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REVIEW

Revisiting respiratory syncytial virus's interaction with host immunity, towards novel therapeutics

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Abstract

Every year there are > 33 million cases of Respiratory Syncytial Virus (RSV)-related respiratory infection in children under the age of five, making RSV the leading cause of lower respiratory tract infection (LRTI) in infants. RSV is a global infection, but 99% of related mortality is in low/middle-income countries. Unbelievably, 62 years after its identification, there remains no effective treatment nor vaccine for this deadly virus, leaving infants, elderly and immunocompromised patients at high risk. The success of all pathogens depends on their ability to evade and modulate the host immune response. RSV has a complex and intricate relationship with our immune systems, but a clearer understanding of these interactions is essential in the development of effective medicines. Therefore, in a bid to update and focus our research community's understanding of RSV's interaction with immune defences, this review aims to discuss how our current knowledgebase could be used to combat this global viral threat.

Keywords RSV · Interferon · Immunity · Therapeutics

Introduction

Respiratory syncytial virus (RSV) remains a significant burden to global health, with nearly all children thought to be exposed to this virus by the age of two, making it the most common cause of paediatric respiratory tract infection (RTI) and the biggest risk factor for severe infection in infants [1]. Elderly individuals are the second major group at risk of severe infection, with similar rates of intensive care admission and mortality to influenza. Indeed, RSV poses a particular threat to residents of long-term care facilities [2]. RSV outbrakes are observed worldwide and follow a seasonal pattern in most parts of the world, with significant occurrence during winter months [3, 4]. However, in some global regions, RSV infections occur throughout the year but still peak during the winter [5, 6].

Virus structure and epidemiology

RSV is a 15.2 kb, negative sense, RNA virus, with 10 genes producing 11 proteins. The viral envelope has three transmembrane proteins: the fusion glycoprotein (F), attachment glycoprotein (G) and the small hydrophobic (SH) protein. Other structural proteins are nucleoprotein (N), large RNA polymerase (L), phosphoprotein (P), matrix protein (M) and transcription factors (M2-1 & M2-2). In addition to these, RSV produces two non-structural (NS) proteins, NS1 and NS2 (Fig. 1) [7].

There are two major antigenic strains of RSV, RSV-A and RSV-B, which have distinct epitopes in the F and G proteins, as well as molecular differences in several genes [8, 9]. Both strains co-circulate, often alternating dominance annually [4]. There is some evidence that RSV-A is associated with higher morbidity, though other studies have shown no significant variation between strains [10–15]. The majority of RSV-infected patients recover after mild illness, however, a study in the UK reported a small percentage (6.9%), developed an acute respiratory infection (ARI) and required

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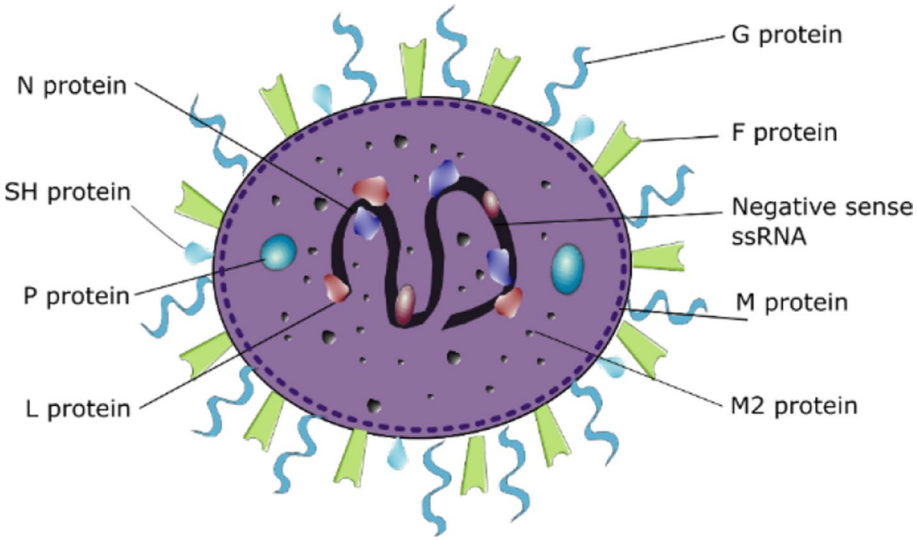

Fig. 1 RSV structure. The RSV genome encodes for 10 genes, giving rise to 11 proteins. The non-structural proteins, NS1 and NS2, are not present in the viron, but are expressed in high levels on infection of the host cell

hospital admission, 2.7% needed intensive care and 1.5% required a ventilator [16]. It is estimated, that by the age of two, nearly all children have had at least one RSV infection, which could equate to 3.4 million children/year needing hospitalisation [17]. Large scale European epidemiological studies, showed that outbreak patterns vary between countries, with those further east seeing a later start to the RSV season, while more northerly countries have a longer RSV season [4]. Variation in disease burden is tied to metrological conditions; RSV fairs best in cool temperatures (6.3 °C) and high humidity (84%), meaning cool, dry winters are predicted to have less RSV-related illness, than warm, wet conditions [18].

