

# Human Respiratory Syncytial Virus: Infection and Pathology

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## Abstract

### Keywords

- ▶ human respiratory syncytial virus
- ▶ virulence factors
- ▶ innate immune response
- ▶ adaptive immune response
- ▶ extrapulmonary manifestations
- ▶ central nervous system

The human respiratory syncytial virus (hRSV) is by far the major cause of acute lower respiratory tract infections (ALRTIs) worldwide in infants and children younger than 2 years. The overwhelming number of hospitalizations due to hRSV-induced ALRTI each year is due, at least in part, to the lack of licensed vaccines against this virus. Thus, hRSV infection is considered a major public health problem and economic burden in most countries. The lung pathology developed in hRSV-infected individuals is characterized by an exacerbated proinflammatory and unbalanced Th2-type immune response. In addition to the adverse effects in airway tissues, hRSV infection can also cause neurologic manifestations in the host, such as seizures and encephalopathy. Although the origins of these extrapulmonary symptoms remain unclear, studies with patients suffering from neurological alterations suggest an involvement of the inflammatory response against hRSV. Furthermore, hRSV has evolved numerous mechanisms to modulate and evade the immune response in the host. Several studies have focused on elucidating the interactions between hRSV virulence factors and the host immune system, to rationally design new vaccines and therapies against this virus. Here, we discuss about the infection, pathology, and immune response triggered by hRSV in the host.

Acute lower respiratory tract infections (ALRTIs) are the major cause of morbidity and mortality in young children, the elderly, and immunocompromised individuals worldwide.<sup>1</sup> Importantly, the human respiratory syncytial virus (hRSV) is the principal microbial agent known to cause ALRTIs.<sup>2–5</sup> Most clinical manifestations caused by hRSV range from mild symptoms, such as rhinorrhea, cough, congestion, low-grade fever, reduced appetite, and respiratory distress, to

severe alveolitis, bronchiolitis, and pneumonia.<sup>6</sup> The heterogeneity of the diseases caused by hRSV depends, among others, on host risk factors, including preterm birth,<sup>7</sup> congenital heart disease,<sup>7,8</sup> chronic lung diseases,<sup>9</sup> and immunosuppression.<sup>10</sup>

hRSV infections are considered highly contagious, affecting nearly 70% of infants before the first year of life and nearly 100% of children by the age of 2.<sup>11</sup> Worldwide, approximately

34 million new cases of hRSV-associated ALRTI occur in children younger than 5 years and as much as 200,000 deaths are estimated annually.<sup>12</sup> Indeed, hRSV causes a minimum of 3.4 million hospitalizations per year in the United States.<sup>12</sup> Due to the high hospitalization rates and increased health care system costs, hRSV infections are considered a major public health burden globally. For instance, medical costs related to hRSV infection in hospitalized individuals is estimated at 394 million USD annually, just in the United States.<sup>13</sup> Thus, safe and effective vaccines against this virus are urgently needed.

hRSV spreads rapidly and efficiently throughout the population by inhalation of aerosolized droplets of infectious viral particles, or directly through the contact of these droplets with the ocular mucosa.<sup>14,15</sup> One of the most relevant characteristics of hRSV infection is the capacity to produce high reinfection rates in individuals during epidemic outbreaks. For instance, epidemiological studies indicate that approximately 36% of individuals can be reinfected at least once during a given season.<sup>13,16,17</sup> Based on the current evidence, these reinfection episodes may be due to the elicitation of a deficient or hampered cellular and humoral immune memory after a first exposure to the virus.<sup>4,18–20</sup>

Bronchiolitis is one of the most severe illnesses caused by hRSV infection. The term *bronchiolitis* is referred to distal bronchiole inflammation and obstruction. Such an obstruction reduces the airflow into small airways and causes an alteration in the exhalation capacity. This phenomenon can lead to lung hyperexpansion, lung function alterations, increased mucus production, atelectasis, and wheezing.<sup>21,22</sup> Furthermore, infection of alveolar epithelium by hRSV leads to pneumonia, which prevents an efficient gas-exchange process. Such infection triggers distal airway inflammation, leading to severe pulmonary disease.<sup>22</sup> The initial response against hRSV infection is given by the airway epithelial cells (AECs), which promotes the recruitment of effector immune cells at the site of infection. hRSV-associated immunopathology is characterized by the expression of proinflammatory cytokines and the subsequent perivascular/peribronchial infiltration by mononuclear cells, mainly neutrophils and lymphocytes.<sup>21</sup> This exacerbated inflammation is thought to trigger an unbalanced and pathogenic T helper (Th)2 response.

For more than 50 years, most research on hRSV has focused on elucidating the mechanisms involved in respiratory pathology, as well as on the design of vaccines and therapies against hRSV.<sup>23–25</sup> Nowadays, it is known that hRSV is able to migrate from the airways to various tissues in the host, such as heart, kidney, liver, and brain, thereby producing diverse clinical manifestations, including cardiopathy, hepatitis, and encephalitis.<sup>6,26–28</sup> As a consequence, extensive research aimed at understanding the extrapulmonary manifestations of hRSV infection has gained attention in the past few years. For instance, neurological abnormalities associated with hRSV infection in patients with severe bronchiolitis have been detected. Such patients have shown symptoms including seizures, apnea and altered proinflammatory cytokine levels in cerebrospinal fluid (CSF).<sup>26</sup> However, the cellular and

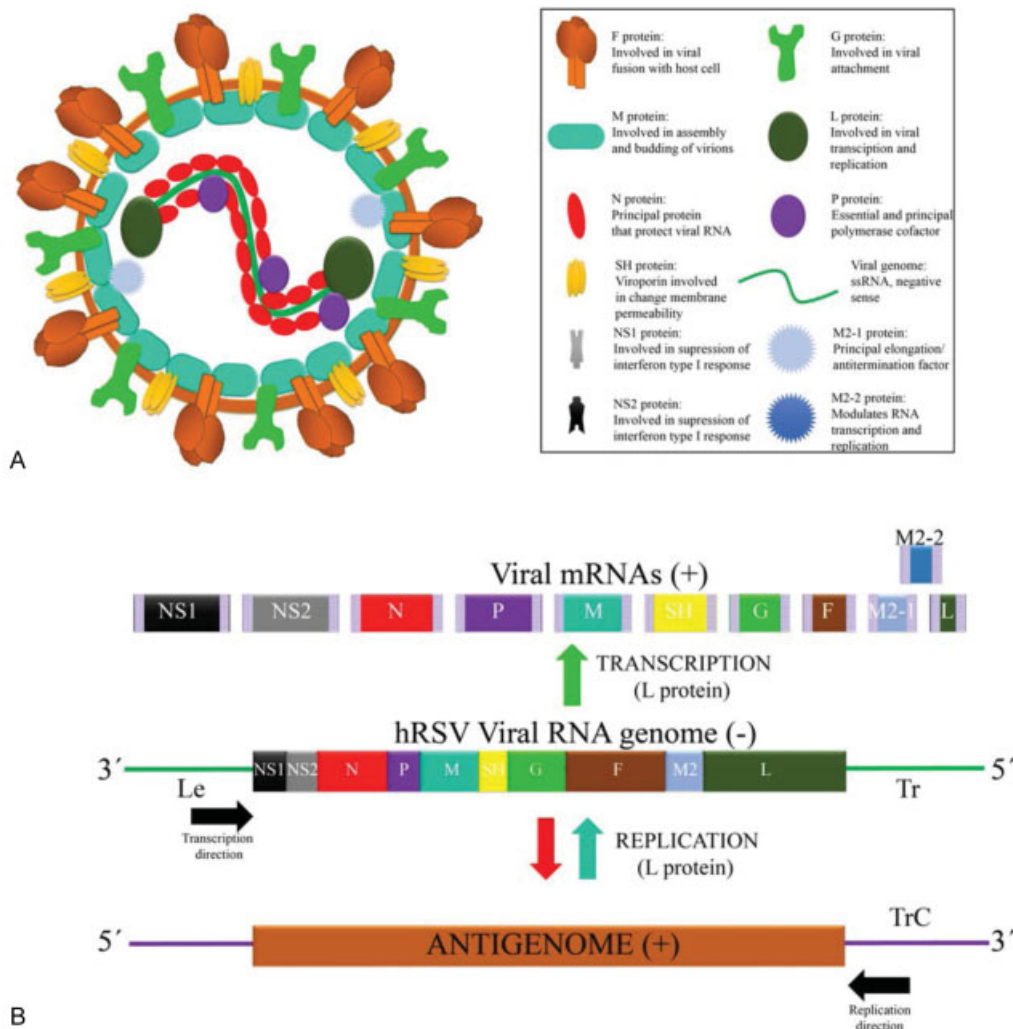
molecular bases for the encephalopathy caused by hRSV remain unknown.

