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REVIEW

Modulation of the host immune response by respiratory syncytial virus proteins

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Respiratory syncytial virus (RSV) causes severe respiratory disease in both the very young and the elderly. Nearly all individuals become infected in early childhood, and reinfections with the virus are common throughout life. Despite its clinical impact, there remains no licensed RSV vaccine. RSV infection in the respiratory tract induces an inflammatory response by the host to facilitate efficient clearance of the virus. However, the host immune response also contributes to the respiratory disease observed following an RSV infection. RSV has evolved several mechanisms to evade the host immune response and promote virus replication through interactions between RSV proteins and immune components. In contrast, some RSV proteins also play critical roles in activating, rather than suppressing, host immunity. In this review, we discuss the interactions between individual RSV proteins and host factors that modulate the immune response and the implications of these interactions for the course of an RSV infection.

Keywords: RSV, virus, T cell, lung, vaccine

Introduction

Respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory tract infection in infants and young children worldwide (Nair *et al.*, 2010). Nearly all individuals become infected with RSV by two years of age (Glezen *et al.*, 1986). RSV represents a significant healthcare burden worldwide, causing three-four million yearly hospitalizations (Nair *et al.*, 2010). Approximately 66,000–199,000 RSV-associated deaths occur annually, with the majority of those mortalities occurring in developing countries (Nair *et al.*, 2010). RSV is also a significant cause of morbidity, hospitalization, and mortality in elderly and immunocompromised individuals (Falsey *et al.*, 2005; Jansen *et al.*, 2007). Additionally, reinfections with RSV are common throughout an individual's lifespan, although disease symptoms are generally milder compared to those observed in young children (Hall *et al.*, 2001).

Due to the significant public health burden caused by RSV infections worldwide, development of an effective vaccine is a high priority. The first RSV vaccine candidate was a formalin-inactivated RSV (FI-RSV) developed for clinical trial in the 1960's. However, vaccinated children under two years of age exhibited enhanced morbidity and mortality following a subsequent natural RSV infection. To this day, despite extensive efforts, there remains no licensed RSV vaccine.

Prophylactic treatment with the RSV fusion protein-specific monoclonal antibody Palivizumab significantly reduces disease severity and hospitalization rates in infants and young children (Feltes *et al.*, 2003). However, Palivizumab treatment comes at a considerable economic cost and is only recommended for use in either high-risk or premature infants with a gestational age of under 29 weeks (American Academy of Pediatrics Committee on Infectious and American Academy of Pediatrics Bronchiolitis Guidelines, 2014). Management of RSV-induced disease severe enough to require hospitalization primarily involves supportive care, including supplemental fluids and oxygen, nasogastric feedings, and nasal suction (Borchers *et al.*, 2013). Other treatments provided to hospitalized individuals include either bronchodilators or ribavirin, however their administration varies widely between treatment centers and conclusive evidence for their effectiveness is lacking (American Academy of Pediatrics Subcommittee on and Management of, 2006; Ventre and Randolph, 2007).

RSV induces a localized pulmonary infection and initiates a host inflammatory response that results in the recruitment of immune cells required for viral clearance. However, the host immune response also contributes to the severity of disease observed in RSV-infected individuals (Bohmwald *et al.*, 2015). Therefore, the immune response is vital for both control of RSV infection and initiation of disease. As such, RSV has evolved several mechanisms for evading the immune response to facilitate prolonged virus replication in the host. Paradoxically, RSV also employs strategies to cooperate with immune cells and help initiate the inflammatory response. Here, we will review the protective and pathogenic interactions individual RSV proteins have with components of the host immune response.

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RSV proteins

RSV is an enveloped virus belonging to the *Pneumovirus* genus in the *Paramyxoviridae* family. Its non-segmented, single-stranded, negative-sense RNA genome is 15.2 kb in length and contains 10 genes. In 3' to 5' order, the genome contains two nonstructural (*NS1* and *NS2*), nucleocapsid (*N*), phosphoprotein (*P*), matrix (*M*), small hydrophobic (*SH*), attachment glycoprotein (*G*), fusion glycoprotein (*F*), *M2*, and polymerase (*L*) genes (Collins *et al.*, 2013). Each gene encodes for its corresponding mRNA that in turn encodes for a single protein, with the exception of *M2*. The M2 mRNA contains two overlapping reading frames, which encode two individual proteins: M2-1 and M2-2 (Collins *et al.*, 1986). RSV therefore encodes 11 known proteins, of which nine are structural proteins that make up the virion. The RSV virion consists of a nucleocapsid surrounded by an outer lipid membrane that is derived from the host cell plasma membrane during viral budding. Three virally-encoded proteins (SH, G, and F) are transmembrane proteins contained within the envelope, while M is present along the inner face of the envelope. The remaining structural proteins (N, P, M2-1, M2-2, and L) are involved in nucleocapsid structure and/or RNA synthesis, while the nonstructural proteins (NS1 and NS2) are not significantly packaged within the assembled virion (Collins and Crowe, 2007).