RSV's primary infection site is the respiratory tract epithelium. The virus is spread by droplet, contact and aerosol transmission [19, 20]. Infection can proceed to the lungs, causing serious disease; with the sloughing of dead cells enabling the virus to spread further into the respiratory airways [21]. There is also evidence that severe RSV infection in early life, increases the likelihood of asthma [22–25]. However, this phenomenon could also be a result of undiagnosed genetic predisposition to respiratory infection, thereby enhancing the observed associated with asthma development, highlighting the need for longer-term studies to fully elucidate this theory [26–28]. Once infected, infants are particularly susceptible to developing a severe infection; the immune system of an infant has fewer degenerative pathways than adults, relying heavily on their innate immune response and maternal antibodies to protect against infection [1, 29, 30]. The bronchial lumens of infants are underdeveloped, and are, therefore, narrower and more likely to be blocked by excessive mucus produced in response to infection, leading to reduced airflow, poor gas exchange in the alveoli and low blood oxygen levels [31]. Efficient clearance of a virus requires a strong Th1 response to activate IFN-γ producing cytotoxic T cells [32, 33]. However, multiple studies have shown that infants with RSV infection have a skewed response, producing a Th2 cytokine profile [34, 35]. RSV infection in young infants increases expression of Thymic Stromal Lymphopoietin (TLSP), which has been shown to be vital for immunopathology in mouse models and has been linked to later asthma development [25, 35]. TLSP alters T cell differentiation via dendritic cells (DCs), with TLSP primed DCs causing CD4 + T cells to express Th2 characteristic cytokines [36, 37]. TLSP is also able to induce type two innate lymphoid cells (ILC2) which play a significant role in allergy [25, 38]. RSV infection also increases expression of IL-33; this cytokine acts on both DCs and ILC2 to promote the differentiation of Th2 cells, increase mucus production and heighten airway sensitivity [39, 40]. The ability of RSV to increase levels of TLSP and IL-33 and thus promote a Th2 response, may indeed be responsible for negatively influencing the overall antiviral response.

With no vaccines currently available, limiting the spread of RSV infection remains under the control of good hygiene and hand washing; however, the close proximity of individuals at day-care centres and schools make these locations common epicentres of RSV outbreaks [19, 20, 41].

Treatments and vaccines

Despite affecting millions of people each year, there is still no fully effective, curative therapeutic available for RSV. Patients admitted to the hospital are generally given supportive treatments, including oxygen and airway clearance [42, 43]. Ribavirin is the only anti-viral drug on the market for use against RSV, though it is currently not recommended by the American Association of Paediatrics, guidelines vary between regions [42–46]. First developed in 1972, Ribavirin

is a guanosine analogue, which limits the replication of several RNA and DNA viruses. While originally licenced for the treatment of RSV, its most significant use has been in the management of Hepatitis C Virus (HCV) [47]. In HCV treatment, Ribavirin was used to good effect in conjunction with other drugs, including the anti-viral cytokine, Interferon (IFN)-α [47–49]. While not widely prescribed, Ribavirin is still used in "extreme" RSV cases [42, 45, 50–52]. A prophylactic preventative anti-RSV antibody, Palivizumab, is also available. Produced by MedImmune, this monoclonal antibody is administered through monthly intramuscular injections during the RSV season [46, 53]. Because of its preventative nature, accurately measuring the effectiveness of Palivizumab has proved difficult. Evidence suggests that Palivizumab significantly reduces RSV-related hospitalisations [54–56]. However, updated guidelines from the American Academy of Paediatrics stated that Palivizumab "has limited effect on RSV hospitalizations on a population basis, no measurable effect on mortality, and a minimal effect on subsequent wheezing" [57]. Palivizumab's high cost generally constrains its use to only "high risk" children, including infants born prematurely, those with congenital heart disease (CHD) or chronic lung disease (CLD) [58–60]. In addition, a randomized trial showed that Palivizumab should not be used as treatment; administering Palivizumab to infants with RSV bronchiolitis had no impact on outcomes [61]. Palivizumab's effectiveness and cost are major limiting factors in its use against RSV; therefore, global research aims must remain focussed on the development of an effective treatment and preventative vaccine.

RSV vaccine research began soon after the virus was first isolated in 1956, but has proven challenging. The 1966 trial of a formalin-inactivated RSV vaccine (FI-RSV), sensitised children to the virus, leading to enhanced disease in the immunised cohort. Unfortunately, this resulted in hospitalisations and the death of two children [62–64]. This failure of FI-RSV instilled caution over new RSV vaccines and halted the quest for a medicinal solution. However, this period of "reflection" sparked intensive research in the immune evasion and modulatory mechanisms of RSV; generating significant developments in our understanding of the virus, not least the discovery that RSV is attenuated upon deletion of the SH, M or NS1/2 genes, revealing these as prime targets for therapeutic intervention [65–68].

RSV presents a challenging, but essential global threat to harness. Several high-risk patient groups would particularly benefit from an effective RSV treatment. Indeed, RSV can be fatal for neonates and immunocompromised individuals, for whom any vaccines offer little protection. It is RSV's multiple "anti-immune" effects, that cloud our current understanding. Therefore, increased knowledge of RSV's cellular and molecular interactions and subversive mechanisms are fundamental in facilitating future drug and vaccine design.

Immune evasion mechanisms of RSV

The immune response can be split into two branches: Innate and Adaptive. Together they provide comprehensive protection from pathogens, thus limiting damage to the host. The innate response is fast acting and non-specific, while the adaptive response provides targeted clearance of the pathogen and lasting immunological memory, that quickly eliminates the pathogen upon reinfection. However, the key to the success of all pathogens is their ability to evade and subvert the immune response. RSV has multiple mechanisms to limit host immunity, allowing it to replicate unhindered, ultimately leading to tissue damage and subsequent clinical symptoms of the disease. Viral evasion is often mediated via conserved mechanisms and limit the immune response at several stages of the viral life cycle [69, 70]. RSV's interaction with the immune system at specific infection and replication points are key to its survival (Fig. 2). Having a genome that codes for 11 proteins provide RSV with multiple mechanisms to mask its replication and modulate the immune response [17]. While there is still much to elucidate, the effect of several specific RSV proteins upon immunity has been well characterised (Table 1).