## hRSV Characteristics

hRSV is an enveloped, negative-sense, singled, stranded RNA virus, which belongs to the Paramyxoviridae family, *Pneumovirus* genus.<sup>29</sup> The virus was first described as a human pathogen in 1957, after being associated with the chimpanzee coryza virus.<sup>30</sup> The viral genome is nonsegmented RNA, 15.2 kb in length, that encodes 10 genes and 11 proteins, particularly because of the M2 gen, which has two open reading frames.<sup>31</sup> The order of the genes within the genome from 3' to 5' is NS1-NS2-N-P-M-SH-F-G-M2-L, and these genes are transcribed into 10 monocistronic, capped, methylated, and polyadenylated mRNAs (►Fig. 1).<sup>29,31</sup>

Within the viral particle, nine proteins can be found: N, P, M, SH, F, G, M2.1, M2.2, and L.<sup>29,31</sup> Three proteins, F, G, and SH (small hydrophobic), are expressed at the surface of the particle, attached to the virion membrane (►Fig. 1).<sup>31,32</sup> Both F and G proteins are the main antigenic proteins against which the majority of the antibodies are raised after hRSV infection.<sup>2,5</sup> The F protein is highly conserved between hRSV serogroups with less than the 10% of sequence diversity between the A and B groups.<sup>33</sup> The F protein is generated from mRNA at the cytoplasm of the host cell and converted to an active protein after cleavage by furin-like protease in the Golgi apparatus. This proteolytic cleavage generates three polypeptides, in which C- and N-terminal (F1 and F2 subunits) polypeptides are linked by two disulfide bonds.<sup>11,34</sup> The active form of the F protein in the viral particles is a trimer in a prefusion conformation.<sup>35</sup> This protein mediates the fusion of the viral envelope with the host membrane by changing its conformation to a postfusion form after interacting with the receptor.<sup>36</sup> Importantly, the F protein can interact with different proteins on the surface of host cells, such as TLR4,<sup>37</sup> ICAM-1,<sup>38</sup> and nucleolin.<sup>39</sup> Particularly, nucleolin has been described as the main hRSV receptor that interacts with the F protein, because it was shown that nonpermissive cells expressing nucleolin became susceptible to hRSV infection.<sup>39</sup> Furthermore, no new virions could be made after infection with a mutant virus lacking this protein.<sup>40</sup> These data suggest that the F protein is one of the most important hRSV proteins contributing to infection and interaction with host cells.

The G protein is responsible for the attachment of the virus to the host cell.<sup>29,31</sup> This protein is highly glycosylated and can interact with heparin<sup>41</sup> and annexin II,<sup>42</sup> based on sugar interactions. Likely, this interaction allows a proper approach between the F protein and nucleolin.<sup>32</sup> In addition, the G protein exists also as a secreted form, which has been shown to be important for capturing antibodies generated by the host against this protein. This soluble form prevents the opsonization and neutralization of the virus by G-specific antibodies.<sup>43</sup> Furthermore, another important feature of this protein is the capacity to impair the function of chemokines and cytokines due to a CX3C chemokine-like motif that can mimic and compete with these molecules for the interaction

