Modulation of host adaptive immunity by hRSV proteins

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Globally, the human respiratory syncytial virus (hRSV) is the major cause of lower respiratory tract infections (LRTIs) in infants and children younger than 2 years old. Furthermore, the number of hospitalizations due to LRTIs has shown a sustained increase every year due to the lack of effective vaccines against hRSV. Thus, this virus remains as a major public health and economic burden worldwide. The lung pathology developed in hRSV-infected humans is characterized by an exacerbated inflammatory and Th2 immune response. In order to rationally design new vaccines and therapies against this virus, several studies have focused in elucidating the interactions between hRSV virulence factors and the host immune system. Here, we discuss the main features of hRSV biology, the processes involved in virus recognition by the immune system and the most relevant mechanisms used by this pathogen to avoid the antiviral host response.

Introduction

HRSV is the leading cause of lower respiratory tract infections (LRTIs) in infants and young children worldwide.[1] Epidemiological data show that more than 70% of children under 1 y old and 100% of children at age 2 have been infected by hRSV.[2-4] HRSV can also infect the elderly and immunocompromised individuals, however the most severe disease manifestations occur in infants younger than 6 mo.[5,6]

HRSV pathology includes a broad spectrum of disease manifestations, ranging from milder upper respiratory tract infection to severe bronchiolitis, alveolitis, and pneumonia.[7,8] Generally, hRSV infections are not lethal and the virus is eliminated from the airways during disease resolution. Nevertheless, global epidemiological studies have estimated that hRSV causes over 34 million LRTIs and more than 200 000 deaths every year.[9] HRSV spread occurs through contact with large-particle aerosol droplets or direct contact with infected patients. The infection begins in the nasopharynx of the host and progress with the spreading of the virus to the lower respiratory tract.[10-12] At these sites the main target of hRSV are epithelial cells present in the airways, but it also may infect lung resident myeloid cells, as evidenced by the detection of infected mononuclear cells in circulation.[13,14]

Molecular Composition of hRSV

HRSV is an enveloped virus classified in the order Mononegavirales, the *Paramyxoviridae* family, and the *Pneumovirus* genus.[15] HRSV contains a non-segmented, negative-sensed and single-stranded RNA genome of 15.2 kb in length. The hRSV genome has 10 genes in the order 3′-NS1-NS2-N-P-M-SH-F-G-M2-L-5′, which are transcribed into 10 different monocistronic mRNAs. The hRSV genome encodes nine structural proteins and two non-structural proteins. The structural proteins include three envelope glycoproteins (F, G, and SH), the nucleocapsid proteins (N, P, and L), the nucleocapsid-associated proteins (M2–1 and M2–2), the matrix protein (M), and the non-structural proteins NS1 and NS2.[16] Different from other viral mRNAs, the M2 mRNA is translated into two different proteins, namely M2–1 and M2–2, through a process of ribosomal termination-dependent re-initiation mechanism (**Fig. 1**).[17] The M2 gene products, M2–1 and M2–2, are pivotal regulatory proteins that modulate the replication cycle of hRSV. More specifically, M2–1 integrates the ribonucleoprotein complex that mediates transcription of viral mRNAs, whereas the M2–2 protein regulates the switch from transcription to replication.[18,19]

Once hRSV reaches the host target cell, the infection cycle begins with the attachment and entry process, which is mediated by the G and F glycoproteins, respectively.[16] It has been reported that G and F glycoproteins can interact with the cell surface receptors CX3CR1 and TLR4,[20,21] respectively, as well as glycosaminoglycan (GAGs)[22] and C-type lectins[23] to promote viral infection. Further, nucleolin was recently described as another the functional host receptor that interacts with the hRSV F glycoprotein.[24] After fusion of the viral envelope and the cell plasma membrane, the viral nucleocapsid is released into the cytosol of infected cells where transcription of viral mRNAs and replication of the viral genome are initiated.[1,25] HRSV replication requires the synthesis of a complementary, polycistronics ssRNA (+) antigenome, which is used as a template for the synthesis of

new full-length ssRNA(-) genomes.[26] Both, genomes and anti-genomes are independently wrapped by the N protein, forming stable nucleocapsids.[27]

HRSV virions are pleomorphic, spherical structures with a diameter ranging from 100–350 nm to 100–100 nm, which are assembled in cholesterol-enriched domains at the host cell membrane.[28] Recent studies suggest that the majority of hRSV strains form long filamentous projections at sites of virus assembly and budding.[29] Furthermore, assembly and maturation of hRSV filaments on the surface of infected cells depend on the proper destination of M protein to cholesterol rich domains of the plasma membrane.[30] Therefore, during genome packaging and virus assembly the hRSV particle acquires a lipid envelope of host origin.[31] Because glycoproteins located at the viral envelope participate in host cell recognition, attachment and infection, this structure is pivotal for the infectivity of hRSV.[32]

