though essential to an effective vaccine, virus replication.

AUTHOR CONTRIBUTIONS

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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Host Components Contributing to Respiratory Syncytial Virus Pathogenesis

Jonatan J. Carvajal[1†], Andrea M. Avellaneda[1†], Camila Salazar-Ardiles[1], Jorge E. Maya[1], Alexis M. Kalergis[2,3] and Margarita K. Lay[1,2]*

[1] Departamento de Biotecnología, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Antofagasta, Chile, [2] Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad de Chile, Santiago, Chile, [3] Departamento de Endocrinología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

Respiratory syncytial virus (RSV) is the most prevalent viral etiological agent of acute respiratory tract infection. Although RSV affects people of all ages, the disease is more severe in infants and causes significant morbidity and hospitalization in young children and in the elderly. Host factors, including an immature immune system in infants, low lymphocyte levels in patients under 5 years old, and low levels of RSV-specific neutralizing antibodies in the blood of adults over 65 years of age, can explain the high susceptibility to RSV infection in these populations. Other host factors that correlate with severe RSV disease include high concentrations of proinflammatory cytokines such as interleukins (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and thymic stromal lymphopoitein (TSLP), which are produced in the respiratory tract of RSV-infected individuals, accompanied by a strong neutrophil response. In addition, data from studies of RSV infections in humans and in animal models revealed that this virus suppresses adaptive immune responses that could eliminate it from the respiratory tract. Here, we examine host factors that contribute to RSV pathogenesis based on an exhaustive review of *in vitro* infection in humans and in animal models to provide insights into the design of vaccines and therapeutic tools that could prevent diseases caused by RSV.

Keywords: RSV, pathogenesis, innate and adaptive immune response, host factors, disease

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Cecilia Johansson,
Imperial College London, United Kingdom

***Correspondence:**
Margarita K. Lay
margarita.lay@uantof.cl

†These authors have contributed equally to this work

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INTRODUCTION

Respiratory syncytial virus (RSV) is the main viral etiological agent that produces lower respiratory tract infections (LRTI) and is the primary cause of hospitalization due to respiratory diseases in infants (1, 2). RSV infection may lead to bronchiolitis and pneumonia and has been implicated in the development of recurrent wheezing and asthma (3). Milder RSV manifestations include rhinorrhea, cough, congestion, low-grade fever, reduced appetite, and respiratory distress (4). Recent reports of other pulmonary manifestations, such as encephalitis, cardiopathy, and hepatitis, suggest that RSV has a versatile ability to infect tissues of the respiratory tract (5).

RSV is highly infectious and easily spread in hospitals, homes, and nurseries, despite being less cytopathic and less invasive than influenza A virus. Worldwide, RSV affects more than 70% of infants in the first year of life, and nearly 100% of children by 2 years of age (6). The estimated rate of hospitalization due to RSV is 3.4 million/year and between 66,000 and 239,000 deaths occur around the world in children under 5 years of age who have suffered LRTI caused by RSV (7, 8).

During the year 2000 in the U.S., there were approximately 86,000 RSV-associated hospitalizations, 402,000 emergency room visits, 1.7 million office visits, and 236,000 outpatient hospital visits, at an estimated cost of US $652 million (9). Interestingly, the rate of hospitalization for primary RSV infection in Alaska is approximately 0.5%, but can vary by situation, with ethnic group susceptibility as high as 25% (10). In contrast, an estimated of 33.1 million cases of RSV LRTI was reported in children under 5 years of age in 2015. Half of the global RSV burden was contributed by cases in India (7,013,468), China (2,581,262), Nigeria (1,728,622), Pakistan (1,575,051), and Indonesia (1,245,185) (11). RSV is also recognized as a major threat to older adults (>64) (12). Epidemiological evidence indicates that the impact of RSV on these patients may be similar to non-pandemic influenza (12).

Scientific evidence has shown that after the resolution of respiratory diseases associated with RSV infection, the virus interferes with the establishment of immunological memory, which leads to recurrent reinfections (13). Indeed, around 36% of individuals can be reinfected with RSV, at least once, during the winter season (13). These reinfections could result when an initial encounter with RSV fails to initiate adequate humoral and cellular immune responses to generate protective memory lymphocytes (13, 14).

RSV was first isolated in 1956, from throat samples in a colony of chimpanzees that had symptoms such as coughing, sneezing, and purulent nasal discharge (15, 16). These symptoms were quickly observed in other monkeys of the colony, indicating that the pathogen responsible for the disease was highly contagious. Originally, the pathogen was called chimp coryza agent (16). Later, in 1957, a similar viral agent was isolated from the throats of babies who had severe respiratory diseases (17). Interestingly, the isolated pathogen induced syncytia formation that was shown later to be caused by the viral fusion (F) protein (18, 19). Since then, this pathogen was renamed as RSV.

This respiratory virus was recently classified in the *Pneumoviridae* family, *Orthopneumovirus* genus (20). Specifically, RSV is an enveloped, negative sense, single stranded RNA virus with a non-segmented 15.2 kb genome, containing ten genes: non-structural proteins (NS)1, NS2, nucleoprotein (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), fusion (F), glycoprotein (G), M2 and large polymerase (L) (from the 3′ to 5′ end) that encode eleven proteins (21). The M2 gene contains two open reading frames that slightly overlap and encode the M2-1 and M2-2 proteins (22). Further, the F, G, and SH proteins are found on the viral surface, whereas the N, P, L, M, and M2 proteins are located underneath the viral envelope (21, 23). The F protein is essential for union and entry of the virus into the host (24, 25). F and G are the only RSV proteins that induce neutralizing antibodies (26).

A growing concern is that severe RSV infection at an early age, may adversely affect pulmonary development and lead to long-term respiratory disorders. Thus, the development of new treatment strategies to prevent RSV infections is a priority of the World Health Organization (27). To design effective therapeutic tools that thwart viral infection, we need to understand host factors that influence RSV pathogenesis. In this review, we describe mechanisms of RSV pathogenesis, as well as host factors and immune responses that contribute to disease severity caused by this important respiratory virus.

RSV PATHOGENESIS

RSV transmission occurs via air, by contact with epithelium of the nostrils, mouth, or eyes of RSV-infected individuals, or by contact with a surface contaminated with the virus (28). RSV can survive for prolonged periods on the surface of furniture (7 h), skin (30 min), fabrics (2 h), and gloves (5 h), which facilitates its spread (29, 30). With an incubation time of 3–8 days, RSV can infect the lower respiratory tract producing bronchiolitis (inflammation of bronchioles in the small airways) or pneumonia (inflammation of the alveolar spaces in the small airways). In children, pneumonia caused by RSV manifests with fever, chest pain, wheezing, nausea, chills and other respiratory difficulties (31, 32). Likewise, bronchiolitis caused by RSV is characterized by wheezing, dyspnea, tachypnea, fatigue, fever, and cough (33). Because these diseases could be fatal, infants with severe RSV symptoms are hospitalized to receive necessary health care.

Once RSV enters the nostrils or mouth, it begins to infect airway epithelial cells (AECs) of the upper respiratory system (34–36), moving down to the lower respiratory system, and reaching the bronchioles where viral replication is more effective, as observed in both mouse and infant respiratory tissues (37, 38). Specifically, ciliated cells in the bronchial epithelia and type 1 pneumocytes in the alveolus, are the main cells targeted by RSV infection (39–42). RSV has also been reported to infect intraepithelial dendritic cells (DCs) and basal epithelial cells of the conductive airways, using *in vitro* cultures (41). Thus, RSV has a wide range of cellular reservoirs in the respiratory tract that perpetuate its pathogenesis in the human host.

An *in vitro* AEC model was used to show that RSV infection is concentrated in groups of non-continuous cells or small groups of ciliated apical cells located in the epithelium of large airways (40). As this infection progresses, RSV induces sloughing and shedding of specific apical AECs, loss of ciliation, as well as sporadic syncytium formation and mucus hypersecretion, which could lead to formation of thick plugs in the bronchiolar lumen *in vivo* (40, 43, 44). RSV has also been shown to cause detachment of apical AECs *in vivo*, which exposes nociceptive nerve fibers and produces a cough reflex (37).

Well-differentiated primary pediatric bronchial epithelial cells (WD-PBEC) provide a suitable *in vitro* model to study RSV infection (45, 46). WD-PBECs consist of polarized pseudostratified multilayered epithelium containing ciliated, goblet and basal cells and intact tight junctions. Hence, this *in vitro* model imitates the physiological, functional, and morphological environment of the respiratory tract (47).

RSV does not cause the massive airway epithelium destruction observed in post-mortem lung samples from RSV-infected patients in the WD-PBEC *in vitro* model (39, 44, 45). The latter *in vitro* studies demonstrated that the cell monolayer remains intact, even when most of ciliated cells were infected with RSV. Other studies confirmed these results and demonstrated that peak RSV infection in ciliated cells occurs at day 4 post infection

(p.i.) and decreases significantly by day 8 p.i. (48), suggesting that in the absence of immune-cell mediated mechanisms, RSV-infected ciliated cells can be cleared from the epithelium between days 4 and 8 p.i. Detachment and apoptosis of ciliated cells in the epithelium of apical airways has also been observed in WD-PBEC cells, which agrees with the results of histopathological studies of infants with fatal RSV, where caspase-3 activity was detected in bronchiolar epithelial cells (49). Using an *in vitro* model, Liesman et al. (48) also found that RSV-infected ciliated cells die when they detach from the epithelium. RSV-infected AECs that have sloughed from the airway epithelium are thought to obstruct the lower airways in RSV-infected individuals, as observed in hospitalized infants (48). RSV NS2 was identified as the viral protein that causes rounding and sloughing of infected ciliated cells by using RSV gene deletion mutants and gain-of-function experiments with recombinant RSV NS2-expressing parainfluenza virus 3 (PIV3-NS2) (48).

An *in vivo* model was also used to show that RSV pathogenesis is characterized by excessive mucus secretion (48). However, RSV does not infect goblet cells, nor does it induce them to secrete mucus. Rather, it infects basal cells of the airway epithelium, which differentiate into mucus-secreting cells, as shown in an *in vitro* culture model (48). Thus, RSV indirectly induces mucus in the bronchial lumen by stimulating goblet cell proliferation, consistent with the presence of goblet cell hyperplasia in lungs of fatal RSV infection cases (49). Therefore, RSV infection in the respiratory tract induces the production of mucus plugs and detached ciliated AECs. Additionally, RSV drastically reduces mucociliary transport (MCT), a unidirectional movement of the airway epithelium that mobilizes mucus plugging out from the airways within 5 days. Thus, these plugs accumulate in the bronchial lumen, leading to the pathogenesis of this viral agent (48). Consequently, RSV infection produces epithelial airway necrosis, submucosal edema, and occlusion of the bronchial lumen (37, 39–41).

RSV was also recently shown to induce production of thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33 (50–54), which are cytokines that play important roles in the development of allergic asthma. *In vivo* and *in vitro* models were used to show that production of these cytokines has important repercussions on RSV pathogenesis. Indeed, upon RSV infection in the lungs, TSLP and IL-33 secretion create an inflammatory environment that directly or indirectly increases mucus secretion, eosinophil and neutrophil numbers, and levels of the T helper (Th) 2 cytokine interleukins IL-5 and IL-13 (55–59), as discussed in more detail in the section below. Concurrently, a decrease in the total CD4+ and CD8+ T-lymphocyte numbers is also observed in RSV-infected individuals (60, 61).

Together, these results suggest that RSV, as a result of its NS2 protein functions, primarily infects ciliated cells in the large airways, inducing their extrusion and cell shedding into large airways. RSV also induces mucus production by causing goblet cell proliferation and proinflammatory cytokine production. Mucus accumulates in the narrow-diameter bronchiolar airway lumen due to RSV inhibition of MCT, which, in turn, is thought to cause acute obstruction in the distal airways. RSV infection of ciliated cells also induces TSLP and IL-33 release, which indirectly induces eosinophil and neutrophil recruitment to the lung. In other respiratory viral infections (62), *in vivo* presence of the latter immune cells correlates with the presence neutrophil extracellular traps (NETs), which are web-like networks of neutrophil DNA covered with histones and cytotoxic microbicidal proteins that trap and eliminate different pathogens (62). The release of NETs by neutrophils has been observed *in vitro* (62). Thus, upon RSV infection, recruited neutrophils and tamponades produce NETs, composed of mucus and dead ciliated cells that appear to exacerbate obstruction of the host upper and lower airways (62, 63). **Figure 1** shows a current RSV pathogenesis model.

RSV Pathogenesis in Infants

RSV is a highly infectious virus, especially in infants and young children. At early ages, most primary RSV infections cause LRTI, resulting in hospitalization for an estimated 2–3% of infected infants. LRTI in infants and young children can result in respiratory diseases including bronchiolitis, pneumonia, wheezing, and even respiratory failure, which likely ends, unfortunately, in death. RSV is considered the second most-likely single pathogen to cause death in children <1 year of age (8). Possible host and virulence factors that determine the outcome of LRTI in infants upon RSV infection will be discussed below.

RSV Pathogenesis in Adults and the Elderly

RSV pathogenesis in adults and the elderly differs from that in infants, displaying symptoms similar to those caused by influenza virus, typically including mild fever, runny nose, nasal congestion, cough, dyspnea, and wheezing (64, 65). A study of adults between 24 and 95 years of age who had been exposed to RSV, showed detectable virus for 10 to 13 days in nasal secretions that in some cases lasted ≥20 days. Levels of RSV viral RNA in sputum were slightly higher than nasal titers, suggesting that viral replication also occurs in the lower airways in adults (12, 66).