Intracellular immunomodulation mechanisms

RSV, like many pathogens, influences cellular signalling pathways, thus disrupting the overall immune response, and limiting the speed and effectiveness of anti-viral clearance.

NS1 and NS2

The two non-structural (NS) proteins of RSV, NS1 and NS2 (which are made up of 139 and 124 amino acids, respectively), are the first proteins to be produced by the virus upon infection [7, 46]. These two proteins show little sequence homology, except for a short region at the C-terminus [71]. However, there is a high level of sequence identity between NS1 and NS2 of circulating RSV strains. Indeed, the conservation of the NS proteins between RSV strains suggests they hold an important role in viral replication.

As their name suggests, the NS proteins have no structural role, with NS1 and NS2 deficient RSV (ΔNS1/2-RSV), still able to replicate, albeit with poor growth in immunocompetent cells [67]. The ΔNS1/2-RSV grows well in IFN receptor-deficient Vero cells [72, 73]. In immunocompetent cells Type I IFNs are released when a pathogen is detected; these IFNs act in an autocrine and paracrine manner, binding to their specific IFN receptors and triggering activation of the Janus kinase/Signal Transducer and Activator of

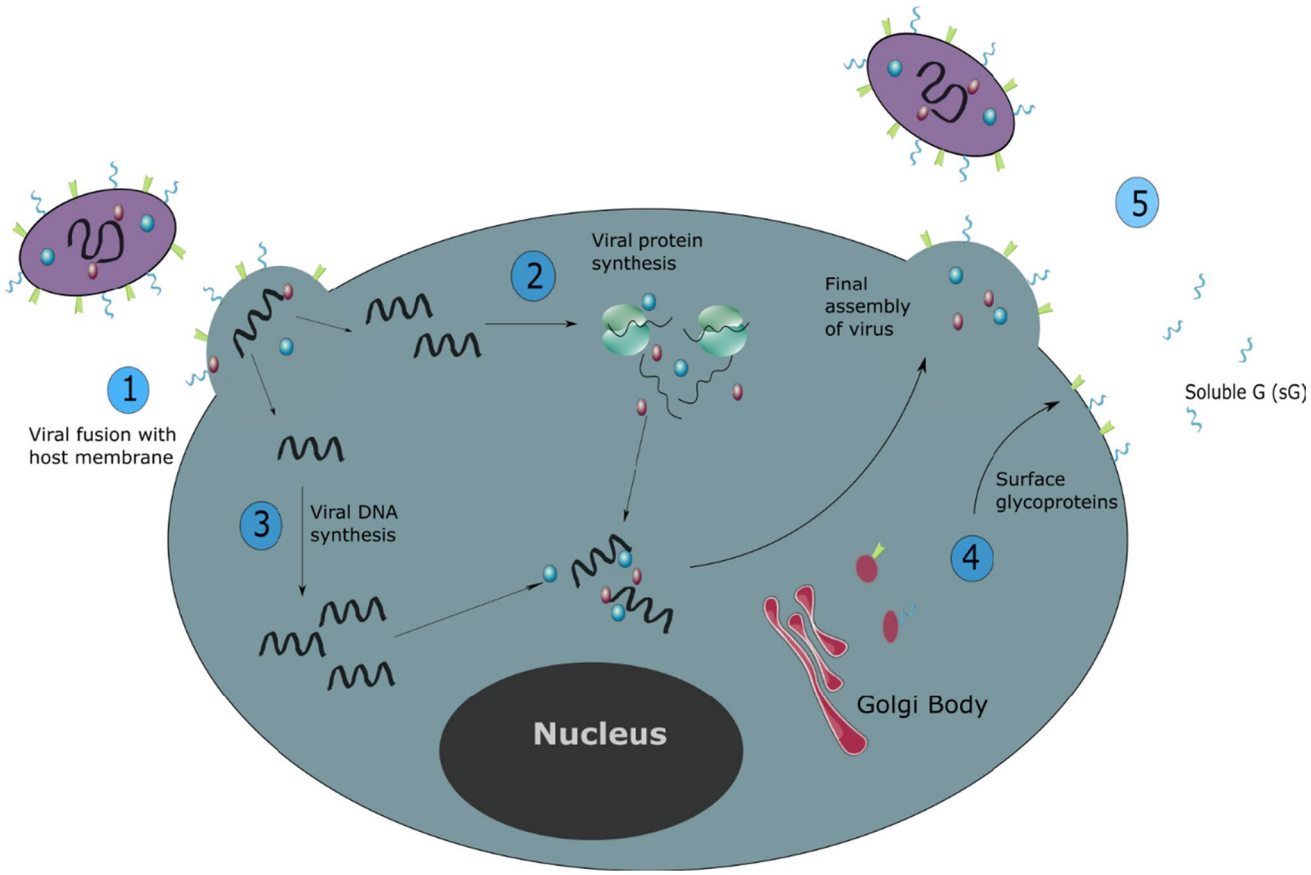

Fig. 2 The RSV Life Cycle. (1) The virion initially binds to the host cell through its G protein and membrane fusion is mediated by the F protein, which anchors into the membrane of the target cell and then folds on itself to bring the viral and host membranes into contact, resulting in membrane fusion. (2) The genome of the virus is used for protein synthesis, with large amounts of NS1/2 and sG protein produced shortly after infection. These proteins protect the replicating virus from the host immune defences. (3) The viral genome is replicated and structural proteins are produced. (4) The surface glycoproteins are synthesised in the Golgi body and deposited in the host membrane. (5) Assembly of the new virion takes place in the cytoplasm, before budding through the host cell membrane, picking up its surface glycoproteins as part of this process. sG protein is also released

Transcription (JAK/STAT) pathway. JAK/STAT signalling promotes the transcriptional upregulation of hundreds of IFN Sensitive Genes (ISGs) (often referred to as the "Interferome"), including cytokines, chemokines and anti-viral mediators [74]. ISGs are translated into effector proteins, which enhance the immune response and limit infection (Fig. 3). The Type I IFN pathway has been shown to be essential in clearing several viruses, including HCV and Influenza A [75, 76]. However, RSV infection only induces a weak anti-viral IFN response, that is insufficient to clear the virus [77–79].