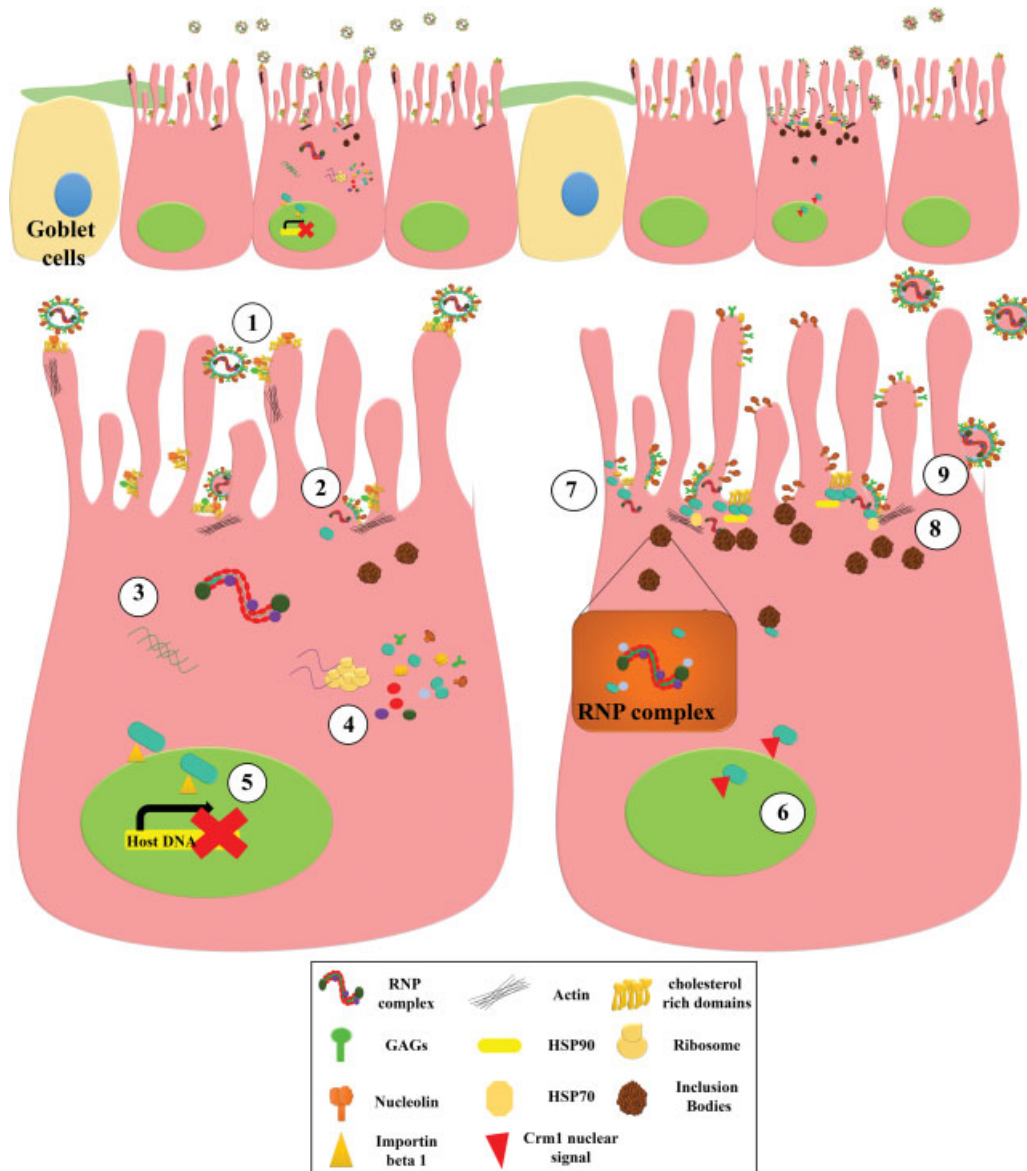


**Fig. 1** hRSV virion and genome structure. (A) Schematic representation of hRSV virion particle. In the rectangle, each protein are represented with their principal associated function. (B) Schematic representation of hRSV genome. Transcription is mediated by L protein which generates 11 viral mRNAs, with cap (vertical bars at the beginning) and polyA (horizontal bars at the ends). One mRNA for each proteins and width of each box represent the quantity of transcription rate of each gene. Replication is mediated by L protein and is necessary for the generation of antigenome product to generate new hRSV RNA. TrC segment in the 3' is where replication promoter is located.

with their receptors and, thus, modulate CD8<sup>+</sup> T-cell responses.<sup>44,45</sup> Moreover, the G protein was shown to display structural homology with tumor necrosis factor (TNF) receptors and is likely to interact with TNF family cytokine members, conducting to a misbalance in the inflammatory response mediated by these molecules.<sup>46</sup> Although G protein is not totally necessary for hRSV infection, it plays an important role at modulating the immune response triggered by hRSV infection.

The SH protein locates at the surface membrane of the virion has been shown to display two different forms that vary in size depending on the hRSV serotype: one of 64 amino acids (serotype A) or 65 amino acids (serotype B).<sup>47,48</sup> SH protein has been described as a viroporin belonging to the family of small/highly hydrophobic viral proteins that are capable of forming ion channels in cellular membranes.<sup>49</sup> In fact, the hRSV-SH protein has been described to allow the entrance of low-molecular-weight compounds and change the permeability of the cellular

membrane.<sup>49-51</sup> In addition, the SH protein seems to be involved in the activation of the inflammasome, particularly through signal 2 by the activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), which triggers the cleavage of pro-IL1 $\beta$  and secretion of this cytokine.<sup>50</sup> Surprisingly, the SH protein has not been described to be involved in virus entry into host cells, as a mutant virus lacking the SH protein can infect and replicate inside permissive cells (in vitro) and generate syncytia similar to wild-type virus.<sup>11,48</sup> However, this mutant virus (hRSV $\Delta$ SH) is attenuated in vivo, which suggests that this protein may work as a virulence factor during hRSV infection.<sup>11,34,47</sup> Other features described for the SH protein include an antiapoptotic effect that promotes viral replication.<sup>48</sup> Indeed, a hRSV $\Delta$ SH mutant virus led to larger syncytia and more apoptosis rates, as compared with WT hRSV.<sup>48</sup> In addition, studies with the hRSV $\Delta$ SH mutant virus showed that the SH protein inhibit NF $\kappa$ B by the overexpression of TNF- $\alpha$ .<sup>48</sup> Although the functions of the



**Fig. 2** hRSV infection of airway epithelial cells: (1) G and F proteins interact with host receptors to initiate virus entry. (2) RNP complex is released by the separation of RNP with M protein. (3) Replication of viral RNA. (4) Transcription and translation of viral proteins. (5) M protein is imported to the nucleus and inhibits host cell transcription. (6) M protein is exported to the nucleus and is transported to cholesterol-rich domains. (7) M protein starts to interact with surface proteins as beginning of assembly. (8) RNP interacts with M protein to finish the assembly process. (9) Budding of nascent virions.

SH protein have not been fully defined, it clearly promotes hRSV replication and dissemination.

Below the virus envelope lie the other viral proteins, namely, proteins N, P, L, M, and M2-1.<sup>29,31</sup> The nucleoprotein N is in close contact with the viral genome and is thought to protect the viral RNA from nucleases and together with P and L proteins constitute the hRSV ribonucleoprotein (RNP), which regulate the transcription and replication of the viral RNA.<sup>52,53</sup> Importantly, the N protein prevents the genomic RNA from forming double-stranded RNA structures, as well as RNA cleavage by host components.<sup>54</sup> Noteworthy, its structure with viral RNA has been recently determined.<sup>55,56</sup> The N protein is generally located within cytoplasmic inclusion bodies, where it interacts with the M2-1, P, and L proteins. During the first hours after infection, the N protein has been

shown to associate within these structures with MDA5 and mitochondrial antiviral signaling (MAVS), which contribute to the innate immune response.<sup>57</sup> The sequestering of these molecules by the N protein would cause a poor detection of viral genome by these nucleic acid sensors, which could dampen the antiviral interferon (IFN) response.<sup>57</sup> Importantly, it has been recently described that the N protein can be expressed on the surface of infected epithelial and dendritic cells (DCs).<sup>58</sup> Expression of this protein impairs the capacity of hRSV-infected DCs to activate T cells, probably due to a blockade of the interaction of peptide-MHC (pMHC; MHC, major histocompatibility complex) complexes with the T-cell receptor (TCR).<sup>58</sup> Such novel role for the N protein has provided new insights relative to the localization of this protein and how hRSV can interfere with the induction of

protective T-cell responses, which is often impaired by hRSV infection.<sup>3,19,58</sup>

The viral phosphoprotein P has been described as a cofactor of the RNP complex and the most important for the L protein. Indeed, the P protein can interact with N protein, allowing it to access the L protein.<sup>52,59</sup> The P protein is highly stable as a tetramer and the C-terminal domain (P<sub>CTD</sub> from the residue 161 to the residue 241) is critical for the interaction with the L and N proteins.<sup>60–62</sup> Consistent with this notion, the phosphorylation of the P protein has been shown to play an important role in the pathogenesis mediated by the virus, as a virus lacking the five phosphorylation sites in this protein shows reduced replication *in vivo* in mice and cotton rats, as well as *in vitro* in HEp-2 sites.<sup>63</sup> However, this recombinant virus can replicate normally in Vero cells, thereby suggesting that the phosphorylation of the P protein is necessary for an efficient viral replication.<sup>63</sup>

The RNA-dependent RNA polymerase (RdRp) L protein is the lesser expressed of all viral proteins in the infected host cells. The principal role of this protein is the replication and transcription of the viral genome, regulated and supported by the RNP complex.<sup>64</sup> Because hRSV is a negative sense RNA virus, the L protein transcribes the genome directly into mRNA for the expression of each hRSV gene.<sup>59</sup> In this process, the L protein recognizes a promoter region in the 3' extreme of the negative RNA strand and starts the transcription of each gene. Accumulating evidence suggests that transcription is modulated by the N protein.<sup>52,53,59</sup> During the replication process, the L protein copies the complete virus genome from a negative sense RNA into a positive sense RNA, which is called an *antigenome*. This RNA is then used as a template to generate new negative sense RNA, which finally will be encapsidated in the virions.<sup>59,65</sup> A typical characteristic of the L protein is that in the process of transcription, it generates a gradient of gene expression, from 3' to 5', producing more mRNAs of the genes 3' as compared with those 5' in the genome.<sup>66,67</sup>

