

## Invited Mini Review

## Innate immune recognition of respiratory syncytial virus infection

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Respiratory syncytial virus (RSV) is the leading cause of respiratory infection in infants and young children. Severe clinical manifestation of RSV infection is a bronchiolitis, which is common in infants under six months of age. Recently, RSV has been recognized as an important cause of respiratory infection in older populations with cardiovascular morbidity or immunocompromised patients. However, neither a vaccine nor an effective antiviral therapy is currently available. Moreover, the interaction between the host immune system and the RSV pathogen during an infection is not well understood. The innate immune system recognizes RSV through multiple mechanisms. The first innate immune RSV detectors are the pattern recognition receptors (PRRs), including toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The following is a review of studies associated with various PRRs that are responsible for RSV virion recognition and subsequent induction of the antiviral immune response during RSV infection. [BMB Reports 2014; 47(4): 184-191]

## INTRODUCTION

RSV is a major cause of respiratory illness in young children, contributing significantly to morbidity and mortality worldwide. Most infants are infected with RSV at least once within first two years of life. In some cases, RSV provokes severe lower respiratory tract illness, such as pneumonia or bronchiolitis. As a result, many of these patients are plagued by recurrent childhood wheezing or asthma. Moreover, RSV infection is well known to recur many times throughout life, but the mechanism of innate immune responses against RSV infection is unclear. RSV is now recognized as an important

cause of respiratory infection in elderly patients who are immunocompromised or have cardiovascular disease.

RSV was first isolated from chimpanzees as a chimpanzee coriza agent in 1955 (1). Shortly after, a similar virus was recovered from infants with respiratory illness and designated RSV (2, 3). RSV is an enveloped negative sense single-strand RNA (ssRNA) virus of the *Paramyxoviridae* family and belongs to the subfamily *Pneumovirinae*. RSV consists of a single serotype and two antigenic subtypes, A or B. The RSV genome is about 15 kb nucleotides in length and encodes nine structural proteins and two non-structural proteins (4). Three envelop glycoproteins, G, F and SH, are present on both the viral membrane and infected cells. F and G are the only RSV proteins that induce neutralizing antibodies (5, 6). The name RSV was given because the F proteins on the viral surface cause the membranes on nearby cells to merge, forming syncytia. NS1 and NS2 inhibit the type I interferon (IFN) host response by interrupting the janus kinase-signal transducers and activators of the transcription 1 (JAK-STAT) pathway (7-9).

The incidence of RSV infection is elevated from winter to early spring. Unlike the seasonal influenza virus, there is no licensed vaccine against RSV. The only prevention method for high-risk infants is monthly injections of palivizumab, RSV neutralizing monoclonal antibodies, during the epidemic season. Many attempts have been made for vaccine development. In the mid-1960s, a formalin-inactivated (FI) vaccine candidate caused the deaths of two infants who were enrolled in a clinical trial (10). Even today, no live attenuated vaccines or live virus vectored vaccines exist. Recently, other types of vaccines, purified proteins, virus-like particles (VLPs), nanoparticles, and DNA or vectors expressing RSV proteins, are being developed for various target populations (11-13). Nonetheless, no vaccine is currently applicable. Moreover, therapeutic methods are limited in that the nucleoside analog ribavirin is only used to treat RSV infection in the pediatric population. In addition, studies have shown that the ribavirin has limited efficacy and can induce hemolytic anemia as a side effect (14).

Similar to other respiratory viruses, RSV infection or uptake by airway epithelial cells, alveolar macrophages, and intraepithelial dendritic cells incites a direct antiviral response through cytokines and chemokines and initiates an adaptive immune response. Sentinel cells, like dendritic cells (DCs) or macrophages, have pattern recognition receptors (PRRs), toll-like receptors (TLRs), retinoic acid-inducible gene-I

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(RIG-I)-like receptors (RLRs), or nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) on the pathogen (15). These PRRs recognize many different PAMPs and activate transcription factor nuclear factor (NF)- $\kappa$ B, a member of the interferon regulatory factor (IRF) family, which regulates the expression of proinflammatory cytokines and type I interferon. This review focuses on the role of these various PRRs, which are responsible for recognizing the RSV virion and inducing the antiviral immune response during RSV infection.