Modulation of Host Cell Biology by hRSV Replication

Upon infection, hRSV modulates several biological processes of the infected cells to enhance their replication. Studies performed in A549 and primary human epithelial cells (PHBE cells) have shown that hRSV infection induces the production of TGFβ1 and the decrease of the p53, which results in G1/S and a G2/M cell-cycle arrest and a subsequent enhancement of hRSV replication.[33] HRSV infection also promotes the formation of host cytoplasmic stress granules (SG) in epithelial cells.[34] Although the formation of SG has been associated with increased viral replication,[34] these structures are also recognized by the cytoplasmic RLR receptor MDA5. This protein is activated by viral dsRNA or by 5′-triphosphorylated un-capped viral RNA,[35] which activates the type I interferon (IFN-α/β) response.[36] Recently, it was described that MDA5 specifically recognizes dsRNA replication intermediates in viral infected cells.[34,37,38] SGs increase in size during the course of infection and contain the N, P, M2–1, L, and M proteins.[37] In addition, hRSV induces changes in the expression of neurotrophic factors and receptors, which are involved in airway inflammation and hyperreactivity. Recent reports have shown that hRSV induces the upregulation of the nerve growth factor (NGF) and their receptor tropomyosin-related kinase A (TrkA), with concomitant downregulation of the low-affinity pan-neurotrophin p75[NTR] receptor.[39] The NGF–TrkA axis prevents apoptosis by increasing the expression of anti-apoptotic Bcl-2 family members, whereas p75[NTR] signaling promotes apoptosis via JNK. These mechanisms keep the infected cells alive and promote viral replication.[39] The same study suggested that upregulation of the NGF–TrKA axis induced by hRSV infection in human bronchial epithelial cell takes place through silencing of miR-221 expression and also induces the downregulation of other 24 miRNAs.[39] MiRNAs are small ssRNA molecules that modulate the gene expression at the post-transcriptional level.[40] The role of these miRNA in the modulation of both innate and adaptive immune responses has been broadly studied.[41] Indeed, miRNAs participate in the maintenance of the airway epithelial barrier and in the modulation of the antiviral defense of epithelial cells.[40] Infection of A549 cell line with hRSV increases the production of miRNA let-7f expression, probably due to the signaling triggered by the G glycoprotein.[8]

Let-7 miRNAs regulate several key host genes induced during hRSV infection that also controls virus replication.[42] Furthermore, let-7f regulates cell-cycle genes (CCND1, DYRK2, and ELF4),[42] the gene encoding the CCL7 chemokine and the gene encoding the suppressor of cytokine signaling 3 (SOCS3).[17,42] Also, the regulation of ELF4 by let-7f modulates the expression of IL-8, which plays an important role in the pathogenesis of hRSV. Also, it has been described that G hRSV glycoprotein can modulate the expression of IL-8 thought let-7f miRNA.[43,44]

HRSV Recognition by PRRs Receptors

Innate immunity is the first line of defense against virus infection, before induction of the adaptive immune response.[45] It is well established that innate immunity is critical to restrain virus spreading and infection, resulting in diminished disease burden. After hRSV infection, the virus infects epithelial cells, alveolar macrophages and dendritic cells, which trigger an innate antiviral response through pattern recognition receptors (PRRs), including toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-biding oligomerization domain (NOD)-like receptors (NLRs). All these proteins recognize pathogen-associated molecular patterns (PAMPs) from the virus or damage-associated molecular patterns (DAMPs) derived from host cells after virus infection.[43,46]

TLRs play an important role in the recognition of hRSV. The TLR-2 and TLR-6 form the cell-surface heterodimer TLR4/TLR6 on immune cells. After activation by hRSV, this complex triggers a signaling cascade in leukocytes that activates innate immunity by promoting the production of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), CCL2, and CCL5.[47] Also, TLR-4 associates with CD14 and the complex recognizes LPS from gram-negative bacteria and the hRSV-F glycoprotein. Activation of TLR4/CD14 complex requires binding of MD2, leading to NFκB activation. The final result of this activation pathway is the secretion of IL-8, IL-10, and IL-6, and also the upregulation of TLR4 on epithelial cells.[48]

HRSV infection is also sensed by the intracellular receptor TLR-3. This receptor localizes on the surface of endosomes and recognizes double-stranded RNA (dsRNA) produced during viral replication.[45] The induction of TLR3 activates the innate immune response through the TRIF-mediated pathway that promotes the production of CCL-5, IFN-α, and IFN-β.[49] TLR7 is also involved in the recognition of hRSV, which binds to the viral ssRNA genome in endosomes during the fusion process. When TLR7 is activated after hRSV infection it signals via the MyD88 pathway.[50]