NS1 and NS2 are small proteins of 139 and 124 amino acids, respectively. As they are the first genes encoded by the genome, NS1 and NS2 mRNAs are abundant viral products produced early following infection (Collins and Wertz, 1983). While NS1 and NS2 are not essential for RSV replication, deletion of either gene significantly attenuates RSV replication *in vitro* and *in vivo* in cotton rats, chimpanzees, and African green monkeys (Jin *et al.*, 2000, 2003; Teng *et al.*, 2000). NS1 and NS2 are able to form both homo- and heteromers, corresponding to their ability to function both independently and cooperatively to exert synergistic effects (Swedan *et al.*, 2009, 2011). The primary function of NS1 and NS2 is to antagonize the type I interferon (IFN) antiviral response, but they also interfere with the host immune response in other ways, as described in detail below.

The G protein is a large 298 amino acid protein that is involved in viral attachment and also serves as a major target for neutralizing antibodies. G is not required for RSV replication *in vitro*, as recombinant RSV strains lacking the G gene replicate to similar levels as wild-type (WT) RSV (Teng *et al.*, 2001). G is expressed in two forms: a transmembrane form (mG) and a secreted form (sG). sG accounts for as much as 80% of the total G protein released during the early stages of RSV infection (Hendricks *et al.*, 1988). mG contains an N-terminal cytoplasmic tail adjacent to a hydrophobic signal anchor that serves as its transmembrane domain. sG is truncated near the N-terminus so it lacks the first 65 amino acids, including the transmembrane domain (Roberts *et al.*, 1994). The ectodomain of G contains two mucin-like domains that vary widely in sequence across RSV isolates and are heavily glycosylated (Johnson *et al.*, 1987). Between the two mucin-like domains lies a highly conserved region of 13 amino acids, which contains both a CX3C motif and a cysteine rich region (GCRR) of four cysteine residues connected by two disulfide bonds (Johnson *et al.*, 1987; Rueda *et al.*, 1994). Both the CX3C motif and the GCRR are critical for the G protein's modulation of the host immune response, as will be discussed below.

The F protein drives viral infection by mediating fusion of the virus with the target cell membrane and syncytia formation between neighboring cells. Given its obligatory role in viral entry, F is essential for RSV replication (Heminway *et al.*, 1994). F is initially formed as an inactivate precursor protein, F_0, that becomes activated through proteolytic cleavage by a furin-like protease encoded by the host cell (Collins and Mottet, 1991; Bolt *et al.*, 2000). The furin-like protease cleaves F_0 twice to generate three individual polypeptides: F_2 (109 amino acids), p27 (27 amino acids), and F_1 (438 amino acids). F_2 and F_1 remain connected by two disulfide bonds following cleavage, and make up the activated form of F (Gruber and Levine, 1983; Day *et al.*, 2006). In contrast to the G protein, F is highly conserved with greater than 90% sequence identity between RSV isolates from either A or B RSV subgroups (Beeler and van Wyke Coelingh, 1989). Combined with its critical function during RSV replication, the F protein's high sequence conservation make it an ideal target for both neutralizing antibodies and small molecule inhibitors (Costello *et al.*, 2012; McLellan *et al.*, 2013).

SH is a small 64 amino acid transmembrane protein that is incorporated into mature virions at very low levels (Rixon *et al.*, 2004). SH is dispensable for RSV replication in cell culture, and recombinant RSV strains lacking SH (ΔSH) replicate more efficiently that WT virus in some cell lines (Bukreyev *et al.*, 1997; Karron *et al.*, 1997; Jin *et al.*, 2000). However, SH appears to play some role in RSV replication *in vivo*, as replication of ΔSH was attenuated in the upper and lower respiratory tract of mice and chimpanzees, respectively (Bukreyev *et al.*, 1999; Whitehead *et al.*, 1999). SH forms a pore-like structure in the membrane of infected cells, which exhibits ion-channel-like activity (Carter *et al.*, 2010; Gan *et al.*, 2012). While the exact role of SH ion-channel activity in RSV infection remains unclear, it appears to function as a cation-selective channel that alters membrane permeability (Gan *et al.*, 2012; Triantafilou *et al.*, 2013). The SH protein's ion-channel activity may have important implications for the immune response, as described below.