## RSV-SPECIFIC PATTERN RECOGNITION RECEPTORS

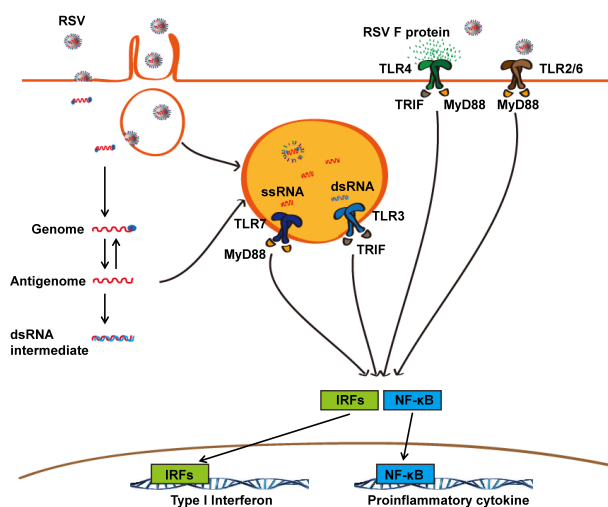
### TLR2/6

TLR2 is located on the cell surface and recognizes cell wall components, such as peptidoglycan or lipoprotein. Recognition leads to subsequent innate immune activation via the MyD88 mediated pathway (Fig. 1). Studies have shown that TLR2 expression is upregulated in RSV-infected airway epithelial cells (16, 17). In addition, TLR2 blocking antibodies suppressed heat-inactivated RSV-induced proinflammatory cy-

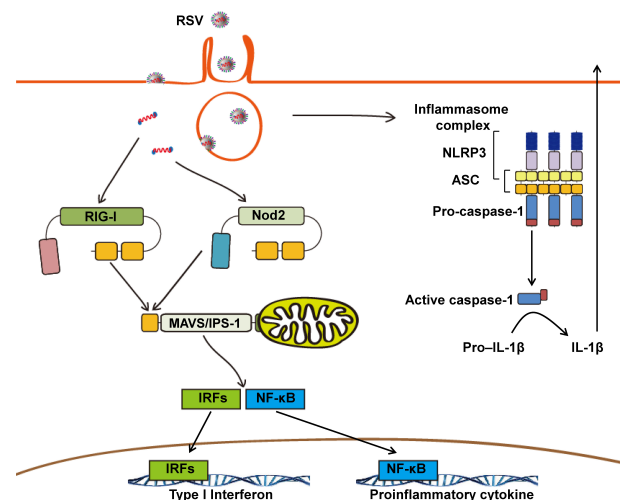
tokines in airway epithelial cells (18). Peritoneal macrophages obtained from TLR2- or TLR6-deficient mice showed impaired production of proinflammatory cytokines and chemokines; however, production of type I IFN was not affected by RSV infection (19). TLR2-deficient mice actually showed uncontrolled viral replication in the RSV-infected lung as well as impaired neutrophil migration and DC activation. It appears that TLR2 plays a role in innate immune activation, but does not contribute to the production of type I IFN. How TLR2 recognizes the RSV virion remains unknown.

### TLR4

Because TLR4 was the first known RSV sensor among the PRRs, it has been studied extensively. TLR4 recognizes gram negative lipopolysaccharide (LPS) on the cell surface. TLR4-mediated signals are transmitted through TRIF- or MyD88-mediated pathways (Fig. 1). Kurt-Jones *et al.* showed *in vitro* that the RSV fusion protein was recognized by TLR4 and CD14 as a co-receptor and produced proinflammatory cytokine IL-6. Furthermore, RSV infected TLR4-deficient mice (C57BL10/ ScCr) showed prolonged viral clearance in the lung (20). RSV infected TLR4-deficient mice (C57BL10/ScNcr)