In adults, it is required that a diagnosis of RSV infection based on laboratory diagnostic tests be confirmed due to the similarity of RSV symptoms with other viral and bacterial agents that cause acute respiratory tract infection. Reverse transcriptase-polymerase chain reaction (RT-PCR) is the reference diagnostic method for RSV detection and is specifically recommended for use in adults because its analytic and clinical sensitivities are superior to those of other diagnostic methods (67, 68). However, a low percentage of clinical laboratories currently use RT-PCR to identify RSV because of its associated costs, specialized equipment, and expertise required (69). Consequently, most RSV disease in adults is not diagnosed early (70, 71). The absence of an easily administered and effective antiviral and a commercially available vaccine has led to a high rate of severe RSV disease in the elderly (72). Indeed, RSV infection rates in nursing homes are ∼5–10% per year, with significant rates of pneumonia (10–20%) and death (2–5%) (73). Data collected in the U.S.A. over 9 years of surveillance indicated that RSV infection causes approximately 10,000 deaths per year in people over 64 years of age. In addition, some *in vivo* studies conducted in this risk group reported high levels of IL-6 and macrophage inflammatory proteins 1 alpha (MIP-1α) upon RSV infection, which directly correlates with the

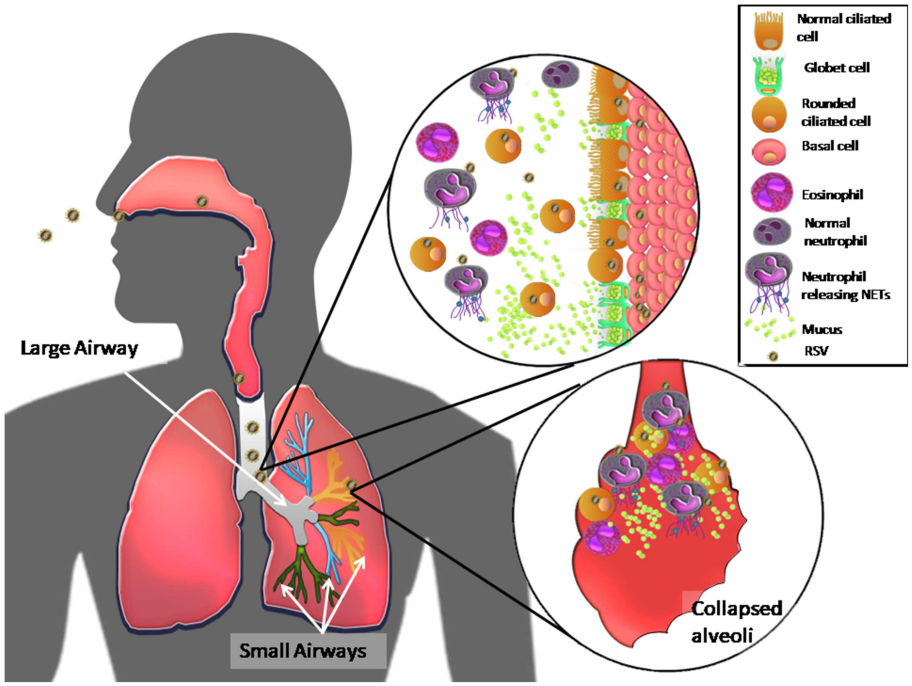

FIGURE 1 | Model of RSV pathogenesis in the human respiratory tract. Once RSV enters the upper respiratory tract, the virus primarily infects ciliated cells in the large airways. The viral NS2 protein induces cell rounding, extrusion, and detachment from the apical zone of the airway epithelium through, generating an accumulation of these cells in the lumen of the airway ducts. RSV also induces proliferation of goblet cells, via infection of basal cells, which differentiate, causing high mucus production and recruitment of eosinophils and massive numbers of neutrophils in the airway ducts. The latter immune cells release NETs in response to RSV infection. All of these components accumulate and move to the distal airways, leading to bronchial obstruction of the narrow ducts, and collapse of alveoli, causing acute inflammation and pathology in the lungs.

severity of patient disease (73, 74). Although the protective or pathological roles of cellular immunity in adults is still unknown, *in vivo* studies indicate that there is decreased production of interferon (IFN)-γ and both CD8+ and CD4+ memory T cells with age, which could influence the severity of RSV disease (75–78). These immune host factors for RSV susceptibility will be discussed more in detail in the next section.

HOST COMPONENTS CONTRIBUTING TO RSV PATHOGENESIS

Several host factors affect RSV pathogenesis and increase the risk of developing severe RSV disease including young age (<6 months), premature delivery (<35 weeks of gestation) (79, 80), malnutrition (81), gender, low titer RSV-specific serum antibodies, and fragile old age (82). Suffering from severe or chronic diseases, including allogeneic bone marrow transplants (83), congenital heart defects (84), chronic lung disease including cystic fibrosis (85), and nervous system and muscle diseases (86, 87) increases the risk of severe RSV disease in older adults and in babies (88). Babies born with Downs syndrome and cerebral palsy have also been shown to have a higher risk of hospital admission with RSV bronchiolitis, although more research is needed to better explain the mechanism behind this risk factor for RSV infection (89). Host conditions that contribute specifically to pathogenesis of severe LRTI caused by RSV (90) include immune system immaturity and immunologic impairment disorders, incomplete development of the respiratory tract, hyperreactivity of the airways, and pulmonary congestion. Epidemiological studies have also established that primary infection at an early age plays a central role in RSV disease severity (91).

Early Age

Inability of the infant immune system to efficiently respond after RSV infection is due, at least in part, to failure of innate antiviral immune responses (92). Studies in infants, including fatal cases, found that after RSV infection, respiratory epithelial cells release chemokines and cytokines that are known to recruit immune cells to the site of infection, such as leukocytes, neutrophils, monocytes, natural killer (NK) cells, macrophages, eosinophils, basophils, and DCs, which contribute to lung inflammation (92). Low expression of Toll-like receptor (TLR) 4 in the neutrophils of infants (93) could also contribute to development of more serious RSV disease caused in this population (93). The status of innate immune cells in the host may also contribute to RSV pathogenesis. In fact, *in vivo* assays of hematopoietic cells found that they are permissive for RSV infection and can serve as an RSV reservoir (93). For example, DCs play a central role in configuring the immune response to, and disease outcome of, RSV infection (94).

Plasmacytoid (p)DCs are thought to be key players in the immune response to different viruses, due to their ability to

produce large amounts of type I IFN (IFN-α and IFN-β) (94). These cells are known to be important in controlling RSV infection in mouse lungs (94, 95). RSV-induced IFN-α, mainly produced by pDCs, is significantly lower in term infants and young children (<5 years of age), than in adults, suggesting that human pDCs have a limited function in early life that could partially explain the severity of RSV disease in infants and young children (96). Further studies are required to fully elucidate the role of pDCs in RSV disease.

Neonatal susceptibility to RSV is intrinsically linked to immunological characteristics of the young pulmonary mucosa. To better understand immune responses to RSV infection in infants, a mouse model of neonatal infection was developed in BALB/c mice (96). Mice infected with RSV within 7 days of birth developed an asthma-like pathology and when these mice were reinfected as adults, they underwent weight loss, airway hyperresponsiveness, mucus hypersecretion, Th2 immune responses, and airway remodeling (95, 96). These results suggest that primary RSV infection at an early age in neonatal mice influences the clinical outcome of RSV re-exposure in adults.

Gender

Gender is another host factor that can affect RSV disease susceptibility. For example, illness caused by RSV infection is more severe in male infants because their airways have a smaller diameter than those of female infants (97, 98). Thus, male infants are more likely than females to have an acute bronchial obstruction upon RSV infection.

Hypersensitivity

Hypersensitivity, such as allergic reactions, is an exaggerated immune response to an antigen. There is a strong epidemiological correlation between severe RSV infection in early life and asthma development later in life (27, 99, 100). Some evidence in infants shows an association between genetic predisposition to asthma and disease severity following RSV infection. During RSV infection of infants, a large amount of specific immunoglobulin (Ig) E is produced (101, 102) and increased sIgE levels correlate with greater severity of RSV infection such as wheezing in babies and asthma in children (101, 103). Similarly, RSV-infected asthmatic patients had higher anti-RSV IgE antibody titers than did non-asthmatic individuals (104), suggesting that RSV infection differs between asthmatic and non-asthmatic individuals.

Atopic hypersensitivity also correlates with severe RSV disease (105). Specifically, 32% of children hospitalized with RSV infection developed atopic sensitization, while only 9% of those who were not hospitalized due to RSV infection developed atopic sensitization after 3 years ($p = 0.002$). In a follow-up study, 34% of patients hospitalized due to RSV infection developed atopic hypersensitivity to allergenic agents by 7 years of age compared to only 15% of those who had not been hospitalized due to RSV infection (106).

Cytokines such as IL-3, IL-4, IL-10, and IL-13 in the lower respiratory tract of infants with RSV bronchiolitis (107) are known to exacerbate allergic processes (108–110). The presence of some of these cytokines correlates with severe RSV infection. Specifically, Bertrand and Lay (107) reported a direct correlation between the number of days hospitalized due to RSV infection and high IL-4 levels (Pearson correlation: $r = 0.52$, $p = 0.05$) (107). They also found a direct correlation between high levels of IL-12p40 and IL-3, and development of recurrent wheezing later in life upon RSV infection (Pearson correlation: $r = 0.68$, $p = 0.0071$, $r = 0.71$, $p = 0.0058$, respectively) (107). These two cytokines were elevated in infants who developed asthma later in life. Interestingly, IL-3 is known to be involved in mast cell infiltration into the airways as well as increased basophil production (111). The same study found elevated expression of IL-33 mRNA in nasopharyngeal aspirates from RSV-infected patients with a family history of atopy (107), suggesting that genetic predisposition of the host promotes Th2 responses and allergic inflammation after RSV infection, similar to previous reports of sensitization to allergens (112–115).

Genetic Factors

Genetic predisposition of the host could affect RSV disease severity (89, 116) and recent studies have proposed that genetic predisposition to asthma could also predispose to severe RSV disease (117, 118). Specifically, a clinical study by Thomsen et al. (117) found that hospitalizations due to RSV and asthma were directly correlated ($r = 0.43$), and that genetic determinants of the two disorders overlap precisely. The same study analyzed the correlation between hospitalization due to RSV and asthma, showing a model, by which asthma "causes" hospitalization due to RSV. This model was adjusted to data significantly better ($P = 0.39$) than one by which RSV hospitalization "causes" asthma ($P < 0.001$). In support of this model, recent studies also showed that asthma increases the risk of RSV hospitalization by 3-fold in a time-independent manner (119, 120). However, the exact mechanisms by which severe RSV infection interacts with asthma inheritance factors at the onset of childhood RSV infection still need to be elucidated.

Other genetic factors associated with severity of RSV disease are two single nucleotide polymorphisms (SNPs) that encode Asp299Gly and Thr399Ile substitutions in the TLR4 ectodomain, which were previously associated with TLR4 and are known to regulate innate and adaptive immune responses by recognizing pathogen-associated molecular patterns (PAMPs) (121). Both SNPs are related to increased severity of RSV infection in premature babies, with 89.5 and 87.6% of heterozygous cases for Asp299Gly and Thr399Ile polymorphisms, respectively (121). These results suggest that heterozygosity of these two TLR4 SNPs is strongly associated with symptomatic RSV disease in high-risk infants, supporting a dual role for TLR4 SNPs in prematurity and increased susceptibility to RSV (121). *In vitro* studies also showed that these SNPs were associated with a decreased response to lipopolysaccharide (LPS) and to purified RSV F protein that activates cells through TLR4 (122). Thus, these mutations may delay and/or attenuate triggering of the innate immune response to RSV (96).

Another genetic factor that has been reported to be involved in RSV disease is the CC genotype of CD14 (−550 C/T), which is associated with development of RSV bronchiolitis in Japanese

populations (123). This study found that CD14 (−550 C/T) is associated with higher serum levels of soluble (s) CD14 in Japanese neonates and children and directly correlates with development of bronchiolitis upon RSV infection. A possible explanation of these associations is that high levels of sCD14, a soluble form of the glycosyl phosphatidylinositol–anchored membrane protein (124), may bind to available LPS and transfer it to membranous CD14, thereby stimulating production of proinflammatory cytokines such as tumor necrosis factor–α (TNF-α) (125–127), which is known to enhance RSV-induced disease (128). RSV bronchiolitis could be triggered by RSV infection and an inflammatory environment caused by high sCD14 levels in the blood.

Although these are very important host factors, several authors have suggested that Th2 cytokine genes such as IL-4, IL-13, and IL-5 contribute to asthma severity. A study of single-strand conformation polymorphism in these genes, which are grouped on chromosome 5, identified point mutations at IL-3 position−68, IL-4 position−590 and IL-9 position−351 (129). The IL-4 promoter polymorphism is associated with increased total serum IgE, which is of special interest, since this group of cytokines is involved in asthma development and could be influenced during and after RSV infection (130). These genes are also associated with exacerbating and perpetuating asthma during RSV infection (129).

Four SNPs of interest have also been shown to be associated with RSV disease severity at allele and at genotype levels. Specifically, a SNP in the vitamin D receptor gene (rs10735810, $P = 0.0017$) (131) has been linked to increased susceptibility to RSV infection (132). In addition, the synthetic nitric oxide 2 (NOS2A) gene (rs1060826; $P = 0.0031$) (131) has been associated with increased chronic respiratory morbidity and reduced lung function in infants who had LRTI caused by RSV (133). Further, the Jun protooncogene product, a subunit of the AP-1 transcription factor (JUN) (rs11688; $P = .0093$) (131) and interferon alpha 5 (IFNA5) gene (rs10757212; $P = 0.0093$) (131) are involved in innate immunity and contribute to the susceptibility to and duration of RSV infection (134, 135).

The olfactory receptor (OR13C5) gene is also involved in RSV pathogenicity, since the olfactory nerve connects the nasal cavity with the central nervous system and thus could be used as a shortcut by RSV (136). This could explain neurological symptoms produced by RSV such as encephalitis, apnea, or seizures, that occur in at least 2% of RSV-infected people (137, 138), and likely cause serious and permanent neurological *sequelae* (139). SNPs in human leukocyte antigen (HLA) HLA-DQA1 and in HLA-DPB1 genes have been associated with the development of bronchiolitis and several types of asthma (140–142). One of the most important SNPs is located in the mucin 4 (MUC4) gene, where three SNPs have been identified: rs201623571 ($P = 3.55 \times 10^{-10}$, OR0.10), rs529417345 ($P = 9.40 \times 10^{-10}$, OR = 0.03) and rs548345415 ($P = 9.40 \times 10^{-10}$, OR = 0.03). These SNPs in MUC4 diminish mucin in the airways upon RSV infection, which can increase the severity of RSV disease (143, 144).

In a severe RSV infection, loss of function (LOF) variants associated with the innate immune response, such as helicase C domain 1 (IFIH1) and other IFN pathway genes become very important (145). IFIH1 encodes a RIG-I-like cytoplasmic sensor that detects viral RNA by interacting with its C-terminal regulatory domain (CTD) and helicase domain with long dsRNA molecules. This ATP-dependent reaction polymerizes IFIH1 molecules into a filament, and assembles IFIH1 caspase activation recruitment domains (CARDs), which in turn induce IFN-β expression and activate antiviral genes (146). IFIH1 has been shown to effectively restrict RSV replication. Specifically, Asgari et al. (145) showed that three IFIH1 LOF variants increase the susceptibility and duration of RSV infection. One of those was a "rare splice" variant rs35732034 (145) that changed the reading frame and produced an early stop codon, whose protein (IFIH1-Δ14) lacks a CTD. IFIH1-Δ14 cannot bind viral dsRNA and has thus lost its main function. The second variant is a "cracking" variant rs35337543 (145), whose protein (IFIH1-Δ8) removes amino acids at the end of the helicase 1 domain and in the helicase 1 and 2 binding site without changing the reading frame. Finally, the "prolonged gain" variant rs35744605 protein (IFIH1-ΔCTD) lacks amino acids at the C-terminus (145). These three variants are unable to induce IFN-β, having lower stability than the normal protein and lacking the characteristic ATPase activity required to polymerize and activate IFH1. These IFIH1 gene variants impair normal function of the viral sensor protein, thus restricting RSV infection (145).

Other genes that contribute to RSV disease severity, duration, and susceptibility include SFPA/D, IL-8, IL-4, and IL4RA, which exacerbate bronchiolitis caused by RSV (147–150); as well as IL-10 and IL-13 genes. Patients with mutations in IL-10 and IL-13 have required mechanical ventilation upon RSV infection (151, 152). In addition, SNPs in genes related to the innate immune response such as IFNA13 (rs643070), IFNAR2 (rs7279064), signal transducer and activator of transcription (STAT) 2 (rs11575234), IL27 (rs181206), Nuclear Factor Kappa B Inhibitor Alpha (rs22333409), C3 (rs22302021), IL1RN (rs315952), and TLR5 (rs5744174), have been associated with failure of the antiviral response against RSV (131, 153). Moreover, the ADAM33 and transforming growth factor beta receptor 1 (TGFBR1) genes participate in respiratory tract remodeling and increase RSV disease severity by favoring viral replication (153–155).