The matrix protein M promotes viral assembly and is essential for hRSV replication.<sup>68</sup> Early after infection, the M protein is located in the nucleus, where it is able to decrease the transcriptional activity of the host cells genes.<sup>69–71</sup> Another role of matrix protein is to arrest the cell cycle in the G1 phase, as shown in A549 cells. It also arrests the G1 and G2/M phases in human bronchial epithelial cells.<sup>72</sup> These actions, which are p53-dependent, increase hRSV replication.<sup>72</sup> In addition, the M protein is directly related to the maturation of viral filaments.<sup>73</sup> In this line, it has been described that hRSV strains that are null for the M protein show significantly lesser infective progeny particles.<sup>73</sup> Moreover, not only lesser new viral particles are generated but also protein trafficking is affected, particularly with the N protein being concentrated in cytoplasmic inclusion bodies, before virus budding. This phenomenon suggests that M protein is important for triggering the trafficking of viral proteins to the budding site.<sup>73</sup> Other studies show that the M protein is expressed in inclusion bodies and interact with the M2–1 protein, as a means to interact with the RNP complex.<sup>74</sup> The M protein is also capable of inhibiting viral transcription and interact with

hRSV G and F proteins to signal the assembly of the virions.<sup>75,76</sup>

The M2–1 protein is involved in the transcription process as part of the RNP complex, and acts as an antitermination/elongation factor promoting the transcription of all hRSV genes, aiding the L protein to proceed with transcription of viral genes.<sup>77</sup> Interestingly, it was also showed that NS1 and NS2 genes can be transcribed by the L protein independently of the hRSV M2–1 protein, suggesting that several transcription mechanisms for viral genes exist.<sup>77</sup> To exert its antitermination functions, M2–1 needs to form as tetramers.<sup>78</sup> Importantly, without this oligomerization, the protein cannot function correctly, which is supported by using a mutant virus for M2–1 protein that cannot generate tetramers.<sup>78</sup> As M2–1 is part of the RNP complex, this protein can interact with different protein of this complex.<sup>74,78–81</sup> The interaction with the N protein is particularly mediated through interactions with the viral RNA, as treatment with RNAses disrupts this binding.<sup>80</sup> Another function of this protein is the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and its association with the RelA protein.<sup>82</sup> On the other hand, the M2–2 protein is involved in the regulation of transcription to the replication by the virus polymerase.<sup>83,84</sup> This effect was discovered by studying an  $\Delta$ M2–2 virus, in which the accumulation of mRNA was higher in cells infected with this mutant virus, as compared with WT virus.<sup>84</sup> In addition, viral titers were reduced over 1,000 times in the first 5 days and over 10 times after 7 to 8 days when the  $\Delta$ M2–2 virus was used.<sup>84</sup> Thus, these two proteins play a critical role in the regulation of transcription and replication of hRSV RNA.

Besides the structural proteins mentioned earlier, the hRSV genome also encodes two nonstructural proteins, namely, NS1 and NS2, both with the capacity to interfere with host type 1 IFN innate response. This process negatively modulates DCs maturation and T-cell responses.<sup>85,86</sup> The NS1 protein interferes with the activation of the IFN gene promoter by inhibiting the phosphorylation of interferon regulatory factor 3 (IRF-3).<sup>87</sup> NS2 also can interfere with the activation of IRF-3 by its interaction with retinoic acid-inducible gene 1 (RIG-I), inhibiting the activation of IFN response genes (IFNRs).<sup>88,89</sup> The NS1 protein is able to interrupt the signaling of JAK/STAT pathways that are activated by IFN receptor pathways, particularly through the degradation of STAT-2.<sup>88,89</sup> Both proteins, NS1 and NS2, are able to promote phosphoinositide 3-kinase (PI3K) pathways promoting the survival of infected cells, increasing viral yield.<sup>90</sup> In this line, interference with the type 1 IFN response by NS1 and NS2 proteins blocks DCs maturation.<sup>86</sup> Concomitantly,  $\Delta$ NS1/NS2 and  $\Delta$ NS1 viruses are able to increase the expression of maturation markers on DCs compared with WT hRSV.<sup>86</sup> Furthermore, this effect in DCs could interfere with their capacity to activate T cells.<sup>85,86</sup> Indeed, human DCs infected with a  $\Delta$ NS1 virus show increased activation and proliferation of CD8<sup>+</sup> T, increase the activation and proliferation of Th17 protective cells, and decrease the activation of IL-4<sup>+</sup> CD4<sup>+</sup> T cells, which are related to increased hRSV pathogenesis.<sup>85</sup> In addition, a recent study showed that the expression of NS1 and NS2 proteins by human bronchial epithelial cells

decreases the polarization of T cells toward Th1, Th2, and Th17 phenotypes by the NS1 protein, and Th2 and Th17 polarization by the NS2 protein.<sup>91</sup> Thus, these two nonstructural proteins are very important virulence factors that directly affect the immune response of the host.

## Viral Infection Cycle

The infection of target cells, such as airways epithelial cells (AECs), starts with the attachment of virions to the cell surface aided by the G protein, which interacts with heparan sulfates and chondroitin sulfate B glycosaminoglycans (GAGs).<sup>92</sup> After this interaction, which helps the virus approach the membrane of the cell to be infected, the F protein contacts its receptor, nucleolin (►Fig. 2).<sup>39</sup> The entry of hRSV has been described to occur particularly in cholesterol-rich microdomains on the cell surface.<sup>93</sup> Further, the fusion membranes require the participation of Pak-1 in the rearrangement of actin filaments.<sup>93</sup> Entry via endocytosis has been discarded because the use of Dynasore shows that, despite dynamin-endocytic process is inhibited, viral fusion still occurs.<sup>93</sup> Therefore, the fusion of the hRSV membrane with the host cell membrane depends on the interactions of hRSV G and F proteins with their receptor and the rearrangement of actin filaments close to cholesterol-rich microdomains (►Fig. 2).<sup>32,93</sup>

The fusion of the viral and cell membranes triggers the release of the viral nucleocapsid content into cytoplasm. Here, the nucleocapsid is dissociated from the RNP complex and repetitions of the M protein, which is mediated by the phosphorylation of the P protein (►Fig. 2).<sup>94</sup> Importantly, this process is mediated not only by viral proteins but also by host cell enzymes, such as glycogen synthase kinase-3 (GSK-3)  $\beta$  and protein phosphatase 2A (PP2A).<sup>94</sup> The transcription process mediated by the function of L protein mainly occurs in cellular inclusion bodies together with N and P proteins.<sup>95</sup> As mentioned earlier, the L proteins associated with the P protein are able to recognize the promoter region on the 3' of viral RNA, and initiate transcription of viral genes.<sup>59,67</sup> The polymerase initiates transcription in gene start (GS) regions carrying out mRNA capping and methylating the 5' of the messenger.<sup>59,67</sup> Then, the L polymerase recognizes a gene end signal at the end of the mRNA and carries out polyadenylation.<sup>59,67</sup> This process goes on again after recognition of a new GS sequence downstream of a previously transcribed gene. It is known that the minimal proteins required for transcription are N, P, and L. The M2-1 protein also appears to be important because of its ability to interact with all RNP proteins,<sup>78-81</sup> included with the M protein.<sup>74</sup> Furthermore, host proteins are also involved in the transcription process; for instance, profilin, an actin-modulatory protein, is required for an optimal transcription.<sup>96</sup> Additionally, host heat shock proteins (HSPs) are also involved in this process, particularly HSP90 and HSP70. Both proteins are expressed in lipid rafts and are associated with the viral RNP complex. Recently, it was described that HSP90 is critical for the stability and functionality of the L polymerase and that HSP70 is necessary for efficient RNA synthesis.<sup>97,98</sup> When the viral genome is repli-

cated, the L polymerase recognizes the TrC promoter region at the 3' of the antigenome and generates genomic hRSV-RNA.<sup>99</sup> This new RNA strand is immediately encapsulated by the N protein.<sup>59</sup>