**Fig. 1.** Role of Toll-like receptors during RSV infection. Stimulation of TLR4 with RSV F glycoprotein in a TRIF- or MyD88-dependent signaling pathway induces production of type I interferon or proinflammatory cytokines. TLR2/6, which are activated by RSV, enhance production of type I interferon or proinflammatory cytokines through a MyD88-mediated pathway. TLR7, localized in the endosome, recognizes the RSV ssRNA genome and activates expression of type I interferon and proinflammatory cytokine genes via a MyD88-mediated signaling pathway. Endosomal TLR3 detects RSV dsRNA, an RSV replication intermediate, and then induces production of type I interferon or proinflammatory cytokines in a TRIF-dependent signaling pathway. Abbreviations: TLR, Toll-like receptor; MyD88, myeloid differentiation primary response 88; TRIF, TIR-domain-containing adaptor-inducing interferon-beta; IRF, interferon regulatory factor; NF- $\kappa$ B, nuclear factor- $\kappa$ B.



**Fig. 2.** Role of intra-cytoplasmic pattern recognition receptors during RSV infection. Activated RIG-I, which forms a panhandle structure with the ssRNA RSV genome, interacts with the adaptor MAVS, and then enhances expression of type I interferon and proinflammatory cytokine genes. Intracellular Nod2 recognizes the ssRNA genome and induces production of type I interferon or proinflammatory cytokines in a MAVS-dependent signaling pathway. In the activated inflammasome complex, active caspase-1 enhances IL-1 $\beta$  production. Abbreviations: Nod2, nucleotide-binding oligomerization domain containing 2; RIG-I, retinoic acid-inducible gene 1; MAVS, mitochondrial antiviral signaling protein; NLRP3, nucleotide-binding domain and leucine-rich-repeat-containing (NLR) family, pyrin domain-containing 3; ASC, apoptosis-associated speck-like protein containing a CARD; IRF, interferon regulatory factor; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

showed diminished NK cell and CD14<sup>+</sup> cell recruitment, impaired cytotoxic NK cell function, decreased inflammatory cytokine expression, and delayed viral clearance (21). In another functional TLR4-deficient mice (C3H/HeJ), the RSV-inducible early NF- $\kappa$ B response was dependent on TLR4 expression in lung (22). However, Ehl *et al.* have shown that the absence of TLR4 (C57BL10/ScN and BALB/c lps) had no impact on NK cell recruitment or the activity and recruitment of other pulmonary inflammatory cells. In addition, TLR4 deficiency did not affect viral clearance or RSV-specific T cell responses (23).

Others have reported that RSV infection induces the upregulated expression and membrane localization of TLR4 in airway epithelial cells (24). In infants with RSV bronchiolitis, TLR4 expression is upregulated in blood monocytes, and the degree of TLR4 expression has been correlated with disease severity (25). However, a biological role for TLR4 in RSV-specific immunity remains a question. Direct interaction between intact RSV particles, not including the RSV F protein, and the human TLR4 receptor complex were not found to play an important role in RSV pathogenesis *in vitro*. In the hTLR4 cell line model, the TLR4 complex played no role in RSV-induced NF- $\kappa$ B activation, viral entry, or replication (26).

Many studies of TLR4 polymorphisms have been conducted compared to other PRRs. Indeed, TLR4 mutations (Asp299Gly and Thr399Ile) have been associated with severe RSV bronchiolitis (27). Both single nucleotide polymorphisms (SNPs) encoding Asp299Gly and Thr399Ile substitutions in the TLR4 polymorphism were highly associated with symptomatic RSV disease in premature infants (28). Others found that the TLR4 amino acid variant (Asp259Gly) is marginally associated with RSV infection (29). However, Paulus *et al.* showed that the Asp299Gly TLR4 polymorphism does not affect receptor function and does not induce severe RSV infection (30). Despite that great effort has been made to define how TLR4 contributes to RSV infection, the biological role of TLR4 in RSV immunity remains controversial.