Malnutrition

Vitamin D plays a major role in innate immunity and influences lung function of asthmatic patients (156). In its active form, vitamin D 25-dihydroxyvitamin D [25(OH)D] helps to modulate inflammatory processes (157–160), promote Treg cell development (160), and acts as an antiviral agent (161). The concentration of 25(OH)D in the blood has been associated with the risk of contracting severe respiratory infection or exacerbating asthma in children and adults (162–164). The risk of contracting a severe respiratory infection decreases by 7% for every 10 nmol/L of 25(OH)D in adults (165). Some studies indicate that the risk rises in children and in infants when 25(OH)D concentrations fall below 75 nmol/L, making these children more vulnerable to bacterial and viral lung infections

(166–168). In recent studies, low levels of vitamin D in cord blood of healthy neonates was associated with an increased risk of severe RSV LRTI in the first year of life (169, 170), suggesting that a low intake of this vitamin by the mother during pregnancy can impact RSV disease severity in infants.

RSV reinfection can occur throughout life, causing winter/early spring epidemics in temperate regions, but synchronization of RSV activity can vary widely depending on geographical location. It should be noted that different RSV strains circulate rapidly throughout the world (171). Environmental factors (temperature and humidity), including those that affect lung function (e.g., smoking at home), external conditions that increase exposure to RSV infection (e.g., daycare, hospitalization, multiple siblings), and lack of lactation, are factors that may indirectly influence RSV disease severity (28).

The host response to RSV infection has largely been studied in infants with comorbidities, but not in healthy infants or in the elderly. Although there is no vaccine or effective antiviral therapy currently, there is much effort to investigate these issues (172–174). Babies at high risk for serious RSV disease can receive passive immunoprophylaxis during an epidemic season by monthly injection of the RSV neutralizing monoclonal antibody, palivizumab (Synagis), which provides a 55% reduction in hospitalization rate associated with RSV (175).

Premature Birth

As mentioned above, infants under 6 months of age have an increased risk of RSV infection. However, premature infants, with a gestational period of <37 weeks are even more likely to develop severe RSV bronchiolitis than full-term infants (176). One reason for this risk is the deficiency in passive immunization by maternal antibodies that are essential to defend against pathogens in the first months of life. Moreover, during gestation antibodies migrate from the mother to the fetus between 26 and 41 weeks of gestation. These antibodies include IgG1 and IgG4 that are efficiently transferred from the mother to the fetus, followed by IgG3 and IgG2 (177–180). Because premature infants with <41 weeks of gestation have not fully acquired maternal antibodies, they have an increased risk of RSV infection. Another important immunological factor in premature infants is the presence of neutrophils, which have a reduced ability to migrate to respiratory tissues than in full-term infants (181). Neutrophils in premature infants also release fewer bactericidal proteins and have decreased pathogen recognition capacities (176, 182). Premature infants also have compromised pulmonary development. One of the complications is bronchopulmonary dysplasia (BPD), which is abnormal development of lung tissue. Infants with this disease upon RSV infection, have a more severe outcome with a much higher rate of hospitalization and death (176, 183).

Microbiome of the Airways

RSV infection in the lower respiratory tract of infants who develop severe bronchiolitis has been associated with a specific microbiota that includes a high abundance of Firmicutes, such as the genus *Streptococcus*. These patients were reported to have a low abundance of Proteobacteria, including the genera *Haemophilus* and *Moraxella* ($P < 0.001$) (183, 184). However, another study reported that children <2 years of age who were hospitalized due to RSV infection had a positive association with the presence of *H. influenzae* and *Streptococcus* and a negative association with *S. aureus* (Firmicutes phylum) abundance (185). A third study showed that nasopharyngeal aspirates of RSV-infected infants (<6 months), with different levels of disease severity, had an abundance of opportunistic organisms like *Haemophilus* and *Achromobacter*. The abundant presence of *Haemophilus* in these RSV-infected patients was associated with increased viral load and mucosal chemokine (C-X-C motif) ligand 8 (CXCL8) responses, which influence RSV disease severity (186). Further studies are needed to elucidate the role of respiratory tract microbiota in RSV disease.

Factors Associated With Age

Aggravating factors such as chronic obstructive pulmonary disease can exacerbate RSV infection in the elderly (184, 187). Being a smoker also increases the chances of developing asthma after RSV infection and exacerbates the pathology if the individual already has asthma (188, 189). Other underlying chronic lung diseases, such as bacterial coinfection in the airways, can increase the severity of RSV infection, including death (189). An acute RSV infection can trigger an acute myocardial infarction in adult patients (189). Immune senescence combined with decreased numbers of RSV-specific neutralizing antibodies in the serum of this patient group (75, 190, 191) can have a detrimental effect on RSV infection.

Role of Regulatory T Cells

Regulatory T cells (Tregs) are immunomodulatory cells that play a key role in tolerance, immune homeostasis, and regulating inflammatory responses by suppressing T-cell proliferation and cytokine production (192). Tregs avoid exacerbating the immune response (193–195), which can be harmful to an individual. Although, Tregs are found in newborns and adults, preterm infants have a higher number of Tregs than do full-term infants (196), while adults have fewer Tregs than do full-term infants (197). However, Tregs from adults more efficiently suppress T-cell responses than do Tregs from children (198). Conversely, Tregs from newborns are more resistant to apoptosis than are Tregs from adults (199). The frequency of activated Tregs was lower in the peripheral blood of infants infected with RSV than in age-matched controls. These results suggest that the reduced number of Tregs in RSV-infected infants precludes their ability to properly control the host inflammatory response leading to severe RSV disease in these patients (200). Further studies still are needed to understand the contribution of Tregs to RSV disease.

HOST IMMUNE RESPONSE TO RSV

Host Innate Immune Response Against RSV

Once RSV enters the host respiratory tract, it begins to infect susceptible target cells in the respiratory epithelium. The host responds through pattern recognition receptors (PRRs) that activate early innate immune responses at the site of infection (47,

201). PRRs can detect PAMPs, including RNA viruses like RSV that infect the respiratory tract (202). These interactions induce cytokine production, including IFN, and antiviral responses (47). A majority of TLRs, RIG-I-like receptors (RLRs), nucleic acid-binding domains, and leucine-rich proteins (LRRs), are involved in antiviral defense and in increasing cytokine production during RSV infection (203, 204). Recognition of RSV by these PRRs is well-studied in humans and in adult mice, but very little is known about their role in neonates.

Early host detection of RSV occurs through three main classes of PRRs. First, TLRs activate the innate immune response via myeloid differentiation primary response 88 (MyD88) (TLRs 2, 4, 7 and 8) or via the TIR-domain-containing adapter-inducing interferon-β (TRIF) (TLR 3 and 4). Once a specific PAMP is recognized (205), RLRs such as RIG-I, melanoma differentiation-associated protein 5 (MDA5), and nucleotide-binding oligomerization domain-containing protein (NOD) 2, activate the innate signaling pathway through the adapter mitochondrial antiviral-signaling protein (MAVS) (206) and NOD-like receptors (NLRs). However, other cellular proteins, such as protein kinase R (PKR) may also recognize RSV in infected cells (207). The signal generated by PRRs activates transcription factors such as the regulatory factors NF-κB, JUN, and different IFN regulatory factors (IRFs). These factors then induce type I IFN expression, DC activation, and expression of proinflammatory cytokines and chemokines, that are produced not only by DCs but also by cells such as alveolar macrophages in the respiratory tract (208). Production of IFN types I and III is induced in this early immune response against RSV, resulting in transcription of IFN stimulating genes and production of proinflammatory mediators. RSV infection activates the inflammasome, cellular stress, and in some cases cell death (209). The role of TLRs in response to RSV infection was evaluated in TLR deficient mice. Peritoneal macrophages from C57BL/6, TLR2 KO, and TLR4 KO mice, previously induced with thioglycolate, were then stimulated with RSV. Mouse macrophages from TLR2 KO and TLR4 KO mice produced lower levels of intracellular TNF-α than did wild type mice after RSV infection. Further, macrophages from TLR2 KO mice generated the lowest TNF-α levels, suggesting that TLR2 plays an important role in proinflammatory cytokine induction after RSV infection (210). Likewise, TLR2 KO mice infected with RSV displayed altered migration of neutrophils to the lung and uncontrolled RSV replication, despite type I IFN production (211). Although TLR3 recognizes viral dsRNA, studies suggest that it may not be required for viral clearance of RSV infection, nonetheless, it is important to maintain an adequate environment in the lung. Similarly, an altered immune environment is induced, affecting the airway epithelium, without TLR7-mediated responses (212, 213).

TLR3 contributes to RSV recognition during infection, since it binds to viral RNA that is generated during replication (47). However, once the viral RNA is detected, both TLRs and RLRs provoke a signaling cascade that activates the transcription factors NF-κB, IRF, and activating transcription factor (ATF)-2 (214).

Another member of the PRR family is the NLRs. These receptors function in cellular processes that are important for immune responses to pathogens (215). Some NLRs, such as NLRP3, are essential for formation of the inflammasome, a protein involved in inflammation and apoptosis by activating host caspases (216). Interestingly, during RSV infection, signaling activated through TLR2 provides the first signal for NLRP3 expression. Once NLRP3 is translated, it forms the NLRP3/ASC inflammasome, a complex that is activated by reactive oxygen species (ROS) (217).

After RSV infects AECs it also induces NF-kB activation causing secretion of cytokines and chemokines, such as chemokine (C-C motif) ligand (CCL-5), CCL2, CXCL8, and CXCL10 (218). These cytokines have chemotactic properties in inflammatory cells and other cell types (47). Secretion of these molecules promotes the recruitment of an arsenal of immune system cells such as neutrophils, eosinophils, monocytes, macrophages, DCs, memory cells, Th1 cells, and NK cells to infected tissues (47). Secretion of TSLP from AECs contributes to an inflammatory environment in the lung. TSLP is a cytokine that plays a critical role in development of allergic asthma in AECs by functioning through the TSLP receptor (TSLPR) on myeloid DCs (219), which then triggers a second round of inflammatory cytokine secretion in RSV-infected tissues, causing lung damage (220).

Type 2 innate lymphoid cells (ILC2) and other cells of the innate immune response are recruited at the alveolarization stage of the lungs (135). After RSV infection, the pulmonary epithelium of neonates can produce large amounts of IL-33, which is associated with ILC2 accumulation during the alveolar period (221, 222). In contrast, IL-33 is not observed in lungs of adult mice in early RSV infection (135). IL-33 increases the production of ILC2 and IL-13 in lungs of neonatal mice, and impacts disease severity in RSV reinfected mice (54). However, the relationship between TSLP produced by respiratory epithelium and ILC2 proliferation/activation is not well-understood in RSV-infected neonatal mice (135).

As mentioned previously, extensive neutrophil accumulation in the lungs after RSV infection and obstruction of the small airways by excess DNA-rich mucus, produces severe RSV-LRTD (223). Although NET formation was initially thought to protect against bacteria and fungi (62), it is also now known to form in response to viral diseases including influenza, human immunodeficiency virus (HIV)-1 and poxviruses. NET formation can capture HIV-1 particles (224) and a similar protective effect is seen in mice infected with poxviruses *in vitro* (225, 226). Cortjens et al. (223) showed that NETs could capture RSV particles, in a functional form, and prevent them from infecting target epithelial cells. The same study also found marked NET formation during RSV infection *in vivo* and an accumulation of NETs in dense structures that obstruct the airways without capturing the viral antigen, indicating an unfavorable response for the host.

NET formation induced by neutrophils may be favorable for the host as a local first-line immune response against RSV. However, the intense response of these cells could worsen the pathology during RSV-LRTD (223, 227, 228). Stokes et al. (228)

stated that depleting these cells induces a decrease in the process of airway inflammation and mucin expression in RSV-infected mice, which supports the fact that neutrophils may be involved in the respiratory tract tamponade during RSV-LRTD (223).

Host Adaptative Immune Response Against RSV

Humoral Response

In addition to innate immune responses to RSV, infants produce antibodies to the majority of RSV proteins (229). RSV infection induces development of IgM, IgA, and IgG antibodies in both blood and mucosa. These antibodies, mediated by the adaptive immune response to RSV, protect the host against reinfections. The primary immune response against RSV is not effective, but when a reinfection occurs, in children for example, IgG and IgA antibody levels increase significantly (230). These antibodies are usually directed against RSV F and G proteins to neutralize the virus (231). However, infants <6 months of age produce less antibody against F protein and thus mount a poor neutralizing response to RSV (229).

The primary humoral immune response against RSV is the induction of the IgM antibody, which is usually detected during the first 5–10 days of the infection and persists in the blood for 1 to 3 months (230). However, in some studies the IgM response remains detectable for at least 1 year (230). Conversely, RSV-specific IgG antibodies are detected in the majority of patients, and peak 20–30 days after the symptom onset (230). Interestingly, 1 year after the patient acquires their first RSV infection, levels of RSV-specific IgG antibodies begin to decline (229). Likewise, a decreased numbers of RSV-specific neutralizing antibodies is observed in the serum of elderly adults that correlate with greater risk of developing symptomatic RSV infection (73).

Some studies showed that production of specific anti-RSV antibodies regulate the T-cell response to RSV (232). Responses mediated by T cells and antibodies are interdependent. During RSV infection of human peripheral blood mononuclear cells (PBMC), the balance between the number of CD4+ and CD8+ T cells directly depends on the relationship between neutralizing and non-neutralizing antibodies *in vitro* (233). In *in vitro* studies, RSV infection significantly increased the proinflammatory effects of substance P, a neuropeptide with bronchoconstrictor effects in animal models, by up-regulating the expression and density of its specific NK1 receptor in target cells (234). RSV not only affects substance P, but also induces specific cellular immune adaptive responses, including lymphocyte transformations, and responses mediated by cytotoxic T cells and by antibodies dependent on cytotoxic T cells (235).

Cellular Response

The cellular immune response to RSV infection is balanced between Th-1 and Th-2 responses (229). The Th-1 response induces IFN-γ release from CD8+ and CD4+ T lymphocytes, neutralizing antibodies, and production of mucosal IgA antibodies. While the Th-2 response induces IL4 secretion of CD4+ T cells, eosinophilia, and high levels of IgE antibody (236–238). Host factors and RSV antigens determine the balance between Th-1 and Th-2 responses (229). For example, the RSV F protein induces a Th-1 response while the G protein stimulates a Th-2 response (239). Interestingly, infants <3 months of age have higher Th-2 cytokines in nasal secretions than do older children (240). A third subset of effector T helper cells that produce IL-17 (Th17 cells), are also involved in RSV infection (241). A study of plasma cytokine profiles in infants infected with RSV (6 months or less) found that patients with a moderate response to the virus had higher IL-17 plasma levels than those with an elevated response to RSV (241). Further, IFN-γ and TNF-α levels were lower in RSV-infected infants than in controls. In contrast, another study examined tracheal aspirates and reported higher IL-6 and IL-17 levels in critically ill ventilated infants upon RSV infection than in healthy infants (242). The role of IL-17 in the respiratory tract remains unclear and further studies are needed to explain why in some cases, but not all, higher IL-17 levels are associated with improvements in RSV-infected infants. It is possible that the immature immune system in newborns presents an altered Th1 response (243) that allows favorable outcomes. Unlike adults, DCs from umbilical cord blood of RSV-infected newborns, produced IL-17 when they were co-cultured with T lymphocytes (244). In the same study, DCs of RSV-infected children were found to produce TGF-β, a cytokine that promotes differentiation of Th17 lymphocytes (244). In another study, human bronchial epithelial cells chronically infected with the long strain RSV A2, promoted differentiation of naïve T lymphocytes to Th2 and Th17 lymphocytes, but not to Th1 lymphocytes (245). Together, these studies indicate that in addition to Th1 and Th2 responses, the Th17 response also occurs in RSV infection, and suggests that the Th17 response is beneficial in some cases of RSV infection. However, Th17 responses have also been linked to respiratory tract pathology during severe asthma dominated by neutrophils. Therefore, more studies and research are needed to identify the consequences of the IL-17 production, its benefits and damages (244).