Similar to MDA5, RIG-I is a receptor belonging to the RLRs family and is activated by viral dsRNA or 5′-triphosphorylated un-capped viral RNA in the cytoplasm.[51] Specifically, RIG-I recognizes ssRNA viral genomes bearing 5′-triphosphates, whereas
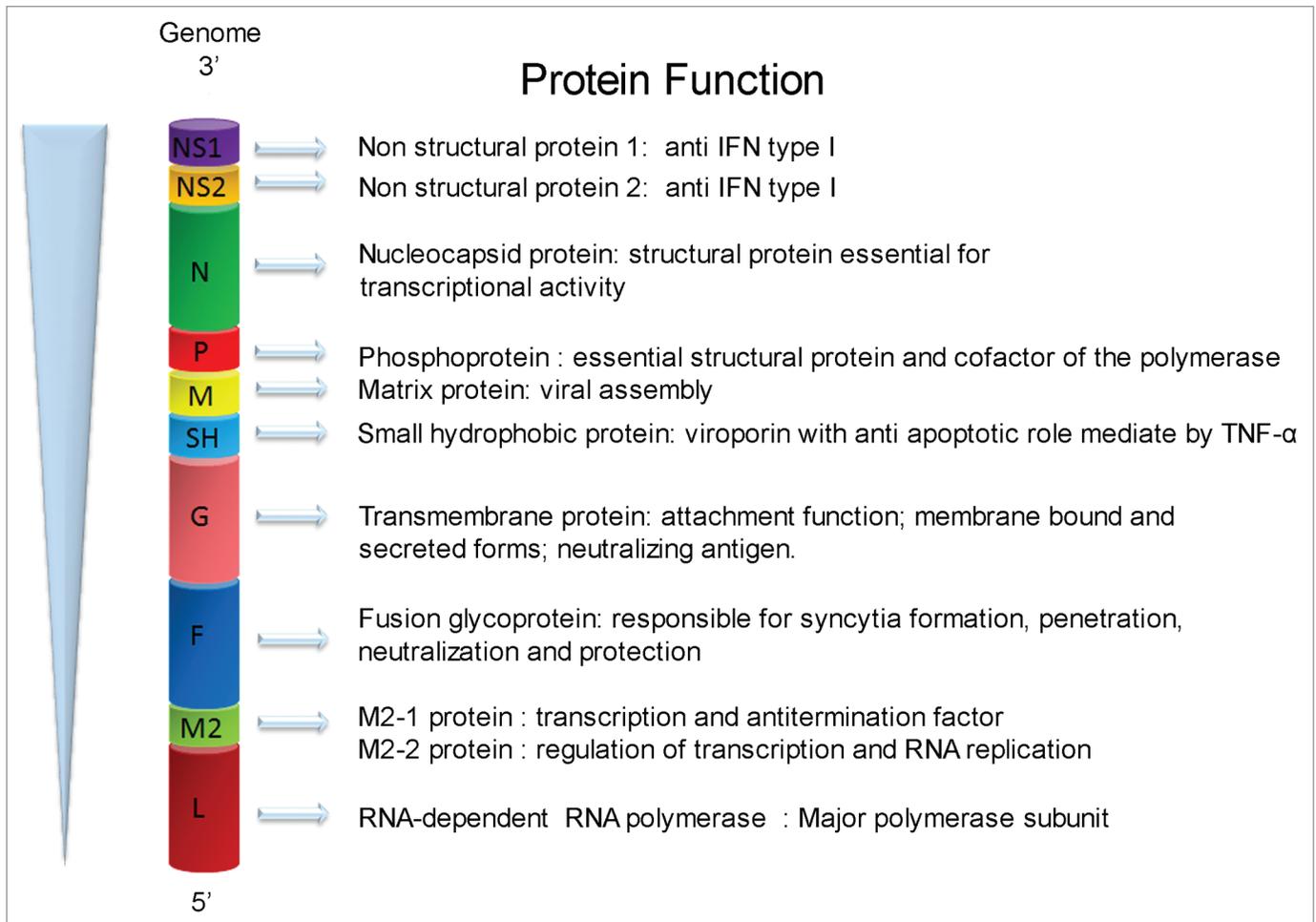
Figure 1. Schematic representation of the hRSV genome indicating the known function for each encoded protein. The figure shows the order of the 10 genes of hRSV in its genome and the known function of the 11 encoded proteins.

MDA5 recognizes long dsRNA molecules.[52] The induction of either RIG-I or MDA5 leads to the activation of downstream NFκB and IRF3 pathways by interacting with the mitochondrial antiviral-signaling protein (MAVS; IFN-β promoter stimulator 1 [IPS-1]). RIG-1 detects hRSV during replication and subsequently activates IFN regulatory factors 3 and 7 (IRF-3 and IRF-7), which are transcription factors for IFN-α and IFN-β.[45] Finally, the nucleotide-binding oligomerization domain 2 (NOD2) detects the ssRNA genome and triggers innate immune activation by binding with the adaptor MAVS. NOD2 is required for IRF3 activation and IFN-β production upon hRSV infection in vitro.[45]

Airway Immune Response against hRSV

The epithelial cells from the airways (tracheal, bronchial, and bronchiolar cells) actively contribute to initiate the immune response after hRSV infection, through the secretion of immunomodulatory molecules with innate antimicrobial activity, as well as secretion of cytokines and chemokines upon infection to recruit immune cells.[53] HRSV infection induces the secretion of surfactant proteins A and D (SP-A and SP-D). Both SP-A and SP-D are polypeptides of the collectin family that bind pathogens and play an important role in host defense and regulation of inflammatory processes in the lung.[54] SPs can act as opsonins, but also stimulate macrophages (Mφ) activation, increasing chemotaxis, phagocytosis and modulating cytokine secretion.[55,56] In vitro infections of human peripheral blood monocytes and HEp-2 cells with hRSV show that SP-A interacts with G and F glycoproteins and favors the binding and uptake of the virus.[57,58] Also, SP-A modulates the activity of Mφ, including activation of the NFκB signaling pathway and the upregulation of cytokine synthesis, mediated by TLR4 complex.[59] SP-A and SP-D bind to CD14,[60] whereas SP-A also binds to TLR2, but does not activate downstream NFκB signaling.[61] Surfactant protein D also binds the hRSV G protein and inhibits hRSV infection in vitro and in vivo.[58]

Recent reports have also shown that infection of airway epithelial cells by hRSV induces the secretion of thymic stromal lymphopoietin (TSLP), an epithelium derived cytokine that plays an important role in the development of allergic asthma

Table 1. HRSV interaction with the host innate and adaptive immune responses

hRSV Proteins	Innate immune response	Adaptive immune response	References
NS1-NS2	Inhibits the induction of IFN-α/β	Suppresses DCs maturation	69–78
	Role in suppressing apoptosis and facilitating virus growth	Suppresses proliferation and activation of two of the protective cell populations (CD103⁺ CD8⁺ T cells and Th17 cells)	
SH	Important role of this protein in the inhibition of apoptosis mediated by TNF-α		84, 85
	Required for the trigger of signal 2 of NLRP3		
G	Inhibits of NK cells infiltration and proinflammatory cytokine secretion	Reduces the Th1 cytokines and promote the Th2 response in pulmonary CD3⁺ T cells	89–100
	Decreases the expression of MIP-1a, MIP-1b, MIP-2, and MCP-1	Induces an exacerbated Th2 type cytokine expression	
F	Induces the secretion of proinflammatory cytokines such as IL-6, IL-1β, and IL-8 mediated by the NFκB pathway.	Activates specific T CD8⁺ cells	103-105