### TLR3

On endosomal compartments, TLR3 recognizes double-stranded RNA (dsRNA) or polyinosine-polycytidylic acid (poly IC), a synthetic analog of dsRNA. It also induces an innate immune response through the TRIF-mediated pathway. Although RSV belongs to the ssRNA virus genetically, it can generate dsRNA intermediates during replication in the cytoplasm. The dsRNA replication intermediates are taken up by the endosome, allowing TLR3 to sense the RSV virus.

Generally, RSV initiates a more severe disease course than human metapneumovirus (hMPV), another common cause of respiratory infection in children. Dou *et al.* showed that severe clinical RSV syndrome was caused by TLR3 activation and production of TNF- $\alpha$  *in vitro* and in mouse lung (31). TLR3 expression was also upregulated in RSV-infected airway epithelial and lung fibroblast cells (32, 33). Moreover, lung cells from RSV-infected BALB/c mice had both upregulated TLR3

expression and NF- $\kappa$ B activation (34). In airway epithelial cells with down-regulated TLR3 through small interfering RNA (siRNA), lesser amounts of chemokines, such as CXCL10 and CCL-5, were produced compared to RSV-infected mock cells (32). However, in these cell lines, RSV infection and replication was not affected by TLR3. Others have shown that siRNA-mediated TLR3 knockdown inhibited IFN- $\beta$ , IFN-inducible protein 10 (IP-10), CCL5, and IFN-stimulated gene 15 (ISG15) expression and significantly reduced NF- $\kappa$ B/RelA transcription (17). NF- $\kappa$ B is known to be a nuclear phosphoprotein with activating sites at serine residues 276 and 536 (35-38). Liu *et al.* have suggested that activation of the TLR3 pathway in airway epithelial cells controls the phosphorylation of RelA at serine 276 (17).

An interaction between the TLR3 signaling pathway and the production of type I IFN is possible. One study has shown that RSV affected type I IFN production through poly IC stimulation in HEK293 cells (39). The authors suggested that RSV G protein or soluble G proteins inhibited TLR3/4-mediated type I IFN production by blocking the TRIF/TICAM-1 pathway.

Viral clearance was not affected, but Th2 biased immune responses, including goblet hyperplasia, eosinophil infiltration, and increased IL-5 and IL-13 production of T cells, occurred in TLR3 deficient mice (40). Taken together, it appears that TLR3 partially contributes to both the innate and adaptive immune responses against RSV infection. However, TLR3 is likely not required for viral clearance. TLR3 may play a supplementary role in pathogen sensing and the initiation of the immune response.

### TLR7

RSV, which contains ssRNA as a genome, enters the cytoplasm through membrane fusion. The ssRNA genome is transferred to the endosome, where it can be detected by TLR7 on the endosomal compartment. In addition, virions engulfed by endocytosis can also be recognized by TLR7 on the endosomal compartment. RSV-activated TLR7 transmits signals via the MyD88 mediated pathway.

Marozin *et al.* have shown that RSV has the capacity to shut down type I IFN production in TLR7/9 agonist stimulated human plasmacytoid dendritic cells (pDCs) (41). This paper suggested an interaction between RSV and the TLR7/9 signaling pathway in pDCs. In a pneumonia virus of mice (PVM) infected mouse model, which mimics RSV infection, the TLR7-MyD88 mediated pathway was required for the CD8<sup>+</sup> T cell response as well as the induction of type I IFN and proinflammatory cytokines in pDCs (42). In addition, upregulated TLR7 expression was observed in splenocytes from hyper-eosinophilic mice (43). This study demonstrated that eosinophils can contribute to innate antiviral immunity and promote virus clearance against RSV *in vivo* through the TLR7-MyD88 mediated pathway. Moreover, lung cells from RSV-infected BALB/c mice exhibit upregulated TLR7 expression and subsequent activation of NF- $\kappa$ B (34).