The inflammatory process generated upon RSV infection, may be influenced by the ability of RSV to induce TSLP production, which polarizes the cellular response to RSV. Qiao et al. (63) suggest that TSLP secretion activates mDCs in AEC caused by RSV infection, which induces polarization toward a Th2 response. This occurs because thymus- and activation- regulated chemokine (TARC/CCL17) is associated with recruitment of Th2 response cells (63). In addition, ILC2 also induces Th2-type cytokines such as IL-4, IL-5, and ILC3 via IL-17 (246) to generate a Th2 response. Activation of mDCs by TSLP allows them to migrate to draining lymph nodes, initiate an adaptive response to allergies, and promote differentiation of naïve CD4+ T cells to Th2 phenotypes, which secrete IL-5 and IL-13 (246). Upon IL-13 induction, eosinophils and neutrophils are recruited to the lung and IL-5 secretion stimulates mucus production by ciliated airway cells (246). A proposed model of this orchestrated cellular immune response to RSV is shown in **Figure 2**.

During RSV LRTI, systemic T-cell lymphopenia can occur due to reduced numbers of CD8+, CD4+, and CD3+ T cells, compared to those present during convalescence and in uninfected infants (241, 247, 248). In circulating T cells, CD119 expression is not increased, suggesting that these cells are not

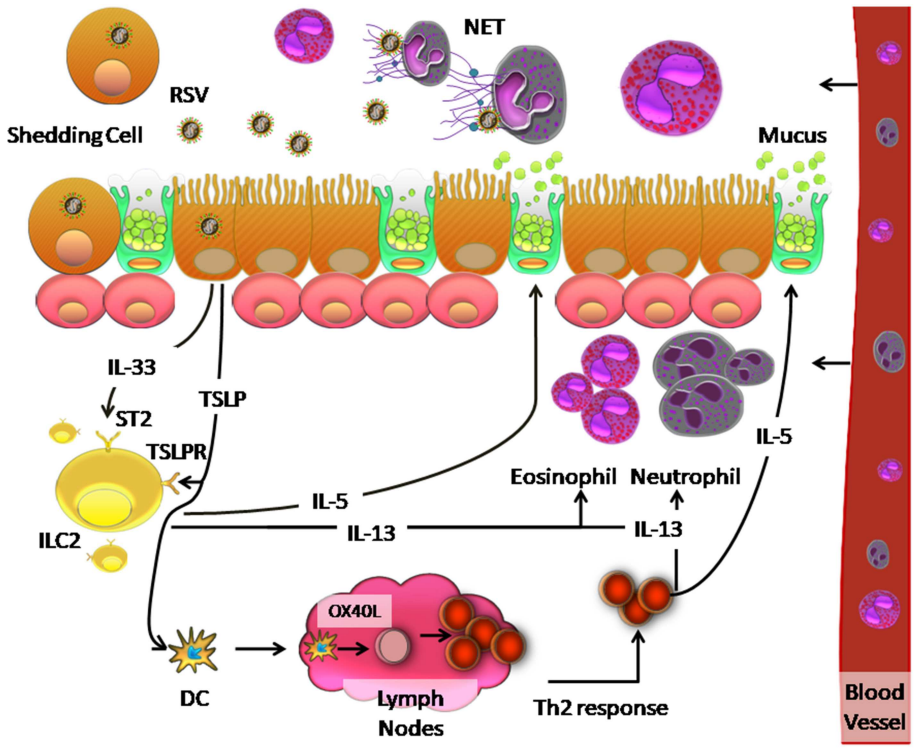

FIGURE 2 | Model of the innate immune response and Th2 response against RSV infection in the human respiratory tract. RSV infection of ciliated cells induces the release of TSLP and IL-33, which are recognized by TSLP and ST2 receptors on ILC2 cells, respectively. This recognition causes the release of IL-5 and IL-13, which induce increased mucus secretion by the goblet cells and recruit neutrophils and eosinophils in the lung, respectively. Neutrophils can generate a network of DNA called NETs, which can trap and eliminate different pathogens. On the other hand, TSLP induces OX40L expression on the surface of dendritic cells, which causes these cells to migrate to the lymph nodes where they interact with naïve CD4+ T cells by binding of OX40 on these cells. The resulting Th2 immune response causes inflammation in the lung.

activated. Also, increased expression of cytotoxic T-lymphocyte antigen (CTLA) 4, a negative regulator of T-cell activation, is observed (249, 250). During RSV infection, T-cell lymphopenia is more pronounced in younger patients (251).

Other studies have considered that adaptive immune responses mediated by T cells and by proinflammatory cytokines, play a major role in RSV pathogenesis in children (28, 252), with little evidence that this occurs in adult patients. Specifically, the severity of RSV disease in adults and the elderly is attributed, among other factors, to low levels of specific serum antibodies against RSV (253, 254). Walsh et al. (255) suggests that the functional capacity of CD8+ T cells in adults is lost over the years (255), which contributes to increased disease severity, while several other studies suggest that immune responses mediated by these cells lose specificity instead (256, 257). Infants under 21 days of age have low numbers of CD8+ and CD4+ T lymphocytes because their adaptive immune system is not yet fully developed. Likewise, adults over 65 years of age, have fewer lymphocytes than do adults under 50 years of age, because lymphocyte numbers decrease with age. These results suggest that CD8+ and CD4+ T lymphocytes play important roles in controlling RSV infection in the host.

RSV pathogenesis in the respiratory system is caused by virulence factors, such as NS2 that provoke cell rounding, detachment of ciliated cells from the airway epithelium and contribute to airway obstruction. In contrast, host factors including age can determine immune system status and thus influence the immune response to efficiently clear RSV with minimal inflammation of lung tissue. Indeed, RSV infection in hosts with immature immune and respiratory systems produces more severe disease. Specifically, RSV infection in the airway epithelium induces TSLP and IL-33 production, which elevates the number of ILC2 at this age. In turn, these cells secrete IL-5 and IL-13, causing mucus secretion by goblet cells and recruiting proinflammatory immune cells, such as eosinophils and neutrophils. TSLP is also known to polarize DCs toward a Th2 immune response, and to stimulate proinflammatory cytokines and immune cells, such as neutrophils, which simultaneously release NETs that may contribute to RSV pathogenesis in the lower airways. A better understanding of host factors that contribute to disease severity caused by RSV will help efforts to develop therapeutic tools, such as vaccines to prevent severe RSV diseases.

TABLE 1 | Summary of vaccines against RSV in different clinical phases according to database.

	Preclinical	Phase I	Phase 2	Phase 3	Market approved
Live-Attenuated/ Chimeric	Codagenix, LID/NIAID/NIH RSV	Intravacc[P] Delta-G RSV			
		Sanofi,[P] LID/NIAID/NIH RSV ΔNS2/ Δ1313/I1314L			
	LID/NIAID/NIH RSV	Sanofi,[P] LID/NIAID/NIH RSV 6120/ΔNS2/1030s			
	LID/NIAID/NIH PIV1-3/RSV	Pontificia[P] Universidad Catolica de Chile BCG/RSV			
		SIIPL, St. Jude[P] Hospital SeV/RSV			
	MeissaVaccines RSV	Sanofi,[P] LID/NIAID/NIH RSV D46/NS2/N/ΔM2-2-HindIII			
Whole-Inactivated	Blue WillowBiologics RSV				
Particle-Based	AgilVax VLP	Novavax[P] RSV F Nanoparticle	Novavax[E] RSV FNanoparticle	Novavax[M] RSV F Nanoparticle	
	Fraunhofer VLP				
	Georgia StateUniversity VLP				
	Icosavax VLP				
	University of*Massachusetts* VLP				
	TechnoVax VLP				
	Virometix VLP				
	Artificial CellTechnologies Peptide microparticle				
Subunit	Instituto de Salud CarlosIII RSV FProtein	Beijing Advaccine[PE] Biotechnology RSV G Protein	Pfizer[EM] RSV FProtein		
	University ofGeorgia RSV GProtein	Immunivaccine, VIB[E] DPX-RSV-SH Protein			
	Sciogen RSV GProtein	NIH/NIAID/VRC[EM] RSV F Protein			
	University ofSaskatchewan RSV FProtein	GlaxoSmithKline[EM] RSV F Protein			
		Janssen[E] Pharmaceutical RSV F Protein			
Nucleic Acid	CureVac RNA				
	InovioPharmaceuticals DNA				
Recombinant Vectors	BravoVax Adenovirus	Vaxart[E] Adenovirus	Bavarian Nordic[E] MVA		
			Janssen[PE]Pharmaceutical Adenovirus		
			GlaxoSmithKline[P] Adenovirus		
Immuno-Prophylaxis/ Combination	Arsanis Anti-F-mAb	Merck[P] Anti-F mAb	MedImmune,[P]Sanofi Anti-F mAb		MedImmune[P] Synagis
	Biomedical ResearchModels DNA prime, Particleboost				
	Pontificia Universidad Catolica deChile Anti-N mAb				
	UCAB,mAbXience Anti-F mAb				

Adequate from the PATH (formerly the Program for Appropriate Technology in Health). Target indication: p, Pediatric; M, Maternal; E,Elderly.

CANDIDATE VACCINES AGAINST RSV

After failure of the first RSV vaccine that used whole RSV inactivated with formalin in the 1960's (258), there have been significant efforts to develop therapies and new vaccine candidates using different approaches. Currently, passive immunity with palivizumab is the mainstay of RSV prophylaxis (259). This therapy, which uses a specific-RSV monoclonal antibody developed by *MedImmune*, was licensed in 1998 and is the only prophylactic tool that is effective and safe to prevent RSV infection (260, 261). It is also used to prevent serious RSV infections in high-risk children, including children born at <35 weeks gestational age (wGA) and <6 months at the onset of the RSV season, children <2 years of age who required treatment for borderline personality disorder within the last 6 months, and children <2 years of age with hemodynamically significant congenital heart disease CHD (HS-CHD) (259). However, more evidence of its usefulness is still needed in certain high-risk populations, such as those with cystic fibrosis.

Vaccine candidates for maternal immunization have been the focus of much effort worldwide (259) because it is a permissible route for childhood prophylaxis against RSV. However, strict safety standards required for any treatment or medication in pregnant women has limited progress of these strategies. Thus, vaccine candidates based on nanoparticles and subunits have been tested in clinical trials, as they would be appropriate for use in pregnant women (259). Vector and subunits vaccines attenuated *in vivo* are considered most suitable for pediatric populations because they are safer than other strategies. The drawback of these vaccine approaches is that they may mount a less robust immune response to RSV (262). Due to the unfortunate experience with the first RSV vaccine, which caused some deaths in vaccinated children upon RSV infection (263), researchers have been very cautious in designing new vaccine candidates for infants (263).

There is consensus that pediatric populations are the main risk group, and that most efforts should focus on developing an effective and safe candidate vaccine for this group. Specifically, there is a high priority to obtain a candidate vaccine for babies in their first 6 months of life, since the risk of acquiring severe RSV disease is greater in this group (263) than in infants older than 6 months of age with a more mature immune system, which reduces their susceptibility to RSV complications (263).

Currently, 84 studies of vaccine candidates against RSV are reported in the *ClinicalTrials* database (https://ClinicalTrials.gov/). Particularly, studies based on RSV subunits are in phase II and those based on RSV particles are already in phase III trials (**Table 1**) (259, 264). Several promising candidate vaccines and therapies based on monoclonal antibody compounds and other strategies in pre-clinical studies are also expected to be available in coming years (259).

CONCLUSIONS

Taken together, the virus and host both contribute to the severity of RSV disease.

RSV pathogenesis in the respiratory system is caused by virulence factors that provoke cell rounding and detachment of ciliated cells from the airway epithelium contributing to airway obstruction. Host factors including age, malnutrition, and premature delivery influence the immune system and its ability to mount an effective response to efficiently clear RSV with minimal inflammation in lung tissue. RSV infection at an early age, when the host has immature immune and respiratory systems, can produce more severe disease. Moreover, the low abundance of RSV-specific memory CD8+ T cells, which decreases with age, in older adults, is a host factor that contributes to severe RSV disease, due to loss of T-cell functional capacity and specific response to RSV in older patients. Therefore, the elderly are more likely than younger adults to present with severe RSV disease. Identification of specific genes that influence the probability of developing severe RSV disease, especially those involved in immune signaling pathways, will be important in ongoing efforts to improve immune responses that promote more efficient clearing of RSV infection from the respiratory tract. A better understanding of host factors that contribute to RSV disease severity will help us develop more effective therapeutic tools and vaccines to prevent severe RSV diseases.

AUTHOR CONTRIBUTIONS

JC, AA, CS-A, and JM wrote the manuscript. AK and ML reviewed the manuscript, and ML reviewed and approved the version to be published. All authors listed have made substantial and intellectual contribution to the work.

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Determining Immune and miRNA Biomarkers Related to Respiratory Syncytial Virus (RSV) Vaccine Types

*Lydia J. Atherton, Patricia A. Jorquera, Abhijeet A. Bakre and Ralph A. Tripp**

Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, United States

Respiratory Syncytial Virus (RSV) causes serious respiratory tract illness and substantial morbidity and some mortality in populations at the extremes of age, i.e., infants, young children, and the elderly. To date, RSV vaccine development has been unsuccessful, a feature linked to the lack of biomarkers available to assess the safety and efficacy of RSV vaccine candidates. We examined microRNAs (miR) as potential biomarkers for different types of RSV vaccine candidates. In this study, mice were vaccinated with a live attenuated RSV candidate that lacks the small hydrophobic (SH) and attachment (G) proteins (CP52), an RSV G protein microparticle (GA2-MP) vaccine, a formalin-inactivated RSV (FI-RSV) vaccine or were mock-treated. Several immunological endpoints and miR expression profiles were determined in mouse serum and bronchoalveolar lavage (BAL) following vaccine priming, boost, and RSV challenge. We identified miRs that were linked with immunological parameters of disease and protection. We show that miRs are potential biomarkers providing valuable insights for vaccine development.