The table summarizes the pieces of evidence supporting the role of NS1, NS2, SH, G, and F proteins in the modulation of both innate and adaptive immune responses.

via activation of RIG-1 antiviral pathway.[62] Additional evidence supports the notion that TSLP induces myeloid dendritic cells (mDCs) to express the OX40 ligand (OX40L), which is a member of the TNF superfamily that has been involved in the B cell–T cell interaction, the DC–T cell interaction, and the initiation of Th2 cell responses through OX40L expressed on these DCs.[63-65] DCs are professional antigen presenting cells (APCs) with superior capacity to activate antigen-inexperienced T cells, thus being an APC subset linking the innate and adaptive immunity.[66] To achieve their function, DCs that are infected by pathogens or that have up-taken antigens at mucosal tissues of the body need to undergo a phenotype change process known as maturation, which has been demonstrated to be a critical response to establish CD8⁺ T cell memory to infections.[67] Along these lines, previous studies have shown that TSLP promotes mDCs maturation, evidenced by the upregulation of major histocompatibility molecules (MHC) class I and II and costimulatory molecules (CD40, CD80, CD83, and CD86).[43,64] In addition, other reports have shown that TSLP can act either in an autocrine or paracrine manner on epithelial cells. Thus, airways cells contribute to the TSLP response and drive the production of the Th2 chemokine CCL17, allowing epithelial cells to induce a Th2-biased response due to hRSV infection.[62] Another study performed in primary rat airway epithelial cells (PRAECs) shows that hRSV induced the production of both TSLP mRNA and protein at 18 h post-infection.[64] In this work, it was shown that hRSV-treated PRAECs induce the maturation of mDCs, which have enhanced levels of OX40L and CCL17 mRNAs.[64] It has been also described that the presence of TSLP in mixed lymphocyte reactions increases the expression of MHC-II and CD86 and promotes enhanced T-cell proliferation.[64] CCL17 have a key role in the Th2 response, because it binds to their chemokine receptor CCR4, which is expressed in almost 100% on Th2 cells that produce IL-4, IL-5, and IL-10.[64,68] This chemokine also participates in the recruitment of Th2 cells and eosinophils into the lungs.[68]

Role of hRSV Proteins in Immune System Evasion

During host-virus co-evolution, several strategies has been developed by viruses to interfere with critical functions of the immune system, including antigen presentation, T-cell activation, and the development of the host humoral response.[69] Among the viral components modulating the host immunity, several hRSV proteins have been attributed with the capacity to modulate the function of either innate or adaptive immune cells (summarized in **Table 1**). In the next sections, we review the virulence determinants used by hRSV to negatively modulate the antiviral immunity, from the early innate responses to the highly specific T-cell responses required to clear the infection.

The Nonstructural NS1 and NS2 Proteins

NS1 and NS2 are two small proteins (139 and 124 amino acids respectively), which are not included as structural elements in the viral particle.[70] NS1 and NS2 are encoded by the first two genes of the hRSV genome and their mRNAs are the most abundant during the infective cycle.[70] Accordingly, NS1 has been shown to be the most abundantly produced protein in hRSV-infected cells, suggesting that these proteins are involved in the modulation of the innate immune response during early stages of the virus replication cycle.[71] In agreement with this notion, both proteins modulate the host immune responses by impairing the induction/signaling of interferons, DC maturation and T lymphocyte activation.[72-74] Also, NS1 and NS2 have been associated with the inhibition of apoptosis, thus prolonging the life of the infected cell and increasing viral yields.[75,76]

Inhibition of the Type I Interferon Response by NS Proteins

The first evidence supporting that NS proteins inhibit the type I interferon pathway showed that virus lacking both NS1 and NS2 failed at preventing the activation of the interferon regulatory factor 3 (IRF-3) and its nuclear translocation.[72,76,77] Further studies demonstrated that NS1 inhibits the phosphorylation of IRF-3, thereby interrupting the binding of this protein to the interferon gene promoter. On the other hand, NS2 causes the degradation of STAT2, an important signaling component of the JAK/STAT cascade that is triggered by interferon receptors. Additionally, NS2 interacts with the RIG-I, preventing the activation of IRF-3 and the interferon stimulated genes involved in the innate antiviral response.[76-78] Moreover, NS1 and NS2 activate the phosphoinositide 3-kinase (PI3K) pathway, which promotes survival of infected epithelial cells and mediates virus maturation and budding.[79-81] In agreement with this notion, suppression of NS1 and/or NS2 expression by either small interfering RNAs (siRNAs) or by viral gene deletion suppressed the activation of the PI3K pathway, which resulted in accelerated apoptosis of hRSV-infected cells and a reduction in virus yield.[78] By activating the PI3K pathway, NS1 and NS2 increased the survival time of the infected cells and increased the yield of viral progeny.[82]

Effects of NS Proteins in the Maturation of Dendritic Cells

It has been demonstrated that hRSV mutants lacking either NS1 or NS1/NS2 have an increased capacity to induce DC maturation as compared with wild-type viruses, as evidenced by an increased expression of maturation markers and secretion of pro-inflammatory cytokines, both of which are known changes associated with DCs maturation.[73] However, NS1 appears to exert most of the modulatory effect over DCs maturation, as evidenced by a non-significant modulation of NS2 knockout hRSV. The upregulation described was inhibited by pretreatment with a blocking antibody against the type I IFN receptor, suggesting that suppression of DCs maturation by NS1/NS2 is associated with antagonism of the type I IFN pathway by these proteins.[73] Furthermore, suppression of DCs maturation negatively affected antigen presentation and T cell activation, suggesting that reduced immune responses against hRSV are at least in part due to the effects of NS proteins over DCs maturation.[73]

