**Role of double-stranded RNA pattern recognition receptors in the response to rhinovirus infection**

by

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## Dedication

To my dear grandparents who will always be with me in my mind.

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## List of Abbreviations

**AIM2**, absent in melanoma 2; **ANOVA**, analysis of variance; **BAL**, bronchoalveolar lavage; **CARD**, N-terminal caspase-recruitment domain; **COPD**, chronic obstructive pulmonary disease; **DAI**, DNA-dependent activator of IFN-regulatory factor; **DAMP**, danger-associated molecular pattern; **DC**, dendritic cell; **eIF4G**, eukaryotic translation initiation factor 4G; **ELISA**, enzyme-linked immunosorbent assay; **EMCV**, encephalomyocarditis virus; **GAPDH**, Glyceraldehyde-3-phosphate dehydrogenase; **GM-CSF**, granulocyte macrophage-colony stimulating factor; **ICAM**, intercellular adhesion molecule; **IFN**, interferon; **IFNAR**, interferon alpha receptor; **IKK**, IκB kinase; **IL,** interleukin**; IN**, intranasal; **IP**, intra-peritoneal; **IP-/CXCL-10**, interferon gamma inducible protein-10; **IPS-1**, interferon-β promoter stimulator 1; **IRES**, internal ribosome entry site; **IRF3**, interferon regulatory factor 3; **ISRE**, interferon stimulated regulatory element; **KC**, keratinocyte chemoattractant; **LDL-R**, low-density lipoprotein receptor; **LPS**, lipopolysaccharide; **LRR**, leucine-rich repeat; **MAVS**, mitochondrial antiviral signaling; **MBP**, major basic protein; **MCP**, monocyte chemotactic protein; **MDA5**, melanoma differentiation associated gene 5; **MEF**, mouse embryonic fibroblasts; **MIP**, macrophage inflammatory protein; **MyD88**, myeloid differentiation primary response gene (88); **NDV**, Newcastle disease virus; **NEMO**, NF-κB essential modifier; **NLR**, NOD-like receptor; **NOD**, nucleotide-binding oligomerization domain; **OAS**, 2′,5′ oligoadenylate synthetase; **ORF**, open reading frame; **OVA**, ovalbumin; **PAMP**, pathogen-associated molecular pattern; **PBS**, phosphate-buffered saline; **poly I:C**, polyinosinic:polycytidylic acid; **PRR**, pattern recognition receptor; **PTK**, protein tyrosine kinase; **PKR**, protein kinase R; **RANTES**, regulated upon activation normal T-cell expressed and secreted**; RIG-I**, retinoicacid-inducible gene I; **RSV**, Respiratory syncytial virus, **RT-PCR**, reverse transcriptase polymerase chain reaction; **RV**, rhinovirus; **SeV**, sendai virus; **Th-2,** T-helper (h)-2; **TIRAP,** Toll-interleukin 1 receptor

domain-containing adapter protein; **TLR**, Toll-like receptor, **TNF**, tumor necrosis factor; **TRAM**, TRIF-related adaptor molecule **TRIF**, TIR domain-containing adapter-inducing interferon-β; **UTR**, untranslated region; **UV**, ultraviolet; **VSV**, vesicular stomatitis virus; **WNV**, West Nile virus.

# Chapter 1

## Introduction

### Rhinovirus

Rhinovirus (RV) is a small positive-stranded RNA virus of the *Picornaviridae* family. It contains a single-stranded positive-sense RNA genome of about 8 kb in length that is enclosed in a non-enveloped icosahedral capsid, which in turn consists of a densely packed arrangement of 60 protomers. Each protomer is comprised of four polypeptides: VP1, 2, 3, and 4 (1-2). VP1, 2, and 3 are located on the capsid surface, whereas VP4 is located on the internal side of the capsid.

RV virion RNA is infectious and serves as both the genome RNA and viral messenger RNA (mRNA). The 5′ end of RV genome is covalently attached to a viral protein, VPg, instead of a methylated nucleotide cap structure, as in eukaryotic RNA. VPg is believed to be important for viral replication (3). A long untranslated region at the 5′ end contains a cloverleaf-like internal ribosome entry site (IRES), which allows direct translation of viral protein (4). A shorter untranslated region at the 3′ end is important for negative-strand synthesis during replication.

Sequencing of the human rhinovirus 14 genome reveals a single large open reading frame of 6536 nucleotides, starting at nucleotide 678 and ending 47 nucleotides from the 3′ end. The open reading frame encodes a single polypeptide that is divided into

functional viral proteins. Starting from the 5′ end, the single open reading frame is translated into a large polypeptide precursor that is divided into three regions: the P1 region, which encodes the capsid proteins VP1, 2, 3, and 4; and the P2 and P3 regions, which encode the non-structural proteins $2A^{Pro}$, 2B, 2C, 3A, VPg, $3C^{Pro}$ and $3D^{Pol}$. Facilitated by viral proteases 2A and 3C, the polypeptide precursor is cleaved and processed into the mature viral proteins by a sequence of proteolytic cleavages. Note that some viral proteases may also interact with the host cell proteins to promote viral replication (5-6). For example, $2A^{pro}$ is responsible for the cleavage of the eukaryotic translation initiation factor 4G (eIF4G), which leads to the prevention of host cell protein synthesis (7).

There are over 100 known RV serotypes, and cross-protection between the serotypes is negligible, preventing both an efficient immune response and development of a cost-effective vaccination strategy. At least 99 RV serotypes that affect humans have been sequenced (8). The major group serotypes (~90%; e.g., RV14, 16, and 39) bind to the intercellular adhesion molecule (ICAM)-1 (9). The minor group serotypes (e.g., RV1B) bind to low-density lipoprotein family receptors (10). A third group of previously unrecognized RV serotypes has been identified and shown to cause respiratory illness, particularly in infants (11-12). However, these serotypes have not been cultured. A recent study reported that VP4 is highly conserved among many RV serotypes, suggesting potential for a pan-serotype RV vaccine (13).

Upon infection, RV binds to its cell surface receptor and is endocytosed. Receptor binding, along with endosomal acidification, triggers the uncoating and subsequent release of viral RNA into the cytosol (14-16). Using the positive-sense RNA genome as

the template, viral RNA-dependent RNA polymerase 3D then forms the corresponding negative strand, which serves as a template for synthesizing a new positive-strand RNA. It has been shown that the RV genome can arrive in the cytosol 10 min post-infection from late endosomal compartments (17). Viral replication may occur as early as 3 h after infection. Finally, at an optimal temperature range of 33ºC–35ºC (the temperature of human nasal passages), assembled mature virions are released from the cell 8–10 h after infection. This temperature requirement was originally thought to exclude RV as a lower respiratory tract pathogen. However, there is increasing evidence that RV is able to replicate in the lower respiratory tract. RV RNA has been detected in lower airway cells from the bronchoalveolar lavage (BAL) of infected subjects (18-19), and RV capsid protein was observed in bronchial biopsies after viral inoculation (20). RV RNA has also been detected in bronchial biopsies of asthmatic subjects long after their exacerbation symptoms have cleared (21). These studies suggest that RV may infect the lower airways *in vivo* and contribute to airway inflammation, although the extent of RV replication remains unclear.

## Innate immunity

### *Interferons (IFNs)*

The innate immune system is no longer considered to be a primitive and weak first line of protection, but rather, it is a highly active antiviral defense that has potential for therapeutic manipulation. The IFN family of cytokines is one of the key components of antiviral innate immunity, and the induction of IFNs is the first line of defense against viral infection (22).

Three types of IFN have been identified and classified according to the receptor complex through which they signal. Type I IFNs, which in humans mainly comprise 13 IFN-α subtypes and IFN-β, engage the ubiquitously expressed IFNAR (IFN-α receptor) complex that is comprised of IFNAR1 and IFNAR2. Type I IFNs induce hundreds of genes that promote a robust antiviral state against viral infection. The importance of the IFN signaling network is illustrated by the extreme susceptibility to viral infection of mice that lack IFN receptors (23-24). IFNAR-deficient mice have increased susceptibility to numerous viruses (25), and humans with genetic defects in components of the IFNAR signaling pathway die of viral diseases (26-27). The type II IFN response is comprised of the single IFN-γ gene product that binds the IFN-γ receptor complex and mediates a broad immune response to pathogens other than viruses. The most recently described are the type III IFNs, which include three IFN-λ gene products that signal through receptors containing IFNLR1 (also known as IL-28Ra) and IL-10R2 (also known as IL-10Rb). Type III IFNs are structurally and genetically distinct from type I IFNs and act through a distinct receptor system. However, with regard to mechanisms of induction, signal transduction, and biological activities, they are surprisingly similar (28).

*Interferon-stimulated Genes (ISGs)*

After binding to their ligands, type I IFN receptors activate the downstream Jak-Stat pathway by associating with the Janus protein tyrosine kinases (Jak PTKs) Tyk2 and Jak1 via their intracellular domains (29). The activation of these Jak PTKs results in the phosphorylation of their downstream transcription factors Stat1 and Stat2. Tyrosine phosphorylation of stat 1 and 2 leads to the formation of the transcriptional activator

complex IFN-stimulated gene factor 3 (ISGF3), which then translocates to the nucleus and binds to the IFN-stimulated regulatory element (ISRE), resulting in the transcriptional induction of a number of ISGs which in turn mediate the antiviral response (30).

The binding of type I IFNs to the IFNAR complex initiates a signaling cascade that leads to the induction of more than 300 ISGs (31), such as ISG15, MxGTPase, 2′,5′ oligoadenylate synthetase (OAS), RNaseL, and protein kinase R (PKR). ISG15, a ubiquitin homologue, can promote antiviral signaling by attaching to effector proteins such as RIG-I, a process called ISGylation (32). The expression of MxGTPase is induced through an ISRE in the gene promoter after stimulation with type I IFNs. The MxGTPase family proteins degrade newly synthesized viral components at early time points (33). OAS accumulates in the cell cytoplasm as an inactive monomer. After the formation of viral double-stranded RNA (dsRNA), OAS forms a tetramer that synthesizes 2′,5′-oligoadenylates. Oligoadenylates, in turn, activate RNaseL. Activated RNaseL forms a homodimer that degrades viral single-stranded RNA (ssRNA) (34). In this manner, OAS, in combination with RNaseL, constitutes an antiviral RNA decay pathway. PKR is a protein kinase that is constitutively expressed in all tissues at a basal level. In response to environmental stresses, PKR inhibits protein synthesis by phosphorylation of eukaryotic translation initiation factor-2α. PKR is directly activated by dsRNA and other ligands such as ceramide (35). Infection of airway epithelium by both major and minor RV groups has been shown to activate antiviral IFN pathways, and the disruption of these pathways enhances RV production (36).

*Interferon-related transcription factors*

Upon viral infection, IFN induction is transcriptionally regulated by the IFN-regulatory factor (IRF) family, as well as other transcription factors, such as NF-κB and ATF2/c-Jun (37-38). The transcription factors bind to specific motifs in the IFN promoter (39-40). The homologous proteins IRF3 and IRF7 are activated in response to IFN and viral stimulation (41). NF-κB and ATF2/c-Jun are activated in response to various stimuli, including viral binding, leading to the expression of genes relevant to inflammation (42). These transcription factors form a transcription regulatory unit called an enhanceosome and cooperatively activate the transcription of IFN genes (43).

Among the nine IRF family members (IRF1-9), an essential role for IRF3 and IRF7 in the activation of IFN-α/β was demonstrated by an analysis of IRF3 and IRF7 knockout mice (41, 44). IRF3, which is constitutively expressed in most cell types, resides in the cytosol in a latent form. It has multiple serine and threonine phosphorylation sites in its C-terminal region (45-46). Upon either viral infection or IFN stimulation, IRF3 is transcriptionally upregulated and undergoes phosphorylation, dimerization, and nuclear translocation (47-49). IRF3 is phosphorylated by two non-canonical IκB kinases: TANK-binding kinase 1(TBK1) and IκB kinase ε/ι (IKKε/ι), which are structurally related and possibly redundant in function (38, 50). IRF7 phosphorylation is similar; however, IRF7 expression is differentially regulated. In fibroblasts and conventional dendritic cells (cDCs), IRF7 is expressed at low levels and can be induced by the trace amount of IFN produced (51-52). Two models of IRF3 activation and dimerization have been proposed: the phosphorylation-induced dimerization model and the autoinhibitory model (53-54). Regardless of the model, the

dimeric form of IRF3 (either a homodimer or a heterodimer with IRF7) then translocates to the nucleus, forms a complex with the coactivators CBP and/or p300, and binds to its target DNA sequence in type I IFN genes, as well as in certain cytokine and chemokine genes, to regulate their expression. Initially, a small amount of type I IFN is produced and secreted. It then stimulates the type I IFN receptor in an autocrine and a paracrine fashion, leading to the activation of the IFN pathway and the transcription of the IRF7 gene. The activation of newly synthesized IRF7 results in further transcription of type I IFN genes; thus, a massive IFN production can be achieved through this positive feedback mechanism (52, 55).

In contrast, IFN-β expression does not strictly require IRF3 and IRF7. The expression of IFNs is usually regulated by a number of transcription factors that form an enhanceosome (43, 56-58). Daffis and colleagues observed that the IFN-β response in the macrophages and dendritic cells of mice lacking both IRF3 and IRF7 was minimally diminished after West Nile virus (WNV) infection (59). This suggests that IFN-β regulation after WNV infection does not always require full occupancy of the IFN-β enhanceosome by canonical constituent transcription factors. Therefore, the participation of various transcription factors in the enhanceosome and their spatial location relative to the promoter region determines the final outcome of viral infection, *i.e.*, the pattern of cytokine expression (60-61).

NF-κB, which consists of p65 (RelA) and p50, is involved in the expression of type I IFNs and proinflammatory cytokines. In unstimulated cells, the p65:p50 heterodimer is retained in the cytoplasm through its binding with the NF-κB inhibitor (IκB) (61-62). In response to viral infection, IκB is phosphorylated by the IκB kinase

(IKK) complex, comprised of IKKα, IKKβ, and the regulatory component IKKγ (NEMO). It is then ubiquitinated and degraded by the proteosome, which leads to the release of the p65:p50 NF-κB dimer into the nucleus.

**Pattern Recognition Receptors**

The discovery of pattern recognition receptors (PRRs) revolutionized the understanding of innate immunity, explaining why and how multiple and diverse infectious agents are recognized by a limited number of innate immune receptors that trigger antimicrobial responses (63). Studies have shown that viral induction of type I IFNs is mediated mainly by the activation of PRRs including Toll-like receptors (TLRs) (64-65), retinoic acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) (66-67). These germline-encoded PRRs are responsible for sensing the presence of microorganisms by recognizing structures conserved among microbial species, namely pathogen-associated molecular patterns (PAMPs), as well as endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Several distinct family members are responsible for the detection of different components of pathogens (68-73). A summary of PRRs and their respective ligands is provided in **Table 1**.

Table 1. Pattern recognition receptors (PRRs) and their ligands

| PRRs | Location | Ligands |
|---|---|---|
| TLR | | |
| TLR1 | Plasma membrane | Triacyl lipoprotein |
| TLR2 | Plasma membrane | Peptidoglycan |
| TLR3 | Endosome | dsRNA |
| TLR4 | Plasma membrane | LPS, viral glycoproteins |
| TLR5 | Plasma membrane | Flagellin |
| TLR6 | Plasma membrane | Diacyl lipoprotein |
| TLR7/8 | Endosome | ssRNA |
| TLR9 | Endosome | unmethylated CpG-DNA |
| RLR | | |
| RIG-I | Cytoplasm | Short dsRNA, 5'-ppp dsRNA |
| MDA5 | Cytoplasm | Long dsRNA |
| LGP2 | Cytoplasm | Unknown |
| NLR | | |
| NOD1 | Cytoplasm | Bacteria peptidoglycan |
| NOD2 | Cytoplasm | Bacteria peptidoglycan |
| NALP1 | Cytoplasm | DAMPs |
| NALP2 | Cytoplasm | DAMPs |
| NALP3 | Cytoplasm | DAMPs, bacteria & virus RNA |
| DNA sensor | | |
| DAI | Cytoplasm | dsDNA (B-form) |
| AIM2 | Cytoplasm | dsDNA |

There are two general principles applied to PRRs that induce type I IFNs: 1) all PRRs that activate the type I IFN response detect microbial nucleic acids due to a lack of conserved features in other viral components; 2) all PRRs trigger the type I IFN response from an intracellular location (22). One possible exception is TLR4, which is believed to be located on the cell surface. TLR4 detects lipopolysaccharides (LPS) and viral glycoprotein derived from gram-negative bacteria and activates type I IFNs in cDCs and macrophages through a signaling pathway that uses TIR domain-containing adapter-inducing interferon-β (TRIF) as an adaptor protein (74-75). However, it has been proposed that TLR4-mediated IFN production occurs only after TLR4 sequentially translocates to early endosomes and activates a set of adaptors after initially signaling at the plasma membrane via a different set of adaptors (76). Similarly, TLR2 is also believed to signal from the plasma membrane. It recognizes cell wall components, such as peptidoglycans, leading to the production of inflammatory cytokines, but not IFNs (77-78). However, when activated by vaccinia virus, TLR2 can also induce type I IFNs after endocytosis, but only in inflammatory monocytes (79). Considered together, these observations challenge the general principle that only nucleic acids can trigger the IFN response, but support the idea that all PRRs that trigger the type I IFN response do so from an intracellular location. Similar to virus-induced responses, a potent type I IFN response can be induced by intracellular bacterial infections through PRRs (80-82). However, whether this IFN induction is beneficial to the host is not clear (83-85).