Keywords: RSV, miR, vaccines, immune, disease, microRNA

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****Correspondence:***
Ralph A. Tripp
ratripp@uga.edu

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INTRODUCTION

Respiratory Syncytial Virus (RSV) is a cause of lower respiratory tract infection (LRTI) worldwide and is responsible for >30 million new LRTI episodes and up to 199,000 deaths in children under 5 years old resulting in more than 3.4 million hospital admission associated with severe RSV disease (1, 2). The elderly population is also markedly affected by RSV (3). Currently, the only approved RSV prophylactic is palivizumab which is used for high-risk patients, but such treatment has limited applicability due to cost and treatment logistics (4–9). Unfortunately, all efforts to develop a safe and effective RSV vaccine have been unsuccessful (10–16). Attempts in the 1960s to develop a formalin-inactivated RSV (FI-RSV) vaccine candidate were hampered by several factors, including lack of protection against RSV infection in infants and young children, and an association with vaccine enhanced disease that resulted in two deaths upon natural RSV infection of vaccinees (10, 17–19). Efforts to develop live attenuated RSV vaccine candidates using cold-passaging, chemical mutagenesis, or reverse genetics have also been unsuccessful largely due to over- or under-attenuation, which currently cannot be precisely predicted (20–30), and natural RSV infection does not provide long-term protective immunity. Several other RSV vaccine platforms have been developed including subunit (31–38), vectored (39–46), particle-based (47–57), or nucleic acid-based (58–63), but none are FDA-approved a feature linked to our incomplete understanding of the host immune response to RSV (10). There are several target populations for RSV vaccines: infants, young children, pregnant women, and the elderly (10). Due to the differences in these target populations, vaccine safety, efficacy, and platform strategies will need to be different (10, 11, 18, 33).

By establishing measures of vaccine protection and disease, a wide range of promising vaccine candidates can be evaluated early in development.

The host immune response is important in the outcome of RSV infections (14, 60, 64, 65), and an imbalance between Th1- and Th2-type cytokines is understood to be responsible for a variety of inflammatory disorders (66, 67). Biomarkers can be surrogates for clinical endpoints and are needed to improve vaccine design and efficacy. Small regulatory microRNAs (miRs) have fundamental roles in regulating the expression and function of key immunological mediators such as cytokines (68–70). miR expression profiles have been identified and shown to be useful predictors for several allergic inflammatory diseases (71–74). In addition, specific miRs have been shown to function in regulating key pathogenic mechanisms in asthma and airway hyperresponsiveness, including polarization of adaptive immune responses, activation of T cells (75–78), regulation of eosinophil development (79–84) and modulation of cytokine-driven responses (68–70). miRs are stable, in a variety of tissues, bodily fluids, and sera allowing for sensitive and accurate measurements regarding the physiological state of the individual (72–74). miRs govern host gene expression by inducing mRNA degradation or translation inhibition and have a prominent role in determining the level of protein expression of host gene targets (85–90). Several miRs can also upregulate target gene expression via regulation of promoter function (91, 92). It has been shown that miR patterns of expression vary for numerous physiological processes that have been deemed useful for diagnosis of neurodegenerative disorders, autoimmune diseases, cardiovascular disease, and cancers; likewise miRs have also been implicated in infectious diseases (42, 93–98). Assessing circulating miRs in the sera of patients has supported miR profiling as a powerful non-invasive biomarker tool.

Previously, it was shown that RSV infection of normal tracheal epithelial cells (NTECs) with GFP-expressing RSV (rgRSV) downregulated the expression of multiple miRNAs (99). Of the 24 miRNAs, miR-221 was shown to regulate nerve growth factor (NGF), a key neurokinin that prevents apoptosis in respiratory cells (99). Later, RSV infection of type II respiratory epithelial cells was shown to induce expression of five and down-regulation of three microRNAs via an RSV G protein regulated mechanism (100). RSV deregulated miRNAs were demonstrated to regulate several key immunological pathways. In a follow-up study, RSV infection of normal human bronchoepithelial (NHBE) cells, miRNA deregulation was tied to mechanisms involving IFN beta and the transcription factor NF-κβ (101). We showed that RSV G and NS1/NS2 proteins can modulate miRNA expression (102, 103). Several studies have investigated differential expression of miRNAs in clinical RSV infections and shown deregulated patterns that can be used as potential biomarkers of infections (104–109). While these data show miRNA deregulation during infection, miRNA expression following vaccination with different RSV vaccine candidates under investigation is not well-understood and has the potential to identify safe vs. unsafe vaccine candidates.

As miRs regulate host gene responses, it is important to determine if miR profiles serve to predict safe, efficacious, or diseased vaccine outcomes, particularly since RSV lacks a licensed vaccine. To determine if patterns of miR expression may serve as a surrogate of RSV immunity or disease requires proof of biological relevance. Therefore, we identified miR biomarkers and immune correlates associated with RSV vaccination to establish baselines for biomarker expression across different vaccination types and strategies. Since serum miR profiles provide indications of how miRs may regulate the immune response induced by RSV vaccination or infection (50), serum miR profiles may also suggest vaccine disease outcomes. We hypothesized that RSV infection or vaccination would alter the pattern and tempo of miR expression and that this would be reflected in changes by the host immune or disease response. In the present study, we examined serum miRs in BALB/c mice at various time-points post-RSV vaccination or RSV challenge using several RSV vaccine types. A miR PCR array was used to identify miRs post-vaccination, post-boost, or post-RSV challenge, and correlated with immune parameters and markers of disease.

MATERIALS AND METHODS

Mice

Specific-pathogen-free, 6-to-8 weeks old female BALB/c mice (The Jackson Laboratory) were used. Mice were maintained in microisolator cages with sterilized water and food *ad libitum*. All experiments were approved by and performed in accordance with the guidelines of the University of Georgia Institutional Animal Care and Use Committee (IACUC).

Viruses and Cell Culture

CP52 was a gift from Stephen Whitehead and Brian Murphy at LID, National Institute of Allergy and Infectious Diseases, Bethesda, MD. CP52 is a cold-passaged live attenuated vaccine strain that lacks the RSV G and SH genes and is derived from RSV B1. RSV A2 and CP52 were propagated in mycoplasma-free Vero E6 cells (ATCC CRL-1586) using DMEM (Gibco) containing 5% FBS (Hyclone) at 37°C/5% CO_2 and 32°C/5% CO_2, respectively (110). Viral titers were determined by plaque assay on Vero E6 cells, and plaques were enumerated by an anti-F protein (clone 131-2A) immunostaining assay (111, 112). Infections were performed in serum-free DMEM (SF-DMEM).

FI-RSV Preparation

The preparation of formalin-inactivated RSV (FI-RSV) vaccine was adapted from the FI-RSV Lot 100 method (113). Briefly, strain A2 was used to infect Vero E6 cells (MOI = 0.1), and at day 4 pi, the cells were lysed following scraping, sonicated, and clarified by centrifugation at 600 x g for 15 min at 4°C. The supernatant was transferred to a tube and filter sterilized using a 2 μm filter; the final protein concentration (determined by BCA) was adjusted to 1 mg/ml. Viral stocks were inactivated by the addition of 37% formalin (final dilution 1:4,000) and incubated at 37°C for 3 days in agitation. FI-RSV was pelleted by ultracentrifugation for 2 h at 25,000 rpm, re-suspended in SF-DMEM at 1/25th of the original volume and adsorbed overnight at room temperature in 4 mg/mL aluminum hydroxide. The

compound material was pelleted by centrifugation and the pellet was suspended in SF-DMEM and total virus inactivation was confirmed via plaque assay on Vero cells. This procedure resulted in an FI-RSV vaccine that is concentrated 100-fold and contains 16 mg/ml alum. The vaccine was aliquoted in 1 ml volumes and stored at 4°C.

RSV GA2 Microparticle-Based Vaccine

A microparticle-based RSV G protein vaccine consisting of 3 μm $CaCO_3$ cores was prepared using alternating poly-I-glutamic acid (PGA, negative charge) and poly-I-lysine (PLL, positive charge) layering to build up to seven layers with an RSV G peptide CX3C motif linked to a cationic sequence added as the outermost layer (4). The composition of the seven-layer film was determined using amino acid analysis, which showed that a comparable amount of the peptide component was present in each vaccine batch. Endotoxin levels by limulus amebocyte lysate (LAL) assay were <0.1 EU/μg. The dispersity of the particle vaccines was monitored by dynamic light scattering (DLS). DLS is used to determine the size distribution profile of small particles in suspension or polymers in solution. This layer-by-layer microparticle vaccine has an apparent diameter of ∼150 nm for uncoated particles to about 400–500 nm for fully coated particles. Some particle aggregation was detected in each batch with a second population of particles in the 1,500–2,000 nm range.

Vaccine Delivery

We examined three vaccine types: (1) live-attenuated (CP52), (2) inactivated (FI-RSV), and (3) an RSV G peptide microparticle-based (GA2-MP). The GA2-MP vaccines were suspended in PBS and dispersed by water bath sonication immediately prior to immunization. Doses were adjusted to deliver 50 μg designed peptide (DP)/100 μl/mouse. Mice were subcutaneously (s.c.) immunized with GA2-MP without adjuvant between the shoulder blades. 10^6 PFU equivalents of FI-RSV was used to intramuscularly (i.m.) vaccinate mice. Mice received a 1:25 dilution of FI-RSV in PBS by i.m. injection in a final volume of 50 μL/mouse. FI-RSV was a positive control for vaccine enhanced disease. 10^6 PFU of live CP52 diluted in PBS was used to vaccinate mice by intranasal (i.n.) instillation in a final volume of 50 μL/mouse. CP52 was a positive control for vaccine protection. PBS vaccinated mice received 50 μL of PBS (vehicle control) by s.c. injection. Mice were anesthetized by i.p. administration of 2,2,2- tribromoethanol (Avertin; 200 μg/kg Sigma) and a portion of vaccinated mice were i.n. challenged with 10^6 PFU A2 diluted in PBS.

Lung Virus Titers and Disease Endpoints

Lung virus titers were determined in treatment and control mice by plaque assay on Vero E6 cells (111). Briefly, lungs were aseptically removed from mice at day 5 post-RSV (10^6 PFU/mouse) challenge, and individual lung specimens were homogenized at 4°C in 1 mL of SF-DMEM using a gentleMACS™ Dissociator (Miltenyi Biotec). Samples were clarified by centrifugation for 10 min at 200 × g and supernatants were transferred and stored at −80°C. For the plaque assay, 10-fold serial dilutions of the lung homogenates were adsorbed to 90% confluent Vero E6 cell monolayers for 2 h, at 37°C, overlaid with 1% methylcellulose medium and incubated at 37°C for 5 days. RSV plaques were enumerated by immunostaining with monoclonal antibodies against RSV F protein (clone 131-2A) as previously described (112). Lungs from vaccinated and challenged mice were examined for disease pathogenesis, and as anticipated (54, 114, 115), only the lungs from FI-RSV vaccinated mice challenged with RSV showed substantially enhanced disease (data not shown).

Microneutralization Assay

Two-fold serial dilutions (1:50-1:1,600) of mouse serum in SF-DMEM were incubated with 10^5 PFU of A2 for 1 h at 37°C, 5% CO_2. Palivizumab (MedImmune) was used as positive control for neutralizing activity, and positive control wells of virus without sera and negative control wells without virus or sera were included in triplicate on each plate. The antibody-virus mixtures were transferred to 80–90% confluent monolayers of Vero E6 cells in 96-well-plates and incubated for 2 h at 37°C, 5% CO_2. The virus overlay was aspirated, and 150 μl/well of DMEM-10% FBS was added and plates were incubated for 3–4 days at 37°C, 5% CO_2, and the plates were fixed with cold 80% acetone in PBS for 10 min, rinsed twice with PBS followed by three washes with 150 μl/well of wash buffer (PBS + 0.1% Tween-20). A monoclonal antibody to the RSV F protein (clone 131-2A) was diluted in PBS with 0.5% gelatin + 0.15% Tween 20 and incubated for 1 h at 37°C, 5% CO_2. RSV plaques were enumerated using horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Southern Biotech), developed using TMB substrate (Sigma), and absorbance measured at 450/650 nm dual-wavelength (BioTek Epoch™ microplate spectrophotometer) and Gen5 Data Analysis software. The percentage of neutralization was calculated, and all samples were normalized to the average value from the no serum control wells.

Indirect ELISA

RSV A2-specific and B1-specific IgG antibodies were detected by ELISA using 96-well-high binding plates (Corning) coated with 10^6 PFU/mL A2 or B1 in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Sera were added to plates in serial dilutions. RSV-specific antibodies were detected with HRP-conjugated antibodies specific for mouse IgG (Southern Biotech) followed by the addition of SureBlue TMB-peroxidase substrate (KPL, Inc.) for 15 min. Antibody titers were determined as the last sample dilution that generated an OD450 reading of >0.2 (mean OD value of background plus 2 standard deviations of the mean).

ELISPOT Analysis

MultiScreen filter 96-well-plates (Millipore) were coated with the anti-mouse IL4 or anti-mouse IFNγ capture antibody (R&D Systems) and incubated overnight at 4°C. The plates were then blocked with 200 μL of RPMI-10 medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine) and incubated for 2 h at 37°C. In parallel, spleens were harvested from mice at day 5 post-A2 challenge and

prepared to a single cell suspension. The cell suspensions were collected by centrifugation for 10 min at 200 × g and suspended in RPMI-10 at 10^7 cells/mL. Spleen cell suspensions were added to the wells, and cells were stimulated with either 10 μg/mL RSV M2 $_{(82-90)}$ peptide, 10 μg/mL RSV F $_{(51-66)}$ peptide, 10 μg/mL RSV G $_{(183-198)}$ peptide, or 10 μg/mL eGFP $_{(200-208)}$ (irrelevant peptide control) for 24 h at 37°C and 5% CO_2. Plates were washed 4 times with wash buffer (0.05% Tween-20 in PBS), anti-mouse IL4 or anti-mouse IFNγ detection antibody (R&D Systems) was added, and plates were incubated overnight at 4°C. Detection antibody was removed, plates were washed, and cytokine spots were developed using NBT/BCIP substrate (R&D Systems). Spots were enumerated using an ELISPOT reader (AID, San Diego).

Quantification of Cytokines

At day 3 post-A2 challenge, a subset of mice from each group was sacrificed and BAL and sera were collected. The mouse lungs were flushed three times with 1 ml of PBS and the retained BAL was centrifuged at 400 × g for 5 min at 4°C. The recovered supernatants were collected and stored at −80°C until assessed for cytokine concentration, and the cell pellet was suspended in 200 μL of FACS staining buffer (PBS containing 1% BSA). Total cell numbers were counted using a hemocytometer. The Luminex® xMAP system using a MILLIPLEX MAP mouse cytokine immunoassay (Millipore) was used to quantitate cytokines in cell-free BAL supernatants and sera according to the manufacturer protocol. Briefly, beads coupled with anti-IFNγ, anti-IL1α, anti-IL2, anti-IL4, anti-IL5, anti-IL6, anti-IL9, anti-IL10, anti-IL12p40, anti-IL13, anti-IL15, anti-IL17A, anti-MCP1, anti-RANTES, anti-TNFα, and anti-eotaxin monoclonal antibodies were sonicated, mixed, and diluted 1:50 in assay buffer. For the assay, 25 μL of beads were mixed with 25 μL of PBS (for BAL samples) or serum matrix (for serum samples), 25 μL of assay buffer and 25 μL of BAL supernatant or serum and incubated overnight at 4°C. After washing, beads were incubated with biotinylated detection antibodies for 1 h and the reaction mixture was then incubated with streptavidin-phycoerythrin (PE) conjugate for 30 min at room temperature, washed, and suspended in PBS. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using Luminex xPONENT 3.1 software.