Negative Modulation of T-Cell Responses by NS Proteins

The hRSV NS proteins can modulate the activation and proliferation of T cells. As described by Munir and coworkers, deletion of NS1, but not NS2, produced an increased activation and proliferation of CD8+ T cells expressing the tissue homing integrin CD103. Because this integrin leads CD8+ T-cell recruitment into the respiratory tract mucosa, which favors its cytolytic activity in infected airways, it is thought that NS1 negatively modulates cytotoxicity in vivo.[74] Also, NS1 knockout mutants display increased activation and proliferation of Th17 cells within the

lungs, which have anti-viral effects and also indirectly attract neutrophils; and decreased activation of IL-4-producing CD4+ T cells and reduced proliferation of total CD4+ T cells.[74] Except for total CD4+ T-cell proliferation, none of the T-cell effects appeared to be due to increased type I IFN signaling. Data from a previous study show that in infected DCs, deletion of the NS1 and NS2 genes strongly upregulated the expression of cytokines and other molecules involved in DCs maturation.[73] This was partly IFN-I-independent, and thus might account for the T-cell effects. Taken together, these reports demonstrate that the NS1 protein suppresses proliferation and activation of two protective T-cell populations (CD103+ CD8+ T cells and Th17 cells), and promotes proliferation and activation of deleterious Th2 cells that may enhance the pulmonary immunopathology.[74]

The Small Hydrophobic (SH) Glycoprotein

The SH glycoprotein gene encodes 64 or 65 amino acids depending of the hRSV serotype (A or B),[83-85] which is highly conserved among all hRSV A subtypes.[84] In cells infected with hRSV strain A2, the SH glycoprotein can adopt several forms, such as SH 0, SH g, and SH p,[84,85] while in cells infected with B1 strain similar glycosylated and non-glycosylated forms are found.[85] Depending of the glycosylation pattern of the SH glycoprotein, three forms have been described, which are: a 7.5 kDa non-glycosylated form (SH0), a 13–15 kDa N-linked glycosylated form (SHg), and a polylactosaminoglycan-modified form of the protein (SHp), which varies between 21 and 30 kDa.[84] The SH glycoprotein is found anchored at the cellular membrane by the N-terminus and the C-terminal amino acids are extracellular.[84] Several reports indicate that SH glycoprotein can form pentamers and when expressed in *Escherichia coli*, it changes the membrane permeability of the bacteria, allowing the entry of low-molecular-weight compounds.[86-88] Structural modeling analyses have demonstrated that this glycoprotein is an ion channel-forming viroporin.[84,85,88,89] Viroporins belong to a group of small/highly hydrophobic virus proteins that can oligomerize forming pores in the cell membrane, causing varying effects in the physiology of infected cells.[86]

Currently, it is know that SH glycoprotein is not important for the viral replication but hRSV lacking SH glycoprotein was attenuated in mouse and chimpanzee models, which indicates that SH glycoprotein is important for hRSV pathogenesis.[82-84,88] Mutant viruses generated by reverse genetics, in which the SH gene has been deleted, suggest that it is dispensable for virus growth, virus entry into host cells or syncytium formation, but may be necessary for the evasion of the host immune system.[84,85] To evaluate the participation of the SH glycoprotein as a virulence factor, experiments has been performed comparing the hRSV SH protein with another member of the *Paramixoviridae* family, Parainfluenza virus 5 (PIV5), because hRSVΔSH shows a phenotype similar to rPIV5ΔSH: a normal growth in vitro but attenuated growth in vivo.[85] Also, studies of rPIV5ΔSH have shown the role of this protein in the inhibition of apoptosis mediated by TNF-α.[85] In the absence of SH glycoprotein during

PIV5 infection, there is an increase of production of TNF-α and activation of NFκB, due to the translocation of the p65 subunit of NFκB into the nucleus of PIV5ΔSH-infected L929 cells.[85] A similarly ability to inhibit the activation of NFκB by TNF-α in the L929 cells has been observed for hRSVΔSH, independent of the strain used.[85] Other characteristic of hRSVΔSH infection is the high cytophatic effect and the high rate of the apoptosis produced in infected cells, compared with the wild-type hRSV.[85] This observation suggests that hRSV SH glycoprotein plays an important role in the inhibition of apoptosis during the infection, to favor the viral replication.[85]

The hRSV infection induces the secretion of IL-1β in the respiratory tract in mouse and humans and its secretion is relevant for the anti-viral immune response to clear viruses.[90] Recently, the activation of the nucleotide binding oligomerization domain like receptor (NLR) inflammasome, principally NOD-like receptor family, pyrin domain containing 3 (NLRP3) has been described following infection with hRSV, resulting in the pro-IL1β cleavage and secretion of the processed cytokine.[90] Given that the triggering of NLRP3 inflammasome requires the permeability of cellular membrane, the participation of the SH glycoprotein was evaluated.[87] In this study it was observed that a mutant strain of hRSV lacking SH glycoprotein fails at triggering inflammasome activation.[87] This result suggests that SH glycoprotein is required for the triggering signal 2 (**Fig. 1**), due to the formation of a pore or channel on the plasma membrane.[87] More studies are required to understand the role of SH glycoprotein as a hRSV virulence factor.