The most common nucleic acid ligands stem from the genomes of infecting viruses or from the intermediates involved in viral replication. The receptors that sense nucleic acids fall into two categories. The first class of PRRs, specifically TLRs, detects

viral nucleic acids in endosomes and in specialized cell types (22). The second class of PRRs is expressed ubiquitously; they are localized to the cytosol where they detect viral nucleic acids produced during infection.

*Toll-like receptors*

TLRs, numbered 1-11, are a family of single-transmembrane proteins expressed predominantly on key sentinel cells of the innate immune system, such as macrophages and DCs (86). All TLRs have an extracellular leucine-rich repeat (LRR) motif for the recognition of specific PAMPs and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain to transmit intracellular responses through the recruitment of TIR-containing adaptors (87). These adaptors, including MyD88, TRIF (TICAM-1), TRAM, and TIRAP, mediate cellular events that lead to the induction of inflammatory genes (88). Among the TLR family members, TLR1, 2, 4, 5, and 6 are expressed on the cell surface and are responsible for the detection of extracellular PAMPs, such as bacterial and fungal cell wall components; whereas TLR3, 7, 8, and 9 recognize viral nucleic acid ligands in intracellular compartments, such as endosomes (89-94). This intracellular localization is thought to enable the discrimination between self and non-self nucleic acids: relocating TLR9 to the cell surface abolishes its ability to respond to virus-encapsulated DNA but enables recognition of self-derived genomic DNA in the extracellular milieu (95). The endosomal TLRs usually require endoplasmic reticulum protein UNC-93B to deliver them to the endosomal compartment (96).

TLR3 recognizes viral dsRNA, which is found in the genome of dsRNA viruses or in replication or transcription intermediates of ssRNA viruses or DNA viruses. The

expression of TLR3 is predominantly observed in the intracellular compartments of cDCs and macrophages; some fibroblasts also express TLR3 on their cell surface (68). After ligand binding, TLR3 is activated and binds to its adaptor protein TRIF via a TIR-TIR homotypic interaction, which then recruits a set of signaling molecules, including tumor necrosis factor (TNF) receptor-associated factors (TRAF)3, TRAF6, receptor interacting protein 1 (RIP1), and transforming growth factor-α-activated kinase 1 (TAK1), leading to the activation of NF-κB, IRF3, and IRF7, and eventually, the engagement of the antiviral IFN pathway (97-98).

*Cytoplasmic PRRs*

Numerous observations suggest that host cells express additional receptors that detect actively replicating viruses in the cytoplasm. Recently, RIG-I and MDA5 have been characterized as cytoplasmic dsRNA receptors that play a vital role in the antiviral innate immune response. RIG-I, whose expression is induced by retinoic acid, IFN, and viral infection, is a member of the DExD/H box RNA helicases (99).

Human RIG-I encodes for a protein of 925 amino acids. Its N-terminal region is characterized by the presence of two caspase recruitment domains (CARD), and its C-terminal helicase domain harbors potential ATP-dependent RNA helicase activity. Over-expression of the N-terminal twin CARD domains is sufficient to activate NF-κB and IRF3 in the absence of a viral challenge, whereas the full length of RIG-I is activated only in the presence of dsRNA. Thus, the binding of dsRNA to the RNA helicase domain of RIG-I likely induces a conformational change that exposes the N-terminal CARD domains to initiate the recruitment of downstream signaling proteins (100). The

functional significance of RIG-I in antiviral immunity was first shown by RNA interference (RNAi) studies and subsequently confirmed by mouse knockout studies (101-102). RNAi of RIG-I in a mouse fibroblast cell line inhibited not only IRF3 activation but also subsequent induction of type I IFNs in response to RNA viruses (the specificity of RIG-I for particular viruses is discussed below). The embryos of RIG-I knockout mice displayed severe liver degeneration. However, studies also showed that pretreatment of lung fibroblasts with IFN-β increased the resistance of RIG-I-deficient fibroblasts to vesicular stomatitis virus (VSV), indicating that RIG-I is required for the induction of IFN-β, but RIG-I deficiency does not necessarily affect the downstream IFN-β amplification signaling (101).

MDA5 is structurally related to RIG-I, as it contains two N-terminal CARD-like domains and a single C-terminal helicase domain (103-104). Like RIG-I, MDA5 is also an IFN-inducible gene. Overexpression of MDA5 leads to enhanced antiviral responses to infection with Newcastle disease virus (NDV), VSV, or encephalomyocarditis virus (EMCV), as evidenced by increased expression of type I IFNs; whereas knockdown of MDA5 blocks NDV-induced activation of type I IFN promoters (67).

RIG-I and MDA5 both use their CARD domains to signal downstream events, which suggests they may signal through a common CARD adaptor molecule. Such an adaptor was identified on the mitochondria membrane and named IFN-β promoter stimulator-1 (IPS-1, also called MAVS, Cardif, or VISA) (105-108). It has been confirmed that both RIG-I and MDA5 signaling converge at IPS-1 before activating downstream transcription factors IRF3 and NF-κB (109). Interestingly, when IPS-1 is released from the mitochondria to the cytoplasm or when it is targeted to another

organelle such as the endoplasmic reticulum, it no longer mediates downstream IRF and NF-κB activation, suggesting a role for mitochondria in antiviral immunity (106). The signaling schematic of RV-induced IFN responses is outlined in **Figure 1-1**.

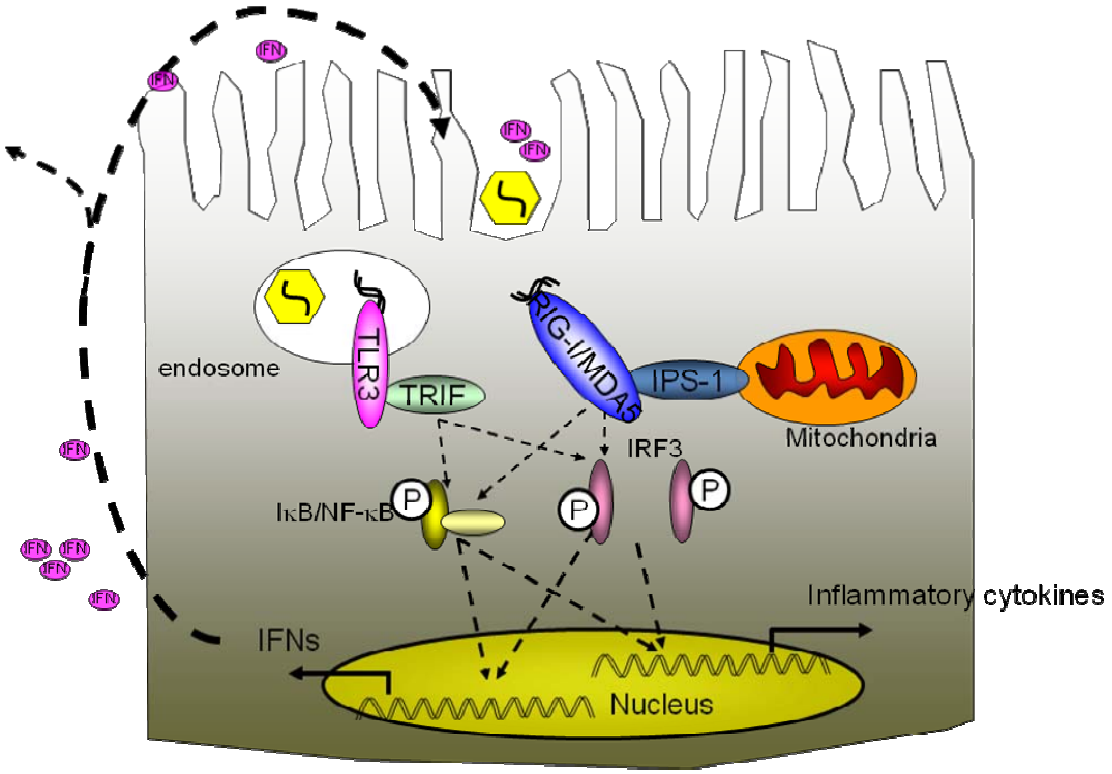


Figure 1-1. The schematic of IFN signaling in response to RV infection in an airway epithelial cell.

Even though both the cytoplasmic RIG-I/MDA-5 and endosomal TLR pathways can activate IFN signaling, they are not functionally redundant. Mice deficient in IPS-1 are highly susceptible to infection despite an intact TLR system (109-110). Kato and colleages clarified the relative contribution of each in antiviral defense by showing their cell type-specific requirements (102). TLRs are activated when they recognize nucleic acids within the endosomal compartments of plasmacytoid DCs (pDCs), whereas RIG-I defends the host against viral infections, including NDV, VSV, and Sendai virus (SeV), within the cytoplasm of mouse embryonic fibroblasts (MEFs) and cDCs. Hence, cell type specificity may reflect the differential ability of infected cells to recognize viral-derived nucleic acids in different compartments and to preferentially trigger TLR- or RIG-I-dependent responses.

RIG-I and MDA5 each have unique specificity in ligand binding. RIG-I and MDA5 knockout mice show impaired antiviral responses against different viruses. RIG-I, but not MDA5, mounts antiviral responses against the positive-strand ssRNA Japanese encephalitis virus, and against a set of negative-strand ssRNA viruses including NDV, VSV, SeV and influenza virus. In contrast, MDA5 detects the presence of EMCV, a member of the picornavirus family (102, 111). RIG-I recognizes relatively short dsRNA (up to 1 kb), especially dsRNA with a 5′ triphosphate, which greatly enhances type I IFN-inducing activity (112-113). MDA5, in contrast, recognizes long dsRNA (more than 2 kb) such as poly I:C. Shortening the length of poly I:C by a dsRNA-specific nuclease converts the poly I:C from an MDA5 ligand to a RIG-I ligand (69). Therefore, recognition is specific at the level of both cell type and pathogen. The receptors required

to recognize RV dsRNA and to trigger the downstream immune response in airway epithelial cells have not been identified.

**Animal models of RV infection**

Species differences in ICAM-1 represent the main challenge in developing an animal model of a human major group RV infection. Recently, we (114) and others (115) showed that minor group serotype RV1B, which binds proteins of the low-density lipoprotein receptor family, infects C57BL/6 and Balb/c mice, thereby providing an animal model to study RV-induced airway inflammation. We reported evidence of human RV1B replication in mouse lungs as follows: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice, 2) the transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells, and 3) the induction of a robust lung IFN response (114). Replication-deficient UV-irradiated virus elicited none of these effects. RV infection also caused a moderate increase in airway resistance to methacholine, suggesting a role for RV-induced airway inflammation in airway hyper-reactivity.

The airway response to the major group virus RV16 was recently studied using a transgenic mouse expressing humanized ICAM-1 (115). The effects induced by RV16 were indistinguishable from those induced by RV1B. Studies indicate that major and minor group viruses induce nearly identical patterns of gene expression in cultured airway epithelial cells (36). Furthermore, recent analysis of all known HRV genomes has revealed that HRV1 and HRV16 are highly homologous and respond similarly to small-molecule antiviral compounds (116). Thus, the distinction between at least some major

and minor group strains may not be clinically relevant. Therefore, we believe that mouse models of human RV1B infection hold promise for the study of RV-induced exacerbations of chronic airway diseases, such as asthma.

**RV-induced airway inflammation**

Another main component of the innate immune response, besides the IFN response, is the inflammatory response. The expression of cytokines from a small number of RV-infected epithelial cells can orchestrate the proliferation, chemotaxis, and attraction of peripheral inflammatory cells to the infection site, initiating the inflammatory response. RV infection of primary epithelial cells and epithelial cell lines is accompanied by the release of inflammatory mediators *in vivo* and *in vitro*; in particular, the pro-inflammatory cytokines, TNF, CXCL8/IL-8, IL-6, CCL5/RANTES, CCL2/MCP-1, and CXCL10/IP-10 (42, 117-118). Consequently, common cold symptoms are now considered to result from an inflammatory cytokine disease of the host in response to the virus and not from the virus itself (119).

Attracted by chemokines, inflammatory leukocytes, granulocytes, DCs, and monocytes migrate to the site of infection (120). Neutrophil infiltration, in particular, into the submucosa and epithelium during the common cold is likely caused by the secretion of IL-8, which is a potent chemoattractant and mediator of neutrophils (121). The increased presence of neutrophils suggests that they are the predominant inflammatory cells during virus-induced asthma exacerbations, whereas eosinophils are responsible for allergen-provoked asthma (122). However, it has been reported that eosinophils are increased in biopsies of bronchial epithelium taken from healthy and asthmatic persons

after experimental RV infection (123). These observations suggest that both neutrophils and eosinophils are necessary for RV-induced asthma exacerbation.

**The role of RV-induced IFN responses in asthma exacerbation**

Viral respiratory tract infections are responsible for up to 85% of asthma exacerbations (124-125). RV is the main virus associated with asthma exacerbations, responsible for up to 80% of virus-induced asthma exacerbations (126). Corne and colleagues were the first to suggest there may be inherent differences in the way asthmatic and non-asthmatic individuals respond to respiratory viral infections, given that asthmatic individuals have more severe symptom scores and a greater decrease in lung function compared to non-asthmatic individuals, despite similar rates of acute viral infection (127). A subsequent study found that asthmatic bronchial epithelial cells produced less type I IFN-β, exhibited higher levels of RV replication, but similar levels of pro-inflammatory cytokine induction (128), suggesting that the differences between asthmatic and non-asthmatic individuals are more specific to antiviral IFNs. Another group showed similar phenotypes using a human experimental infection model of adults with mild-to-moderate asthma (129). These studies collectively established a striking link between asthma and a deficiency in the innate immune response, and formed a new paradigm for the role of innate immune responses to viruses in asthma. However, the mechanisms behind this paradigm are provoking vigorous debate. First, several groups have failed to confirm a deficient IFN response in asthmatic airway epithelial cells. Indeed, one group found elevated type III IFN mRNA in the sputum of children and adults with asthma (130). Clearly, confirmation of a deficiency in IFN responses in

asthmatics requires further investigation. Second, and of equal significance, it remains unclear whether this deficiency in IFN responses, if present, is causative of asthma or is merely a consequence of asthma. More studies are required to answer these important questions.

Our studies aim to examine the mechanisms of the RV-induced innate immune response both *in vivo* and *in vitro*, using a cultured airway epithelial cell line, primary bronchial epithelial cells, and knockout mice. Further, using an allergic airway disease mouse model established previously in the laboratory, we aim to determine the role of the IFN response in RV-induced asthma exacerbation. The results of this program of study will elucidate the mechanisms underlying the innate immune response to RV infection and the contribution of RV infection to asthma exacerbation. The overall goal will be resolved by the following specific aims:

1. To determine the contribution of RIG-I, MDA5, and TLR3 in RV-induced IFN responses *in vitro* (Chapter 2);
2. To determine the contribution of MDA5 and TLR3 in RV-induced innate immune responses in naïve mice (Chapter 3);
3. To determine the role of IFN responses in a mouse model of asthma (Chapter 3).

## References


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# Chapter 2

## Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses


### Summary

Rhinovirus (RV), a single-stranded RNA virus of the picornavirus family, is a major cause of the common cold as well as asthma and chronic obstructive pulmonary disease (COPD) exacerbations. Viral double-stranded RNA produced during replication may be recognized by the host pattern recognition receptors Toll-like receptor (TLR)-3, retinoic acid inducible gene (RIG)-I and melanoma-differentiation-associated gene (MDA)-5. No study has yet identified the receptor required for sensing RV double-stranded (ds)-RNA. To examine this, BEAS-2B human bronchial epithelial cells were infected with intact RV-1B or replication-deficient UV-irradiated virus, and interferon (IFN) and IFN-stimulated gene expression determined by quantitative PCR. The separate requirements of RIG-I, MDA5 and IFN response factor (IRF)-3 were determined using their respective siRNAs. The requirement of TLR3 was determined using siRNA against the TLR3 adaptor molecule TRIF. Intact RV-1B, but not UV-irradiated RV, induced IRF3 phosphorylation and dimerization, as well as mRNA expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, RIG-I,

MDA5, IP-10/CXCL10, IL-8/CXCL8 and GM-CSF. siRNA against IRF3, MDA5 and TRIF, but not RIG-I, decreased RV1B-induced expression of IFN-β,.IFN-λ1, IFN-λ2/3, IRF7, RIG-I, MDA5 and IP-10/CXCL10, but had no effect on IL-8/CXCL8 and GM-CSF. siRNAs against MDA5 and TRIF also reduced IRF3 dimerization. Finally, in primary cells, transfection with MDA5 siRNA significantly reduced IFN expression, as it did in BEAS-2B cells. These results suggest that TLR3 and MDA5, but not RIG-I, are required for maximal sensing of RV dsRNA, and that TLR3 and MDA5 signal through a common downstream signaling intermediate, IRF3.