RNA Isolation

Blood was collected from mice via axillary vessels in 1.5 ml microcentrifuge tubes (Fisher), allowed to clot for 30 min at room temperature, and centrifuged at 900 × g for 10 min and 4°C. Serum layer was transferred to a new microcentrifuge tube and centrifuged for 10 min at 16,000 × g and 4°C, and the cleared supernatant was transferred to a new microcentrifuge tube without disturbing the pellet. One hundred microliter of serum sample per mouse was processed for RNA isolation using miRNeasy Serum/Plasma Kit (Qiagen) as per manufacturer's recommended protocol or stored at −80 till processing. Serum/Plasma *C. elegans* miR-39 Spike-In Control (Qiagen) was spiked into each sample prior to RNA purification as an internal control for miR expression profiling in serum to allow for monitoring of RNA recovery and purity, and reverse transcription efficiency. The RNA concentration and purity were determined by Qubit RNA assay broad range (Qubit RNA assay BR, Invitrogen) fluorometry. This reagent specifically binds to RNA only and does not detect DNA, protein or free nucleotides. Additionally, spectrophotometric analysis of all samples using Epoch Gen 5 spectrophotometer (Biotek) showed that all RNA samples had A260/280 ratios ≥1.8.

miR PCR Arrays and Data Analysis

First-strand cDNA synthesis was performe4d with 200 ng/total RNA from each sample using the miScript II RT kit with miScript HiSpec Buffer (Qiagen) following manufacturer protocol. Briefly, cDNA synthesis was performed at 37°C for 60 min followed by inactivation at 95°C for 5 min. First-strand cDNA was diluted 1:10 in molecular grade water and expression of 84 miRs having a role in T or B cell function was assessed using a miScript miR PCR Array Mouse T cell and B cell Activation (Qiagen) array following the manufacturer's protocol on a Stratagene Mx3000P/3005P real-time instrument. Each array plate contains oligos specific to 84 mature miRs validated to regulate T cell or B cell development and function as well as oligos for spike in (*C. elegans* miR-39), six housekeeping genes [small nucleolar/nuclear RNA (snoRNAs) SNORD61, SNORD95, SNORD96A, SNORD68, SNORD72, and RNU6B] and positive and negative controls for reverse transcription and PCR. Data obtained were analyzed with miScript miR PCR analysis template (Qiagen) using the $\Delta\Delta C_T$ method (116). miRs with fold change ≥2 were considered upregulated while miRs with fold change ≤0.5 were considered downregulated. RT-qPCR using miR-specific primers was then performed on differentially expressed miRs between treatment groups using a PCR array. The specificity of amplification was validated by dissociation curve analysis.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (ver 5.0; GraphPad). Statistical significance was determined using a one-way ANOVA or two-way ANOVA followed by Bonferroni's *post-hoc* comparisons tests; a $p \leq 0.05$ was considered significant.

Pathway Analysis

Analysis of pathways regulated by differentially expressed miRs was carried out using DIANA miRPath ver 3.0 (117–119) using the microT-CDS database. The significance of pathway association was determined using a *p*-value threshold ≤0.05 and microT threshold of 0.8 using Fisher's exact test. When multiple miRs were analyzed together, data were filtered to identify pathway intersections instead of unions to identify common core pathways regulated by the miRs. Pathway hits were corrected for false discovery rate.

RESULTS

To assess the serum miR profiles in RSV-vaccinated or challenged mice, the mice were vaccinated (primed) with a live attenuated vaccine (CP52), an inactivated vaccine (FI-RSV), or a microparticle peptide-based vaccine carrying the G CX3C motif (GA2-MP) and boosted 3 weeks later. Three weeks post-boost

vaccinated mice were i.n. challenged with 10^6 PFU A2, and the sera and lung tissues were collected from vaccinated and mock-treated mice at several time-points. Antibody responses to RSV were assessed to confirm that antigens induced a recall response upon vaccination and challenge. RSV A2 or B1-specific serum IgG was determined at 2 weeks post-boost and 5 days post-RSV challenge, and the levels of neutralizing antibody determined at day 5 pi. Lung viral titers were determined at 5 days post-challenge. Broadly, vaccination with CP52 or FI-RSV elicited a cross-reactive humoral response to either A2 or B1 relative to mock (**Figures 1A,C**, respectively). CP52 (but not FI-RSV) vaccination followed by A2 challenge elicited a strong antibody response (**Figures 1B,D**, respectively), which was neutralizing (**Figure 1E**) and correlated with a statistically significant ($p \leq 0.05$) reduction in lung viral titers (**Figure 1F**). Despite the G protein CX3C motif having intra-strain conservation (120), it was less immunogenic as indicated by the lower anti-A2 and B1 IgG serum levels. These data show the prototypical responses associated with CP52, GA2-MP, and FI-RSV vaccination in mice (121, 122).

RSV Vaccine Types and the Th1- and Th2-Type Response

Th1- or Th2-type responses were assessed by ELISPOT assays, and levels of IFNγ or IL4 expression (representing Th1- or Th2-type responses, respectively) were evaluated at day 14 post-boost vaccination by re-stimulated splenocytes with RSV M2, F or G peptides. As expected, CP52 vaccinated mice had the highest frequency of IFNγ expressing cells compared to IL4 expressing cells (**Figure 2A**), while splenocytes from FI-RSV vaccinated mice had the highest frequency of IL4 expressing cells compared to IFNγ expressing cells (**Figure 2B**). GA2-MP vaccinated mice had higher levels of G protein-specific IL4 secreting cells than IFNγ expressing cells, however this difference was not statically significant ($p > 0.005$). MCP1 and RANTES are chemokines involved in leukocyte recruitment to the airway, and to sites of inflammation in response to RSV infection (123–125). Given the role of these immune cell types in disease pathogenesis, levels of MCP1 (**Figure 2C**) and RANTES (**Figure 2D**) in sera and BAL were determined by multiplex cytokine/chemokine assays. MCP1 was localized to the lungs, with the highest level in the BAL from FI-RSV vaccinated mice although the level of expression was not substantially different between CP52 and GA2-MP vaccinated mice. In contrast, RANTES was expressed systemically, as evident by a higher level in the sera compared to BAL, with higher levels for FI-RSV and CP52 immune mice compared to GA2-MP vaccinated mice at day 3 post-RSV challenge. As anticipated, no detectable cytokine expression occurred following mock (PBS) treatment (data not shown). These data indicate that CP52 or FI-RSV vaccinated mice have an overall higher level of inflammation than mice vaccinated with GA2-MP upon RSV challenge. Other Th2-specific cytokines (IL4, IL5, IL6, IL10, and IL3) were higher in the sera (data not shown) and BAL of FI-RSV vaccinated

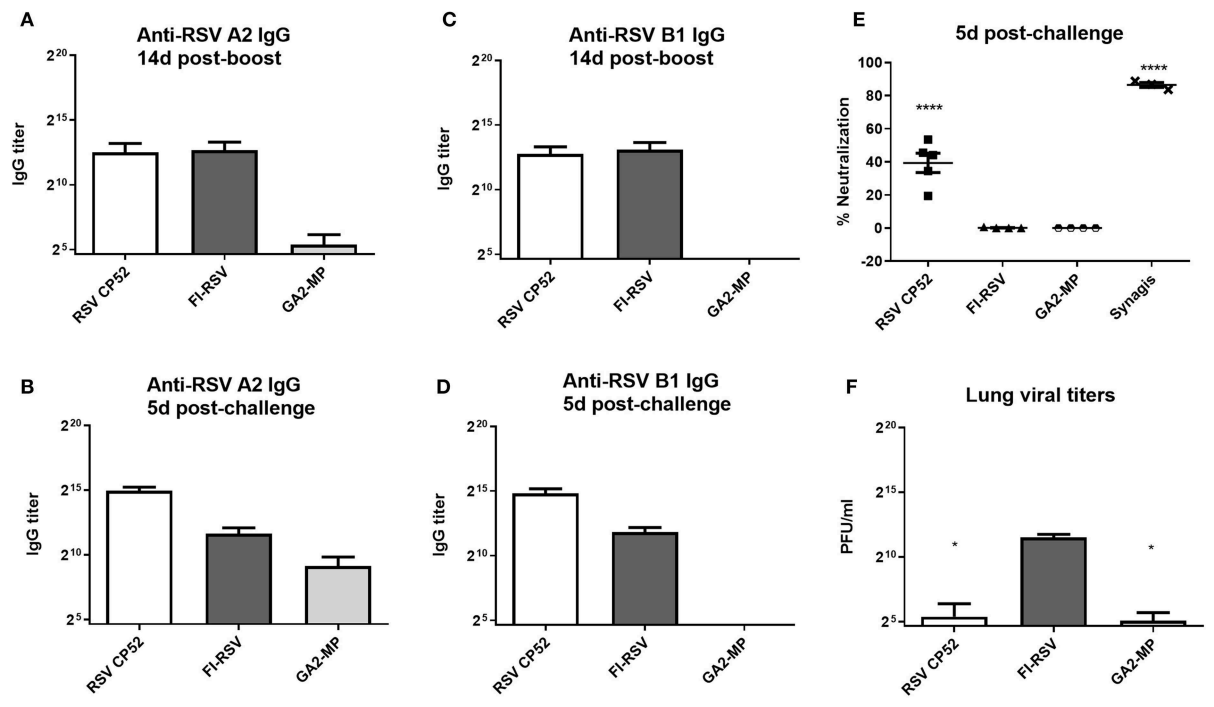

FIGURE 1 | RSV vaccines types, serum IgG, and virus clearance. Sera at day 14 **(A)** and 5 **(B)** post-RSV A or B challenge of prime-boosted mice **(C,D)**; IgG reactivity was determined against A2 **(A,C)** and B1 **(B,D)**. Three weeks after the boost-vaccination mice were i.n. challenged with 10^6 PFU of A2. **(E)** RSV neutralizing antibody levels were measured by microneutralization assay at day 5 post-RSV challenge. **(F)** Lung virus titers were determined 5 days post-challenge by plaque assay. PBS only-treated groups treated had no detectable effect and are not included. All samples were assayed in duplicate and $n = 4$ mice/group. Error bars represent the SEM and results were considered significant with a $^*p \leq 0.05$ and $^{****}p \leq 0.0001$ as determined by one-way ANOVA and Bonferroni's test.

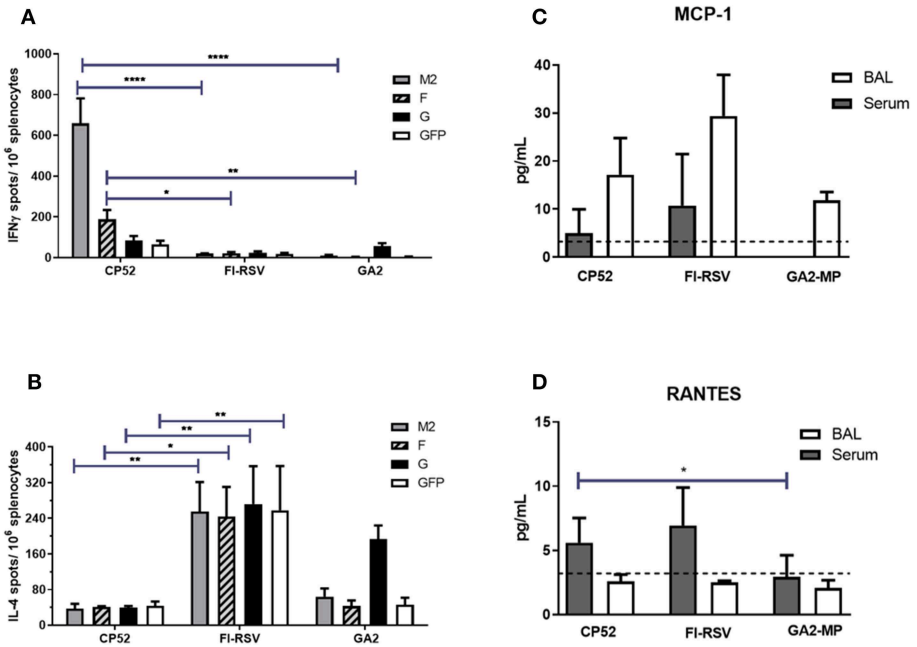

FIGURE 2 | Vaccine types and Th1/ Th2 memory responses. The number of $M2_{82-90}$, F_{51-66}, $G_{183-198}$, and $eGFP_{200-208}$-specific (irrelevant peptide control) IL4- and IFNγ- producing splenocytes were determined by ELISPOT harvested at 14 days post-boost vaccination. **(A)** IFNγ- producing splenocytes and **(B)** IL4-producing splenocytes. The data are presented as ELISPOTS/10^6 splenocytes. Three weeks after the boost mice were i.n. challenged with 10^6 PFU of A2. The level of **(C)** MCP1 and **(D)** RANTES were measured in sera and BAL supernatant by multiplex cytokine/chemokine assay and the data are presented as pg/mL of cytokine in BAL supernatant at day 3 post-challenge ($n = 4$–6 mice/group). The dashed line indicates the limit of detection (LOD) = 3.2 pg/ml. Error bars represent the SEM from $n = 4$ mice/group and results were considered significant with a $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{****}p \leq 0.0001$ as determined by two-way ANOVA and Bonferroni's test using GraphPad Prism ver. 8.0.

mice compared to all other vaccinated groups which further confirm the biased Th2-type cytokine response associated with FI-RSV vaccination.

The Type of Vaccination Is Linked With Different miR Expression Patterns

Evaluating the Th1- or Th2-type cytokine response using accompanying assays is not efficient for testing of multiple vaccine candidates; however, the examination of miR biomarkers as correlates of the host immune response may aid vetting of safe or disease vaccine types, and considerably accelerate RSV vaccine research. The pattern of miRs can be readily evaluated using PCR (126) in a variety of fluids and tissues (127–133), there is sequence conservation across species (134), and miRs regulate key immunological processes (135). These features can be used to determine baseline data that may differentiate safe from disease risk vaccine types to aid the development of vaccine candidates. Since the memory T cell response is pivotal to development of immunity and disease, we analyzed 84 key miRs connected with T cell function in the sera from vaccinated mice, pre- and post-RSV A2 challenge, at several time-points, e.g., 1-week post-prime/boost or 3d post-challenge. Total RNA was isolated from sera, used for cDNA synthesis, and miR expression was assayed using optimized primer-probes. Fold-changes in miR expression was plotted after normalization.

Analysis of the 84 miRs across all treatments identified miR expression signatures unique to prime-boost vaccination and RSV challenged mice, and those miR signatures conserved among all treatments (**Figure 3**). In general, each vaccine type, i.e., CP52, FI-RSV, or GA2-MP induced temporal and vaccine-specific miR expression patterns (**Figure 3**) where miR expression levels were heightened post-boost relative to prime and challenge. Given the differences in vaccine type and vaccination routes, differences were expected and emphasize miR patterns for their utility as potential vaccination biomarkers. Analogously, miR responses following A2 challenge of CP52 vaccinated mice compared to FI-RSV vaccinated mice correlated with safe vs. disease phenotypes of Th1- vs. Th2-type cytokines. Intergroup comparisons identified 58, 70 and 65 miRs differentially expressed in sera from CP52, FI-RSV, and GA2-MP vaccinated mice, respectively. Within groups, 8, 18, and 11 miRs were conserved between prime, boost and 3 days post-challenge for CP52, FI-RSV, and GA2-MP vaccinated mice, respectively. Of the miRs examined, let-7a-5p expression was upregulated >2SD in all vaccinated mice, thus this is likely not a biomarker for distinguishing vaccine-specific responses, but instead a general inflammatory biomarker (**Table 1**).