The Attachment (G) Glycoprotein

The G hRSV glycoprotein contains 298 amino acid residues and besides of its role in the attachment process, it seems to have additional functions, unrelated with attachment proteins from other *Paramyxoviridae* family members.[91] The G glycoprotein has a trans-membrane domain near the N-terminus and the major part of the molecule, including the C-terminus, is external. However, the G glycoprotein exists also in a secreted form lacking this trans-membrane domain.[91] The G glycoprotein has a central conserved cysteine region that contains a CX3C chemokine motif at amino acid positions 182–186.[92] This CX3C motif interacts with the CX3CR1 receptor, whose ligand is CX3CL1, also known as fractalkine. Therefore, the G glycoprotein establishes a competitive inhibition with CX3CL1 for the binding to CX3CR1, facilitating infection and impairing CX3CL1-mediated responses.[92]

CX3CL1 has several functions related to leukocyte biology, including adhesion, chemoattraction and immunomodulation of T cells.[93] The membrane-anchored form of CX3CL1 promotes cell adhesion with CX3CR1 expressed primarily on cytotoxic cells, e.g., T cells, natural killer NK cells, and monocytes/macrophages, and the soluble form acts as a chemoattractant for CX3CR1+ cells.[94] Studies of the interaction between CX3CL1-CX3CR1, using either blocking antibodies for CX3CL1/CX3CR1 or knockout mice for CX3CR1, have shown a high

inhibition of leukocyte migration and chemotaxis after hRSV infection.[20,93-95] Also, it has been suggested that G glycoprotein is important for the development of enhanced pulmonary disease in the vaccination model of formalin-inactivated hRSV,[95] and also increases the expression of the pro-inflammatory tachykinin sustance P during hRSV infection.[96] Indeed, antibodies that block hRSV G glycoprotein CX3C–CX3CR1 interaction prevent many of the immunomodulatory effects associated with RSV G glycoprotein,[97] supporting the idea that the interaction of CX3 mimetic domain in the G glycoprotein with CX3CR1 receptor have an important role in the hRSV infection and disease pathogenesis.[98]

Modulatory Effects on the Host Innate Immune Response by the G Glycoprotein

HRSV G glycoprotein has the ability to modify the immune response at different levels, affecting the function of chemokines, cytokines and leukocytes. Competition with the CX3C chemokine is one of the most described effects of G glycoprotein attributed to the central conserved region, which contains a CX3C motif. Also the G glycoprotein presents a structural homology with the fourth subdomain of the tumor necrosis factor receptor (TNFr).[89] TNF-α and TNF-β are important cytokines of the inflammatory response, and the structural homology with the TNFr of the G glycoprotein suggest that this protein could bind to TNF-α and TNF-β, affecting the antiviral response against hRSV.[89]

Experimental approaches using mutant hRSV virus lacking the G gene showed a increases in the recruitment of natural killer cells into the lungs, as well as increases in the production of IFN-γ and TNF-α, supporting the involvement of the G glycoprotein in the inhibition of NK cell infiltration and proinflammatory cytokine secretion.[99] The G glycoprotein has been associated with the induction of a Th2 response and the increased recruitment of eosinophils into the lungs after hRSV infection. The secreted form of G glycoprotein increases the IL-5 levels, producing a more severe immunopathology due the activation and migration of eosinophils.[100] Other studies have also shown that G glycoprotein decreases the expression of macrophage inflammatory protein (MIP-1a), MIP-1b and MIP-2 and monocyte chemoattractant protein (MCP-1), which have attracting function over NK cells into the lungs.[98]

Modulation of the Host Adaptive Immune Response by the G Glycoprotein

Several pieces of evidence support the notion that hRSV G glycoprotein has important immune modulatory effects. For instance, it has been shown that during hRSV infection the G glycoprotein promotes a Th2 immune response in pulmonary CD3+ T cells (high expression of IL-4 and IL-5) by negatively modulating Th1 cytokines, including IFN-γ and IL-2. It is possible that this phenotype is due to alterations in DCs
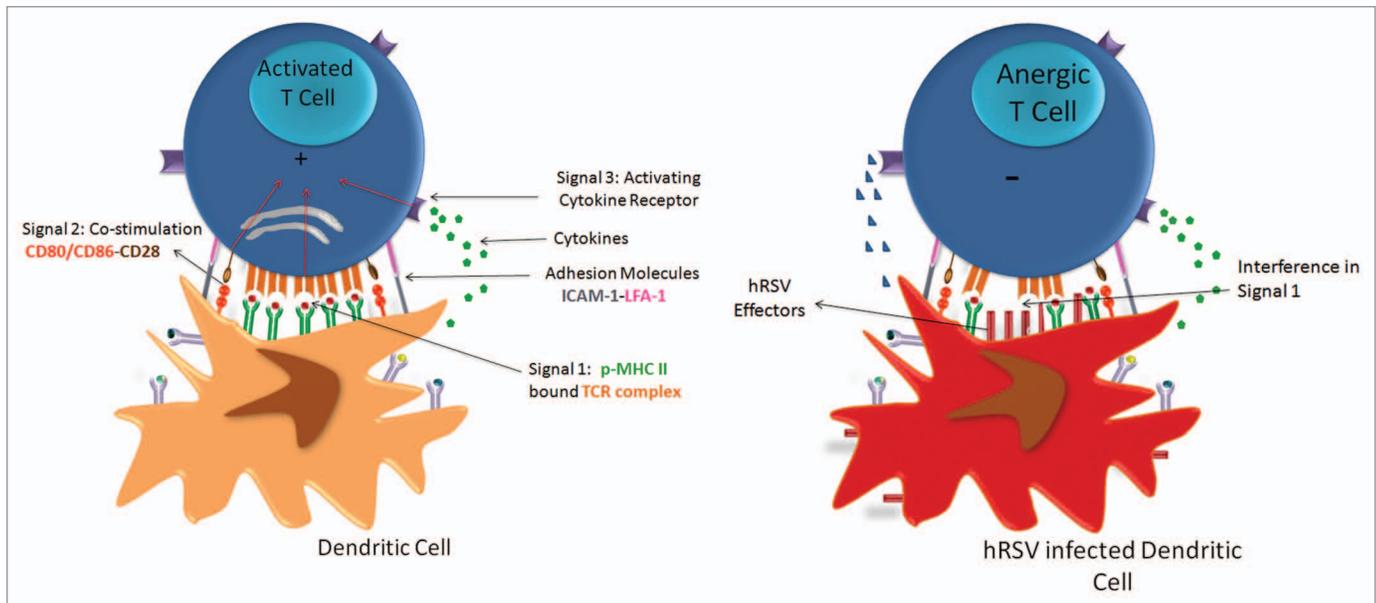
Figure 2. HRSV infection blockade of IS assembly between infected DCs and T cells as a novel RSV virulence mechanism. A novel mechanism to avoid the host immune response elicited by hRSV is the impairment of immunological synapse (IS) assembly between hRSV-infected DCs and naïve T cells that impairs the activation of T cells. During IS assembly DCs provide three different signals to promote the T-cell activation: Signal 1 (antigenic presentation in p-MHC), signal 2 (Co-stimulation) and signal 3 (cytokines) represented in the left part of cartoon. In hRSV infected DCs, as shown in the right part of the cartoon, hRSV effectors are expressed in the surface membrane interfering with the molecular interaction between the T-cell receptor (TCR) and antigenic pMHCs impairing the T-cell activation. This phenomenon is evidenced by a reduction in the Golgi apparatus polarization toward the DC and decreases in IL-2 secretion by T cells.