## Introduction

Viral infections, most commonly caused by rhinovirus (RV), are a frequent cause of asthma and chronic obstructive pulmonary disease exacerbations (1). RV is a non-enveloped, positive, single-stranded RNA virus from the *Picornaviridae* family. RV is internalized by receptor-mediated endocytosis and undergoes a conformational change at endosome low pH, leading to insertion of viral RNA into the cytosol. After entry, replication occurs entirely in the cytoplasm, where single-stranded RNA forms a double-stranded (ds)-RNA intermediate, the main form of viral RNA genome inside the cell.

dsRNA produced during viral infection represents an important stimulus of the host innate immune response. It is recognized and engaged by three pattern recognition receptors. Toll-like receptor (TLR)-3 is localized to the endosomal and plasma membranes. TLR3 senses dsRNA released from dying cells and signals through its unique adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) (2).

The cytoplasmic proteins retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA)-5 have recently been identified as intracellular receptors for viral dsRNA (3, 4). RIG-I and MDA5 are homologous cytoplasmic helicases containing two amino-terminal caspase activation and recruitment domains (CARDS) and a carboxy-terminal DExD/H-Box RNA helicase domain. They bind to dsRNA through the helicase domain and signal through CARD domains to a common adaptor molecule, interferon-beta promoter stimulator (IPS)-1 (also called VISA) (5, 6). Engagement of TLR3, RIG-I or MDA5 initiates signaling through two protein kinase complexes, TANK-binding kinase (TBK1)/IκB kinase-ε (IKKε) and IKKα/IKKβ, leading to activation of interferon regulated factor (IRF)-3 and nuclear factor (NF)-κB, respectively (7). Transcription factor activation, in turn, induces expression of IFNs and pro-inflammatory cytokines.

Although all three receptors can recognize viral dsRNA, they appear to be specialized in their recognition of particular viruses. RIG-I and TLR3 are required for respiratory syncytial virus (RSV)-induced expression of IFN-β and IP-10 in airway epithelial cells (8). RIG-I-deficient mice fail to produce type I IFNs in response to the negative-sense single-stranded RNA (ssRNA) viruses Newcastle disease virus, Sendai virus, vesicular stomatitis virus and influenza virus, and to the positive-sense ssRNA Japanese encephalitis virus, whereas MDA5-deficient mice fail to detect encephalomyocarditis (EMCV), a positive-sense ssRNA picornavirus (9). The engagement of PRRs is also cell-type specific: for example, while MDA5 is essential for induction of type I IFNs after infection with EMCV in fibroblasts and conventional dendritic cells (cDCs), plasmacytoid DC use the TLR system for viral detection (9).

Little is known about the contributions of the various pattern recognition receptors to RV-induced responses in bronchial epithelial cells. Primary human bronchial epithelial cells express TLR3, and the TLR3 ligand polyI:C elicits a strong pro-inflammatory response in these cells (10, 11). In 16HBE14o- human bronchial epithelial cells, TLR3 is primarily localized in the endosomes, not on the cell surface (12). TLR3 is partially required for RV39-induced IL-8 expression in 16HBE14o- cells (12) and RV1A-induced MUC5AC expression in NCI-H292 mucoepidermoid carcinoma cells (13). However, the requirement of either RIG-I or MDA5 for RV-induced responses has not yet been tested. In the present study, we found that MDA5 and TLR3, but not RIG-I, are required for RV-induced IFN responses in human airway epithelial cells.

## Materials and Methods

### *Cell culture*

BEAS-2B human bronchial epithelial cells, a SV-40-transformed airway bronchial epithelial cell line, were purchased from ATCC (Manassas, VA). Cells were grown on collagen-coated (5 μg/cm$^2$) plates in Bronchial Epithelial Growth Medium (BEGM, Lonza, Conshohocken, PA) containing epidermal growth factor (25 ng/ml), bovine pituitary extract (65 ng/ml), all-*trans* retinoic acid (5 x $10^{-8}$ M), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5μg/ml), triiodothyronine (6.5 ng/ml), gentamycin (50 μg/ml) and amphotericin (50 μg/ml).

Primary tracheal epithelial cells were all isolated from the tracheas of lung transplant donors, as described (14, 15). Submerged cells were grown as monolayers to

80-100% confluence in BEGM containing epidermal growth factor (25 ng/ml), bovine pituitary extract (130 ng/ml), all-trans retinoic acid (5 x $10^{-8}$M) and bovine serum albumin (1.5 μg/ml).

*RV infection*

RV1B and RV39 were obtained from ATTC. Viral stocks were generated by incubating HeLa cells with RV in serum-free medium until 80% of the cells were cytopathic. Viral stocks were concentrated, partially purified and titered as previously described (14, 15). RV1B was irradiated with UV light at 100 μJ/cm$^2$ for 10 min on ice, using a CL-1000 crosslinker (UVP, Upland, CA).

*Quantitative real-time PCR*

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), then transcibed to first-strand cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). First-strand cDNA is then used to quantify the expression of IFN-α, IFN-β, IFN-λ1, λ2/3, IRF-7, IP-10/CXCL-10, IL-8/CXCL8 and GM-CSF mRNA levels by quantitative real-time PCR (qPCR) using specific primers and probes.

*Measurement of cytokine protein levels*

BEAS-2B cells were grown to 80% confluence and infected with RV1B or medium alone for 1 h. Inoculum was then replaced BEGM. Twenty-four h later, supernatant was collected for the measurement of IFN-λ1, IP-10/CXCL-10 and IL-8/CXCL-8 protein by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN).

*Immunoblotting*

Cells were lysed with RIPA buffer (50 mM Tris, pH7.4; 150 mM NaCl; 5 mM EDTA; 50 mM NaF; 1% NP-40; 10% glycerol). 30 μg of protein lysate was loaded in each well. Lysates were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose or PVDF membrane. Membranes were blocked in 5% milk for 1 h at room temperature and probed with either mouse anti-RIG-I (Alexis Biochemicals, Plymouth Meeting, PA), goat anti-MDA5 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-IRF3 (IBL America, Minneapolis, MN), rabbit anti-TRIF (Cell Signaling, Danvers, MA) or rabbit anti-IRF7 (Abcam, Cambridge, MA). Antibody binding was detected with a peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat IgG and chemiluminescence.

*Native PAGE to determine IRF3 dimerization*

BEAS-2B cells were lysed with native PAGE sample prep kit (Invitrogen, Carlsbad, CA). Native PAGE was performed using 10% Ready Gel (Bio-rad, Hercules, CA), as described (16). The gel was pre-run with 25 mM Tris and 192 mM glycine, pH 8.4, with and without 1% deoxycholate in the cathode and anode chamber, respectively, for 30 min at 40 mA. Samples in the native sample buffer (10 μg protein, 62.5mM Tris-Cl, pH 6.8, and 15% glycerol) were applied to the gel and electrophoresed for 60min at 25 mA. Immunoblotting of IRF3 was performed as described above.

*siRNA knockdown of RIG-I, MDA5, IRF3 and TRIF.*

19-bp duplex of targeting siRNA or non-targeting siRNA (Dharmacon, Lafayette, CO) was transfected into subconfluent BEAS-2B cells while cell seeding using RNAiMAX in OptiMEM (Invitrogen, Carlsbad, CA). A pool of double-stranded siRNAs

containing equal parts of the following antisense sequences was used to knockdown RIG-I: 1, 5'- GCACAGAAGUGUAUAUUGG-3'; 2, 5'- CCACAACACUAGUAAACAA-3'; 3, 5'- CGGAUUAGCGACAAAUUUA-3'; 4, 5'-UCGAUGAGAUUGAGCAAGA-3'. The non-targeting siRNA sequence was 5'-CGAACUCACUGGUCUGACCdtdt-3'(sense), 5'-GGUCAGACCAGUGAGUUCG-dtdt-3'(antisense). For knockdown of MDA5, a pool of the following sequences was used: 1, 5'-GAAUAACCCAUCACUAAUA -3'; 2, 5'-GCACGAGGAAUAAUCUUUA -3'; 3, 5'- UGACACAAUUCGAAUGAUA -3'; 4, 5'-CAAUGAGGCCCUACAAAUU'-3'. For knockdown of IRF3, a pool of the following sequences was used: 1, 5'-CGAGGCCACUGGUGCAUAU-3'; 2, 5'-CCAGACACCUCUCCGGACA-3'; 3, 5'-GGAGUGAUGAGCUACGUGA-3', 4, 5'-AGACAUUCUGGAUGAGUUA-3'. For knockdown of TRIF, a pool of the following sequences was used: 1, 5'-GGAGCCACAUGUCAUUUGG-3'; 2, 5'-CCAUAGACCACUCAGCUUU-3'; 3, 5'-GGACGAACACUCCCAGAUC-3'; 4, 5'-CCACUGGCCUCCCUGAUAC-3'. The next morning, cells were incubated in fresh BEGM containing for 24 h. Finally, cells were treated with the relevant stimulus in BEGM medium for one day prior to harvest.

*Data analysis*

SigmaStat computing software (SPSS, Chicago, IL) was used for data analysis. Data are represented as mean±SEM. Normality was tested using the Kolmogorov-Smirnov test. Statistical significance was assessed by either one-way analysis of variance (ANOVA) or the Kruskal-Wallis one-way ANOVA based on ranks, whenever appropriate. Differences identified by ANOVA were pinpointed by the Student Newman-Keuls' multiple range test.

## Results

*RV1B-induced IFN expression in BEAS-2B human bronchial epithelial cells*

To test whether RV induces an IFN response in human bronchial epithelial cells, BEAS-2B cells were infected with intact RV1B or UV-irradiated RV-1B for 1 h at 33ºC. Cellular total RNA was extracted from lysates to measure the gene expression at 1, 8, 18, 24, 48 and 72 h post-infection by quantitative PCR. Compared with replication-deficient UV-irradiated virus, intact RV1B increased the mRNA expression of IFN-β, IFN-λ1 and IFN-λ2/3, as well as the expression of several interferon-stimulated genes (ISGs) including IRF7, RIG-I, MDA5 and TLR3 (Figure 2-1). The peak level of mRNA expression was 24 h post-infection, except in the case of IFN-β, which was 48 h post-infection. The fold-induction varied widely, from 4-fold (TLR3) to approximately 27,000-fold (IFN-λ2/3). Fold-increases in IFN and ISG mRNA expression tended to be higher for genes expressed at lower levels as baseline, as measured by cycle number (Table 2-1). RV1B infection also increased the protein expression of IFN-λ1, IP-10/CXCL-10 and IL-8/CXCL-8 (Figure 2-2 A-C). However, there was no induction of IRF7 protein expression (Figure 2-2D). Nevertheless, these data, combined with increases in RIG-I and MDA5 protein abundance (see below), demonstrate that RV induces a robust, replication-dependent innate immune response at both the mRNA and protein levels. RV1B infection also increased IFN and ISG mRNA expression in primary tracheal epithelial cells (Figure 2-3, Table 2-2).

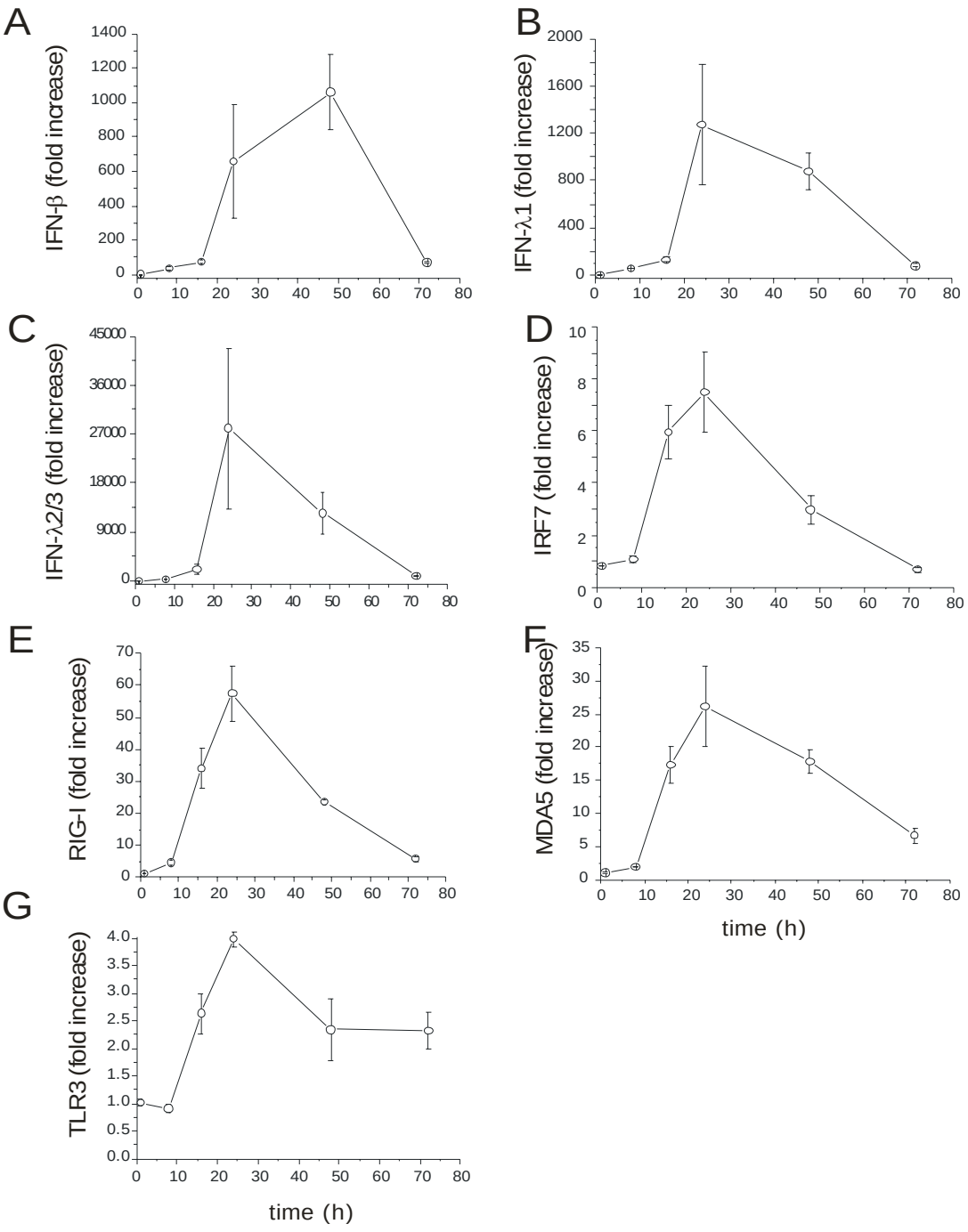


**Figure 2-1.** ***Fold increase of RV1B-induced IFN and ISG responses in cultured airway epithelial cells.*** BEAS-2B cells were infected with RV1B or sham (1 h at 33ºC). Total RNA was extracted at 1, 8, 16, 24, 48 and 72 hours after infection. A-G. The expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, RIG-I, MDA5 and TLR3 at each time point was determined by qPCR. The expression of each target gene was normalized to GAPDH. Expression levels are represented as the ratio of the response to intact RV vs. the response to sham. Data represent mean ± SEM for three experiments.

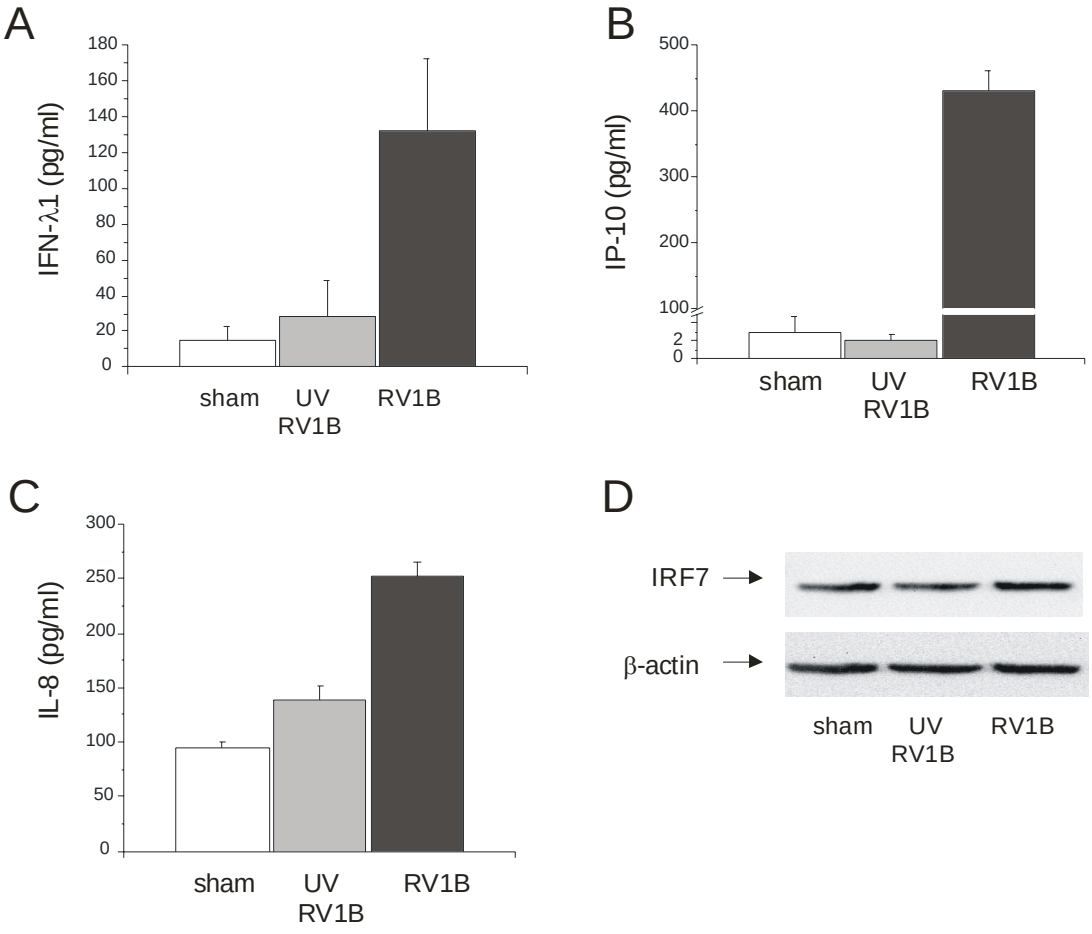


**Figure 2-2.** ***RV1B-induced protein expression of IFNs and ISGs.*** BEAS-2B cells were grown to near confluence and infected with RV1B, UV-irradiated RV1B or sham. A-C. Medium supernatants were extracted twenty-four hours after infection for ELISA to determine the expression of IFN-λ1, IP-10 and IL-8. D. Protein lysates were used to determine IRF7 expression by immunoblotting.