CP52 vaccination resulted in lower expression of miR-466f-3p and miR-467b-3p and did not induce any miRs. FI-RSV vaccination repressed miR-365-3p and miR-762 expression post-priming, but led to >2.0-fold induction of multiple miRs e.g.,

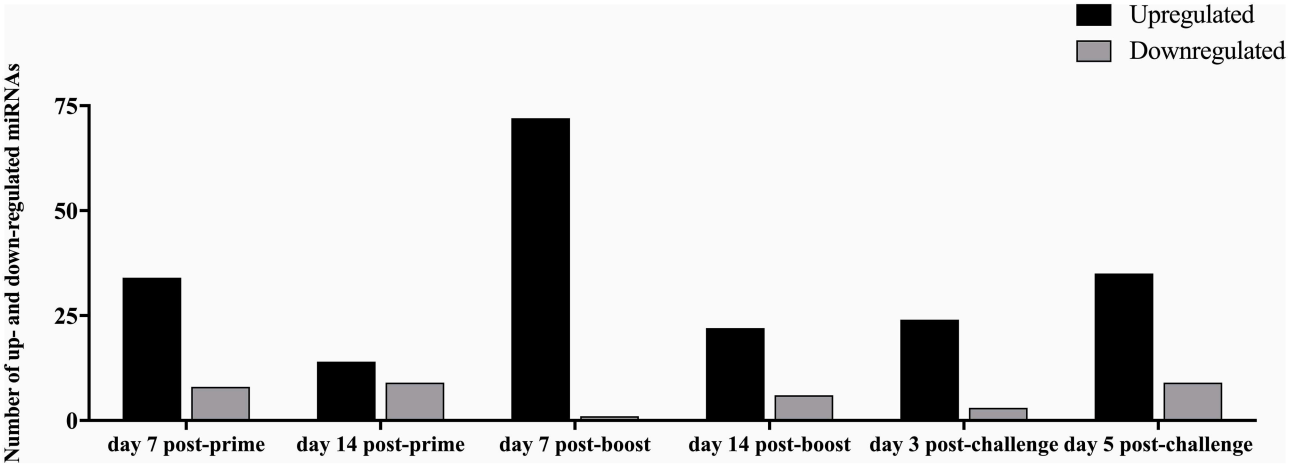

FIGURE 3 | The number of differentially expressed miRNAs during vaccination and post-RSV challenge. Sera miRNA profiles of vaccinated mice ($n = 4$/group) were evaluated at day 7 post-prime, day 14 post-prime, day 7 post-boost, day 14 post-boost, day 3 post-challenge, and day 5 post-challenge using a miRNA PCR array. The y-axis indicates the number of differentially expressed miRNAs. Significance was determined using a fold-change threshold of >2, the result was reported as a fold-upregulation. If the fold change was <0.5, the result was reported as a fold-downregulation.

TABLE 1 | Differentially expressed miRs expressed by the various vaccines.

Vaccine type	Number of miRs	Conserved miRs
GA2-MP	3	miR-26b-5p, miR-346-5p, miR-142a-5p
FI-RSV	9	miR-31-5p, miR-30c-5p, let-7d-5p, miR-326-3p, miR-93-5p, miR-30e-5p, miR-483-5p, let-7g-5p, miR-106b-5p
FI-RSV and GA2-MP	4	miR-20b-5p, let-7f-5p, miR-103-3p, miR-15a-5p
FI-RSV and CP52	4	miR-20a-5p, miR-195a-5p, miR-17-5p, miR-106a-5p
GA2-MP and CP52	3	miR-467f, miR-182-5p, let-7e-5p
GA2-MP, FI-RSV, and CP52	1	let-7a-5p

Common and unique differentially expressed miRs for GA2-MP, FI-RSV, and CP52 vaccinated mice conserved amongst post-prime (7 and 14 days), post-boost (7 and 14 days), and post-RSV challenge (3 and 5 days) are shown. miRNA expression levels are normalized by SN1/2/3/4/5/6 expression and n = 4 mice/group.

let-7d-5p, miR-326-3p, miR-331-3p, miR-16-5p, miR-103-3p, miR-30a-5p, miR-93-5p, miR-181a-5p, miR-101a-3p, miR-15b-5p, miR-15a-3p, miR-106b-5p, miR-142a-3p, miR-19a-3p, miR-30c-5p, miR-101b-3p, miR-25-3p, miR-31-5p, let-7i-5p, let-7g-5p post-prime (**Table 2**), and miR-326-3p, miR-145a-3p, miR-466f-3p, miR-24-3p, miR-181a-5p, miR-27a-3p, miR-125b-5p, miR-31-5p, miR-214-3p, miR-466f-5p, miR-365-3p, miR-146b-5p, miR-30c-5p, miR-466h-5p, miR-126a-3p post-boost (**Table 3**). In contrast, GA2-MP vaccination induced only miRs let-7e-5p and miR-26b-5p post-prime and miR-669f-3p, miR-142a-3p post-boost (**Table 3**). miR-466f-3p had divergent expression between CP52 and FI-RSV vaccinated mice, while miR-142a-3p showed early induction post-FI-RSV but was induced in GA2-MP vaccinated mice post-boost (**Table 3**).

miR profiling for each vaccine type post-challenge showed unique patterns and tempos of expression. For example, CP52 vaccinated mice had higher expression of let-7f-5p, miR-103-3p, miR-15b-5p, miR-101a-3p, miR-16-5p, miR-20a-5p, miR-106a-5p, miR-98-5p, miR-30a-5p, miR-17-5p, miR-195a-5p, miR-142a-5p, miR-181a-5p, miR-714, miR-31-5p, miR-101b-3p, miR-25-3p, let-7i-5p, miR-130b-3p, and reduced miR-182-5p post-challenge (**Table 4**). In contrast, FI-RSV vaccinated mice showed repression of miR-483-5p post-challenge, while GA2-MP vaccinated mice had induction of miR-145a-5p, miR-346-5p, miR-146b-5p, and repression of miR-669e-5p post-challenge. Thus, comparing between vaccine groups at 7 days post-prime, 7 days post-boost, or 3 days post-RSV challenge showed miRs profiles as related to safe or disease responses (**Table 5**).

miR Patterns Specific to the Vaccine Type

To determine if serum miR profiles were specific to a vaccine type e.g., live (CP52) i.n. delivered, or killed (FI-RSV) i.m. delivered, or subunit (GA2-MP) s.c. delivered, the miRs were evaluated from vaccinated mice at day 7 post-vaccination. Interestingly, we observed nearly twice as many miRs expressed in the sera at day 7 post-vaccination compared to 14 days post-vaccination (**Supplementary Table 1**). The sera miR profiles showed that CP52 vaccinated mice had decreased miR-466f-3p and miR-467b-3p expression at 7 days post-vaccination, whereas sera miR expression from FI-RSV-vaccinated mice had two downregulated miRs (miR-365-3p and miR-62) while sera from GA2-MP vaccinated mice expressed higher miRs, i.e., let-7e-5p and miR-26b-5p at 7 days post-prime (**Table 2**). Interestingly, miR-467f expression was downregulated for all vaccine types at day 7 post-vaccination. Additionally, several miRs were identified in all vaccinated mice, e.g., let-7a-5p, miR-142a-5p, and miR-20b-5p which were upregulated (**Table 1**). The results showed that several miRs were expressed specifically to CP52 (miR-466f-3p,

TABLE 2 | miRs induced by the vaccines at post-prime.

Time-point	Vaccine types	Upregulated miRs	Downregulated miRs
Day 7 post-prime	GA2-MP, FI-RSV, and CP52	let-7a-5p, miR-142a-5p, miR-20b-5p	miR-467f
	FI-RSV and GA2-MP	let-7f-5p, miR-15a-5p, miR-98-5p	None
	FI-RSV and CP52	miR-106a-5p, miR-195a-5p, miR-30e-5p, miR-20a-5p, miR-17-5p, miR-19b-3p	miR-182-5p, miR-466j, miR-483-5p
	GA2-MP	let-7e-5p, miR-26b-5p	None
	FI-RSV	let-7d-5p, miR-326-3p, miR-331-3p, miR-16-5p, miR-103-3p, miR-30a-5p, miR-93-5p, miR-181a-5p, miR-101a-3p, miR-15b-5p, miR-15a-3p, miR-106b-5p, miR-142a-3p, miR-19a-3p, miR-30c-5p, miR-101b-3p, miR-25-3p, miR-31-5p, let-7i-5p, let-7g-5p	miR-365-3p, miR-762
	RSV and CP52	None	miR-466f-3p, miR-467b-3p

Sera miR profiles of vaccinated mice (n = 4/group) were evaluated at day 7 post-prime using a miR PCR array. The relative expression levels of candidate miRs selected from the PCR array analysis were validated by RT-qPCR. Values are represented as fold-change/mock (PBS vaccinated/RSV A2 challenge). miR levels were normalized by RNU6B gene expression and all samples were run in duplicate. Fold-change was calculated using $2^{(-\Delta\Delta CT)}$ method. Differential expression was determined using the following criteria, if the fold change was >2, the result was reported as a fold-upregulation. If the fold-change was <0.5, the result was reported as a fold-downregulation.

TABLE 3 | miRs induced by the vaccines at post-boost vaccination.

Time-point	Vaccine type	Upregulated-miRs	Downregulated-miRs
Day 7 post-boost	GA2-MP, FI-RSV, and CP52	miR-195a-5p, miR-320-3p, let-7a-5p, miR-181b-5p, miR-672-5p, let-7e-5p, miR-17-5p, let-7c-5p, miR-714, let-7d-5p, let-7f-5p, miR-574-5p, miR-182-5p, miR-16-5p, miR-467f, miR-21a-5p, miR-130b-3p, miR-1187, miR-15b-5p, miR-26b-5p, miR-20a-5p, miR-184-3p, miR-762, miR-20b-5p, miR-25-3p, let-7i-5p, let-7g-5p	None
	FI-RSV and GA2-MP	miR-331-3p, miR-103-3p, miR-29a-3p, miR-30e-5p, miR-23b-3p, miR-101a-3p, miR-106b-5p, miR-142a-5p, miR-19b-3p, miR-19a-3p, miR-101b-3p, miR-30b-5p, miR-221-3p, miR-106a-5p, miR-30a-5p, miR-346-5p, miR-93-5p, miR-29b-3p, miR-466j, miR-15a-3p, miRR-15a-3p, miR-29c-3p	None
	GA2-MP and CP52	miR-223-3p, miR-669e-5p, miR-98-5p, miR-26a-5p, miR-155-5p	None
	FI-RSV and CP52	miR-483-5p, miR-1196-5p	
	GA2-MP	miR-669f-3p, miR-142a-3p	None
	FI-RSV	miR-326-3p, miR-145a-3p, miR-466f-3p, miR-24-3p, miR-181a-5p, miR-27a-3p, miR-125b-5p, miR-31-5p, miR-214-3p, miR-466f-5p, miR-365-3p, miR-146b-5p, miR-30c-5p, miR-466h-5p, miR-126a-3p	None

Sera miR profiles of vaccinated mice (n = 4/group) were evaluated at day 7 post-boost using a miR PCR array. The relative expression levels of candidate miRs selected from the PCR array analysis were validated by RT-qPCR. Values are represented as fold-change/mock (PBS vaccinated/RSV A2 challenge). miR levels were normalized by RNU6B gene expression and all samples were run in duplicate. Fold-change was calculated using $2^{(-\Delta\Delta CT)}$ method. Differential expression was determined using the following criteria, if the fold change was >2, the result was reported as a fold upregulation. If the fold-change was <0.5, the result was reported as a fold-downregulation.

TABLE 4 | miRs at post-RSV challenge.

Time-point	Vaccine type	Upregulated-miRs	Downregulated-miRs
Day 3 post-challenge	GA2-MP and RSV CP52	miR-467f, miR-184-3p	None
	GA2-MP	miR-145a-5p, miR-346-5p, miR-146b-5p	miR-669e-5p
	FI-RSV	None	miR-483-5p
	CP52	let-7f-5p, miR-103-3p, miR-15b-5p, miR-101a-3p, miR-16-5p, miR-20a-5p, miR-106a-5p, miR-98-5p, miR-30a-5p, miR-17-5p, miR-195a-5p, miR-142a-5p, miR-181a-5p, miR-714, miR-31-5p, miR-101b-3p, miR-25-3p, let-7i-5p, miR-130b-3p	miR-182-5p

Sera miR profiles of vaccinated mice (n = 4/group) were evaluated at day 3 post-challenge using a miR PCR array. The relative expression levels of candidate miRs selected from the PCR array analysis were validated by RT-qPCR. Values are represented as fold-change/mock (PBS vaccinated/RSV A2 challenge). miR levels were normalized by RNU6B gene expression and all samples were run in duplicate. Fold-change was calculated using $2^{(-\Delta\Delta CT)}$ method. Differential expression was determined using the following criteria, if the fold change was >2, the result was reported as a fold-upregulation. If the fold change was <0.5, the result was reported as a fold-downregulation.

TABLE 5 | Patterns of miR expression following prime, boost, and challenge.