The N protein is a 391 amino acid protein that encapsidates both the viral genome and its positive-sense counterpart, the antigenome. This encapsidation protects the genome and may also reduce recognition by host pattern recognition receptors. The N protein's structure allows for the formation of flexible templates for the RNA polymerase. Individual N monomers can bind 7 nucleotides of RNA, with the RNA groove located at the hinge between N- and C-terminal domains of the monomer (Tawar *et al.*, 2009). The orientation of multiple monomers together in the same direction allows the polymerase access without disassembling the helix, further protecting the genomic RNA (Collins *et al.*, 2013).

While the remaining RSV proteins serve important functions during infection, they do not significantly interfere with the host immune response and will therefore only briefly be discussed here. The M protein lines the inner side of the

virion envelope and is critical for RSV assembly in host cells, as mature virions cannot form in its absence (Mitra *et al.*, 2012). The L protein is the major subunit of the RNA-dependent RNA polymerase and contains the catalytic domain. The P protein serves as an important co-factor for the polymerase complex and also assists in protecting the RSV genome by delivering free N protein to genomes or antigenomes that are not encapsidated (Grosfeld *et al.*, 1995; Castagne *et al.*, 2004). M2-1 functions as an essential transcription factor that binds RNA and may preferentially bind RSV mRNAs (Cartee and Wertz, 2001; Blondot *et al.*, 2012). In contrast, M2-2 appears to negatively regulate RNA synthesis, as recombinant RSV strains lacking M2-2 exhibit increased and prolonged transcription compared to WT RSV (Bermingham and Collins, 1999).

Host immune response to RSV

The innate immune response is critical for early control of RSV, prior to the induction of the adaptive response. The innate antiviral response is initiated in the airways following infection of ciliated epithelial cells, alveolar macrophages, and dendritic cells (DCs). Viral particles are recognized by host pattern recognition receptors (PRRs) on infected cells, including toll-like receptors (TLRs), retinoic acid-inducible gene-I-(RIG-I-) like receptors (RLRs), and nucleotide-binding oligomerization domain-(NOD-) like receptors (NLRs) (Zeng *et al.*, 2012). Signaling through these receptors induces the production of several chemokines and pro-inflammatory cytokines, including IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, IL-1β, IL-6, IL-8, and TNF-α (McNamara *et al.*, 2005; Yoon *et al.*, 2007). Expression of these molecules leads to the recruitment and activation of important innate cell subsets, including monocytes, macrophages, natural killer (NK) cells, neutrophils, and DCs. Additionally, infected cells produce the type I IFNs, IFN-α, and IFN-β, which give rise to an antiviral state in the airways by inducing transcription of many IFN-stimulated genes (ISGs) (Sen and Sarkar, 2007).

Following the initial innate response, the adaptive immune response is initiated and plays an important role in viral clearance as well as protection against subsequent RSV infection (Varga and Braciale, 2013). DCs are critical for activation of the host adaptive response. Following uptake of viral antigen, airway-resident DCs migrate to the lung draining lymph nodes and activate RSV-specific CD4 and CD8 T cells. CD8 T cells are critical for viral clearance in both primary and secondary RSV infections (Cannon *et al.*, 1987; Graham *et al.*, 1991). CD4 T cells also play an important role during RSV infection through their ability to differentiate into multiple subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs). Th1 CD4 T cells support viral clearance through their production of IFN-γ, while Th2 cells that produce IL-4, IL-5, and IL-13 are associated with more severe RSV disease (Lukacs *et al.*, 2006; Boyoglu-Barnum *et al.*, 2014). IL-17-producing Th17 cells are associated with mucus production and airway hyperreactivity, however, they may also contribute to viral clearance (Fujisawa *et al.*, 2011; Mukherjee *et al.*, 2011; Zhang *et al.*, 2016). Tregs are crucial for limiting lung inflammation during RSV infection, as there is significantly reduced immune cell infiltration in the lungs of mice in the absence of Tregs (Fulton *et al.*, 2010; Lee *et al.*, 2010; Durant *et al.*, 2013). In addition to T cells, B cells are also vital for the adaptive immune response to RSV through the production of neutralizing antibodies specific to the F and G proteins. In humans, both serum IgG and mucosal IgA antibodies play important roles in protection against subsequent RSV infection. However, both anti-RSV IgG and IgA titers wane rapidly over time in humans. Neutralizing antibody titers were undetectable in