Virus assembly, after viral RNA transcription and replication, depends on the M protein localization and occurs at cholesterol-rich domains.<sup>73,100</sup> As described earlier, at the beginning of the infection cycle, the M protein is transported to the nucleus by the interaction with importin- $\beta$ 1, where M protein can interfere with cellular transcription (►Fig. 2).<sup>69,70</sup> The M protein is exported from the nucleus to the cytoplasm by a Crm1-dependant nuclear signal so that it localizes to lipids rafts.<sup>71</sup> When the M protein is associated with these domains, the assembly and budding process begins and involves interactions with surface proteins F, G, and SH.<sup>101</sup> Accordingly, a recent report showed that the F and G proteins are expressed on the surface of ciliated cells.<sup>102</sup> Thereafter, the interaction of the F protein with the M proteins promotes assembly of the new virions (►Fig. 2).<sup>103</sup> On the other hand, the M2-1 protein has been shown to bind to the M protein, promoting its assembly with the RNP complex.<sup>74</sup> The formation of filaments that contain the virions is regulated by the hydroxymethylglutaryl coenzyme A reductase enzyme, which mediates changes in F-actin to generate viral filamentous projection that are involved in cell-to-cell transmission.<sup>104</sup> Finally, the budding process is not regulated by the endosomal sorting complex required for transport machinery, as occurs for other enveloped RNA viruses. In its place, hRSV budding is controlled by the RAB11 family interacting protein 2 (FIP2),<sup>105</sup> which has been described as a novel pathway for this type of process. Taken together, the hRSV-infective cycle depends of three main processes: (1) hRSV protein localization, where inclusion bodies and rich cholesterol sites are principal places where the hRSV proteins can be founded; (2) hRSV protein interaction, the particular interaction between RNP complex and accessory proteins for replication and transcription and the interaction of M protein to surface protein triggering the virion budding; and (3) interaction of host cell proteins with hRSV proteins and structures, principally how host cells help in the release of nucleocapsid at the beginning of the process and how a novel process of budding depends of host proteins (►Fig. 2).

## Innate Immune System against Respiratory Syncytial Virus

Upon infection, AECs, DCs, and macrophages play a key role in the innate response against hRSV in the lungs.<sup>106</sup> Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family members, and NOD-like receptors (NLR) are activated following hRSV infection.<sup>107</sup> TLRs have been shown to be fundamental for the recognition of hRSV.<sup>108-110</sup> TLR4/TLR6 triggers a signaling cascade that activates innate immune responses by enhancing the production of TNF- $\alpha$ , interleukin (IL)-6, CCL2, and CCL5.<sup>110</sup> hRSV is also sensed by endosomal TLR3 and TLR7, which triggers CCL5, IFN- $\alpha$ , and IFN- $\beta$  production by TRIF-mediated- and MyD88 pathways,

respectively.<sup>108,109</sup> The NLRP3 inflammasome, which belongs to the NOD-like receptor family, senses the SH protein of hRSV and triggers pro-IL- $\beta$  cleavage and secretion of IL-1 $\beta$  cytokine.<sup>50</sup>

After PRRs are activated, NF- $\kappa$ B, IRFs, and ATF-2/cJun are promoted.<sup>111</sup> As a consequence, the expression of type 1 IFNs and the production of inflammatory cytokines, such as IL-8 (IL-8/CXCL8), IL-4, IL-5, IL-6, and IL-10, as well as chemokines and adhesion molecules are triggered.<sup>112,113</sup> Such signaling cascades also prompt the recruitment of immune cells, such as eosinophils, monocytes, and neutrophils to the lungs.<sup>114</sup> As a result, exacerbated Th2-mediated airway inflammation is triggered, which contributes to lung damage (**►Fig. 3**).

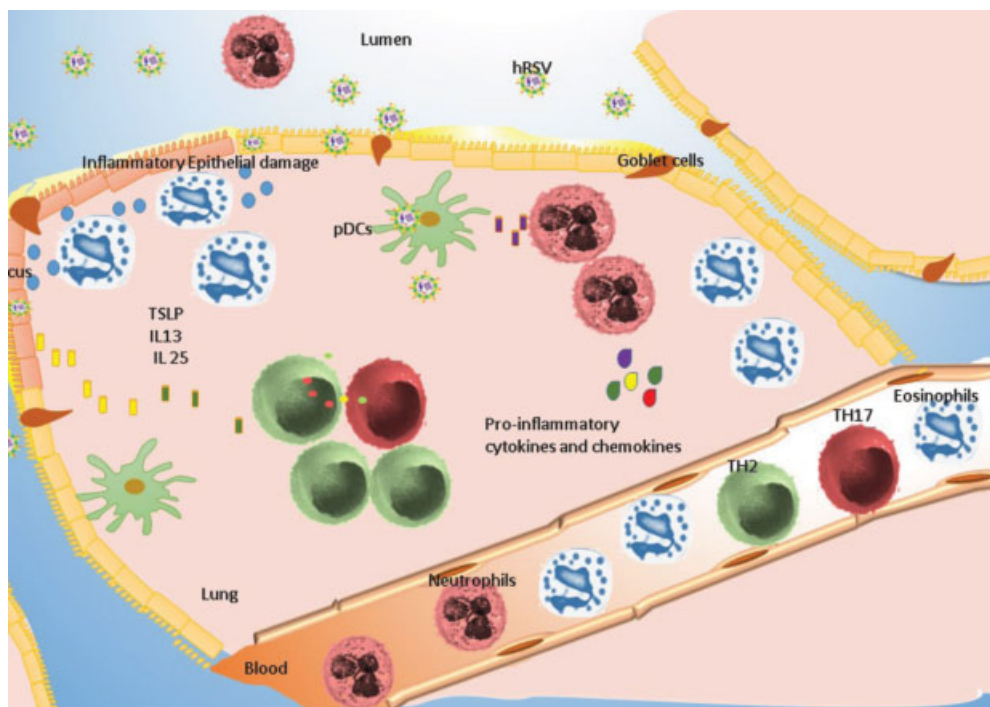
hRSV also induces the secretion of both surfactant proteins A and D (SP-A and SP-D) in the airways.<sup>115</sup> These proteins play an important role in the regulation of the immune response in the lung.<sup>116,117</sup> Indeed, SP-A and SP-D can also stimulate macrophage activation by increasing chemotaxis, phagocytosis, and increase cytokines secretion.<sup>118</sup> Interestingly, SP-D is able to bind hRSV G protein, thereby inhibiting hRSV infection in vivo and in vitro.<sup>119</sup> Recently, hRSV infection of AECs has been shown to be involved in the production of thymic stromal lymphopoietin (TSLP) and epithelial cell-derived IL-7, an IL-7-like cytokine.<sup>120,121</sup> Interestingly, these cytokines, together with IL-25 and IL-33, are related with acute exacerbations of asthma and Th2 inflammatory responses triggered by viruses.<sup>122,123</sup> Importantly, Qiao et al showed the induction of functional maturation of myeloid DCs (mDCs) in hRSV-infected AECs through Th2-polarizing

molecules, such as thymus activation-regulated chemokine (TARC/CCL17) and OX40 ligand (OX40L) activation.<sup>121</sup> Indeed, they suggested that mDCs activation was mediated by TSLP, as TSLP-targeted siRNA abrogated mDCs activation.<sup>121</sup>