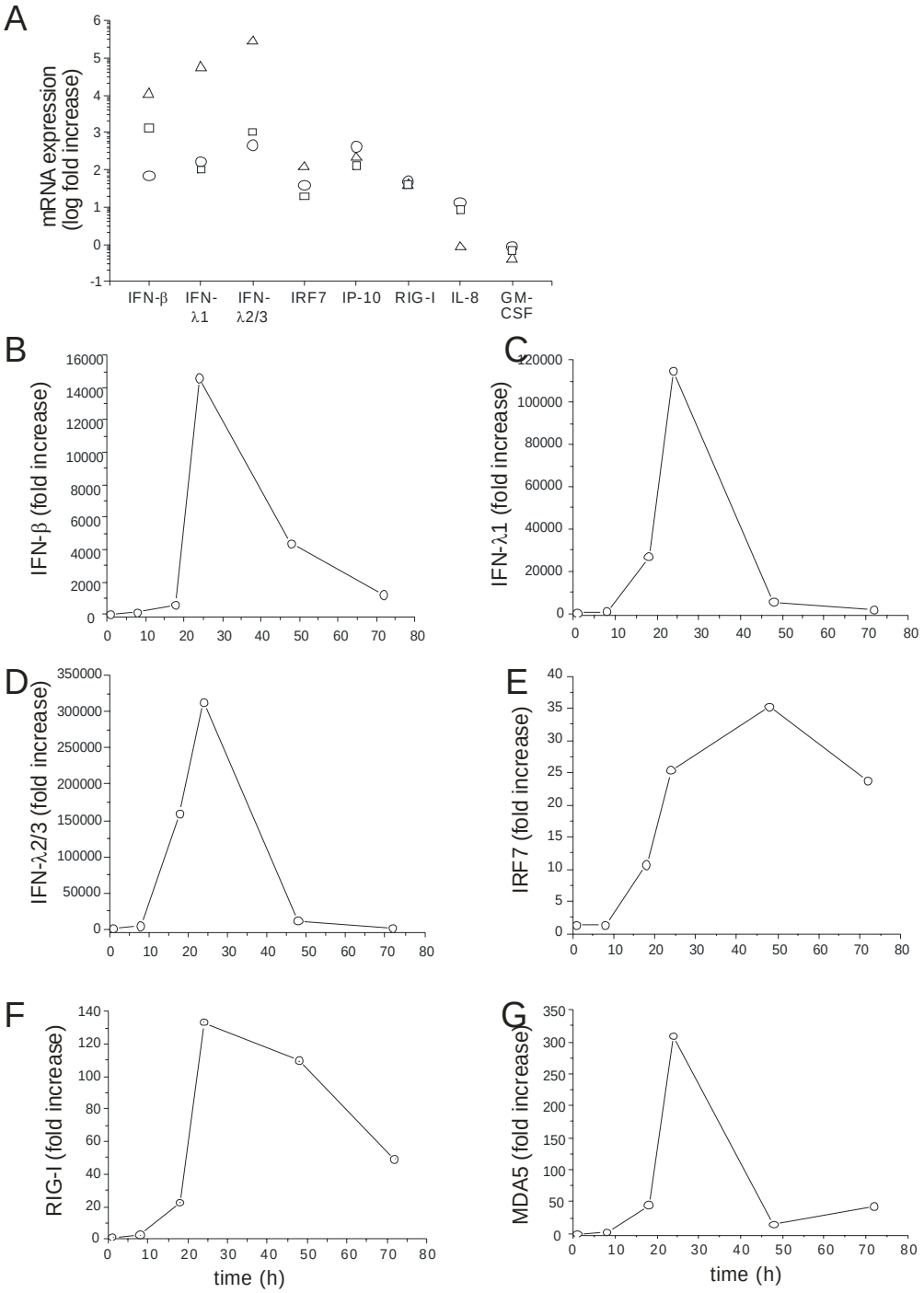


**Figure 2-3.** ***RV1B-induced expression of IFNs, ISGs and chemokines in primary tracheobronchial epithelial cells.*** Primary tracheobronchial epithelial cells were grown to near confluence and infected with RV1B or sham. A. Total RNA was extracted twenty-four hour after infection, and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF determined by qPCR. Expression levels are represented as the ratio of the response to intact RV vs. the response to sham. The y-axis is in log scale. B-G. Time course of RV1B-induced responses in primary cells.

**Table 2-1. The effect of RV1B infection on mean cycle threshold (Ct) values of IFN and ISG mRNA expression in BEAS-2B cells.**

| | sham | | RV1B | |
|---|---|---|---|---|
| | Ct average | SE | Ct average | SE |
| **IFN-β** | 39.47 | 0.53 | 30.91 | 0.30 |
| **IFN-λ1** | 34.80 | 0.16 | 24.23 | 0.20 |
| **IFN-λ2/3** | 39.67 | 0.33 | 25.30 | 0.21 |
| **IRF7** | 24.54 | 0.20 | 21.38 | 0.15 |
| **RIG-I** | 26.97 | 0.29 | 21.19 | 0.19 |
| **MDA5** | 26.50 | 0.20 | 21.64 | 0.11 |
| **TLR3** | 25.47 | 0.16 | 22.80 | 0.04 |

**Table 2-2. The effect of RV1B infection on mean Ct values of IFN and ISG mRNA expression in primary cells.**

| | sham | | RV1B | |
|---|---|---|---|---|
| | Ct average | SE | Ct average | SE |
| **IFN-β** | 35.97 | 2.14 | 32.75 | 2.72 |
| **IFN-λ1** | 33.68 | 1.69 | 29.06 | 2.02 |
| **IFN-λ2/3** | 35.51 | 1.64 | 28.47 | 2.18 |
| **IRF7** | 29.94 | 1.26 | 29.87 | 0.16 |
| **RIG-I** | 28.43 | 0.31 | 28.22 | 0.59 |
| **IP-10** | 30.78 | 1.00 | 28.04 | 0.02 |

*MDA5 and TLR3, but not RIG-I, are required for RV-induced innate immune responses*

Viral dsRNA generated during replication may be detected by the PRRs, RIG-I, MDA5 and/or TLR3. To determine which PRR is responsible for sensing RV dsRNA and inducing the innate immune response, we employed siRNA against RIG-I, MDA5 and the TLR3 adaptor protein TRIF/TICAM. Forty-eight h later, cells were infected with RV1B, UV-irradiated RV-1B or sham HeLa cell lysate, and the expression of IFNs and ISGs was measured by qPCR 24 h after infection. RIG-I expression was knocked down by 80-90% following treatment with RIG-I siRNA (Figure 2-4A). Immunoblots also showed a significant increase in RIG-I protein expression with RV1B treatment, suggesting that expression of RIG-I is inducible. RIG-I siRNA had a slight inhibitory effect on the expression of its homologue protein, MDA5 (Figure 2-4B). However, RIG-I siRNA failed to decrease RV-induced expression of IFNs or ISGs compared to non-targeting siRNA, suggesting that RIG-I is not required for sensing RV dsRNA (Figure 2-5).

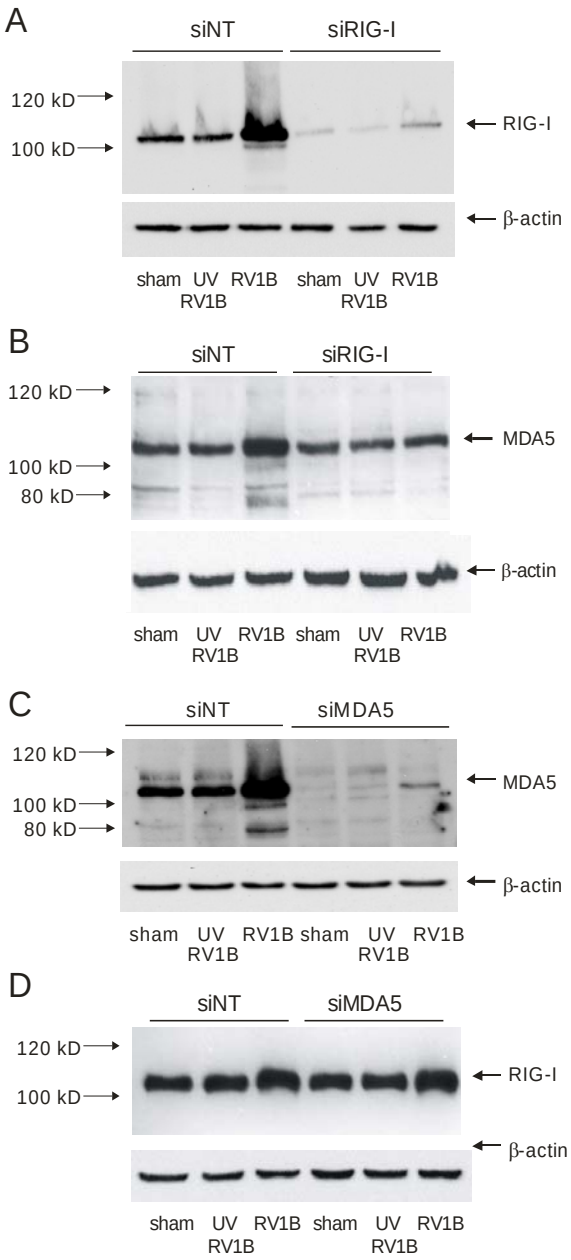


**Figure 2-4.** ***RIG-I and MDA-5 siRNA knockdown efficiencies.*** A, B. RIG-I or non-targeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B) or sham. After infection, cell lysates were probed with antibodies against RIG-I (A) or MDA-5 (B). Note the inductions in RIG-I and MDA5 expression with intact RV, as well as the apparent degradation of MDA-5 following viral infection. C, D. MDA5 or non-targeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B) or sham. After infection, cell lysates were probed with antibodies against either MDA5 (C) or RIG-I (D). (The blots shown are a representative of three separate experiments.)

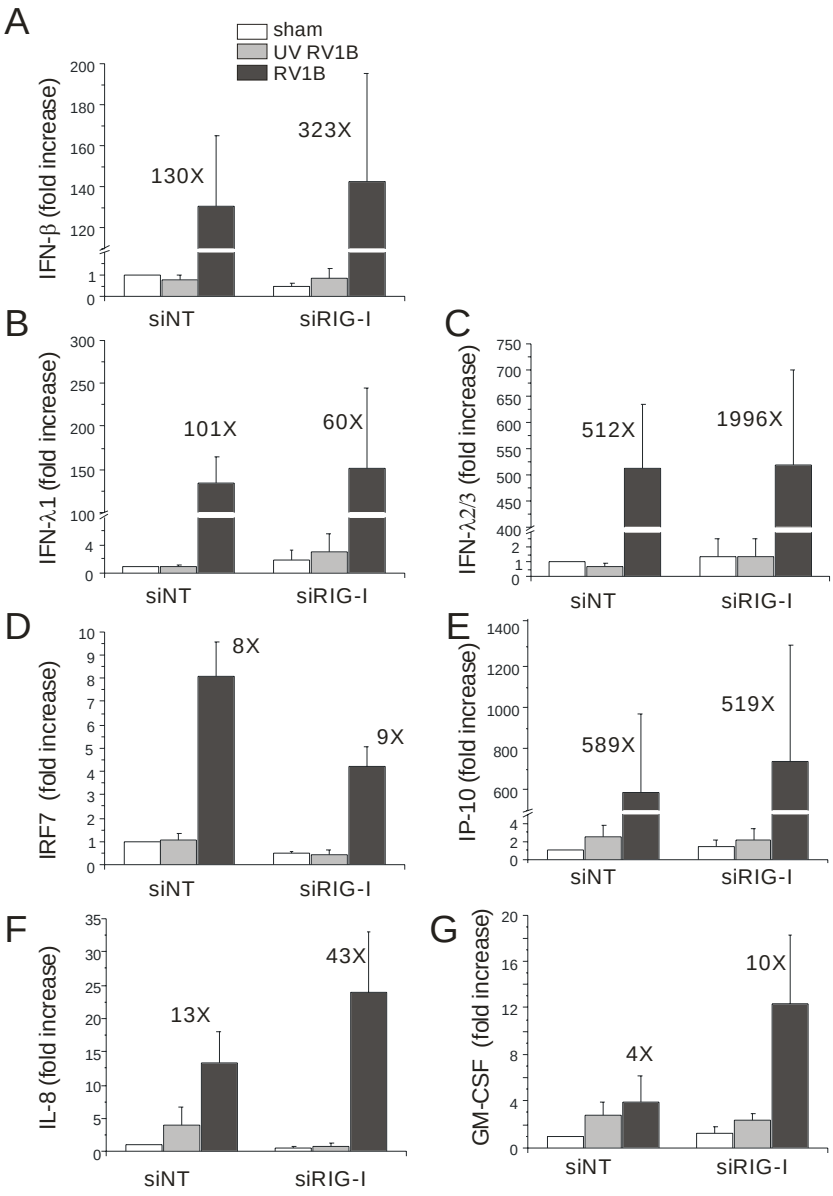


**Figure 2-5.** ***siRNA against RIG-I does not block RV1B-induced IFN and ISG expression.*** RIG-I-specific or non-targeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B) or sham. A-G. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. Expression levels are represented as the fold increase vs. sham-infected, non-targeting siRNA-transfected cells. The y-axis has been broken in order to show the effects of siRNA on both basal and maximal gene expression. Bars represent mean ± SEM for four experiments; numbers on top of bars indicate the fold increase compared to sham-infected sample within its own siRNA group. These notions will apply to similar data in the whole chapter. (*$p<0.05$ vs. RIG-I siRNA-transfected RV1B-infected sample, ANOVA.)

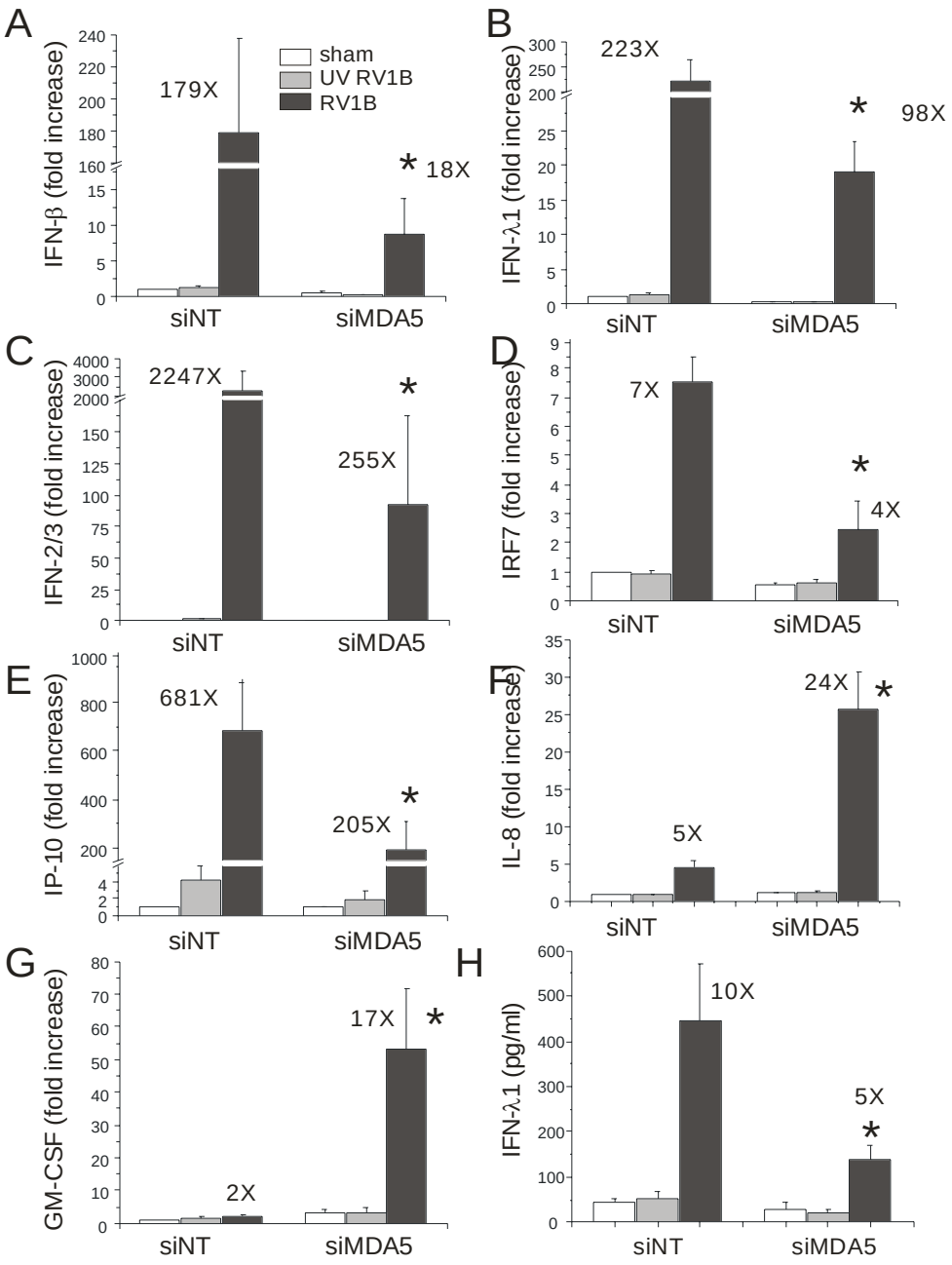


**Figure 2-6.** ***siRNA against MDA5 blocks RV1B-induced IFN and ISG expression.*** MDA5-specific or non-targeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B) or sham. A-G. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. H. To determine the protein expression of IFN-λ1, cell supernatants were collected twenty-four hours after infection for ELISA. Bars represent mean ± SEM for 3-5 experiments. (*$p<0.05$ vs. MDA5 siRNA-transfected RV-infected sample, ANOVA.)