A more pathologic RSV infection has been observed in TLR7 deficient mice, including mucus production, goblet cell hyperplasia, and pulmonary inflammation. TLR7 deficient bone marrow derived DCs (BM-DC) preferentially produced Th17 promoting cytokines, but decreased Th1 promoting cytokines. After restimulating lung cells obtained from RSV-infected TLR7 deficient mice with RSV, a significant increase in the Th17 cytokine IL-17A production was observed, as well as elevation of Th2 cytokines IL-4 and IL-13 (44). However, IRF-1 expression was not affected by TLR7 deficiency. Considering the RSV life cycle, TLR7 likely plays a crucial role in RSV detection and subsequent innate immune initiation. Further study is necessary to determine whether the TLR7-MyD88 mediated pathway is required for type I IFN induction during RSV infection.

### RIG-I and MAVS (IPS-1)

RLRs, which consist of melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene-I (RIG-I) and laboratory of genetics and physiology 2 (LGP2), are a type of intracellular pattern recognition receptor. The RLRs, including RIG-I and MDA5, bind viral dsRNA or 5'-triphosphorylated uncapped viral RNA in the cytosol (45-47). In particular, RIG-I recognizes ssRNA viral genomes bearing 5'-triphosphates (48). Viral RNA can be detected by RIG-I or MDA5, which activate the downstream NF- $\kappa$ B and IRF3 pathways by interacting with the mitochondrial antiviral-signaling protein (MAVS; IFN- $\beta$  promoter stimulator 1 (IPS-1)) adaptor molecules (Fig. 2). In theory, the RSV genome consists of ssRNA, and RSV replicates in the cytoplasm. Therefore, RIG-I has the advantage of detecting actively replicating RSV.

In a mouse fibroblast model, RIG-I was essential for initiating innate immune defenses against RSV, but MDA5 was not (49). Liu *et al.* have shown that RSV induced IFN- $\beta$ , IP-10, CCL5, and ISG15 expression levels were decreased in a RIG-I-silenced airway epithelial cell line very early in the infection (17). In nasal pharyngeal washes from infants with RSV associated bronchiolitis, gene expression levels for MDA-5, RIG-I, TLR-7, and TLR-8 were relatively higher in infants with RSV infection. However, there was a significant positive correlation only between RSV viral load and RIG-I mRNA levels (50). In blood mononuclear cells from neonates, young children, and adults, human pDCs from healthy adult donors produced IFN- $\alpha$  in RSV infection through a RIG-I dependent pathway. However, those from young and premature infants did not produce IFN- $\alpha$  in a RIG-I dependent manner (51).

The other paper mentioned that MAVS was required for RSV-induced type I IFN and proinflammatory lung cytokine responses *in vivo* as well as in lung fibroblasts, bone marrow derived macrophages (BM-DM) and BM-DCs *in vitro* (52). However, RSV viral clearance and CD8<sup>+</sup> T cell responses were not affected in MAVS deficient mice during RSV infection. Interestingly, in IPS-1 deficient mouse lungs, RSV infection reduced inflammatory resolution, prolonged viral clearance, and increased the activation of T cells with a predom-

inant Th1 profile (53). However, in bone marrow chimera experiments that restored IPS-1 signaling, viral clearance and lung pathology were rescued in the nonimmune and immune compartments.

We previously mentioned that RIG-I and the MAVS complex activate the downstream NF- $\kappa$ B and IRF3 pathways. NF- $\kappa$ B is controlled either by a canonical pathway, which releases sequestered RelA complexes from the I $\kappa$ B $\alpha$  inhibitor, and by a noncanonical pathway, which releases RelB from the 100-kDa NF- $\kappa$ B2 complex (54-57). However, RelA activation in response to RSV infection was induced, in part, by a cross-talk pathway involving the noncanonical NIK-IKK $\alpha$  complex downstream of RIG-I and the MAVS adaptor (58). Other study showed that RelA activation was mediated by RelA Ser-276 phosphorylation and cytoplasmic mitogen- and stress-related kinase-1 (MSK1) activation (59). In addition, when RSV is sensed by RIG-I, it triggers a signaling cascade involving the MAVS and TRAF6 adaptors ultimately leading to p65 phosphorylation at Ser536 by IKK $\beta$  kinase (60). Thus, RSV mediated NF- $\kappa$ B p65 phosphorylation at Ser536 is dependent on RIG-I, TRAF6 and IKK $\beta$ . Collectively, it appears that the RIG-I and MAVS pathway plays a crucial role in activating the innate immune response. However, further study is necessary to understand the function of RIG-I and MAVS in RSV infection.