Table 1. Summary of RSV protein interactions with the host immune response

RSV protein	Modulation of the immune response	References
NS1/NS2	Inhibits type I IFN induction	Kotelkin *et al.* (2000), Spann *et al.* (2004), Munir *et al.* (2008)
	Prevents transcription of IFN-stimulated genes	Lo *et al.* (2005), Ramaswamy *et al.* (2006)
	Suppresses type III IFNs	Spann *et al.* (2004)
	Inhibits activation and proliferation of protective T cell populations ($CD103^+$ CD8 T cells & Th17 cells)	Munir *et al.* (2011)
	Suppresses DC maturation and cytokine production	Munir *et al.* (2008)
	Prevents apotosis of infected cells	Bitko *et al.* (2007)
G	Mimics fractalkine to reduce immune cell migration	Tripp *et al.* (2001), Harcourt *et al.* (2006)
	Inhibits DC maturation and cytokine production	Johnson *et al.* (2012b)
	Suppresses the activity of Tregs	Boyogla-Barnum *et al.* (2014)
	Promotes Th2 cells and suppresses Th1 cells	Johnson *et al.* (1998), Tripp *et al.* (1999), Boyogla-Barnum *et al.* (2014)
	Enhances the CD8 T cell response	Bukreyev *et al.* (2006), Melendi *et al.* (2011), Boyogla-Barnum *et al.* (2014)
F	Induces production of pro-inflammatory cytokines	Kurt-Jones *et al.* (2000)
	Promotes neutrophil extracellular trap formation	Funchal *et al.* (2015)
SH	Reduces NF-κB promoter activty downstream of TNF-α	Fuentes *et al.* (2007)
	Mediates NLRP3 inflammasome activation	Triantafilou *et al.* (2013)
	Prevents apotosis of infected cells	Fuentes *et al.* (2007)
N	Interferes with the immunological synapse to interfere with CD4 T cell activation	Cespedes *et al.* (2014)

25-50% of young children, while over 75% of adults observed a four-fold reduction in neutralizing anti-RSV IgG titers within a year following natural RSV infection (Murphy *et al.*, 1986; Brandenburg *et al.*, 1997; Falsey *et al.*, 2006). Therefore, the RSV antibody response elicited does not confer lifelong protection, contributing to the ability of RSV to cause repeated infections.

Modulation of the host immune response by RSV proteins

NS1/NS2

NS1 and NS2 modulate the host immune response to RSV infection in a wide variety of ways, both independently and cooperatively (summarized in Table 1). Their primary and most well characterized function is the inhibition of the type I IFNs IFN-α and IFN-β. NS1 and NS2 cooperate to suppress the mRNA and protein of IFN-α and IFN-β in A549 human epithelial cells and human monocyte-derived macrophages (Spann *et al.*, 2004). NS1 alone is the primary driver of type I IFN suppression in human DCs (Munir *et al.*, 2008). In contrast, NS2 dominates the suppression of IFN-α by murine fibroblasts (Kotelkin *et al.*, 2006). As type I IFNs are critical antiviral cytokines, their inhibition by NS1 and NS2 is crucial for RSV replication and pathogenesis.

One of the primary pathways by which RNA viruses induce type I IFNs involves signaling through the PRR RIG-I. In this pathway, RIG-I recognizes viral RNA products, then interacts with the mitochondrial protein MAVS, ultimately resulting in the phosphorylation and activation of the transcription factor IRF3 by IRF3 kinases (Vareille *et al.*, 2011). Activated IRF-3 dimerizes, translocates to the nucleus, and works together with other transcription factors, including NF-κB, to induce transcription of IFN-β (Vareille *et al.*, 2011). NS1 and NS2 have been shown to target multiple areas within this pathway to inhibit IFN-β production. First, NS2 interacts with the N-terminal CARD domain of RIG-I, while NS1 binds with MAVS (Ling *et al.*, 2009; Boyapalle *et al.*, 2012). Together, these interactions prevent RIG-I and MAVS from binding together, resulting in the inhibition of the downstream activation of IRF-3. Phosphorylation and nuclear translocation of IRF-3 is inhibited by NS1 and NS2, highlighting the importance of the interactions between NS1 and NS2 with RIG-I and MAVS (Spann *et al.*, 2005; Ling *et al.*, 2009). Alternatively, NS1 can also inhibit IRF-3 by directly binding to IRF-3 and its transcriptional coactivator CBP/p300 (Ren *et al.*, 2011). As CBP/p300 is required for the DNA-binding activity of IRF-3, disruption of this interaction by NS-1 prevents IRF-3 from binding to and activating transcription from the IFN-β promoter (Suhara *et al.*, 2002; Ren *et al.*, 2011).