Both hRSV NS1 and NS2 proteins inhibit the secretion of type 1 IFNs in host cells by decreasing the levels of TNF receptor-associated factor 3.<sup>124</sup> Furthermore, survival of infected epithelial cells is achieved, thanks to NS1 and NS2, which activate the PI3K pathway as previously mentioned.<sup>108,125</sup> Consistent with this notion, suppression of these proteins resulted in accelerated apoptosis in hRSV-infected cells and consequently reduction in the virus yield.<sup>90</sup> Importantly, the hRSV nucleoprotein could also attenuate the IFN response, as colocalization of this protein with RIG-1 and MAVS protein were found 6 hours postinfection.<sup>57</sup>

### Adaptive Immune Response against Respiratory Syncytial Virus

T cells play an important role in hRSV infection. CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been shown to play pivotal roles in both hRSV clearance and pathogenesis. Such a dichotomy in the role of virus-specific T cells has been observed for infection, and also lung damage after challenge with the virus.<sup>126</sup> For instance, T cells expanded in mice experimentally infected with hRSV have been shown to be essential for the clearance of the hRSV, although this immune response causes an exacerbated activation of the immune system within the airways.<sup>127</sup> Similarly, mice immunized with a vaccine



**Fig. 3** Immune response triggered by hRSV infection in respiratory airways. hRSV reaches the lower respiratory tract and is recognized for respiratory epithelial cells by pattern recognition receptors (PRRs) expressed leading to the secretion of innate cytokines and chemokines such as TSLP, IL-13, and IL-25. Inflammatory cytokines and chemokines promote the recruitment of innate immune cells into the lungs, such as eosinophils, neutrophils, and monocytes. The inflammatory environment induced by the innate immune cell recruitment and mucus production together with an excessive Th2 and Th17 response generate destruction of the respiratory epithelium and the obstruction of distal bronchiolar airways.

consisting of formalin-inactivated virus suffered “vaccine-enhanced disease.”<sup>128</sup> Pathology was observed as an exacerbated increase in the immunological response of vaccinated mice to the virus upon challenge, which was manifested by increased eosinophil infiltration and Th2-like responses in the lungs.<sup>128</sup> Importantly, in this scenario, T cells were described as a critical cell subset mediating the “vaccine-enhanced disease.”<sup>129</sup> Furthermore, Th17 cells have also been shown to contribute to hRSV airway pathology in human newborns.<sup>130</sup> On the contrary, mice immunized with BCG expressing either the hRSV nucleoprotein (BCG-N) or M2 protein (BCG-M2) showed a significant recruitment of IFN- $\gamma$ -producing T cells in the lungs, promoting a Th1-response, which was protective and led to virus clearance without detrimental inflammation.<sup>131</sup>

Cytotoxic CD8<sup>+</sup> T cells (CTLs) are usually responsible for viral clearance by recognizing the F and N proteins of the hRSV.<sup>132</sup> However, hRSV-specific CD8<sup>+</sup> T cells have also been shown to play a role in a detrimental immune response. Consistent with this notion, depletion of CD8<sup>+</sup> T cells reduced the severity of hRSV-induced disease during primary and secondary infection.<sup>133</sup> Such detrimental responses have been suggested to occur because CD8<sup>+</sup> T cells play a role in the regulation and activation of the CD4<sup>+</sup> T cells toward Th2 polarized phenotypes.<sup>134</sup> On the other hand, a reduction of intracellular granzyme B content, diminished secretion of IFN- $\gamma$ , and impairment of perforin expression have been observed in CD8<sup>+</sup> T cells in the lungs of hRSV-infected individuals.<sup>135,136</sup>

Inefficient T cells against hRSV have been reported in infected individuals.<sup>137,138</sup> Such suppression of T-cell activation is thought to be due to an impairment of DC-T cell immunological synapse assembly, which has been observed by a decrease in Golgi polarization in T cells cultured with hRSV-infected DCs<sup>3</sup> (–Fig. 4). This impairment of immunological synapse assembly also causes improper TCR engagement, leading to the failure of antigen-specific T cells priming.<sup>3</sup> Interestingly, these authors reported that DC-derived soluble factor mediators were not involved in this suppression.<sup>3</sup> By contrast, alteration of cytokine secretion

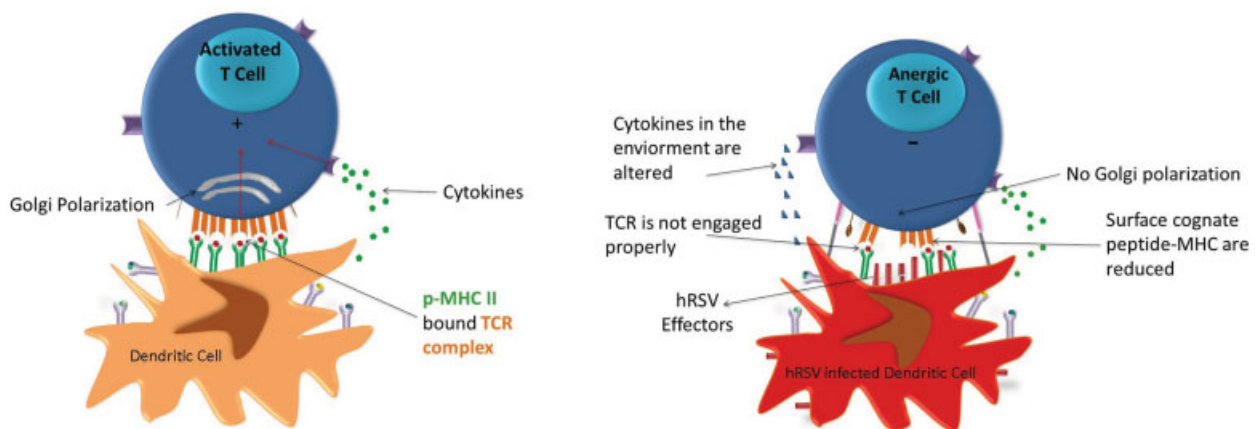
surrounding the DC-T cell environment by hRSV have been shown to modulate T cell response.<sup>139</sup> Furthermore, hRSV has also been observed to alter the quantity of surface cognate peptide-MHC, impairing T cell activation.<sup>140</sup> Likewise, secretion of Th1-like cytokines is reduced in hRSV-infected DCs and may decrease cytotoxic T cell activity.<sup>3</sup> Taken together, immunological synapse is a fine system that could be exploited by hRSV at multiple levels. Thereafter, further research is needed to elicit mechanisms of hRSV to interfere in the immunological synapse.

Regulatory T cells (Tregs), characterized by the expression of forkhead box transcription factor (Foxp3), have emerged as key cells in preventing hRSV inflammatory-associated disease.<sup>141,142</sup> This notion is supported by studies in which depletion of CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> cells prior to infection results in increased hRSV-associated pathology.<sup>141</sup> Likewise, these mice display enhanced weight loss, cellular influx in the lungs, and high eosinophils in the airway after infection.<sup>142</sup> Interestingly, this was associated with an increase of IL-13<sup>+</sup> T cells and enhanced expression of the Th2-like transcription factor GATA-3 in the airways.<sup>142</sup> Taken together, Tregs play an advantageous role against hRSV infection by downregulating unfavorable proinflammatory cytokines, thereby reducing lung damage.