	Fold-change					
	Prime		Boost		Challenge (day 3)	
	>2.0	≤0.5	>2.0	≤0.5	>2.0	≤0.5
CP52		**miR-466f-3p,** miR-467b-3p		NA	let-7f-5p, miR-103-3p, miR-15b-5p, miR-101a-3p, miR-16-5p, miR-20a-5p, miR-106a-5p, miR-98-5p, miR-30a-5p, miR-17-5p, miR-195a-5p, miR-142a-5p, miR-181a-5p, miR-714, miR-31-5p, miR-101b-3p, miR-25-3p, let-7i-5p, miR-130b-3p	miR-182-5p
FI-RSV	Let-7d-5p, miR-326-3p, miR-331-3p, miR-16-5p, miR-103-3p, miR-30a-5p, miR-93-5p, miR-181a-5p, miR-101a-3p, miR-15b-5p, miR-15a-3p, miR-106b-5p, **miR-142a-3p**, miR-19a-3p, miR-30c-5p, miR-101b-3p, miR-25-3p, miR-31-5p, let-7i-5p, let-7g-5p	miR-365-3p, miR-762	miR-326-3p, miR-145a-3p, **miR-466f-3p**, miR-24-3p, miR-181a-5p, miR-27a-3p, miR-125b-5p, miR-31-5p, miR-214-3p, miR-466f-5p, miR-365-3p, **miR-146b-5p,** miR-30c-5p, miR-466h-5p, miR-126a-3p	NA		miR-483-5p
GA2-MP	Let-7e-5p, miR-26b-p		miR-669-3p, **miR-142a-3p**	NA	miR-145a-5p, miR-346-5p, **miR-146b-5p**	miR-669e-5p

miR expression for the top 96 miRs associated with T cell development and function were evaluated in sera obtained day 7 post-prime, day 7 post-boost, or day 3 post-challenge using miR qPCR arrays. Fold-change was calculated using ΔΔCt method relative to several reference genes that showed no change in expression across time-points and treatments. All data represent >3 independent experiments. miRs common between time-points or treatments are highlighted in bold. NA, not applicable.

miR-467b-3p), to FI-RSV (let-7d-5p, miR-326-3p, miR-331-3p, miR-16-5p, miR-103-3p, miR-30a-5p, miR-93-5p, miR-181a-5p, miR-101a-3p, miR-15b-5p, miR-15a-3p, miR-106b-5p, miR-142a-3p, miR-19a-3p, miR-30c-5p, miR-101b-3p, miR-25-3p, miR-31-5p, let-7i-5p, let-7g-5p) and to GA-M2 vaccines types (let-7e-5p, miR-26b-5p).

miRs Patterns Induced by Post-Boost Vaccination and RSV Challenge

Serum miR profiles were examined to determine the miR profiles by the vaccine types post-boost (**Table 3**). Of 75 differentially expressed miRs evaluated (**Table 3**; **Figure 3**), the miRs commonly expressed were miR-195a-5p, miR-320-3p, let-7a-5p, miR-181b-5p, miR-672-5p, let-7e-5p, miR-17-5p, let-7c-5p, miR-714, let-7d-5p, let-7f-5p, miR-574-5p, miR-182-5p, miR-16-5p, miR-467f, miR-21a-5p, miR-130b-3p, miR-1187, miR-15b-5p, miR-26b-5p, miR-20a-5p, miR-184-3p, miR-762, miR-20b-5p, miR-25-3p, let-7i-5p, let-7g-5p which were induced by all vaccine types. As these miRs are commonly expressed it is likely their expression is linked to a general response, i.e., the pro-inflammatory response associated with vaccination. The serum miRs upregulated specific to CP52 vaccinated mice were miR-98-5p, miR-26a-5p, miR-155-5p, miR-223-3p, miR-669e-5p, for GA2-MP vaccinated mice miR-669f-3p and miR-142a-3p, and fifteen miRs were upregulated in the sera of FI-RSV vaccinated mice. Two miRs, miR-669f-3p and miR-142a-3p, were commonly expressed in CP52 and GA2-MP vaccinated mice, and 50 miRs were differentially expressed post-RSV challenge of vaccinated mice. All data are shown in **Supplementary Tables 2**, **3**. At day 3 post-RSV challenge, 24 miRs were upregulated for all vaccinated mice types compared to mock-vaccinated (**Table 4**). For CP52-vaccinated mice, several serum miRs were upregulated (e.g., let-7f-5p, miR-103-3p, miR-15b-5p, miR-101a-3p, miR-16-5p, miR-20a-5p, miR-106a-5p, miR-98-5p, miR-30a-5p, miR-17-5p, miR-195a-5p, miR-142a-5p, miR-181a-5p, miR-714, miR-31-5p, miR-101b-3p, miR-25-3p, let-7i-5p, miR-130b-3p), for FI-RSV-vaccinated mice only miR-483-5p was upregulated, and for GA2-MP vaccinated mice miR-145a-5p, miR-346-5p, and miR-146b-5p were upregulated. Interestingly, miR-184-3p was expressed in the sera by all vaccine groups suggesting that this miR is not vaccine-specific. The data from these studies is summarized in **Table 5**.

miRs and the Host Pathway

miRs act as rheostats to subtly regulate aspects of the host immune response to virus infection and vaccination (64). They fine-tune responses, adjust functions, and bolster or dampen immune operations to maintain homeostasis. The pattern of miR expression highlights their function, i.e., constrain or enhance responses in a temporal fashion. As this study examines the pattern and tempo of miRs expressed in the response to vaccination and challenge, it is not surprising to identify unique and common miR profiles and those that are differentially expressed during and after vaccination or challenge. A goal of

these studies is to determine if the miR expression patterns can be used to predict safe or unsafe responses to vaccination or challenge. Viral infection and vaccination induce inflammation and determining miR pathways that are induced or repressed in mice can help differentiate safe vs. disease risk vaccines. As we examined the miR expression pattern linked to the type of vaccine and the cytokine response to RSV vaccination and challenge, we analyzed the gene pathways that could be regulated by miRs that are induced or repressed following CP52, FI-RSV or GA2-MP priming, prime-boost, and RSV challenge using DIANA miRPath (117–119). The intersecting pathways were selected for examination having a *p*-value cutoff of $p < 0.05$ (**Table 5**). CP52 vaccination downregulated miR-466f-3p and miR467b-3p which is known to regulate genes of the TGFβ signaling which has been shown to have an important role in RSV replication and inflammation leading to lung injury, fibrosis, and remodeling (136–144). Additionally, these miRs are predicted to regulate cancer-related pathways (**Table 6**; **Supplemental Table 1**) which contain many of the top genes involved in cell cycle control, a feature live RSV infection is known to regulate (65, 102, 142, 145–147). FI-RSV priming induced miRs predicted to regulate mucin biosynthesis, axon guidance, and other pathways in cancer (**Table 2**) while GA2-MP primed miRs were predicted to regulate Lysine degradation, proteoglycan expression and function and FoxO signaling (**Table 6**). FoxO signaling pathway has been shown to regulate the innate immune pathways in respiratory epithelium following infection (148). Analysis of miR at 7d post-boost showed distinct miR pathway profiles among candidate vaccines. While both CP52 and GA2-MP boosting did not alter miR expression, FI-RSV boosting led to many deregulated miRs (**Table 5**). In particular, the fatty acid metabolism pathway is predicted to be regulated by these miRs. Fatty acid metabolism is essential for RSV replication (149). Additional pathways predicted to be regulated by miRs and linked to FI-RSV boosting include lysine degradation and steroid biosynthesis (**Table 6**). GA2-MP boosting affected miRs patterns predicted to regulate adherens junction signaling which are associated to disruption of the airway barrier during infection [**Table 7**; (150–152)]. miRs deregulated following RSV challenge in CP52 or GA2-MP vaccinated mice were predicted to regulate multiple pathways in fatty acid metabolism and pluripotency, likely related to cell cycle (**Table 8**). Additionally, TGFb and Hippo signaling pathways were also shown to likely regulated by the miR expression patterns (**Table 8**). The Hippo pathway is thought to be involved in modulating the potency of anti-viral response particularly in a nutritional deprivation state (153).

The results indicate that the miR profiles and their tempos of expression are adjusted to the type of vaccine and challenge, an effect linked to both non-specific responses (e.g., inflammation) and specific immune responses (e.g., T cell activation or memory). It is important to note that some miRs are unaffected by vaccination while others undergo a global up- or down-regulation upon vaccination or challenge. For reasons of brevity, we have focused on understanding those miR expression patterns induced ≥2SD above the control. It is important to note that several immune regulatory molecules are miR targets. Specifically, cytokines/chemokines are immune effector molecules and are integrated in the net responses to vaccination and challenge. miRs control the activation and integration of the pathways to support T cell responses while maintaining homeostasis. Additional information regarding the

TABLE 6 | Pathway analysis following priming by different vaccine types.

Treatment	Pathway name	*p*-value	# of miRs
Vaccine Type			
CP52	TGFb signaling pathway	3.55e-12	18
	Endometrial cancer pathway	1.04e-09	11
	Prostate cancer pathway	8.35e-08	12
FI-RSV	Mucin type O glycan biosynthesis	3.63e-13	6
	Axon guidance	9.07e-13	7
	Pathways in cancer	3.99e-11	7
GA2-MP	Lysine degradation	3.09e-14	2
	Proteoglycans in cancer	2.24e-06	2
	FoxO signaling pathways	1.44e-05	2

*Pathways regulated by miRs in **Table 1** were analyzed using DIANA miRPath (117–119) using the microT-CDS database. E-values designate statistical confidence ascribed to gene hits for pathways using standard hypergeometric distribution and meta-analysis statistics ($p < 0.05$, Fisher's exact t-test).*

TABLE 7 | Pathway analysis of miRs deregulated following boosting by different vaccine types.

	Pathway name	*p*-value	# of miRs
Post-boost			
CP52	No deregulated miR	NA	NA
FI-RSV	Fatty acid metabolism	1.33e-13	3
	Lysine degradation	2.13e-10	7
	Steroid biosynthesis	3.99e-06	4
GA2-MP	Adherens junction signaling	0.012	2

*Pathways regulated by miRs in **Table 1** were analyzed using DIANA miRPath (117–119) using the microT-CDS database. E-values designate statistical confidence ascribed to gene hits for pathways using standard hypergeometric distribution and meta-analysis statistics ($p < 0.05$, Fisher's exact t-test). NA, not applicable.*

TABLE 8 | Pathway analysis of miRs deregulated following challenge of vaccinated mice.

	Pathway name	*p*-value	# of miRs
Post-challenge			
CP52	Fatty acid metabolism	6.22e-16	15
	Prion diseases	9.06e-09	13
	Fatty acid degradation	3.12e-08	14
FI-RSV	No deregulated miR	NA	NA
GA2-MP	TGFb signaling	6.17e-05	2
	Hippo signaling pathways	0.000474	2
	Signaling pathways regulations pluripotency of stem cells	0.009	2

*Pathways regulated by miRs in **Table 1** were analyzed using DIANA miRPath (117–119) using the microT-CDS database. E-values designate statistical confidence ascribed to gene hits for pathways using standard hypergeometric distribution and meta-analysis statistics ($p < 0.05$, Fisher's exact t-test). NA, not applicable.*

miR host pathway analysis can be found in **Data Sheet 1** of the Supplementary Material section.

DISCUSSION

The development of safe and effective RSV vaccine candidates can be assisted by a better understanding of biomarker expression. Biomarkers may allow for the prediction of probable vaccine candidate outcomes. Additional analyses are needed to further aid decisions regarding vaccine candidates, but ways to improve RSV vaccine candidate selection has become paramount after more than 5 decades of unsuccessful research efforts. We hypothesized that assessment of miR profiles with general Th1/Th2 cytokine responses would enable correlations with safe, live vaccines (CP52), subunit vaccines (GA-M2), and disease-enhancing vaccines (FI-RSV) to help develop baselines for a better understanding of prospective RSV vaccine candidates. In this study, show vaccine-specific and temporal miRNA expression profiles relating to efficacy or vaccine-associated disease. We examined miR expression profiles of vaccinated mice pre- and post-RSV challenge were determined for 84 miRs associated with T cell responses and function. We showed that while both CP52 and FI-RSV vaccination induce a humoral response, only CP52 induced a neutralizing antibody response leading to reduction in RSV replication (**Figure 1E**). Further, splenocytes from CP52, FI-RSV, or GA2-MP vaccinated mice were stimulated with RSV-specific peptides then assayed for Th1-type or Th2-type cytokines. The cytokine and miR expression showed that $M2_{82-90}$ re-stimulation of splenocytes from CP52 but not FI-RSV vaccinated mice led to a strong induction of IFNg which is characteristic of a Th1 response. In contrast, peptide stimulation of splenocytes from FI-RSV vaccinated mice led to a strong induction of IL4, a cytokine characteristic of a Th2-type response. Peptide stimulation of splenocytes from GA2-MP vaccinated mice led to a higher number of $G_{183-198}$ IL4- and IFN-specific secreting cells, characteristic of a balanced Th1-/Th2-type response to the G protein. Taken together, these results led to the assessment that CP52 vaccination primes for a safe response while FI-RSV primes for disease following vaccination and GA2-MP primes for a mostly balanced cytokine response.

The miR PCR array showed differential expression of a conserved set of miRs across prime-boost vaccination and RSV-challenge, more specifically 11 miRNAs in GA2-MP vaccinated mice, 18 miRs in FI-RSV vaccinated mice, and 8 miRs in RSV CP52 vaccinated mice. Several of these miRs have been shown to participate in the regulation of the immune response, and in some cases are associated with RSV infection. FI-RSV vaccinated mice had let-7d-5p, let-7f-5p, and let-7g-5p miR expression at post-prime, post-boost, and post-challenge. GA2-MP vaccinated mice had similar results with let-7e-5p and let-7f expression. Members of the let-7 family target IL-6 expression, and has an extensive list of other experimentally validated targets including SOCS4, caspase-3, p27, TLR4, IL-13, and IL10 (101). Let-7 could be a mechanism of IL-6 regulation during RSV infection (101). RSV infection induces secretion of numerous pro-inflammatory cytokines, including type I and types II IFNs, TNFα, IL-12, and IL-6 (101, 154–156). Mice vaccinated with CP52 or GA2-MP induced differential miR-467f expression during prime-boost vaccination and post-challenge. Previous miR screens for respiratory viruses have not previously identified miR-467f; however, a microarray-based approach to evaluate the miR profile of HIV-associated nephropathy in a mouse model showed that treatment with rapamycin (an mTOR inhibitor) to halt disease progression induced upregulation of miR-467f expression (157). Interestingly, rapamycin inhibits RSV-induced mTOR activation and increases the frequency of RSV-specific CD8 T cells and RSV-specific memory T cell precursors in mice (158). Therefore, miR-467f may have a role in cellular immunity during vaccination and RSV infection. miR-106a-5p and miR-106b-5p expression levels were upregulated in FI-RSV vaccinated mice during prime-boost vaccination and post-challenge with RSV. Interestingly, allergic airway inflammation in mice has been associated with increased miR-106a expression and decreased IL-10, suggesting that miR-106a may regulate IL-10 expression and Th2-type responses (159, 160). Expression levels of miR-30c-5p and miR-30e-5p (from the miR-30/384-5p family) were upregulated in FI-RSV vaccinated mice at prime and boost-vaccination, and post-challenge. Upregulation of miR-30c-5p expression in the airway wall has been shown in a BALB/c mouse model of chronic asthma (161). The miR profiles identified for vaccine-induced protection and vaccine-enhanced disease appear to correlate with protective immune functions and airway inflammation, respectively. Although this study produced a list of miRs that may regulate RSV vaccine efficacy, additional studies are warranted to clarify the mechanisms behind how these miRs mediate host-virus interactions.

Overall, the results from these studies show that vaccine candidates associated safe or disease responses exhibited differential miR profiles following boosting which were higher in magnitude compared to priming or RSV challenge sera specimens. The results demonstrate that a considerable number of miRs are different between vaccine types, and a common set of miRs is expressed for all vaccine treatments. Pathway analysis of miR targets identified pathways correlated with inflammation particularly those that may contribute to airway inflammation, leukocyte recruitment and alveolar infiltration (12, 162, 163). The miR profiles from vaccinated mice were linked to cytokine phenotypes of protection or disease and appear to correlate with miRs that regulate protective immune functions or airway inflammation. Additional studies are warranted to validate miR phenotypes to determine the mechanisms of action linked to host gene regulation, and the associated immune response to determine their value as predictive biomarkers. These studies show that serum miR profiles may offer a proxy to assist vaccine development and facilitate a better understanding of vaccine studies.

ETHICS STATEMENT

Specific-pathogen-free, 6-to-8 weeks old female BALB/c mice (The Jackson Laboratory) were used. Mice were maintained in microisolator cages with sterilized water and food *ad libitum*. All experiments were performed in accordance with the guidelines

of the University of Georgia Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

LA, PJ, AB, and RT conceived, designed, performed, analyzed the experiments, and wrote the manuscript. RT contributed reagents, materials, and analysis tools.

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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.2019.02323/full#supplementary-material

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