Following their secretion, type I IFNs signal through the IFN-α-receptor (IFNAR) to induce the expression of many antiviral genes. IFN-α and IFN-β bind IFNAR, which multimerizes and becomes phosphorylated by the tyrosine kinases Tyk2 and Jak1 (Hoffmann *et al.*, 2015). Phosphorylated IFNAR becomes a docking site for signaling proteins STAT2 and STAT1, which become activated, associate with IRF-9, and translocate into the nucleus to activate the transcription of several ISGs (Hoffmann *et al.*, 2015). NS1 and NS2 have been shown to prevent the transcription of ISGs by inhibiting STAT2. NS1 and NS2 act cooperatively to reduce the levels of STAT2 total protein and phosphorylated STAT2, although both NS1 and NS2 can inhibit STAT2 to some degree alone (Lo *et al.*, 2005; Ramaswamy *et al.*, 2006; Elliott *et al.*, 2007). The observed inhibition of STAT2 occurs through proteasomal degradation, as the addition of protease inhibitors prevents NS1/NS2-mediated reduction of STAT2 (Elliott *et al.*, 2007). NS1 can interact with E3 ubiquitin ligase proteins Elongin C and Cul2, suggesting that NS1 may act as an E3 ubiquitin ligase to degrade STAT2 (Elliott *et al.*, 2007). NS1-mediated STAT2 degradation is dependent on Cul2 and another E3 ubiquitin ligase complex component Rbx1, supporting this hypothesis (Elliott *et al.*, 2007). NS2 may also assist in STAT2 degradation as a ubiquitin ligase, as NS2 was shown to have ubiquitin and monoubiquitin activity of target proteins (Whelan *et al.*, 2016). Downstream of STAT2, NS1 and NS2 have also been shown to inhibit IFN-dependent signaling by reducing the expression of ISGs (Lo *et al.*, 2005; Ramaswamy *et al.*, 2006).

In addition to the inhibition of type I IFNs, NS1 and NS2 also suppress the functions of many immune cell types including DCs, CD8 T cells, and CD4 T cells. NS1, alone or in combination with NS2, can suppress the maturation of human DCs by inhibiting their upregulation of the costimulatory molecules CD80 and CD86 and receptors critical for DC-T cell interactions, including CD38, CD54, and CD83 (Munir *et al.*, 2008). Additionally, NS1 and NS2 cooperate to suppress DC cytokine and chemokine expression, including the production of IL-6, IL-8, RANTES, and TNF-α (Munir *et al.*, 2008). However, antibody blockade of IFNAR ameliorates the suppressive effects of NS1 and NS2 on DCs, suggesting that they occur, at least in part, as a consequence of type I IFN inhibition (Munir *et al.*, 2008). Another consequence of type I IFN suppression is the inhibition of RSV-specific CD8 T cells by NS2 following RSV infection in mice, which is abrogated in STAT1 knockout mice (Kotelkin *et al.*, 2006). Using cocultures of human DCs and T cells, an IFN-independent role of NS1-mediated inhibition of specific T cell subsets has also been identified (Munir *et al.*, 2011). NS1 inhibits CD8 T cells positive for the tissue-residency marker CD103 by reducing their numbers and suppressing their IFN-γ production and expression of the degranulation marker CD107a (Munir *et al.*, 2011). NS1 also reduces the frequency of IL-17-producing CD4 T cells, or Th17 cells (Munir *et al.*, 2011). Therefore, NS1 inhibits protective cell subsets, as CD8 T cells are critical for RSV clearance, and IL-17 reduces viral load in the lungs of mice (Graham *et al.*, 1991; Zhang *et al.*, 2016). Lastly, NS1 enhances IL-4-producing Th2 CD4 T cells, suggesting that NS1 promotes a Th2 bias, which has been implicated in RSV-induced disease (Munir *et al.*, 2011).

NS1 and NS2 have also been shown to mediate other general antiviral activities. Similar to type I IFNs, NS1 and NS2 are also able to suppress the type III IFNs IFN-λ1, -2, and -3 following RSV infection to inhibit their antiviral activity (Kotenko *et al.*, 2003; Spann *et al.*, 2004). NS1 and NS2 also suppress the antiviral response of host cells by preventing the apoptosis of infected cells (Bitko *et al.*, 2007). Expressed

early following RSV infection, NS1 and NS2 activate pro-survival molecules of the AKT pathway, including AKT, PDK, and GSK (Bitko *et al.*, 2007). The AKT pathway prevents apoptosis of infected cells in part through the activation of NF-κB, which is also activated by NS1 and NS2 (Spann *et al.*, 2005; Bitko *et al.*, 2007). Therefore, NS1 and NS2 are critical for suppressing antiviral functions of the host immune response, which promotes sustained RSV replication.