Several studies have investigated the ability of the RSV NS proteins to limit IFN signalling, with both NS1 and NS2 documented to suppress ISGs [80–83]. NS1 and NS2 are key to RSV's regulation of the IFN response. NS1 harnesses a specific cellular E3 ligase, which selectively targets STAT2 for ubiquitination and proteasome-mediated degradation [81, 84]. In removing STAT2, RSV acts to limit anti-viral JAK/STAT signalling, thereby blocking the normal function of Type 1 IFNs and ultimately reducing ISG transcription [81, 82]. The presence of NS1 also modulates the T cell response, reducing the number of anti-viral CD8 + T cells and Th17 cells [85, 86], while also bolstering the activation of Th2 cells [34, 87], which, if uncontrolled, progresses the physiological symptoms of the disease. Additionally, the NS1 protein is associated with Mitochondrial Anti-viral Signalling Protein (MAVS), which may block its interaction with Retinoic Inducible Gene I (RIG-I), reducing IFN production in infected cells [88]. NS2 has also been implicated in targeting RIG-I, although this study was unable to show an interaction with MAVS [89].

Type 1 IFNs also sensitise infected cells to programmed cell death through the Fas-Associated protein with Death Domain (FADD) and Caspase-8 pathways. The death of infected cells effectively removes the virus's "life-line", limiting viral production and preventing infection of

Table 1 RSV proteins with documented immune evasion roles

RSV protein	Primary function	Immune evasion
F protein	Fusion Protein (Targeted by Palivizumab)	Contains variable regions to limit immune memory response between stains [161]
G protein	Attachment to cell membrane	Heavily Glycosylated, limiting recognition by antibodies [118]
		Contains variable regions to limit immune memory response between stains [122]
		CXC3R1 binding ability [132]
		Soluble 'decoy' form to limit immune cell migration [131,162]
		Promotes a Th2 response [129,163]
SH protein	Unknown	Reduces TNF-α sensitivity [111]
		Reduces IL-1β sensitivity [146]
		Inhibition of apoptosis [111]
		Disruption of cell surface membrane [143,164]
N protein	Nucleoprotein	Co-localised with RIG-I & MAVS to attenuate IFN response [150]
		Impairs immunological synapse formation [149]
NS1 protein	Immune evasion	Limits IFN signalling [67,80–82,84,92]
		Targets STAT2 for ubiquitination and degradation [81,82,84]
		Co-localised with MAVS to attenuated the IFN response [88]
NS2 protein	Immune evasion	Limits IFN signalling [67,80,85,89,92]
		Limits CD8+ memory [85]
		Delays cell death [71]

RSV interacts with the host through several of its proteins. Extracellular proteins highlighted in yellow and intracellular proteins in blue. Through each of its proteins RSV manipulates the immune response, creating a favourable environment for its replicative lifecycle and making the host more susceptible to infection

surrounding tissue [90]. In addition to limiting IFN signalling, NS2 also stimulates the phosphoinositide 3-kinase (PI3K) pathway, leading to delayed cell death and enhanced cell survival [71]. This prevents the action of NK and CD8 + T cells, allowing the virus to continue replicating in infected cells [71, 85]. RSV is also able to upregulate the expression of the Programmed Cell Death Ligand (PD-L)1, enabling infected cells to attenuate CD8 + T cell-mediated killing [91]; indeed, these discoveries reveal that the virus uses multiple molecular mechanisms to limit cell death. As well as the direct ubiquitination of STAT2, NS1 and NS2 have been linked to the reduction of several signalling molecules, including RIG-I, IRF3, TRAF3 and IKKε, further highlighting the broad spectrum of immune signalling components RSV targets to ensure the propagation of its infection and replication lifecycle [89, 92, 93].

Interestingly, type III IFNs have also been shown to be important in RSV infection. Much like type I IFNs, type III IFNs induce an anti-viral state, however, while type I IFNs are recognised by almost all cell types, type III IFNs are only detected by a subset of cells, typically those in the mucosal membrane, including macrophages, lymphocytes, Plasmacytoid DCs.

(pDCs) and epithelial cells [94]. This restricted expression of IFN III receptors allows enhanced response in specific cells without stimulating neighbouring cells. It is postulated that the restricted response to type III IFNs may have evolved to enable a selective anti-viral response in cells most likely to encounter viral infection [95, 96]. Although they bind different receptors, both type I and III IFNs activate the formation of Interferon Stimulated gamma factor 3 (ISGF3) (Fig. 3), leading to ISG production. Both type I and III IFNs are produced by and can act on airway epithelial cells, revealing type III IFN's importance in the context of viral respiratory infections [94, 95]. Both type I and III IFNs are produced at low levels in response to RSV infection in the A549 cell line, however, the removal of the NS proteins causes an increase in all IFNs, highlighting the role of these RSV proteins in suppressing the innate