To examine whether MDA-5 is also required for major group RV-induced IFN responses, we repeated our experiment using RV39. BEAS-2B cells were transfected with either MDA5 or non-targeting siRNA and then infected with RV39, UV-irradiated RV39 or sham HeLa cell lysate. Twenty-four h after infection, cellular RNA was extracted to measure the expression of IFNs and ISGs by quantitative PCR (Figure 2-7). There was a significant decrease in mRNA expression of IFN-β, -λ1 and -λ2/3 in cells transfected with MDA5 siRNA compared to cells transfected with non-targeting siRNA. MDA5 siRNA also decreased RV39-induced mRNA expression of the ISGs, IRF7 and IP-10/CXCL10.

We then sought to determine whether MDA-5 is required for the recognition of viral dsRNA in primary tracheal epithelial cells as in BEAS-2B cells. Primary cells were cultured until 70% confluent and then transfected with siRNA against MDA5 or non-targeting siRNA. Forty-eight h later, cells were infected with RV1B or UV-irradiated RV1B. Cellular mRNA was extracted 24 h after infection to determine mRNA expression by qPCR. Compared to non-targeting siRNA, MDA5 siRNA decreased RV1B-induced expression of IFN-β,.IFN-λ1, IFN-λ2/3, IRF7 and IP-10/CXCL10 (Figure 2-8). These data confirm that MDA5 is required for sensing RV dsRNA and induction of the subsequent IFN response in primary cells.

Next, we blocked TLR3 signaling using siRNA against TRIF/TICAM, the TLR3 adaptor molecule. Again, a high knockdown efficiency was verified by immunoblotting (Figure 2-9). Like MDA5 siRNA, TRIF siRNA abolished RV-1B induced expression of IFN-β,.IFN-λ1, IFN-λ2/3, IRF7 and IP-10/CXCL10, suggesting TLR3 signaling is also required for maximal RV1B-induced IFN responses.

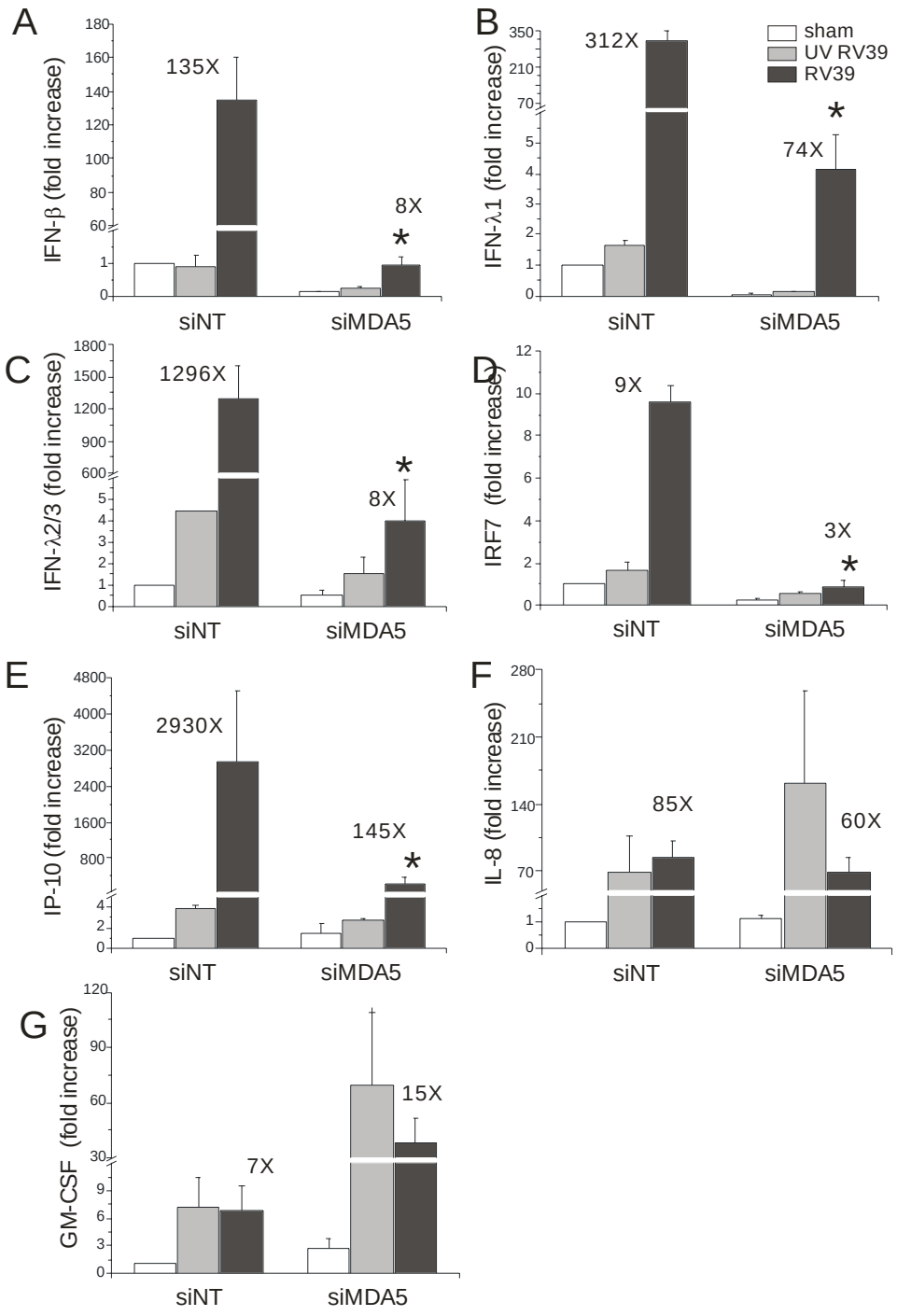


**Figure 2-7.** ***siRNA against MDA5 blocks RV39-induced IFN and ISG expression.*** A-G. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. Bars represent mean ± SEM for three experiments. (*$p<0.05$ vs. MDA5 siRNA-transfected RV-infected sample, ANOVA.)

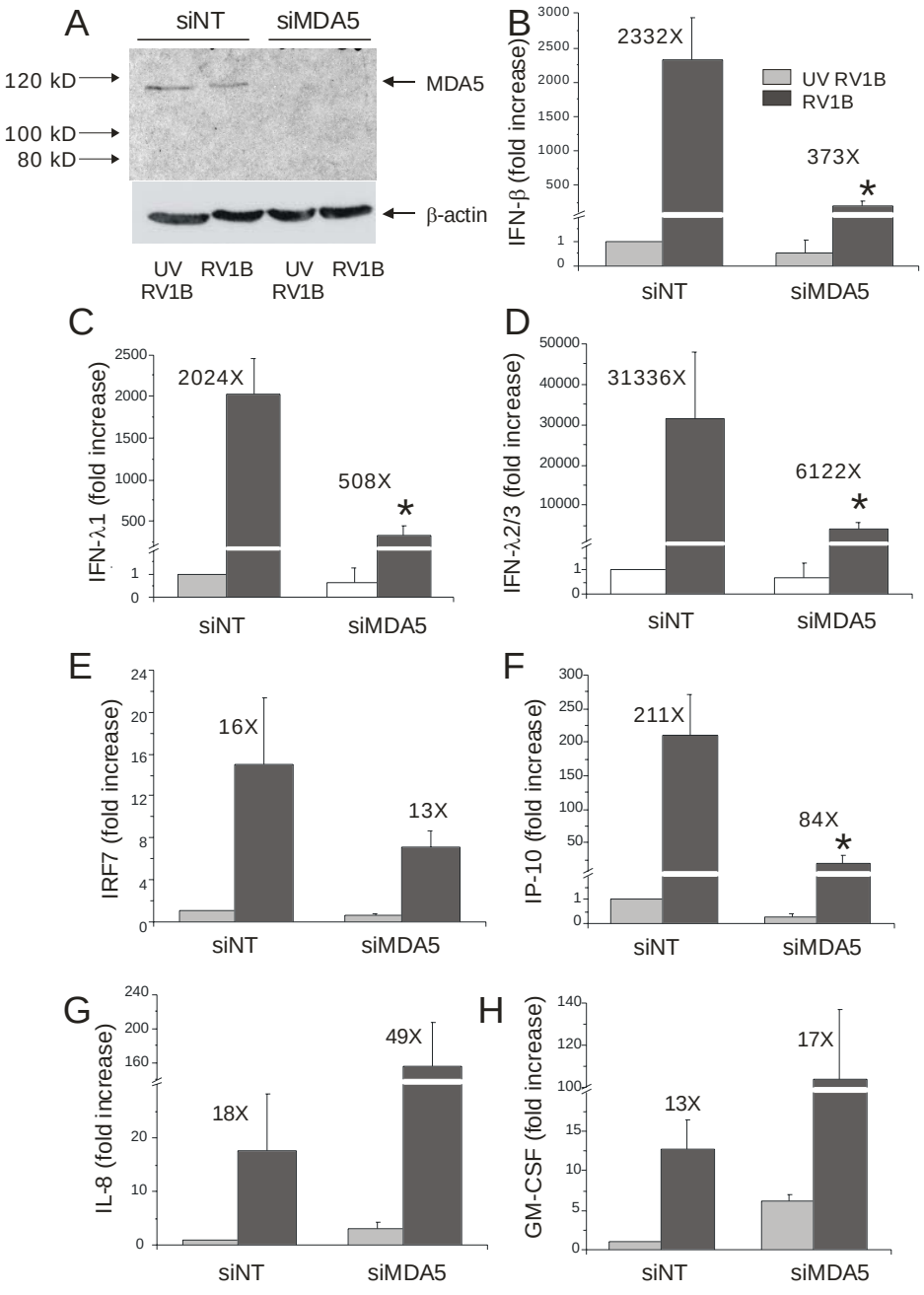


**Figure 2-8.** ***siRNA against MDA5 blocks RV1B-induced IFN responses in primary tracheal epithelial cells.*** A. MDA5 siRNA or non-targeting siRNA was transfected into primary tracheobronchial epithelial cells. After transfection, cells were infected with RV1B or UV-irradiated RV1B. After infection, cell lysates were probed with anti-MDA5 antibody. The blot shown is typical for three experiments. B-H. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. Bars represent mean ± SEM for three experiments. (*$p<0.05$ vs. MDA5 siRNA-transfected RV-infected sample, ANOVA.)

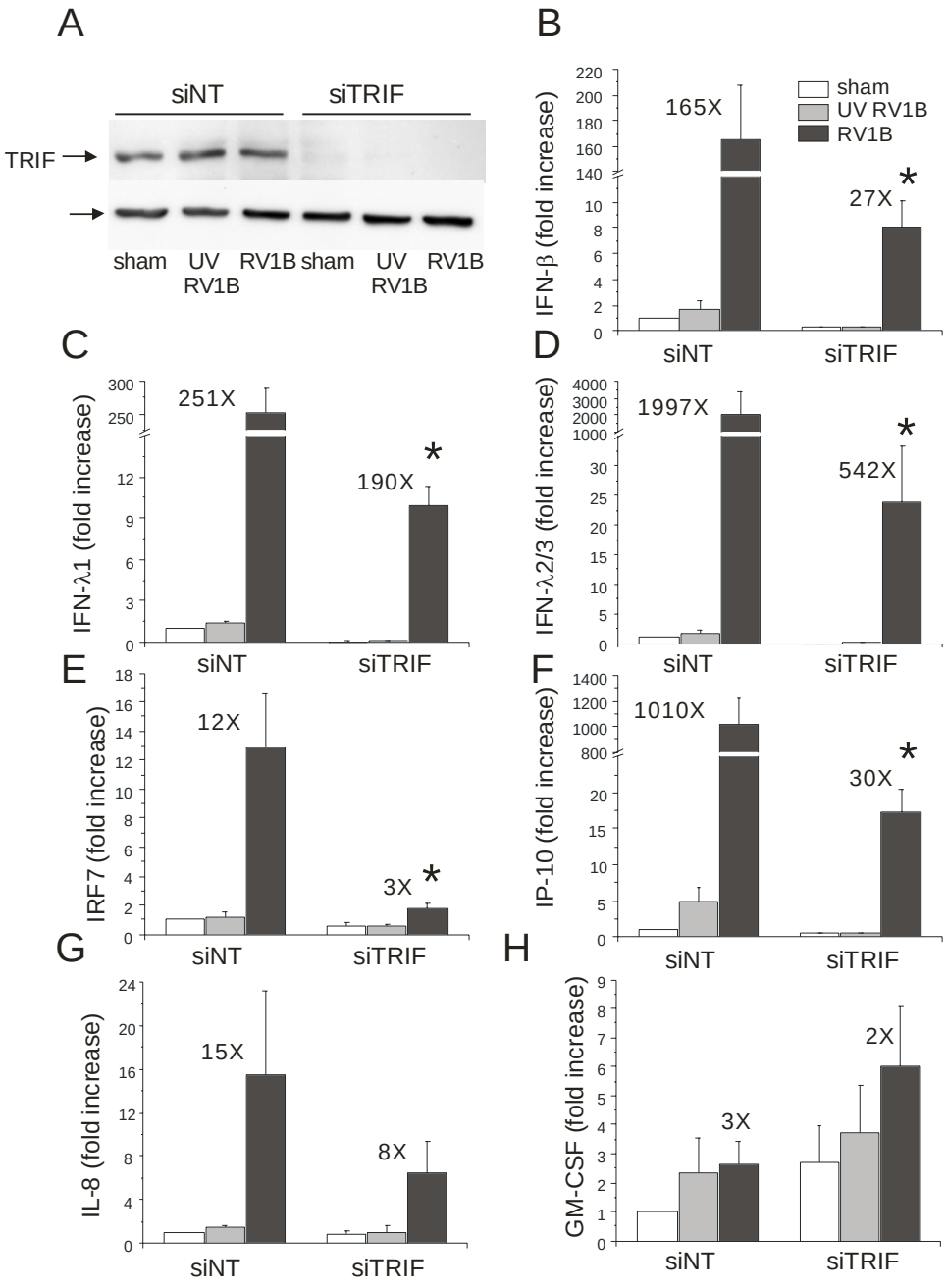


**Figure 2-9.** ***siRNA against TRIF blocks RV-induced IFN and ISG expression.*** A. TRIF siRNA or non-targeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV irradiated-RV1B or sham. After infection, cell lysates were probed with anti-TRIF antibody. B-H. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. Expression levels are represented as the fold increase vs. sham-infected, non-targeting siRNA-transfected cells. Bars represent mean ± SEM for 4 experiments. (*$p<0.05$ vs. TRIF siRNA-transfected RV-infected sample, ANOVA.)

*IRF3 is required for RV1B-induced IFN responses*

IRF3 is a ubiquitously-expressed transcription factor which regulates type I IFN production. To test whether RV1B induces IRF3 activation, BEAS-2B cells were infected with RV1B or UV-irradiated RV-1B. Cell protein lysates were collected 12 h after infection. IRF3 phosphorylation shift was determined by SDS-PAGE followed by immunoblotting using anti-IRF3 antibody (Figures 2-10A, B), and IRF3 dimerization determined by native PAGE (Figure 2-10C). PolyI:C, a synthetic dsRNA which induced both IRF3 phosphorylation and dimerization, served as a positive control. We found that RV1B, but not UV-irradiated RV-1B, induced IRF3 phosphorylation and dimerization. Similar results were observed in primary tracheal epithelial cells (Figure 2-10D).