In addition to the role of T cells in hRSV infection, macroautophagy in DCs has emerged as a key process contributing to proper antiviral adaptive responses against hRSV.<sup>143,144</sup> Mice deficient for autophagy processes (beclin +/- mice) display higher weight loss, elevated Th2 cytokine production, and eosinophil infiltration in the lungs.<sup>143</sup> Indeed, DCs with impaired autophagy machinery presents an amelioration of IFN- $\gamma$  and IL-17 stimulation in CD4<sup>+</sup> T cell cocultures.<sup>143</sup>

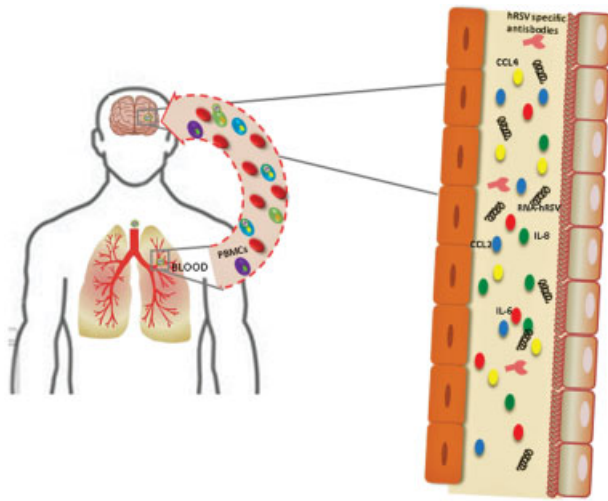
### Extrapulmonary Manifestations Caused by Severe hRSV Infection

Despite human AECs are the main target for hRSV, several reports have shown that this virus can also infect immune cells, such as macrophages, monocytes, DCs, and B lymphocytes.<sup>3,145–147</sup> Moreover, endothelial and neuronal



**Fig. 4** hRSV blocks the DC-T-cell synapse assembly. hRSV elicits immune host system by impairment T-cell activation. The DC-T-cell synapse assembly is interfered by decreasing Golgi polarization, altering the cytokines secreted in the environment, reducing the surface cognate peptide MHC and impairing the TCR engagement.





**Fig. 5** Model for hRSV spread from the lungs to the CNS. The figure shows the possible hRSV spread from the lungs to CNS through the hematogenous pathway.

cells have also been shown to be infected by this pathogen *in vitro*.<sup>113,148</sup> The infection of nonepithelial cells by hRSV has been related to the expression of the hRSV receptor nucleolin, as well as other surface molecules, such as GAGs and TLR4, which interact with hRSV proteins.<sup>145</sup> Importantly, infected immune cells are detectable in systemic blood, as shown in infected infants by RT-PCR (reverse transcriptase-polymerase chain reaction) (►Fig. 5)<sup>149,150</sup> and in PBMCs in BALB/c mice.<sup>146</sup> Such evidence supports the notion that hRSV is able to spread through the hema-

togenous pathway, thereby reaching distant organs (►Fig. 5).

hRSV infections in peripheral lungs have been associated with severe bronchiolitis in hospitalized children.<sup>26,151</sup> For instance, myocardial disease has been extensively associated with severe hRSV bronchiolitis in infants who do not necessarily carry congenital heart diseases.<sup>151–153</sup> Consistent with this notion, elevated levels of cardiac troponin T (cTnT), a sensitive and specific marker of myocardial damage, have also been detected in severe hRSV-infected infants with hypotension (low blood pressure).<sup>152,154</sup> The first report of myocardial failure during an hRSV-driven bronchiolitis was described in 1972 and ended with a fatal case of interstitial myocarditis, an inflammation of the myocardium.<sup>155</sup> Importantly, cardiac alterations during hRSV infection can range from arrhythmias or irregular heartbeat to mechanical dysfunction.<sup>153,156</sup> Noteworthy, hRSV-RNA has been detected in the myocardium by PCR in a case report of myocarditis, thereby suggesting that such alteration can be a direct effect of viral infection.<sup>157</sup> Additional evidence of the cardiovascular manifestation of hRSV infection has shown that 76.5% of positive patients for severe hRSV bronchiolitis present sinoatrial blocking, characterized by interference in the passage of impulses from the sinoatrial node, and this manifestation is common in patients with elevated viral load ( $\geq 100,000$  copies per mL).<sup>27</sup> In addition, clinical manifestations of pericardial effusion, an abnormal amount of fluid in the pericardial space, were associated with severe bronchiolitis in a 1-month-old infant (►Table 1).<sup>158</sup>

Hepatic alterations have also been related to hRSV infection, as evidenced by the detection of elevated levels of transaminase in patients with hRSV-associated bronchiolitis.<sup>159</sup> Additionally,

**Table 1** Extrapulmonary complications associated with hRSV infection

Complications due to hRSV infection	Clinical manifestations	Findings	References
Cardiovascular	Heart block Ventricular tachycardia Ventricular fibrillation Myocarditis Pericardial effusion	hRSV-RNA in a patient with myocarditis and a correlation between viral load and sinoatrial blocks	27,158,177–179
Hepatic	Hepatitis	hRSV particles were detected in culture of liver from an immunocompromised patient and elevated transaminase levels	26,28
Endocrine	Hyponatremia	Patients with hyponatremia and hRSV bronchiolitis show elevated ADH levels	26,56
Renal	Steroid-responsive simple nephrotic syndrome (SRSNS)	hRSV-RNA and antigens were detected by RT-PCR and alkaline phosphoesterase–anti-alkaline phosphoesterase enzyme-linked assay (APAAP) in the urines, respectively	161
Neurological	Apneas Status epilepticus Seizures Encephalopathy Encephalitis Strabismus	hRSV-RNA by RT-PCR antibodies and elevated proinflammatory cytokines in CSF, such as IL-6	6,26,164,165,167–170,180,181

Abbreviations: ADH, antidiuretic hormone; CSF, cerebrospinal fluid; hRSV, human respiratory syncytial virus; RT-PCR, reverse transcriptase-polymerase chain reaction.

severe hepatitis characterized by elevated alanine aminotransferase levels up to 3,000 IU/L have also been described and further associated with impairment in coagulation.<sup>152</sup> Moreover, high levels of transaminases were associated with severe-hRSV disease in a study comprising 54 children who needed mechanical ventilation.<sup>159</sup> Here, hRSV liver infection was confirmed for one of the hRSV-infected immunocompetent infants in a liver biopsy.<sup>160</sup> Additionally, a case of adipose hepatic infiltration has been reported in a fatal case of Reye syndrome associated with hRSV infection,<sup>26</sup> as well as in developed hepatitis during hRSV infection (►Table 1).<sup>28</sup>

On the other hand, alterations of the endocrine system have also been associated with hRSV infection, as evidenced by a clinical study that showed that 33% of patients with severe hRSV bronchiolitis who were under intensive care manifested low sodium concentration in the blood, condition named *hyponatremia*,<sup>26,56</sup> and that 11% presented sodium levels below 130 mM/L in the serum.<sup>56</sup> Patients with hyponatremia and hRSV bronchiolitis have also been shown to display elevated levels of the antidiuretic hormone (ADH).<sup>56</sup> Furthermore, it has been reported that ADH levels are significantly higher in patients with bronchiolitis, as compared with patients with apneas or upper respiratory tract infections with hRSV.<sup>56</sup> Further, increased ADH levels have been associated with higher carbon dioxide arterial partial pressure and excessive expansion of the lungs or hyperinflation which was visible in chest X-rays (►Table 1).<sup>56</sup>