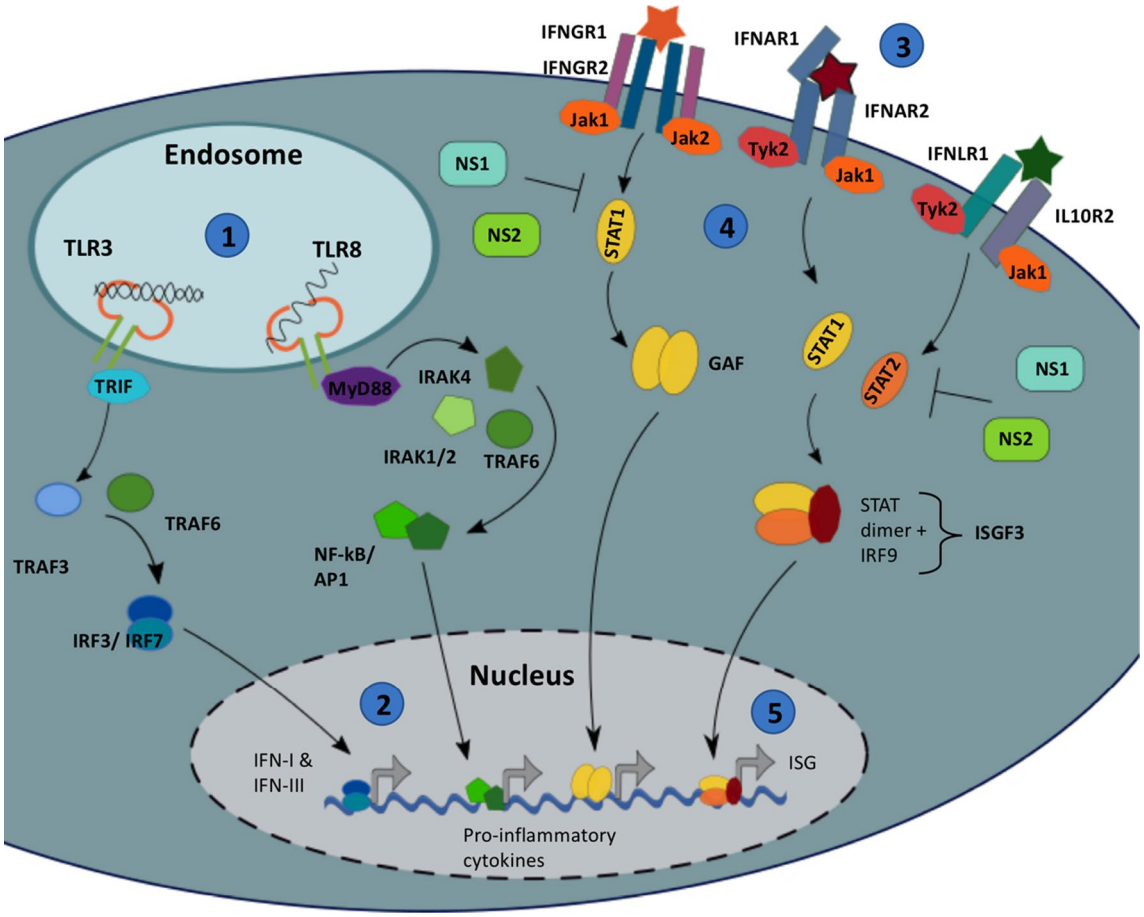

Fig. 3 TLR & IFN signalling. (1) Toll-like Receptor 3 & 8 detect intracellular pathogens by detecting dsRNA and ssRNA, respectively. Once initiated, signalling cascades activate transcription factors, (2) which upregulate anti-viral IFNs (Type I, II and III) and pro-inflammatory cytokines. (3) IFNs act on the infected and neighbouring cells by binding the Interferon receptors (e.g. IFNAR). (4) Change in receptor conformation allows the receptor-associated kinases, Tyk and Jak1, to trans-phosphorylate, which in turn phosphorylate receptor subunits, providing docking sites for STAT proteins. (5) Receptor-associated STATs become phosphorylated, dissociate from the receptor and form homo- or hetero-dimers. The IFN-α-activated STAT1:STAT2 dimer binds IRF9, forming a complex that translocates to the nucleus and stimulates the expression of Interferon Sensitive Genes (ISGs). RSV NS proteins have been shown to inhibit IFN signal transduction by impairing STAT activation

immune signalling that induces these anti-viral cytokines [97, 98]. Additionally, investigating IFN production in primary nasal epithelial cells showed type III IFN production, but not type I, is induced by RSV [99]. This suggests that primary human nasal epithelial cells behave differently to cell lines, and that specific cell lines also mount differential responses; a factor that should be considered when reviewing the literature and designing physiologically relevant experiments [99–101].

While NS1 and NS2 together severely impair the IFN response in humans, it is thought that the scope of their interplay is still not fully understood. Indeed, how these proteins function in different species further confuses the issue, with bovine RSV (bRSV), NS proteins appearing to target IFN production by blocking IRF3 activation, rather than blocking the IFN-mediated JAK/STAT signalling [102].