We then examined the requirement of IRF3 in RV-induced IFN responses using siRNA against IRF3. IRF3 protein abundance was substantially knocked down by IRF3 siRNA (Figure 2-11). IRF3 siRNA nearly abolished RV-1B-induced expression of IFNs β, λ1 and λ2/3, IRF7 and IP-10/CXCL10. However, there was no effect on IL-8 or GM-CSF expression. Taken together, these data suggests that IRF3 is activated by RV1B, and that IRF3 is required for RV-induced IFN responses, as well as the expression of ISGs such as IRF7 and IP-10.

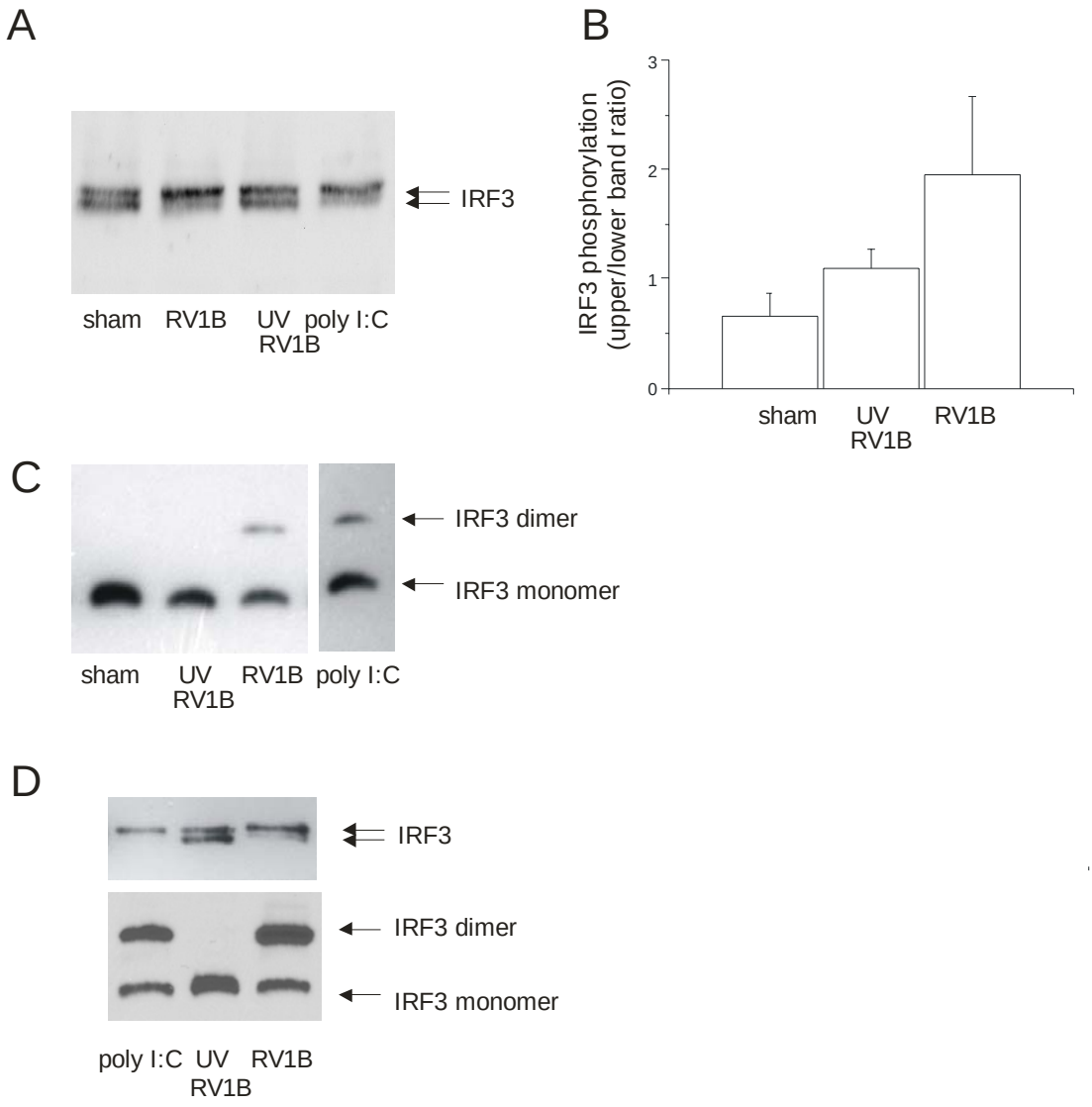


**Figure 2-10.** ***RV-induced IRF3 activation in cultured BEAS-2B and primary airway epithelial cells.*** A. BEAS-2B cells were infected with RV1B or UV-irradiated RV1B (1 h at 33ºC) at MOI=10. Cell lysates were collected and probed with anti-IRF3 antibody. Poly I:C served as a positive control. IRF3 protein is visualized as two bands, an upper phosphorylated form and a lower unphosphorylated form. B. Densitometry of IRF3 phosphorylation is provided. Bars represent mean ± SEM for 3 experiments. C. Cellular proteins were also subjected to native-PAGE to resolve the dimerization of IRF3. Poly I:C served as a positive control. IRF protein is visualized as two bands, an upper dimer and a lower monomer. (The blot shown is a representative of five individual experiments.) D. Primary tracheobronchial epithelial cells were infected with RV1B or UV-irradiated RV1B. Upper panel: cell lysates were collected twelve h after infection and probed with anti-IRF3 antibody. Poly I:C served as a positive control. Lower panel: cellular proteins were subjected to native-PAGE to resolve the dimerization of IRF3. Poly I:C served as a positive control. (The blots shown are representative of three experiments.)

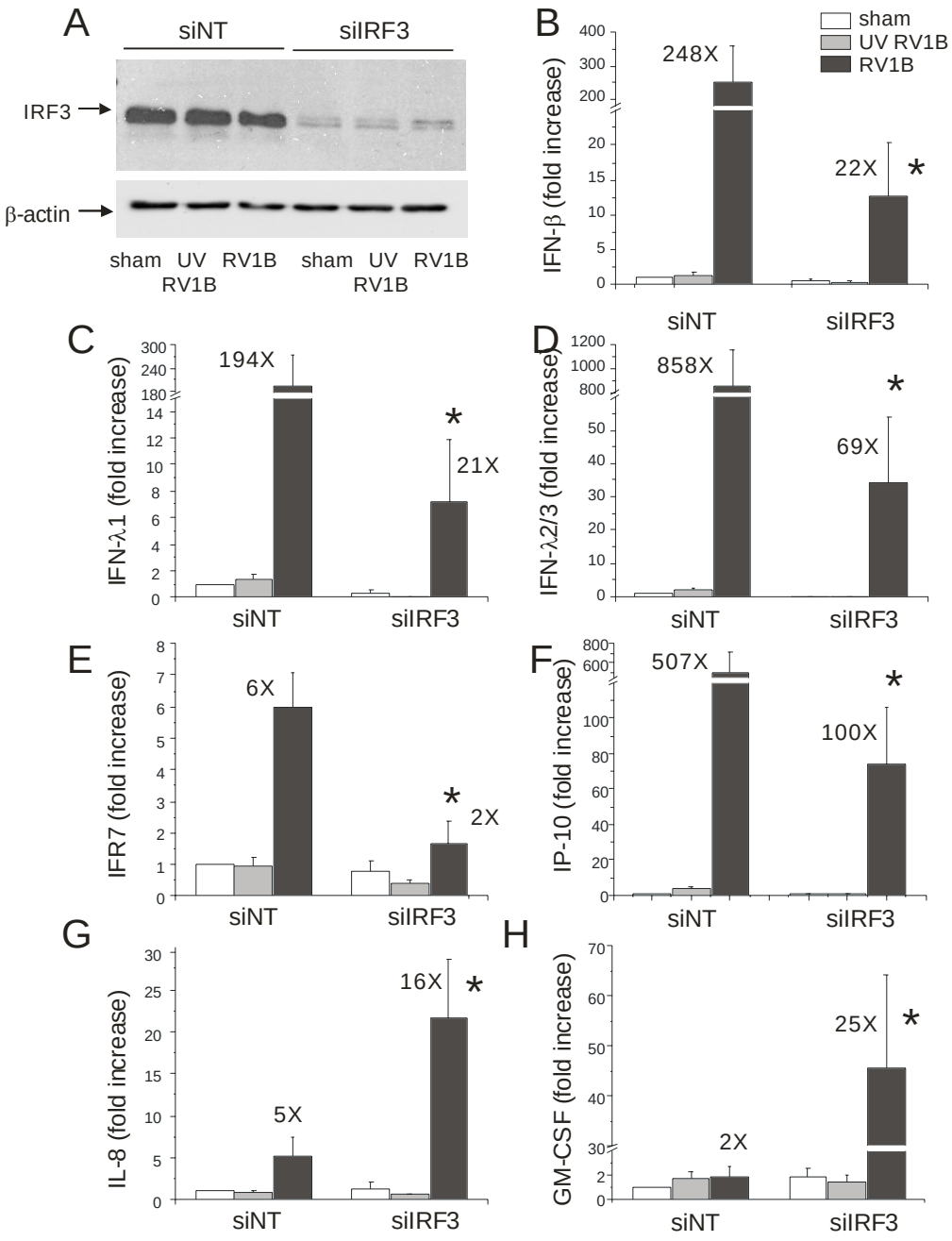


**Figure 2-11.** ***siRNA against IRF3 blocks RV-induced IFN and ISG expression.*** A. IRF3 siRNA or non-targeting siRNA was transfected into BEAS-2B while seeding. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B) or sham. A. Cell lysates were probed with anti-IRF3 antibody. (The blot shown is representative of 3 separate experiments.) B-H. Total RNA was extracted and the expression of IFN-β, IFN-λ1, IFN-λ2/3, IRF7, IP-10, IL-8 and GM-CSF was determined by qPCR. The expression of each target gene was normalized to GAPDH. Bars represent mean ± SEM for four experiments. (*$p<0.05$ vs. non-targeting siRNA-transfected RV1B-infected cells, one-way ANOVA.)

*IRF3 functions downstream of MDA5 signaling*

RIG-I/MDA5 and TLR3 signaling pathways converge on a common TRAF3 (TNF receptor associated factor 3) adapter complex, which then activates two IRF3 kinases, TBK1 and IKK-ε (7). To examine whether IRF3 functions downstream of MDA5 in airway epithelial cells, BEAS-2B cells were transfected with either MDA5 siRNA or non-targeting siRNA, and then infected with RV-1B, UV-irradiated RV-1B or sham HeLa cell lysate. IRF3 dimerization was resolved by native PAGE (Figure 2-12). We found that MDA5 and TRIF siRNA each caused a partial reduction in RV-induced IRF3 dimerization compared to non-targeting siRNA-transfected cells, confirming in airway epithelial cells the general notion that IRF3 functions downstream of MDA5 and TRIF.

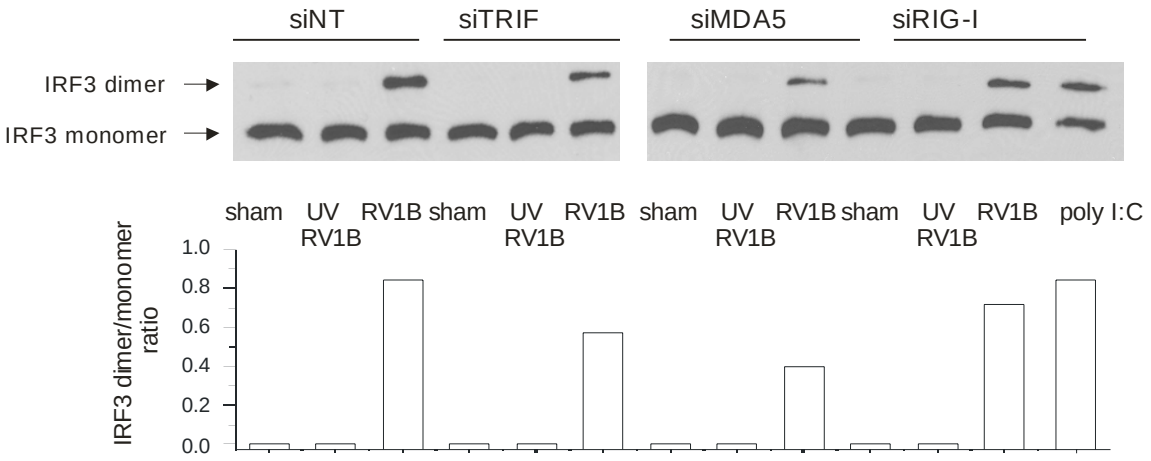


**Figure 2-12.** ***siRNA against TRIF, MDA5, but not RIG-I, reduced RV1B-induced IRF3 dimerization.*** A. RIG-I, MDA5, TRIF siRNA or non-targeting siRNA was transfected into BEAS-2B. After transfection, cells were infected with RV1B, UV-irradiated RV1B or sham. After infection, cell lysates were probed with anti-IRF3 antibody. Poly I:C served as a positive control. B. IRF3 dimer to monomer ratio was quantified by densitometry. (The blot shown is a representative of two experiments.)

**Discussion**

Host pathogen recognition, as reflected by the induction of type I interferons is mediated by activation of pattern recognition receptors. The membrane dsRNA receptor, TLR3, and the recently-identified cytoplasmic dsRNA receptors, RIG-I and MDA5, are responsible for sensing viral dsRNA (24). Although all three receptors can recognize viral dsRNA, the engagement of receptor and viral dsRNA seem to be cell type and virus-specific. RIG-I-deficient mice fail to produce type I IFNs in response to the negative-sense single-stranded RNA (ssRNA) viruses Newcastle disease virus, Sendai virus, vesicular stomatitis virus and influenza virus, and to the positive-sense ssRNA Japanese encephalitis virus, whereas MDA5-deficient mice fail to detect EMCV, a positive-sense ssRNA picornavirus (9). Whereas MDA5 is essential for induction of type I IFNs after infection with EMCV in fibroblasts and conventional dendritic cells, plasmacytoid DC use the TLR system for viral detection (9). While these results are compelling, it seems premature to conclude that all picornaviruses are sensed by MDA5 in all cell types, because only cardioviruses have been studied.

In this manuscript, we found that infection of BEAS-2B and primary tracheal epithelial cells with RV induced substantial increases in IFN and ISG mRNA expression. In limited studies, we also found similar changes in gene expression following infection with major and minor group virus. Due to the low level of baseline IFN expression, the fold-increases in IFN expression were quite high, perhaps artificially so. However, increases in IFN-λ1, IP-10/CXCL-10, RIG-I and MDA-5 were verified by ELISA and immunoblotting, implying that RV significantly increases IFN responses in airway epithelial cells. Further, we demonstrate for the first time that MDA5 and TRIF, but not

RIG-I, are required for maximal sensing of RV dsRNA in human airway epithelial cells. Transfection of both a human bronchial epithelial cell line (BEAS-2B cells) and primary tracheobronchial epithelial cells with siRNA against MDA5, but not non-targeting siRNA, significantly inhibited RV1B-induced expression of a type I IFN, IFN-β, as well as a number of ISGs. Knockdown of MDA5 also attenuated expression of the type III IFNs IFN-λ1 and -λ2/3, which functionally resemble type I IFNs (17) and are also induced by RV infection (18). These data are in agreement with previous data suggesting MDA5 is required for sensing picornavirus dsRNA (9). On the other hand, our data contrast with previous data from A549 alveolar type II epithelial cells showing that RIG-I and TLR3, but not MDA5, are required for sensing human RSV, a paramyxovirus (8). Thus, in the airway epithelium, recognition of viral dsRNA is indeed virus type-specific.

We also examined the contribution of IRF3 to RV-induced responses in airway epithelial cells. IRF3 siRNA nearly abolished IFN and ISG expression. MDA5 and TRIF knockdown also decreased IRF3 dimerization. These data are consistent with the idea that TLR3 and MDA5 regulate IFN expression via a common downstream intermediate, IRF3.

In contrast to siRNA against MDA5, siRNA against RIG-I had no effect on RV-induced IRF3 dimerization or IFN expression. The divergent roles of RIG-I and MDA5 in the context of RV infection suggest that the two homologous helicases function distinctly from each other. Further, though RIG-I expression was induced after RV infection, RIG-I apparently cannot compensate for reduced expression and/or function of MDA5.

Using siRNA against MDA5 and IRF3, we found that MDA5/IRF3 signaling is required for RV-induced IFN, but not IL-8 expression. Indeed, expression of IL-8 and GM-CSF were often paradoxically increased. Previous studies have shown that RV-induced IL-8 expression is strictly regulated by the transcription factor NF-κB (14, 19). The initial phase of IL-8 expression is also replication-independent (14, 20-22). Inhibition of dsRNA sensing would therefore not be expected to reduce IL-8 expression. Further, the upregulation of pro-inflammatory cytokine expression following MDA5 and IRF3 knockdown may represent a compensatory mechanism by which the airway epithelial cells increase immune surveillance when the IFN response is suppressed.