recruitment/activation or to effects in signaling add pathways important in T-cell activation, such as substance P.[99] Moreover, recent studies have suggested a possible mechanism by which the G glycoprotein may be interfering with the cytotoxic T-cell response (which is essential for viral clearance and the control of virus replication) by antagonizing the activities of the chemokine CX3CL1 over the CX3CR1+ cells, characterized by a Th1 response.[94] Several studies performed in the murine model suggest that hRSV suppresses the effector activity of CD8+ T cells and the development of pulmonary CD8+ T-cell memory,[101] which can be recovered by exogenous IL-2 treatment.[102] These findings are consistent with hRSV G glycoprotein-associated reduction of Th1-type cytokine responses.[97] Because CX3CR1 plays an important role as a chemotactic and adhesion receptor for CX3CL1+ cells, it is though that the hRSV G glycoprotein through its CX3C motif may be involved in the negative regulation of T-cell function observed in vivo. Indeed, the G glycoprotein differentially affects the trafficking of CD8+CX3CR1+ T cells into the lungs and the mediastinal lymph nodes (MLN) of hRSV infected mice. Furthermore, additional evidence suggest that the hRSV G protein may affect the antiviral response through the modulation of perforin and granzyme B expression in cytotoxic CX3CR1+ cells[94] and also expression of the G glycoprotein during hRSV infection induces an exacerbated Th2 type cytokine expression.[98]

The Fusion (F) Glycoprotein

The F hRSV glycoprotein is a type I integral membrane protein of 574 amino acid similar to the fusion proteins of other *Paramyxoviridae* family members, which participates in both the fusion of the viral envelope with the host cell membrane during viral entry and the formation of syncytia.[103] The F glycoprotein is highly conserved among hRSV genogroups, displaying amino acid sequence identities of 90% or higher between serogroups A and B.[104] The F glycoprotein is synthesized as an inactive F0 precursor; three F0 monomers assemble into a trimer, which is further modified and activated in the Golgi apparatus by the host furin-like protease.[82,83] This protease cleaves at amino acid positions 109 and 136, therefore forming three polypeptides. Finally, the N-terminal and C-terminal polypeptides (named F2 and F1 subunits) are linked by two disulfide bonds.[83] During the replication of hRSV, the F mRNA is produced in the cytosol and the functional F protein exists as a trimer located in the virion membrane as a metastable pre-fusion form, which upon binding to its relevant ligand; the nucleolin, undergoes a refolding process into a post-fusion conformation.[105] This conformational change promotes the fusion of the virus and cell membranes, allowing virus entry and the initiation of the hRSV replication cycle (reviewed in[103]). Because of this, the F glycoprotein is essential for the infective cycle, as evidenced by studies showing complete loss of virus

infectivity both in vivo and in vitro in hRSV strains lacking the F glycoprotein (RSVΔF).[106]

As the major protein mediating infection of target cells, the F protein has been attributed as a major target for the pattern recognition receptors (PRRs) of the innate immune system. Indeed, through binding to the TLR4/CD14 complex expressed on the surface of monocytes, the F protein triggers the NFκB pathway and the secretion of pro-inflammatory cytokines, including IL-6, IL-1β, and IL-8 in vitro.[107] Because these cytokines act as chemoattractants, they promote the recruitment of neutrophils and macrophage cells into injured tissues.[108] Furthermore, the TLR4 pathway appears to be essential for the efficient elimination of hRSV from the infected lungs, as evidenced by studies showing severe impairment of viral clearance in TLR4 null mice. Although the mechanism underlying increase susceptibility to hRSV in TLR4 null mice is not fully understood, studies with these mice show an overall impairment of the innate antiviral immunity, including IL-12 expression and diminished numbers of NK cells and CD14+cells in hRSV-infected lungs. Also, the infiltrating NK cells have a significantly diminished cytotoxicity.[108]