Mechanisms to limit antigen recognition

The surface of each virion holds the F, G and SH proteins. The G protein enables attachment of the virus to the host cell and the F protein initiates fusion of the host and viral membranes [103]. To enable viral entry into the target cell, initial contact is made through the G protein which allows the engagement of a secondary receptor, causing the activation of the F protein and membrane fusion (Fig. 4). Which cell surface proteins are used by G and F are debated. Extensive studies using submerged cell lines have shown that heparan sulfate is needed for RSV entry [104, 105], while other studies have shown that CX3CR1 is sufficient for viral entry [106, 107]. Investigations using human airway epithelial (HAE) cultures have seen low levels of heparan sulfate expressed on the apical cell surface [108], suggesting

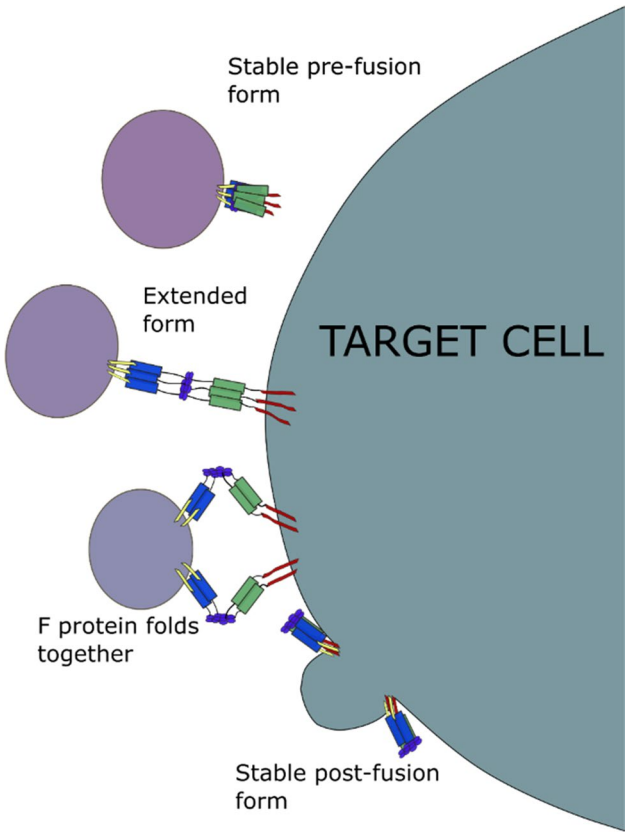

Fig. 4 The RSV F protein facilitates the fusion of the viral and host membranes. The stable form of the F protein anchors its hydrophobic N-terminus into the membrane of the target cell. The extended form of the F protein is not stable and the coils of the Heptad repeat A (blue) and Heptad repeat B (green) domains fold together. This overcomes the hydration force, to allow the viral capsid to fuse with the cell

that the use of heparan could be less clinically relevant [107–110].

Less is known about the role of the SH protein; while it is not needed for viral growth, the SH protein is conserved across all strains of RSV and is thought to influence virulence [46, 111, 112]. Their prominent external placement of the G and F proteins makes them key antigens for immune recognition, as a result, RSV has developed several mechanisms to limit host detection of both proteins.

G protein

The G protein is the major attachment protein of RSV and has been shown to bind heparan sulfate on the surface of immortalised cell lines and CX3CR1 on primary ciliated cells [107, 110, 113]; indeed, heparin reduces RSV infection of cell [114], and antibodies that prevent G protein–CX3CR1 interaction reduce RSV infection of mice [115]. While low levels of CX3CR1 are seen in both the upper and lower respiratory tract of infants, RSV has been shown to preferentially infect CX3CR1-expressing cells [116]. Another study found that removing RSV's CX3C domain had no impact on the infective potential and replication of RSV [117]. To protect the G protein from detection it is heavily glycosylated. Glycosylation is a common viral strategy used to protect the antigenic protein from antibody recognition [118, 119]. This mechanism allows the viral glycan structure to change, frequently altering the macrostructure of the G protein and thus masking the protein backbone from antibodies, which effectively limits their affinity.

Large variation in oligosaccharide arrangement across RSV strains also generates antigenic variation, which, in turn, limits the efficiency of immune memory, as previously generated antibodies have a poor affinity to seasonal variants of the G protein [120]. In addition, G protein frameshifts, point mutations and premature stop codons are regularly observed between seasonal strains [121]. These "immune avoidance" strategies decrease the likelihood of neutralising antibodies (against the G protein), being protective between seasons, effectively rendering immunological memory redundant against RSV infection. To avoid the adaptive immune response further, the G protein contains two hypervariable regions within its ectodomain, allowing regular mutation of antigenic epitopes and preventing recognition by antibodies selected for during a previous infection, confounding immune memory further [11]. These variations in RSV are used as the basis for its strain classification; specifically into RSV-A and RSV-B genotypes [12, 122]. As well as suppressing an effective humoral response, antigen variability enhances virus pathogenicity; in 2010, a new genotype of RSV-A was discovered in Ontario, Canada, named RSV-A ON1. RSV-A ON1 contains a 72 nucleotide duplication at the C terminus of the G protein [123]. This strain is now observed worldwide; a study in Vietnam saw that as RSV-A ON1 spread, there was an increased risk of lower respiratory tract infections and pneumonia [124].

As well as limiting the antibody response, heavy glycosylation of the G protein also hampers antigen presentation to T cells. To activate T cells viral antigen are presented by antigen-presenting cells (APC), such as DCs, through the major histocompatibility complex (MHC) and costimulatory molecules. The resulting structure forms an immunological synapse [125]. This stabilises the interaction between the MHC and T cell receptor, allowing correct alignment and activation of cognate T cells. If the presented antigen has sufficient affinity to the three complementary-determining regions of the T cell receptor, the T cell is activated and begins affinity maturation. Heavy glycosylation of proteins can disrupt this process, curtailing T cell activation [118]. During processing for presentation, antigens have some or all of the oligosaccharide groups removed by N-glycanase, before proteolytic cleavage within lysosomes. Any remaining

large oligosaccharides can affect the proteolytic cleavage, impacting the T cell repertoire produced. These short antigens bind to vacant MHC molecules in the lysosome, before being trafficked to the surface of the cell [126]. As a result, RSV infection generates a limited T cell response against the G protein. As CD4 + T cell help is critical in the affinity maturation of B cells, this process further hinders the generation of high affinity neutralising antibodies that can target the G protein.