We previously showed that TLR3 was partially required for RV39-induced IL-8 expression in 16HBE14o- cells (12). In the present study, we could not verify TLR3 knockdown by immunoblotting or flow cytometry in BEAS-2B cells (not shown), leading us to employ siRNA against TRIF. In contrast to MDA-5 knockdown, inhibition of TLR3 signaling using TRIF siRNA inhibited both IFN and IL-8 expression. Based on our previous result, the complete effect of TRIF siRNA on IFN signaling was unexpected. It is conceivable that TRIF is coupled to other, as yet unknown pattern recognition receptors, and that the reduction in IFN expression induced by TRIF knockdown is not solely due to TLR3-linked signaling. In any event, the differential effects of MDA-5 and TRIF siRNA on IL-8 expression suggest that, although NF-κB and IRF3 are both components of the same transcriptional enhanceosome in the regulation of IFNs, ISGs and inflammatory cytokine IL-8 (23, 24), their requirement for gene expression may vary for different target genes, perhaps depending on the organization of IFN-stimulated and NF-κB response elements in the promoter.

It has recently been reported that picornaviruses may develop strategies to escape host immune surveillance. Hepatitis A virus has been shown to suppress RIG-I-mediated signaling in fetal rhesus monkey kidney (FRhK-4) cells (25). Poliovirus infection induces cleavage of MDA5 in HeLa cells (26). In A549 human alveolar type II alveolar epithelial cells, RV14 infection fails to induce high levels of IFN, apparently by interfering with IRF3 dimerization (27). In this study, RV1B induced robust IRF3 dimerization and IFN responses in both BEAS-2B and primary bronchial epithelial cells. Also, while we did indeed observe apparent degradation of MDA5 following viral infection (Figure 5), the total protein abundance of MDA5 was still substantially increased 24 h after RV inoculation. Taken together, these data suggest an intact host innate immune defense against RV1B dsRNA.

We confined most of our studies of RV-induced IFN responses to the minor group serotype RV1B. However, IFN expression by RV39, a major group virus, was also blocked by MDA-5 but not RIG-I siRNA. This was not unexpected, as RV1B and RV16, another major group serotype, have been shown to induce nearly identical patterns of gene expression in primary cultured airway epithelial cells (28). We have found that infection with RV1B and RV39 induce similar levels of Akt phosphorylation and IL-8 expression in cultured 16HBE14o- human bronchial epithelial cells, and that inhibition of PI 3-kinase blocks both RV1B- and RV39-induced IL-8 expression induced by either virus (29). Thus, there are ample data suggesting that major and minor subgroup RV stimulate similar airway epithelial cell signaling pathways and elicit similar immune responses.

In conclusion, we have shown for the first time that MDA5, TLR3 and IRF3 are each required for maximal RV-induced IFN responses. Viral infections, most commonly caused by RV, are the most frequent cause of asthma exacerbations, and account for a substantial percentage of chronic obstructive pulmonary disease exacerbations (1). Bronchial epithelial cells isolated from patients with asthma have been demonstrated to have an incomplete innate immune response to rhinovirus infection, with deficient type I IFN-β and type III IFN-λ production (18, 30). Further understanding of the biochemical signaling pathways regulating RV-induced IFN expression may therefore provide insight into the pathogenesis of human airway diseases and new therapeutic targets for treatment.

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# Chapter 3

## MDA5 and TLR3 signaling initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness


### Summary

Rhinovirus (RV) is the most frequent cause of asthma exacerbations. We previously demonstrated in cultured human bronchial epithelial cells that TRIF, the adaptor protein for TLR3, and MDA5 are each required for maximal RV1B-induced IFN responses. However, in vivo, the overall airway response to viral infection likely represents a coordinated response integrating both antiviral and pro-inflammatory pathways. Therefore, we examined the airway responses of TLR3- and MDA5-deficient mice to infection with RV1B, a minor group virus which replicates in mouse lungs. TLR3 null mice showed essentially normal IFN responses and unchanged viral titer. MDA5 mice showed a delayed type I IFN and attenuated type III IFN response to RV1B infection, leading to a transient defect in viral clearance. Further, RV-infected TLR3- and MDA5-deficient mice showed reduced lung inflammatory responses and reduced airways cholinergic responsiveness. Finally, RV-infected MDA5 null mice with allergic airways disease showed lower viral titers despite deficient IFN responses, as well as decreased

airway inflammatory and contractile responses. Together, these results suggest that, in the context of RV infection, TLR3 and MDA5 individually initiate pro-inflammatory signaling pathways leading to airways inflammation and cholinergic hyper-responsiveness, implying that TLR3 and MDA5 signaling might be maladaptive following RV infection.

## Introduction

Rhinovirus (RV) is the most frequent cause of acute respiratory tract infection in humans. RV infection is typically responsible for upper respiratory symptoms including rhinorrhea, sore throat, nasal congestion, sneezing, cough, and headache. More importantly, RV has emerged as the most frequent pathogen associated with exacerbations of asthma (1, 2).

RV is a positive sense single-stranded RNA (ssRNA) virus from the *Picornaviridae* family. After endocytosis, RV RNA is inserted into the cytosol, where viral replication occurs. During replication, a double-stranded RNA (dsRNA) intermediate is formed, the main form of viral genome inside the cell. The mechanisms by which RV causes asthma exacerbations are not fully established, but current evidence indicates that the immune response is critical in this process. The first line of defense against RV infection is the innate immune system. Innate pathogen sensors detect viral products and respond by initiating a signaling cascade that leads to a rapid antiviral response involving secretion of IFNs and inflammatory cytokines. RV induces the

expression of both type I (*e.g.*, IFN-α, IFN-β) and type III IFNs (in mice, IFN-λ2/IL-28A, IFN-λ3/IL-28B). Though they signal through the engagement of different receptor complexes, the intracellular signaling program activated by type I and type III IFNs is very similar, as evidenced by the cellular gene expression profiles induced after stimulation with IFN-λ versus IFN-α (3). However, whereas IFN-α receptor complex is expressed ubiquitously, the IL-28 receptor is only expressed in few cell types, notably epithelial cells (4). The preferential expression of IFN-λ receptors on epithelial surfaces may allow the host to rapidly eliminate viruses at the major portals of entry into the body before infection is established, and without activating other arms of the immune system.

Several pattern recognition receptors have been shown to be responsible for binding viral dsRNA and initiating the IFN response. RIG-I and MDA5 are homologous proteins located within the cytoplasm, whereas TLR3 is located mainly on the endosomal membrane and plasma membrane. RIG-I has been shown to preferentially recognize 5' phosphorylated short ssRNA, whereas MDA5 recognizes long dsRNAs (3-6). Thus, RIG-I has been shown to detect negative-strand viruses such as influenza, paramyxovirus and RSV (7), as well as some positive-strand Flaviviruses. In comparison, MDA5 has been shown to selectively detect positive-strand viruses including picornaviruses (EMCV, Mengo virus and Theiler's virus). Previously, we demonstrated in cultured human bronchial epithelial cells that MDA5 and TIR-domain-containing adapter inducing interferon-β (TRIF), the adaptor protein for TLR3, are each required for maximal RV1B-induced IFN responses (8). Knockdown of RIG-I had no effect on IFN responses. TRIF, but not MDA5, was required for maximal pro-inflammatory cytokine expression (8). TLR3 is partially required for HRV39-induced IL-8 expression in 16HBE14o- human

bronchial epithelial cells (9), as well as HRV1A-induced MUC5AC expression in NCI-H292 mucoepidermoid carcinoma cells (10).

However, in vitro studies may not truly represent the complicated situation in vivo, where multiple cell types are involved. Further, the overall airway response to viral infection likely represents a coordinated response integrating both antiviral and pro-inflammatory pathways. For example, it has been proposed that asthmatics are susceptible to RV infection due to deficient IFN production. RV-infected airway epithelial cells from asthmatic subjects show impaired production of IFN-β and -λ (11, 12) and asthmatics experimentally infected with RV16 showed a reduced IFN-γ/IL-5 mRNA ratio in their sputum (13). According to the theory, reduced IFN responses, in turn, lead to increased viral-mediated inflammation. However, it is also conceivable that reduced RV-induced IFN responses are coupled with attenuated airways inflammation and hyperresponsiveness. For example, pneumovirus-infected IFNαβ receptor null mice show fewer BAL leukocytes and prolonged survival despite increased virus titers (14). To address these questions, we examined the airway responses of TLR3- and MDA5-deficient mice to infection with RV1B, a minor group virus which replicates in mouse lungs (15).

## Materials and methods

*Animals*

B6;129S1-Tlr3$^{tm1Flv}$/J (TLR3 -/-) and B6;129SF2/J control mice were purchased from Jackson Laboratories (Bar Harbor, MA). MDA5-/- mice were bred on a >99.5% C57BL/6 background, as described (16). Control C57BL/6J mice were also purchased from Jackson Laboratories. Six-to-eight week old female mice were used in this study. All mice were housed in a specific pathogen-free area within the animal care facility at the University of Michigan. The protocol was approved by the Institutional Animal Care and Use Committee.

*Generation of RV and titer determination.*

RV1B (ATCC, Manassas, VA) was concentrated, purified and titered as described previously (17, 18). Viral titer was determined by plaque assay (19). To produce replication-deficient virus, RV1B was UV-irradiated using a CL-1000 crosslinker (UVP, Upland, CA).

*RV exposure and ovalbumin (OVA) sensitization/challenge.*

Mice were inoculated intranasally with 45 μl of 1x10$^8$ $TCID_{50}$/ml RV1B, UV-irradiated RV or an equal volume sham HeLa cell lysate (15). For OVA sensitization, mice were injected intraperitoneally on days 1 and 7 with 0.2 ml PBS or a solution of alum and 100 μg endotoxin-free OVA (Sigma-Aldrich, St. Louis, MO). Next, mice were challenged intranasally with 50 μl of PBS or 100 μg OVA on days 14, 15 and 16. Selected mice were infected with RV1B immediately following the last OVA or PBS

treatment.

*Lung inflammation*.

To quantify inflammatory cells, lung digests were obtained by mincing the tissue, proteolysis in collagenase type IV (Gibco Invitrogen, Carlsbad, CA) and straining through a 70 μm nylon mesh (BD Falcon, San Jose, CA), as described (20). The resulting pellet was treated with red blood cell lysis buffer (BD Pharmingen, San Diego, CA) and leukocytes were enriched by spinning the cells through 40% Percoll (Sigma-Aldrich). The total cell count was determined on a hemocytometer. Cytospins were performed from lung digested leukocytes and were then stained by Diff-Quick method (Dade Behring, Newark, DE). Differential counts were determined by counting 200 cells per slide.

*Measurement of airways responsiveness.*

Mice were anesthetized with sodium pentobarbital (50 mg/kg mouse, intraperitoneal injection) and a tracheostomy performed. Mechanical ventilation was performed and total respiratory system measured using a Buxco FinePointe operating system (Wilmington, NC). Airway responsiveness was assessed by measuring changes in resistance in response to increasing doses of nebulized methacholine, as described (15).

*Histology*.

Lungs were fixed in 10% formalin at an inflation pressure of 30 $cmH_2O$ overnight, transferred to 70% ethanol and paraffin embedded. Five μ sections were stained with hematoxylin and eosin.

*Cytokine/chemokine expression.*

Total lung RNA was extracted using the RNeasy kit (Qiagen, Alameda, CA) and then transcibed to first-strand cDNA using Taqman reverse transcription reagents (Applied Biosystems Life Technologies, Carlsbad, CA). First-strand cDNA was then used to quantify the expression of IFN-α, IFN-β, IFN-λ2, IFN-λ3, IFN response factor (IRF)-7, CXCL10/IP-10, IFN-γ, CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, and CCL11/eotaxin-1 by quantitative two-step real time PCR using specific Syber green primers. All primers were designed and purchased from IDT (Coralville, IA). The signal was normalized to GAPDH and expressed as fold-increase over sham.

*Cytokine production*

Lungs were homogenized in 1 ml PBS with protease inhibitor cocktail, spun for 15 minutes at 1500 g, and the supernatant assayed for CXCL1, CXCL2 and IFN-β by ELISA (R&D Systems, Minneapolis, MN; PBL InterferonSource, Piscataway, NJ).

*Presence of viral RNA*

RNA was extracted from lungs of mice using Trizol reagent (Sigma-Aldrich, St. Louis, MO) and analyzed for the presence of viral RNA by reverse transcriptase-PCR. uantitative one-step real time PCR for positive-strand viral RNA was conducted using RV-specific primers and probes (forward primer: 5'-GTG AAG AGC CSC RTG TGC T-3'; reverse primer: 5'-GCT SCA GGG TTA AGG TTA GCC-3’; probe: 5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3’ (12). Copy numbers of positive strand

viral RNA were normalized to 18S RNA, which was similarly amplified using gene-specific primers and probes.

*Data analysis*

SigmaStat computing software (SPSS, Chicago, IL) was used for data analysis. Data are represented as mean±SEM. Statistical significance was assessed by one- or two-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by the Student Newman-Keuls' multiple range test.

## Results

*TLR3 deficiency does not alter RV1B-induced type I IFN responses.*

Our previous studies in cultured airway epithelial cells demonstrated that TRIF, the adaptor protein for TLR3, is required for maximal RV-induced IFN expression. However, while the airway epithelium is a major target of RV infection, other cell types such as monocytes and macrophages may also be infected (21). To determine whether TLR3 is required for RV1B-induced airway IFN responses *in vivo*, we studied TLR3-/- mice and their strain- and age-matched controls. Mice were inoculated intranasally with sham, replication-deficient UV-irradiated RV1B or intact RV1B. Lungs were harvested at 4-96 h after infection. Four h after infection, there was no induction of IFN-α gene expression in RV1B-infected control mice or TLR3-/- mice (Figure 3-1a). By 24 h, compared to sham- or UV-RV1B-infected mice, RV1B-induced IFN-α expression was significantly elevated in both control and TLR3-/- mice. There was no difference in IFN-α expression between strains. IFN-α mRNA expression peaked 24 h post-infection and decreased thereafter. The mRNA expression pattern of another type I IFN, IFN-β, was similar to that of IFN-α (Figure 3-1b). TLR3-/- mice did not show a lower level of IFN-β mRNA expression compared to control mice. Accordingly, there was no difference in lung IFN-β protein between RV1B-infected control and TLR3-/- mice (Figure 3-1f).

Type III IFNs utilize a receptor complex different from that of type I IFNs, but both types of IFN induce STAT1, 2 and 3 activation. We therefore measured the expression of IFN-λ2 and IFN-λ3 in RV1B-infected wild-type and TLR3 -/- mice

(Figures 3-1c, 1d). mRNA levels of both IFN-λ2 and IFN-λ3 were decreased in RV1B-infected TLR3-/- mice compared to controls, though the change did not achieve statistical significance. By 48 h, differences in IFN expression between strains were less apparent. The expression of IRF7, an IFN-stimulated gene, was not different between control and TLR3-/- mice at any time point (Figure 3-1e).

*TLR3-deficiency does not change viral titer in the lung*

To determine whether TLR3 plays a role in viral clearance, we determined viral titer in the whole lung by plaque assay. Control and TLR3-/- mice showed a whole lung viral titer of approximately $10^3$ PFU/ml 24 h after infection (Figure 3-2a). Viral copy number at various time points was also determined by qPCR (Figure 3-2b). There was no difference in viral copy number between the control and TLR3-/- mice. Together, these data suggest that TLR3 is not required for viral titer change.