Renal manifestations have also been described in hRSV infections. hRSV-RNA and viral antigens have been detected by RT-PCR and alkaline phosphoesterase-anti-alkaline phosphoesterase enzyme-linked assay (APAAP) in urine samples of children with active, steroid responsive, simple nephrotic syndrome (SRSNS) (►Table 1).<sup>161,162</sup> Consistent with this notion, Liu et al evaluated the association of hRSV infection with nephropathy in a rat model. In this study, hRSV-infected rats showed a gradual increase in proteinuria and alterations in tubular epithelial cells with slight inflammatory cell infiltration in the renal interstitium. Also, both hRSV-RNA and viral titers were detected in the renal tissue with a peak at 8 days postinfection. These data suggest that hRSV can cause nephrotic syndrome, although minimal in an hRSV-infected animal model.<sup>162</sup>

## Neurological Manifestations of hRSV Infection

Epidemiological data suggest that hRSV infection may cause neurological complications in 1.2 to 1.8% of the cases.<sup>6,163</sup> These neurological alterations include seizures, central apnea, lethargy, feeding or swallowing difficulties, tone abnormalities, strabismus, abnormalities in the CSF, and encephalopathy.<sup>26,163-165</sup>

Acute encephalopathies resulting from viral infection have been classified into three mayor types: metabolic error, cytokine storm, and excitotoxicity.<sup>166</sup> The etiology of the encephalopathies induced by hRSV infection remain unclear. However, an association between the overproduction of inflammatory cytokines and free radicals in the CSF has

been extensively associated with neurological complications. For instance, IL-6, IL-8, and nitrogen oxide are increased in the CSFs of hRSV-infected patients, thereby suggesting that a cytokine storm may be involved in the pathogenesis of hRSV encephalopathy (►Fig. 5 and ►Table 1).<sup>163,166,167</sup> Indeed, increased protein levels, cell infiltration, and low glucose levels have been observed in the CSF of hRSV-infected patients.<sup>26</sup> Interestingly, CSF abnormalities have also been detected in infants with no apparent neurological symptoms, suggesting that hRSV can induce subclinical neurological alterations.<sup>168</sup> Furthermore, brain imaging of patients with acute hRSV encephalopathy is also unclear. These findings include diffuse edema, an abnormal shifts of fluid in various compartments of the brain parenchyma, that involves the entire cerebral cortex and subsequent diffuse brain atrophy, which results in loss of neurons and the connection between them.<sup>163</sup>

Central apnea and seizures are the most frequent neurological alterations described in clinical reports.<sup>169</sup> Nevertheless, the frequency of neurological complications can reach up to 40% in children younger than 2 years with critical consequences due to severe hRSV infection.<sup>6</sup> Supporting evidence for an association between neurological alterations and hRSV infection is the detection of viral RNA and specific antibodies against hRSV in CSF of patients with neurological alterations (►Fig. 5). Furthermore, several studies have shown that encephalopathy-associated hRSV patients display altered cytokine profiles, as compared with patients infected with hRSV and bronchiolitis, but without neurological alterations.<sup>167,170</sup>

Central apnea can occur in up to 21% of children admitted into the clinic after hRSV infection. It has been proposed that hRSV infection can cause disruptions in neural control pathways by reducing nonadrenergic and noncholinergic inhibitory responses, which eventually may cause the central apnea symptom.<sup>171,172</sup> Further, hRSV causes abnormal cholinergic responses in an animal model, which suggests that hRSV can directly alter specific central nervous system (CNS) responses<sup>173</sup> by participating in CNS inflammation. An alternative explanation could be the significantly prolonged laryngeal chemo reflex observed in sleeping infants with hRSV bronchiolitis, as compared with those infected with hRSV but that do not develop central apnea.<sup>172</sup>

Other extrapulmonary clinical manifestations by hRSV, besides seizures, include recurrent neurological abnormalities. Such abnormalities have been described in two different types: generalized tonic-clonic and partial seizures with altered consciousness and focal motor features or eye deviation (►Table 1).<sup>165,168</sup> Similar to hRSV encephalopathy, seizures have also been related to the overproduction of cytokines and free radicals.<sup>163,167</sup> Importantly, hyponatremia can also contribute to seizures.<sup>165,168</sup> The direct infection of cranial nerves can also result in less common neurological alteration as reported for hRSV infection-induced strabismus in the form of esotropia.<sup>165</sup>

Taken together, extrapulmonary manifestations occurring in severe cases of hRSV infections are not isolated cases. Thus, there is an urgent need to evaluate the occurrence of these

events in hospitalized children with hRSV bronchiolitis and to study the possible consequences of acute neurological abnormalities due to the infection with this virus. Furthermore, it is imperative to determine whether these extrapulmonary effects are due to direct effects of tissues with hRSV or by inflammatory mediators dispersed from the airways or responding immune cells.

### Possible Mechanisms behind the Neurological Alterations Caused by hRSV

The neurological complications observed upon hRSV infection<sup>167</sup> have encouraged researchers to understand the mechanisms involved in CNS dysfunction. Studies performed in BALB/c mice and Sprague Dawley rats have detected hRSV-RNA and viral proteins in the brain of animals previously infected intranasally with this pathogen.<sup>174</sup> Studies have found that immune cells are associated with hRSV in peripheral blood from hRSV-infected patients. Consistent with this notion, hRSV-infected immune cells would migrate to the CNS by the hematogenous pathway and trespass the blood–brain barrier.<sup>174</sup> An unexpected and important finding regarding the access of hRSV to the CNS was the description that impairment in cognitive function is observed after pulmonary disease was resolved in mice and rats.<sup>174</sup> Indeed, our group recently described that mice and rats infected with hRSV have a deficient performance in tests that evaluate these abilities. hRSV-infected mice performed significantly worse than noninfected mice, both in the Marble Burying (MB) and Morris Water Maze (MWM) tests, several weeks after viral challenge.<sup>174</sup> The MB test consists in measuring the ability of rodents to dig and hide marbles, which is controlled by hippocampal function.<sup>175</sup> In addition, the MWM evaluates the animal's ability for spatial learning through spatial localization of relevant visual cues that are subsequently processed, consolidated, retained, and then retrieved in the brain to successfully navigate and thereby locate a hidden platform to escape from the water.<sup>176</sup> In both tasks, hRSV-infected animals showed significant alterations in behavioral and learning processes, as compared with control animals. Moreover, electrophysiological assays suggested that impaired cognitive function was due to a failure to efficiently induce long-term potentiation responses in the stratum radiatum in the hippocampus area. Our study supports the previously proposed idea that hRSV can alter CNS function. Accordingly, hRSV has been shown to infect primary neuronal cells in vitro, as well as neural processes innervating the lungs.<sup>148</sup>

The association of an exacerbated immune response against hRSV together with hRSV-induced cognitive impairment is supported by the observation that a vaccine that induces protective T cell immunity prevents virus spread into the CNS, as well as neurological alterations caused by infection.<sup>174</sup> A possible explanation is that hRSV may enter the CNS associated with leucocytes or freely, triggering an elevated secretion of proinflammatory cytokines that affect normal neuronal function.

In summary, hRSV infection can cause important extrapulmonary symptoms, which can lead to important and long-

lasting health sequelae in children affected by this virus. Therefore, significant research efforts are required for the generation of vaccines and therapies to prevent or treat the infection caused by this virus in the most susceptible population.

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