The RSV G protein can also be produced in a soluble form (sG), which acts as an immune "decoy" (Fig. 2). The sG protein is produced in large quantities at the beginning of the viral life cycle, flooding the surrounding area and limiting the effectiveness of G-specific antibodies upon the actual RSV virion [127]. The creation of sG proteins is achieved by initiating transcription at the second AUG codon; this removes 65 amino acids from the N-terminal, the transmembrane region that normally anchors the G protein in the viral capsid. This shorter, truncated version has a hydrophobic amino terminus and is trafficked out of the infected cell and can be detected in the culture medium [128]. The combination of highly variable glycosylation and sG protein release, could limit the effectiveness of any G-specific antibodies and hinder effective immune memory. When studied in vivo it was found that sG protects RSV neutralisation from both G- and F-specific antibodies [127]; while the antibody decoy model suggests that sG would result in protection from G-specific antibodies, sG also reduced levels of F-specific antibodies, suggesting that sG uses multiple mechanisms to reduce the antibody response. Indeed, these modulatory effects of sG protein could also alter cytokine responses, which, in turn, limit the Th1 response [129, 130]. Interestingly, RSV lacking sG increases pro-inflammatory modulators, such as the chemokines CCL5 and IL-8; these findings suggest a role for the sG in blocking the recruitment of immune cells (specifically T cell and neutrophils), to the site of infection [130]. Furthermore, sG protein also limits the impact of antibody-dependent cell-mediated cytotoxicity and clearance of virus particles through the complement system [131], revealing yet another immune-modulatory effect of this soluble viral protein. Importantly, while these discoveries reveal how the RSV sG protein launches an effective immune evasion strategy, which affects both the innate and adaptive responses, they also highlight that it is not the only mechanism by which RSV suppresses and avoids immunity.

The G protein contains a CX3C motif (in both in its soluble and membrane-bound form), which acts to limit immune cell recruitment and thus modify the immune response [106, 132]. Through its CXC3 motif, RSV's G protein acts as a mimic of the chemokine CX3CL1 (Fractalkine). As well as blocking the action of CX3CL1, the RSV's CX3C motif allows the attachment of viral particles through the CX3CR receptor, aiding RSV infectivity [107, 117]. In addition, the CX3CR region reduces IFN production, with reduced levels of IFN-α2, IFN-λ1 and IFN-λ2 observed from A549 cells infected with WT RSV, compared to a CX3CR motif mutant version of RSV. This reduced IFN response limits the antiviral activity of the cell, increasing opportunity for RSV replication [132]. Co-culturing PBMCs with A549 epithelial cells infected with WT or CX3C-mutated RSV strains influenced the expression of anti-viral cytokines in several immune cell types. A greater proportion of monocytes and pDCs produced IFN-α and TNF-α, and more CD8 + and CD4 + T cells produced IFN-γ, when co-cultured with A549 cells infected the CX3C-mutated RSV, compared to the WT strain [132]. As pDCs are a major source of Type I IFNs and hold an important role in shaping the overall immune response, RSV's influence over this cell type, through its G protein, is of major importance to our understanding of RSV's immune evasion strategy. Furthermore, the presence of the CX3CR motif is also associated with reduced T cell trafficking, CD8 + T cell function and IFN-γ expression, revealing yet another key function for this region in blocking effective immunity [106]. Collectively, these discoveries show the RSV G protein to have a broad role in immunomodulation, allowing the virus to limit the impact of G-specific antibodies, reduce immune cell migration to the site of infection and alter key cytokine production, thus impacting the normal function of several immune cell types.

F protein

The activity of the F protein is thought to be essential for viral replication and is highly conserved between RSV-A and RSV-B strains [122, 133]. Its positioning on RSV's surface and high level of sequence homology between strains makes the F protein a prime target for vaccine development. The monoclonal antibody therapeutic, Palivizumab, targets the F protein and can neutralise RSV replication, offering some protection to high-risk patients. While this indicates that targeting the F protein provides protection against the majority of RSV strains, emerging RSV strains that contain a N276S mutation within the F protein, are resistant to Palivizumab [134, 135]. When the virion comes into contact with the host cell, the F protein undergoes a conformational shift, with the hydrophobic N-terminus anchoring into the target membrane (Fig. 4). The F protein then folds back in on itself to bring the viral and target membranes together causing membrane fusion [136–140]. Initial work on designing antibodies and small molecules against the F protein were hampered by the multiple confirmations the protein forms; indeed, targeting the post-fusion F protein form had little clinical benefit. The crystal structures of both the pre- and post-fusion F protein have now been solved which, it is hoped, will lead to the development of new therapeutics which can inhibit its function [133, 141]. Previous small peptides have been

developed for therapeutic use against the RSV F protein, but high costs and the requirement for frequent injections limits their appeal [113].

Though the G protein is the main attachment protein of RSV, the F protein is also able to bind host cells; this degenerate function protects against potential loss of function mutations in the G protein, which is much less stable than the F protein [137].

The F protein has been shown to allow preferential infection of neonatal B regulatory cells (nBreg). F protein binds to nBregs through the B cell receptor (BCR), causing cellular upregulation of CX3CR1, which is bound by the G protein, through its CX3C domain, allowing infection of the cell. RSV infection of nBregs causes an increase in the production of anti-inflammatory IL-10, thus supressing Th1 activity [142]. This activity was observed to be specific to nBreg cells found in both cord and infant blood immediately after birth but significantly decreased with age, with less than 2% of CD19 + B cells being nBregs by the age of 12 months. Indeed, this observation may explain why younger infants have such a significant risk factor for severe RSV infection.