G

Like NS1 and NS2, the RSV G protein interacts with the immune system in a variety of ways, mostly to evade the host's immune response (Table 1). Structural features of the G protein allow it to interact with immune cells and disrupt cell migration to the lung. The G protein's CX3C motif is structurally similar to the chemokine fractalkine (CX3CL1), which induces leukocyte chemotaxis via its receptor CX3CR1 (Fong *et al.*, 1998). Similar to CX3CL1, the G protein's CX3C motif also binds to CX3CR1 and mediates leukocyte migration (Tripp *et al.*, 2001). Addition of recombinant G protein or its CX3C motif alone reduces $CX3CR1^+$ CD4 and CD8 T cell recruitment to the lung following RSV infection, supporting the hypothesis that the G protein's CX3C motif functions as a CX3CL1 mimic (Harcourt *et al.*, 2006). Accordingly, CX3CR1-deficient mice exhibited reduced $NK1.1^+$ NK cell, $CD11b^+$ macrophage, and $R86\text{-}8C5^+$ neutrophil migration consistent with previous studies with viruses lacking the G gene or with antibody blockade of the CX3CL1-CX3CR1 interaction (Tripp *et al.*, 1999; Haynes *et al.*, 2003; Johnson *et al.*, 2012a). However, contradictory to a previous study in which CD4 and CD8 T cell recruitment was altered by the CX3C motif, CX3CR1-deficient mice did not have any alterations in adaptive immune cell recruitment (Harcourt *et al.*, 2006; Johnson *et al.*, 2012a). Thus, further studies are needed to clarify the role of the G protein's CX3C motif in cell migration to the lung.

sG also plays a critical role in the modulation of the host immune response. sG was originally hypothesized to serve as an antigen decoy molecule for virus-neutralizing antibodies, which would provide a mechanism for immune evasion (Collins *et al.*, 2001). sG does appear to serve as a decoy for neutralizing antibodies *in vitro*, as a recombinant RSV strain mutated to express only mG was more efficiently neutralized by RSV G-specific mouse serum than a WT RSV strain expressing both mG and sG (Bukreyev *et al.*, 2008). However, sG's ability to serve as an antigen decoy is not a significant mechanism by which RSV evades the host immune response, as sG does not function in this manner *in vivo* (Bukreyev *et al.*, 2008). In mice, sG was capable of preventing both G-specific and F-specific antibodies from reducing virus replication, suggesting that sG acts through an alternative mechanism *in vivo* to sustain RSV replication (Bukreyev *et al.*, 2008). Instead of functioning as an antigen decoy, sG prolongs virus replication *in vivo* by inducing Fc-receptor-expressing leukocytes to produce pro-inflammatory cytokines, including IL-6, MIP-1α, RANTES, and IFN-γ (Bukreyev *et al.*, 2008). Similarly, sG was required to induce IL-6 production by human monocytes, which specifically required the GCRR to mediate its effects (Polack *et al.*, 2005). However, sG suppressed the production of inflammatory cytokines IL-8 and RANTES from human lung epithelial cells, suggesting that sG may have different effects on different cell types in humans (Arnold *et al.*, 2004).

The G protein can also interact with several immune cell types to inhibit or enhance their number and/or function. G binds to the C-type lectins DC-SIGN and L-SIGN on DCs to suppress DC maturation as measured by the markers CD209 and CD163 (Johnson *et al.*, 2012b). DC-SIGN and L-SIGN binding of G also inhibits the production of IFN-α, MIP-1α, and MIP-1β from DCs, particularly from plasmacytoid DCs (pDCs) (Johnson *et al.*, 2012b). G may also modulate the activity of Tregs, as prophylactic treatment with the anti-G antibody 131-2G slightly increased the frequency of $Foxp3^+$ $IL\text{-}10^+$ CD4 T cells (Boyoglu-Barnum *et al.*, 2014). In contrast, 131-2G treatment decreased the frequency of CD8 T cells following RSV infection, suggesting that G enhances the CD8 T cell response, consistent with previous studies (Bukreyev *et al.*, 2006; Melendi *et al.*, 2011; Jorquera *et al.*, 2013; Boyoglu-Barnum *et al.*, 2014).

RSV G also modulates host immunity by altering the inflammatory cytokine response. G is critical for the induction of the Th2 cytokine bias associated with RSV-induced disease. G exerts the Th2 bias by promoting IL-4- and IL-5-producing Th2 cells and suppressing IFN-γ-producing Th1 cells (Hancock *et al.*, 1996; Bembridge *et al.*, 1998; Johnson *et al.*, 1998; Tripp *et al.*, 1999; Harcourt *et al.*, 2006; Boyoglu-Barnum *et al.*, 2014). This correlates to an increase in $GATA\text{-}3^+$ cells and a decrease in $T\text{-}bet^+$ cells, the lineage-defining transcription factors for Th2 and Th1 cells, respectively (Boyoglu-Barnum *et al.*, 2014). In DCs, G is also able to suppress type I and type III IFNs through its CX3C motif (Shingai *et al.*, 2008; Chirkova *et al.*, 2013). Additionally, G is able to suppress the induction of ISGs, as ISG15 expression is reduced by G in both mouse and human epithelial cells (Moore *et al.*, 2008; Oshansky *et al.*, 2009).