### Nod2

As a cytoplasmic viral sensor, nucleotide-binding oligomerization domain 2 (Nod2) can detect the ssRNA genome and trigger innate immune activation by binding with the adaptor MAVS. Nod2 was shown to be required for IRF3 activation and IFN- $\beta$  production in RSV infection *in vitro* (61). Moreover, nod2-deficient mice failed to produce adequate type I IFN, induce viral clearance, and resolve pathology against RSV infection *in vivo*.

Another paper focused on muramyl dipeptide (MDP), which is a peptidoglycan constituent of both gram positive and negative bacteria that is recognized by Nod2 (62). Vissers *et al.* insists that IFN- $\beta$  induction by RSV RNA is an essential first step in the augmented nod2-mediated proinflammatory response to MDP (63). This means that a synergistic response to stimulation with RSV and MDP was observed, which was dependent on a Nod2 mediated pathway. The function of Nod2 during RSV infection has not yet been fully elucidated. Thus, further research is warranted to assess the role of Nod2 in the immune response against RSV infection.

### Inflammasome

The inflammasome is a multiple protein complex, including NLRs, their adaptor proteins and pro-caspase-1. The complex stimulates caspase-1 activation to promote the processing and secretion of proinflammatory cytokines IL-1 $\beta$  and IL-18 as well induce cell death. Some research has shown that influenza virus can induce the activation of the inflammasome (64). Others have reported that RSV infection also activate the in-



flammasome (65-67).

In human alveolar epithelial cells, live RSV activated the IL-1 $\beta$ -converting enzyme (caspase-1) gene and led to subsequent protein production, possibly implicating the inflammasome in the response to RSV infection (65). Segovia *et al.* showed that NLRP3/ASC inflammasome activation was crucial for IL-1 $\beta$  production during RSV infection (67). Their studies emphasized the requirement of reactive oxygen species (ROS)/potassium efflux (second signal) as well as the TLR2/MyD88/NF- $\kappa$ B pathway (first signal) for NLRP3/ASC inflammasome formation. This leads to caspase-1 activation and subsequent IL-1 $\beta$  secretion during RSV infection in BM-DMs.

Another group showed that the small hydrophobic (SH) RSV viroporin localized within lipid rafts in the Golgi induced permeability by disrupting membrane architecture and forming ion channels. It then triggered NLRP3 trafficking from the cytoplasm to the Golgi in primary human lung epithelial cells. This SH RSV viroporin was used as viral recognition pathway to signal inflammasome activation (66). Thus, it appears that the inflammasome plays an important role in viral recognition and the initiation of antiviral responses during RSV infection. However, the precise function of the inflammasome in RSV infection is not yet known, and the mechanism of NLRP3 activation also remains unclear.

## CONCLUSION

Over the past several decades, RSV vaccine development studies have advanced. Despite these efforts, there is currently no licensed vaccine against RSV infection besides palivizumab. Recent studies have demonstrated that various PRRs, including extracellular TLRs, endosomal TLRs, RLRs, NLRs and the inflammasome recognize RSV infection and induce immune activation through several pathways. However, limited information is available regarding the role of PRRs in RSV infection. Because cell types express different PRR subtypes, antiviral responses against RSV infection can vary by PRR or cell type. To better understand these processes, it will be necessary to determine which PRRs predominate the antiviral immune response against RSV infection. The interaction between PRRs and RSV likely represents the first step in the immune response leading to viral clearance. Therefore, studies investigating the PRR-mediated antiviral response to RSV will be important for aiding vaccine and therapeutic agent development.

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