SH protein

The final protein on the surface of the RSV capsid is a type II transmembrane protein, the small hydrophobic (SH) protein. Structural studies suggest that SH functions as a viroporin and may be able to form pores in cell membranes, altering membrane permeability [143, 144]. SH deletion mutants (ΔSH-RSV) are still able to enter cells and replicate, however, murine experiments have shown that the ΔSH-RSV is less virulent than the WT strain [111, 112, 143, 145]. Although the SH protein mechanism is not yet fully understood, research suggests that it has a role in prolonging the life of infected cells; this insensitivity to apoptosis permits increased viral replication [111]. The presence of the SH also influences cytokine production, with the ΔSH-RSV inducing increased IL-1β and TNF expression [111, 146]. As a result, SH deletion mutants have been explored as live attenuated vaccine candidates, though, to date, none have been brought to market [147].

N protein

The nucleoprotein, together with the phosphoprotein (P), coats the RSV RNA genome in a nucleocapsid to protect it from degradation [7]. Structural analyses have shown that it forms a left-handed helix around the viral genome [148]. As well as this key structural role, the N protein has also been shown to have an immune evasion function. Despite being a nucleoprotein, N protein is also present on the surface of infected epithelial cells and DCs early during infection [149]. Additionally, the presence of N protein in a bi-lipid membrane prevents the formation of mature immunological synapses, leading to a reduction in T cell activation [149]. The ability of RSV to modulate T cell activation also affects antibody production; with fewer naïve T cells activated, there are fewer T follicular helper cells and consequently a reduction in B cell activation. Lifland et al., found that after 6 h RSV infection, the N protein co-localised with RIG-1 and Melanoma Differentiation Associated Gene 5 (MDA5), and later during infection, viral inclusion bodies were observed containing MAVS and MDA5 [150]. This interaction of the N protein with the RIG pathway components, limits the subsequent anti-viral IFN response, thereby enabling more efficient, undisturbed, RSV replication.

Future vaccine and treatment development

With an estimated 3.2 million RSV-related hospital admissions each year, the development of vaccines is key to protection and control of this virus [151]. The nature of RSV's infectivity and immune evasion strategies has hindered the development of a vaccine that balances safety and efficacy. The spectrum of different immune profiles of the high-risk groups (infants, children, pregnant women and the elderly), adds a layer of complexity, that has stunted successful vaccine development [134]. Where available, the standard treatment of care for children hospitalised with RSV-related LRTI is to mitigate the symptoms of bronchiolitis with fluids and oxygen. Ribavirin is administered in some countries, though its often morbid side effects (such as potential teratogenicity), high cost and poor effectiveness has limited its widespread use against RSV [44, 45, 50–52].

The primary purpose of any vaccine is to generate a significant long-lasting antibody and memory B and T cell response, thus protecting against future infection. However, "natural" RSV infection only generates short-lived antibody responses, with two RSV infections thought to be required to generate protective antibodies, which even then can rapidly wane [103, 152]. Maternal antibodies provide protection for new-born infants, but the rate of infant RSV infections peak around 2–3 months of age, with maternal antibodies declining to seronegative levels at ~ 2.5 months. This process is thought to rely on the mother having had an RSV infection close to birth, thus enabling antibody transfer [153, 154]. However, one study showed that high titres of RSV antibodies in cord blood did not reduce the infant's number of RSV infections, although it did reduce the severity, indicating that maternal anti-RSV antibodies have some protective effect. Interestingly, the antibody transfer rate was lower in male infants than females, leaving the males at higher risk of RSV-related hospital admittance [30].

The first clinically trialled vaccine was formalin-inactivated RSV (FI-RSV) in 1966 [62]. While several other successful formalin-inactivated vaccines exist, including those against polio, hepatitis A and Rabies [155–157], the formalin-inactivated RSV vaccine not only provided no protection in RSV naïve infants but increased the risk of significant infection [134, 158]. The cause of this vaccine-enhanced disease is still not fully understood. This initial clinical trial failure resulted in subsequent caution, which has most certainly contributed to the current absence of a licenced RSV vaccine. Advances in crystallography have given insights into the native form of key RSV proteins, including the structure of the F protein [133, 138, 141], which will likely guide and direct future vaccine development. Indeed, the fact that Palivizumab offers some protection indicates that a targeted antibody response can protect against RSV infection [54, 134, 138], though the emergence of resistant Palivizumab strains of RSV suggests any vaccine will require continued maintenance, similar to that against Influenza.

There are now several dozen RSV vaccines under clinical trial, including some maternal vaccines, which could offer protection for neonates in the future [159, 160]. Use of viral proteins, particularly G and F, should theoretically, generate a strong antibody response. While the design of an effective vaccine is a priority, in its absence, the development of a curative treatment remains key. Indeed, when or if, an effective vaccine is found, treatment will always be essential for those do not have sufficient vaccine protection.

An ideal curative treatment will be a direct-acting antiviral, or a therapeutic that targets a conserved immune evasion mechanism of RSV, thus restoring natural immunity against RSV and limiting viral replication. To better inform the design of these new therapeutics and vaccines the research community must work towards defining the role of all 11 RSV proteins and determine all the processes by which RSV suppresses immunity, while also monitoring the genetic variation of the virus over time. Armed with this knowledge, we will be better placed to develop vaccines and therapeutics that protect our global populations from this ongoing viral threat.

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