F

Unlike the NS1, NS2, and G proteins whose interactions are primarily intended to evade host immunity, the majority of the F protein's interactions with the host serve to promote the immune response to RSV infection (Table 1). Immediately following infection, F interacts with PRRs TLR4 and CD14 to induce the production of pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α (Kurt-Jones *et al.*, 2000). TLR4's recognition of F may also promote the trafficking of NK cells and $CD14^+$ monocytes to the airways, the cytotoxic function of NK cells, and the expression of IL-12, as TLR4-deficient mice are impaired in these functions compared to mice expressing TLR4 (Haynes *et al.*, 2001). Along these lines, TLR4 polymorphisms in the human genome correlate to an increased propensity to develop severe disease following RSV infection, supporting the hypothesis that the F protein's interaction with TLR4 is critical for inducing the innate immune response (Tal *et al.*, 2004; Puthothu *et al.*, 2006).

Recent work has demonstrated that the F protein signals through TLR4 to induce the formation of neutrophil extracellular traps (NETs) by human neutrophils (Funchal *et al.*, 2015). Similar to prototypical NETs, the NETs induced by F protein are composed of chromatin fibers and antimicro-

biocidal products including neutrophil elastase and myeloperoxidase (Brinkmann *et al.*, 2004; Funchal *et al.*, 2015). NET formation has been proposed as a role for viral control in studies with HIV-1 and poxvirus (Saitoh *et al.*, 2012; Jenne *et al.*, 2013). However, as evidenced by influenza virus, NET formation induced by respiratory pathogens may cause more extensive damage in the respiratory tract and not be beneficial to the host (Narasaraju *et al.*, 2011). *In vivo* studies evaluating NET formation in RSV infection are lacking, however, so whether NETs produced in the respiratory environment following an RSV infection are protective versus pathogenic requires further study.

SH

Although it is dispensable for virus replication, SH has been implicated in interfering with the host immune response in a number of ways (Table 1). First, SH has been shown to prevent the apoptosis of infected cells, as a ΔSH RSV strain accelerated apoptosis of both mouse fibroblast and human lung epithelial cells compared to WT RSV (Fuentes *et al.*, 2007). This prevention of apoptosis was also shown using recombinant parainfluenza virus 5 (PIV5) strains either lacking SH (rPIV5ΔSH) or expressing only the RSV SH protein (rPIV5ΔSH-RSV SH). While rPIV5ΔSH induced significantly increased cell death and cytopathic effect (CPE) in infected mouse fibroblasts and bovine epithelial cells, rPIV5ΔSH-RSV SH infected cells were protected from cell death and CPE. Additionally, bovine epithelial cells that were coinfected with rPIV5ΔSH and rPIV5ΔSH-RSV SH were also protected from cell death and CPE, supporting the notion that RSV SH protein prevents apoptosis of infected cells (Fuentes *et al.*, 2007).

The SH protein also interferes with critical signaling pathways following RSV infection, including the NF-κB pathway and NLRP3 inflammasome activation. Mouse fibroblasts transfected with GFP-tagged RSV SH protein had significantly reduced TNF-α-induced NF-κB promoter activity compared to GFP transfected controls, suggesting that SH inhibits the NF-κB signaling pathway (Fuentes *et al.*, 2007). More recently, SH has been shown to be an important mediator of NLRP3 inflammasome activation (Triantafilou *et al.*, 2013). IL-1β and caspase 1 production was significantly reduced following infection with a ΔSH RSV strain compared to WT RSV (Triantafilou *et al.*, 2013). Interestingly, RSV-induced IL-1β production was also reduced in the presence of hexamethylene amiloride, EIPA, or benzamil ion channel inhibitors, suggesting that the ion channel activity of SH is critical for the SH-mediated activation of the inflammasome (Triantafilou *et al.*, 2013).