Besides being a major hRSV signature for the innate immune system, the hRSV fusion protein has been recognized as a major CD8+ T-cell antigen, as evidenced by the identification of several F-derived antigenic peptides both in humans and mice.[83,109,110]

CTL responses against F protein in mice

In agreement with this notion, it has been shown that approximately 4.8% of the pulmonary CD8+ T cells activating the adaptive antiviral response during the infection peak (day 8) are indeed F_{85-93}-specific.[83] Nevertheless, the F-specific T-cell repertory generated during the experimental infection with hRSV display a substantially reduced cytotoxic capacity when compared with the F repertory induced through vaccination with a recombinant influenza or adenovirus vaccine,[111] suggesting that hRSV negatively modulates the expansion and generation of CD8+-specific T cells. Furthermore, F-specific CD8+ T cells isolated from hRSV infected mice exhibited lower IFN-γ synthesis and impaired cytolytic activity ex vivo.[83,112] Although the mechanisms accounting for inefficient cytolytic activity of CD8+ T cells are not fully understood, it has been shown that the heterologous expression of the hRSV F protein in epithelial cells reduced their sensitivity to CD8+ cytotoxicity in vitro.

Recently, studies reported the interaction of F glycoprotein with the intracellular adhesion molecule (ICAM)-1 expressed on the cell surface, suggesting a possible role of this molecule in the viral fusion. Indeed, hRSV infection increases expression of ICAM-1 on epithelial cells and the ICAM-1 cross-linking of human epithelial cells induced the expression of IL-1β, which is also induced by hRSV infection, suggesting the participation of ICAM-1 in this process.[113] The high conservation of the F protein and its pivotal role in the infection of target cells and the modulation of the host immune response, make the pre-fusion form of the F glycoprotein an ideal target for neutralizing antibodies and the development of antiviral therapies.[104]

The Nucleoprotein (N) as a Novel Modulator of T-Cell Activation

The hRSV N protein is a non-glycosylated, 43 kDa protein with essential roles in virus transcription and replication, acting as a scaffold for the assembly of the hRSV ribonucleoprotein complex. Although its major role in infected cells has been attributed to the protection of viral RNA species, including the wrapping of genomes and polycistronic antigenomes,[114,115] a recent work of our group has proposed that N protein could be expressed in the surface of infected cells at early stages of the viral replication cycle (Céspedes et al., manuscript accepted). Although the mechanism of surface N protein destination is still elusive, it may occur through interactions with the M and P proteins.[116] M protein has a highly positive face able to interact directly with the inner leaflet of the plasma membrane,[117] and the P protein serving as chaperone of N protein that prevents its interaction with RNA.[118] Furthermore, surface expression of N was shown to interfere with the assembly of the T-cell activating immunological synapse (IS) when expressed in the surface of infected APCs, mainly by interfering with the molecular interaction between the T-cell receptor (TCR) and antigenic pMHCs. This observation was in agreement with our previous work showing impairment of IS assembly between hRSV-infected DCs and naïve T cells, which was characterized as a contact-dependent mechanism[119] as shows the **Figure 2**.

Because the priming of T cells largely depends on the assembly of the IS with DCs (and in a lesser extent with other APCs), these observations suggest that the major mechanism explaining the broad impairment of CD4+ and CD8+ T-cell activation by hRSV is due to expression of hRSV proteins in APCs (**Fig. 2**).

Concluding Remarks

HRSV has been recognized as the major viral agent causing severe acute respiratory infections in children. However, and despite considerable efforts made to understand the molecular mechanisms explaining the immunopathology and the evasion of the host-immune response, there is still no appropriate vaccine for hRSV prophylaxis. It is known that hRSV pathology is due to an excessive inflammation of the respiratory tract, characterized initially as a Th2, allergic-like immune response. Because the hRSV-elicited immune response is non-optimal for virus clearance (due to broad impairment with T-cell functions), infected individuals can eliminate the virus at the expense of an exaggerated inflammatory response in the lungs. Furthermore, the aberrant immune response observed in the airways of hRSV-infected animals have been associated with several viral proteins, each of them with unique complementary functions in the modulation of the innate antiviral response of the host, thus facilitating viral propagation. Indeed, hRSV use multiple mechanisms to avoid the host immune response including interference with type I IFN responses (mediated by hRSV NS1 and NS2 proteins) and the antagonism of CX3CL1 chemokine mediated by the mimetic

domain of the hRSV G glycoprotein. The pieces of evidence suggesting immune regulatory roles for hRSV F and SH glycoproteins is not conclusive yet. However, our knowledge is still insufficient to understand the complete picture of hRSV infection necessary to design an effective and safe vaccine available for the population most affected by this pathogen.

Considering the accumulated knowledge of hRSV proteins that act as negative modulators of T-cell physiology, we propose that during host-virus co-evolution the hRSV has selected/evolved virulence determinants with complementary functions at inhibiting T-cell activation and effector functions. Indeed, hRSV proteins impair several critical steps occurring during the development of T-cell responses; from naïve T-cell priming (by hRSV N protein), the acquisition of proper Th1 anti-viral CD4^{+} responses (by hRSV NS1 protein), the proper recruitment of CTLs and helper T cells within infected lungs (by hRSV G glycoprotein) and the execution of antigen-specific CD8^{+} cytotoxicity (by hRSV F glycoprotein). Accordingly, new, rational vaccine design strategies should consider the development of T-cell responses with the capacity to circumvent the mechanisms imposed by hRSV to restraint the host adaptive immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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