N

While it is most commonly known for its role in protecting the viral RNA, N has recently been reported to localize to the surface of infected cells where it can inhibit T cell activation following RSV infection (Cespedes *et al.*, 2014). Soluble N protein impaired the activation of naïve OT-II transgenic CD4 T cells by OVA-pulsed DCs *in vitro* as measured by a reduced upregulation of surrogate activation marker CD69. Additionally, DCs transfected with lentivirus expressing RSV N protein induced significantly less CD69 upregulation and IL-2 and IFN-γ production from OT-II CD4 T cells following coculture (Cespedes *et al.*, 2014). Alternatively, the authors used an *in vitro* supported lipid bilayer system coated with peptide-MHC complexes, ICAM-1, and the RSV N protein to mimic an infected antigen presenting cell surface. Using this system, mature immunological synapse formation between CD4 T cells and the lipid bilayer was significantly reduced compared to controls lacking the N protein, supporting the role for surface N protein in the inhibition of T cell activation (Cespedes *et al.*, 2014). While the mechanism behind the N protein's ability to inhibit T cell activation requires further study, it is likely that N interacts with one or more components of the TCR microcluster complex, such as CD3, CD4, CD28, or the TCR itself, which is present on the surface of the T cell. This hypothesis is supported by the findings that N does not colocalize or move with the peptide-MHC complex on the lipid bilayer. Instead, N colocalizes with the TCR complex on the T cell and can even induce TCR assembly in the absence of peptide-MHC complexes (Cespedes *et al.*, 2014). Future studies identifying which protein N interacts with will be critical for identifying a mechanism mediating the N protein's inhibitory activity. Additionally, whether the N protein also inhibits the activation of CD8 T cells or is specific to CD4 T cells remains unexplored.

Concluding discussion

The RSV NS1, NS2, G, SH, and N proteins are all capable of suppressing the host immune response in a variety of ways. But are these immune interactions with RSV proteins strictly for the benefit of acute virus replication, or do they have long-term implications on the host immune response? In particular, could these interactions ultimately play a role in the poor long-term memory that is generated in RSV-infected individuals? While RSV proteins do not seem to directly suppress the antibody response, these interactions may interfere with the development of long-lived T cell memory. Protective T cell memory may be impacted through the NS1 protein's suppression of lung-resident $CD103^+$ CD8 T cells or the reduced trafficking of CD4 and CD8 T cells into the lung through the actions of the G protein's CX3C motif mimicry of CX3CL1. The reduction of $CD103^+$ CD8 T cells in the lung during a primary infection indicates that the numbers remaining in the lung upon secondary infection are also likely reduced. As lung-resident memory cells are critical for protection in other respiratory infections, such as influenza virus, it is possible that this early suppression of lung-resident T cells impacts the memory response formed during RSV infection (McMaster *et al.*, 2015). It is also possible that the N protein's interference with the CD4 T cell immunological synapse during primary activation prevents the T cell from being adequately primed to differentiate into a long-lived memory cell. However, N exerted a significantly reduced effect on the activation of antigen-experienced CD4 T cells compared to naïve CD4 T cells, so a direct effect on memory cell reactivation by N *in vivo* is unlikely (Cespedes *et al.*, 2014). Alternatively, the disruption of the CD4 T cell immunological synapse by the N protein could alter the induction of

T follicular helper (T_{FH}) cells, which are critical for the development of long-lived antibody-secreting plasma cells. If T_{FH} induction is altered by the N protein, the production of RSV-specific antibodies would be significantly impacted and could provide an explanation for the lack of a long-lived antibody response generated by RSV infection in humans. Whether these RSV protein interactions with the immune response have an impact on the generation of protective memory T cells or T_{FH} induction are interesting topics for future study.

In contrast, RSV F and G proteins cooperate with the immune response to enhance the inflammatory response following RSV infection. F binds to TLR4 and CD14, which is critical for the activation of the innate immune response and promotes inflammatory cell infiltration. Why would it be beneficial to RSV for F to play such a critical role in the host immune response? The F protein's ability to signal through TLR4 to induce the formation of NETs by neutrophils could provide an explanation. If NETs in the lung following RSV infection serve to exacerbate disease, as suspected for influenza virus infection, it is possible that the primary role of F signaling through TLR4 is to be pathogenic through the formation of NETs, rather than by activating the innate immune response. Further studies on the role of pulmonary NET formation following RSV infection could provide clarity on this knowledge gap. Additionally, the G protein promotes the host inflammatory response by enhancing CD8 T cell responses following RSV infection. However, this raises an interesting question as to how the enhancement of CD8 T cells would benefit the virus. As CD8 T cells are vital for RSV clearance, it remains unclear why G would enhance a cell population that is critical for RSV's eventual demise. However, this property of the G protein to enhance CD8 T cell activity may have the potential to be taken advantage of in future vaccine design. Additionally, interference with the immune suppressive functions of RSV proteins should be considered in the design of an RSV vaccine.

Further studies examining the interactions between individual RSV proteins and the host factors responsible for modulating the immune response are likely to provide valuable information to further our understanding of the underlying mechanisms that contribute to RSV pathogenesis. Such insights should also aid in the development of a future RSV vaccine.

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