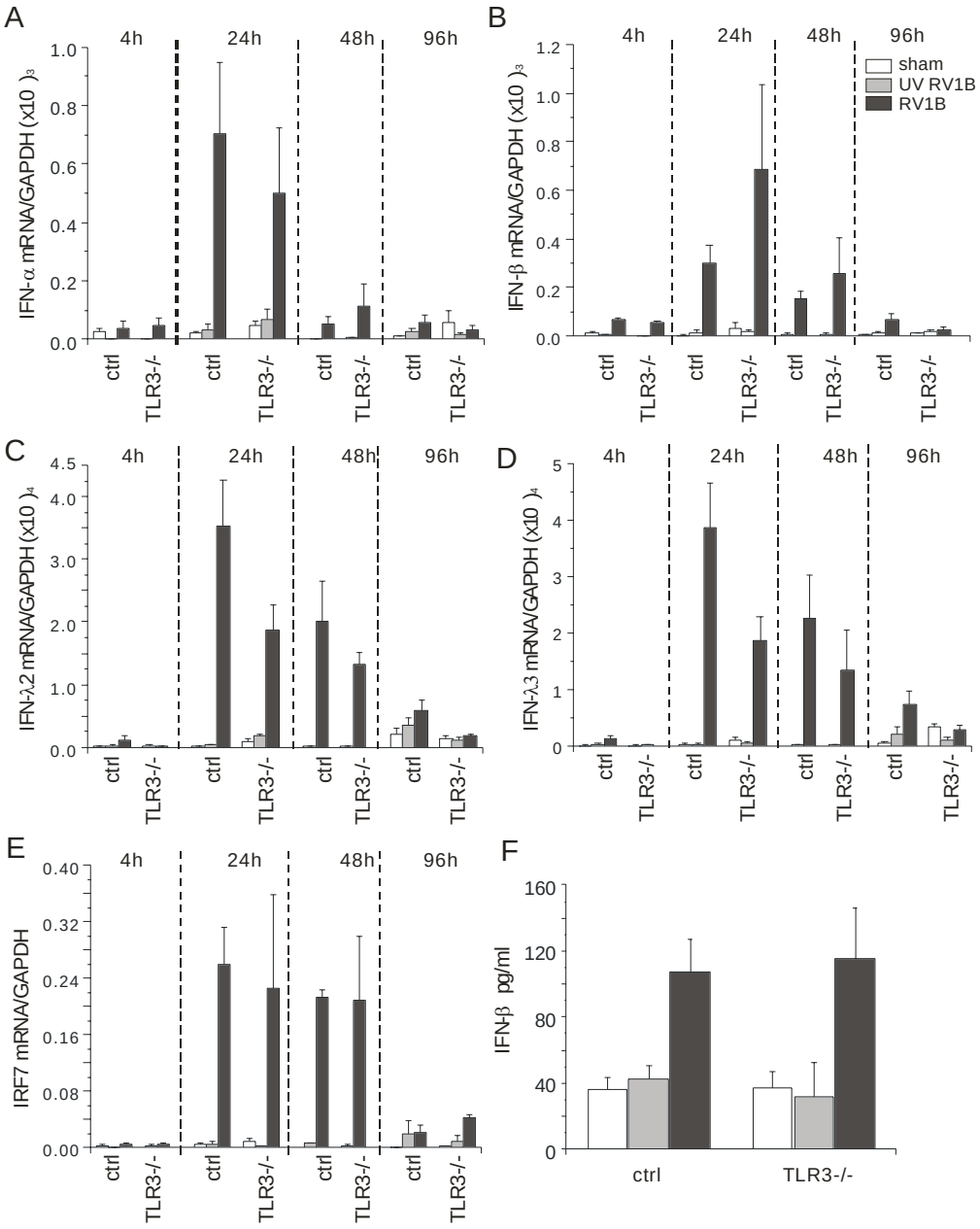


**Figure 3-1.** ***RV1B-induced expression of IFNs and interferon-stimulated genes in TLR3-/- mice.*** TLR3-/- and their control mice were inoculated with sham, UV-irradiated RV1B (UV RV1B), or intact RV1B. Lungs were harvested at 4, 24, 48, and 96 h after infection. A-E. The expression of IFN-α, IFN-β, IFN-λ2, IFN-λ3, and IRF7 at each time point was determined by qPCR. F. IFN-β protein production was measured by ELISA at 24 h post-infection. The expression of each target gene was normalized to GAPDH. Data represent mean±SEM for 3-7 mice.

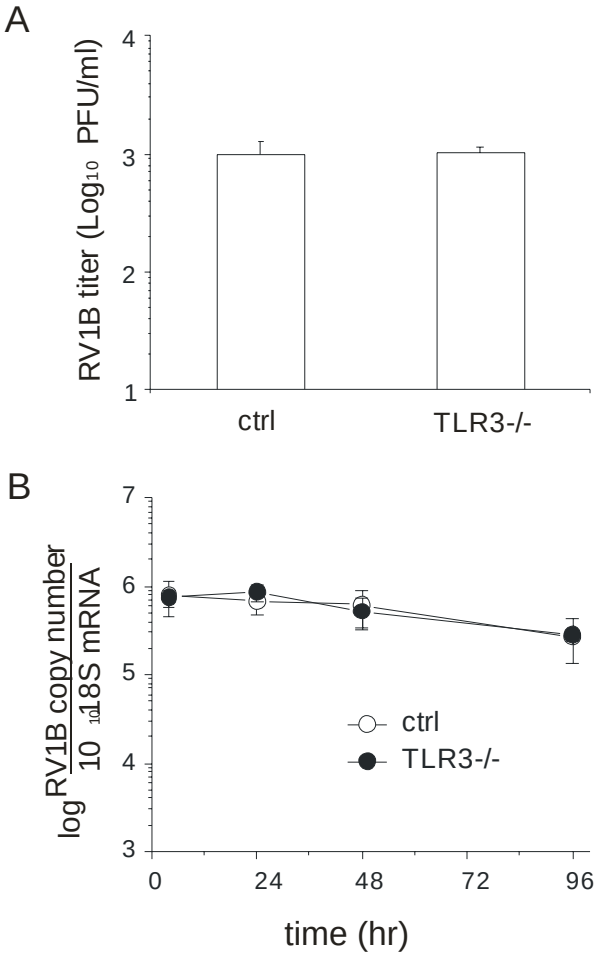


**Figure 3-2.** ***Viral copy number and titer changes in the lungs of control and TLR3-/- mice.*** TLR3-/- and their control mice were infected with RV1B. Lungs were harvested at 4, 24, 48, and 96 h post infection. A. Total lung titer at 24 h post-infection was determined by plaque assay. B. RV1B copy number at each time point was determined by qPCR. RV copy number was normalized to 18S rRNA. Data represent mean±SEM for 3-7 mice.

*TLR3 signaling is required for RV1B-induced inflammatory responses.*

Previously, we showed that TLR3 is required for RV-induced CXCL8/IL-8 expression in cultured airway epithelial cells (9). We found similar results *in vivo* using TLR3-/- mice: Twenty-four h post-infection, RV1B-infected TLR3-/- mice displayed significantly decreased mRNA expression of genes encoding the neutrophil chemoattractants CXCL1/KC and CXCL2/MIP-2 (Figures 3-3a, b). Their lung protein levels were also significantly decreased compared to control mice (Figures 3-3f, g). mRNA expression of three additional pro-inflammatory cytokines, CCL2/MCP-1, CXCL10/IP-10, and CCL11/eotaxin-1, were also significantly reduced (Figures 3-3c-e). These data suggest that TLR3 is required for RV1B-induced inflammatory responses, in particular the expression of neutrophil chemokines. Accordingly, 24 h after infection, there were significantly fewer neutrophils in the lungs of RV1B-infected TLR3 -/- mice compared controls (Figure 3-3h). These results were reflected in hematoxylin- and eosin-stained lung sections (Figure 3-4a). Sham-infected control and TLR3-/- mice showed uninflamed airways, whereas RV1B-infected control mice displayed increased peribronchial inflammation. In contrast, inflammation was significantly alleviated in the lungs of RV1B-infected TLR3-/- mice.

*TLR3 is required for RV1B-induced airway hyper-responsiveness.*

We have previously shown that RV1B-induced airways cholinergic hyper-responsiveness in naïve mice is dependent on CXCR2 and airway neutrophilic inflammation (22). Since RV1B-infected TLR3 -/- mice showed significant reductions in lung neutrophils, CXCL1 and CXCL2 (each of which are CXCR2 ligands), we

hypothesized that RV1B-infected TLR3 null mice would show reduced airway responses to methacholine compared to control mice. As noted previously, RV1B-infected control mice displayed significantly higher airways responses at 20 and 40 mg/ml methacholine compared to sham-infected mice (Figure 3-4b). However, compared to sham-inoculated mice, RV1B-induced airway responses were not elevated in TLR3 -/- mice. Together, these results demonstrate that TLR3 signaling, while not required for viral clearance, initiates a pro-inflammatory signaling pathway leading to airways inflammation and hyperresponsiveness. Thus, TLR3-driven innate immune responses to RV are maladaptive in this model.

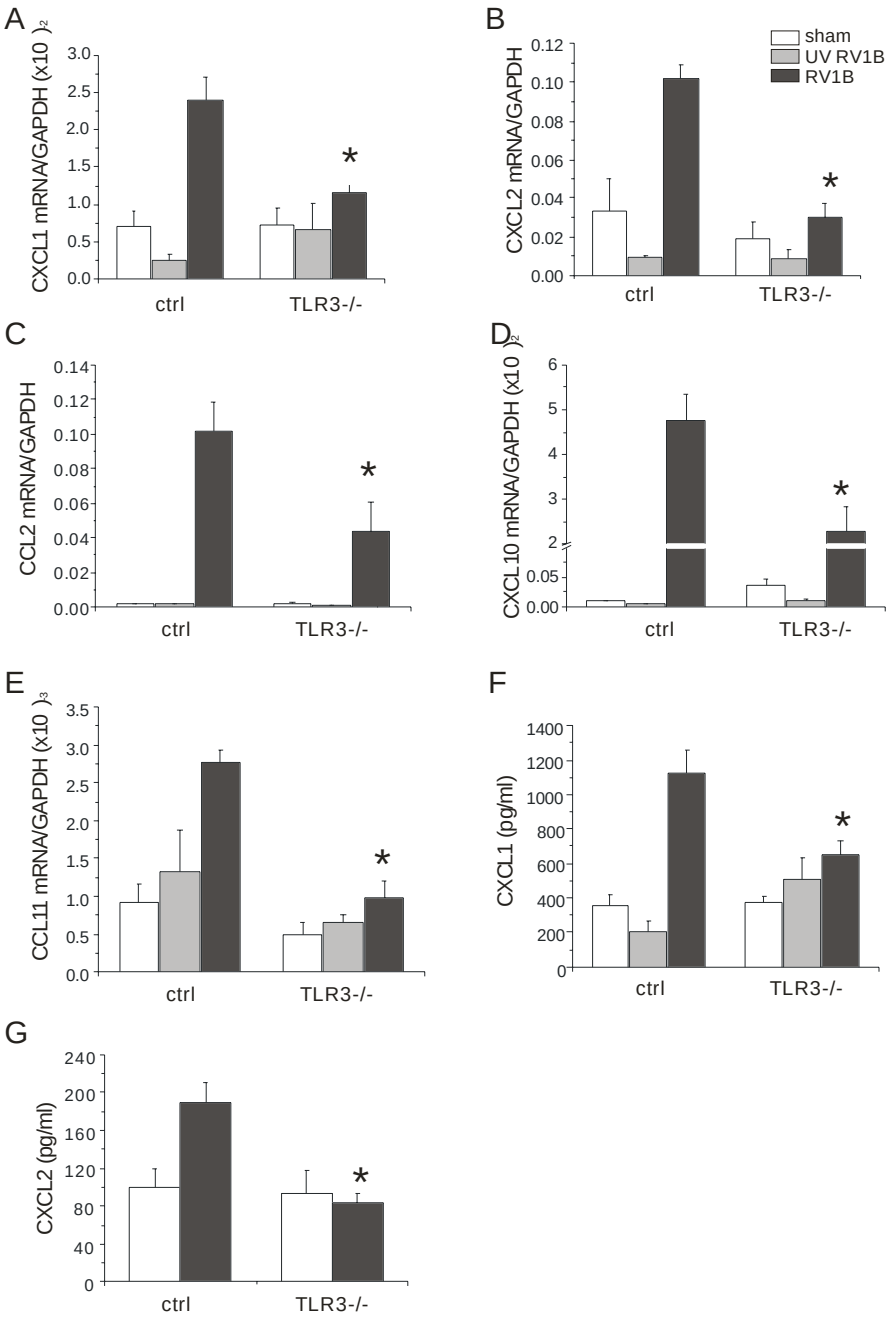


**Figure 3-3.** ***RV1B-induced pro-inflammatory cytokine expression in TLR3-/- mice.*** TLR3-/- and their control mice were inoculated with sham, UV-irradiated RV1B (UV RV1B), or intact RV1B. Lungs were harvested 24 h after infection. A-E. The expression of CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, CXCL10/IP-10 and CCL11/eotaxin-1 was determined by qPCR. F-G. Protein production of CXCL1/KC and CXCL2/MIP-2 was measured by ELISA and bioplex assay. The expression of each target gene was normalized to GAPDH. Data represent mean±SEM for 3-7 mice (*$p<0.05$, one-way ANOVA).

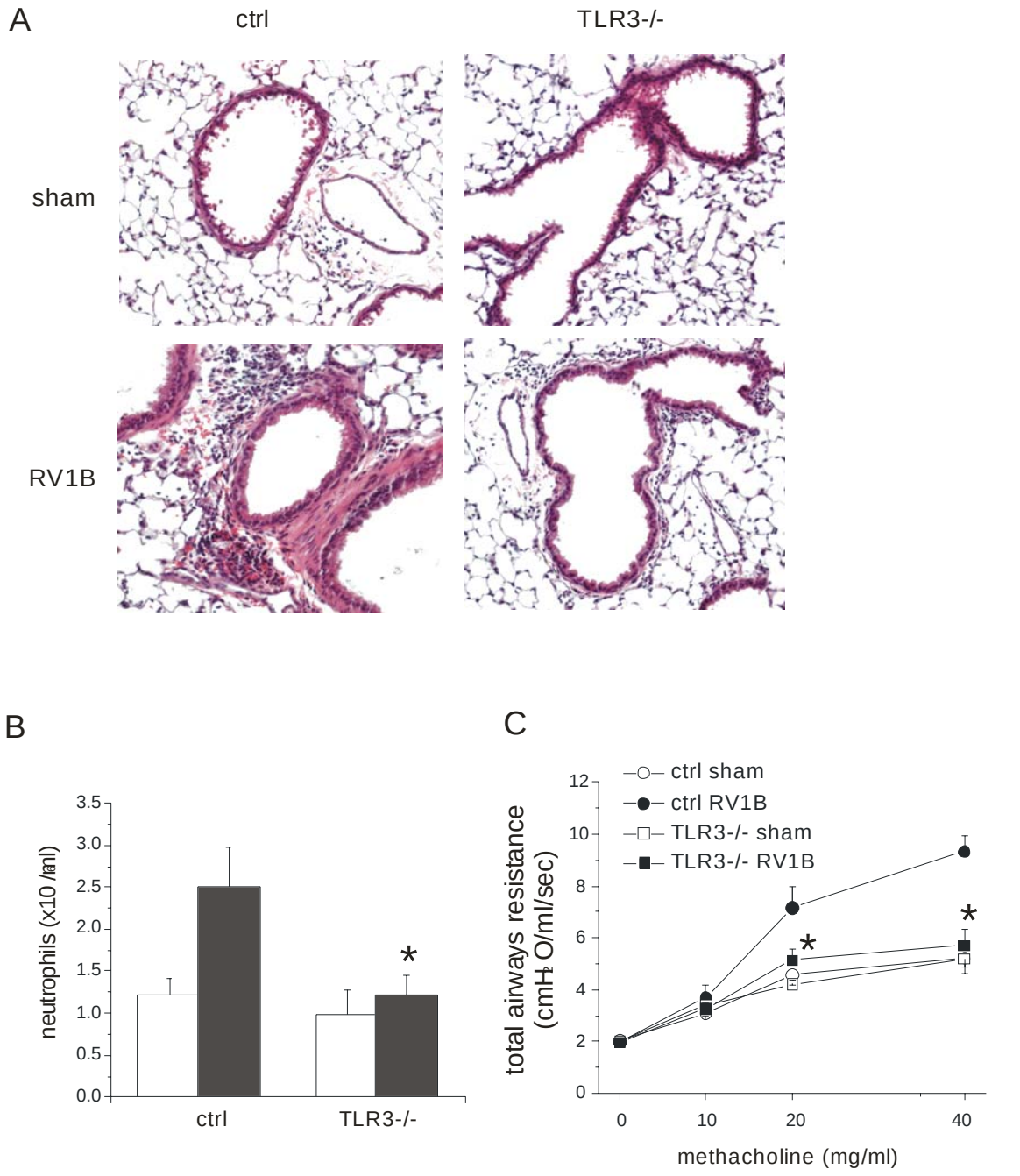


**Figure 3-4.** ***Airway inflammation and responsiveness in RV1B-infected TLR3-/- and control mice.*** TLR3-/- and their control mice were infected with RV1B. A. Twenty-four h after infection, lungs were fixed and stained with hematoxylin and eosin. B. RV1B-induced neutrophil infiltration was determined. Data represent mean±SEM for 3-7 mice (*$p<0.05$, one-way ANOVA).. C. Total respiratory system resistance was determined by plethysmography. Data represent mean±SEM for 3-7 mice (*$p<0.05$, two-way ANOVA).

*MDA5 deficiency significantly decreases RV1B-induced IFN responses.*

The recognition of viral dsRNA is pathogen-type specific. We next examined whether the role of cytosolic dsRNA receptor *in vivo* using MDA5-/- mice and age matched controls. We infected the mice with sham, UV-irradiated RV1B or RV1B intranasally, and harvested the lung at various time points after infection (4-96 h) for total RNA and protein extraction. RV1B infection caused a significant induction of the expression of type I IFNs, namely IFN-α and IFN-β, in control mice compared to sham or UV-RV1B inoculated mice. However, MDA5-/- mice showed no type I IFN response 4 and 24 h post-infection (Figures 3-5a, b). The IFN-β protein response 24 h after infection was also significantly decreased in MDA5 -/- mice compared to control mice (Figure 3-5g). Interestingly, by 48 h post infection, when the type I IFN expression in RV1B-infected control mice was essentially over, MDA5 -/- mice showed robust but delayed type I IFN response. In contrast, the expression of type III IFNs (IFN-λ2 and IFN-λ3) in RV1B-infected MDA5 -/- mice was significantly lower than that of control mice throughout the time course studied (Figures 3-5c, d). These data suggest that the regulation of type III IFN expression is different from that of type I IFNs. The expression patterns of IRF7 in control and MDA5 -/- mice were similar to that observed for type I IFNs, with a delayed response in MDA5-/- mice (Figure 3-5e).

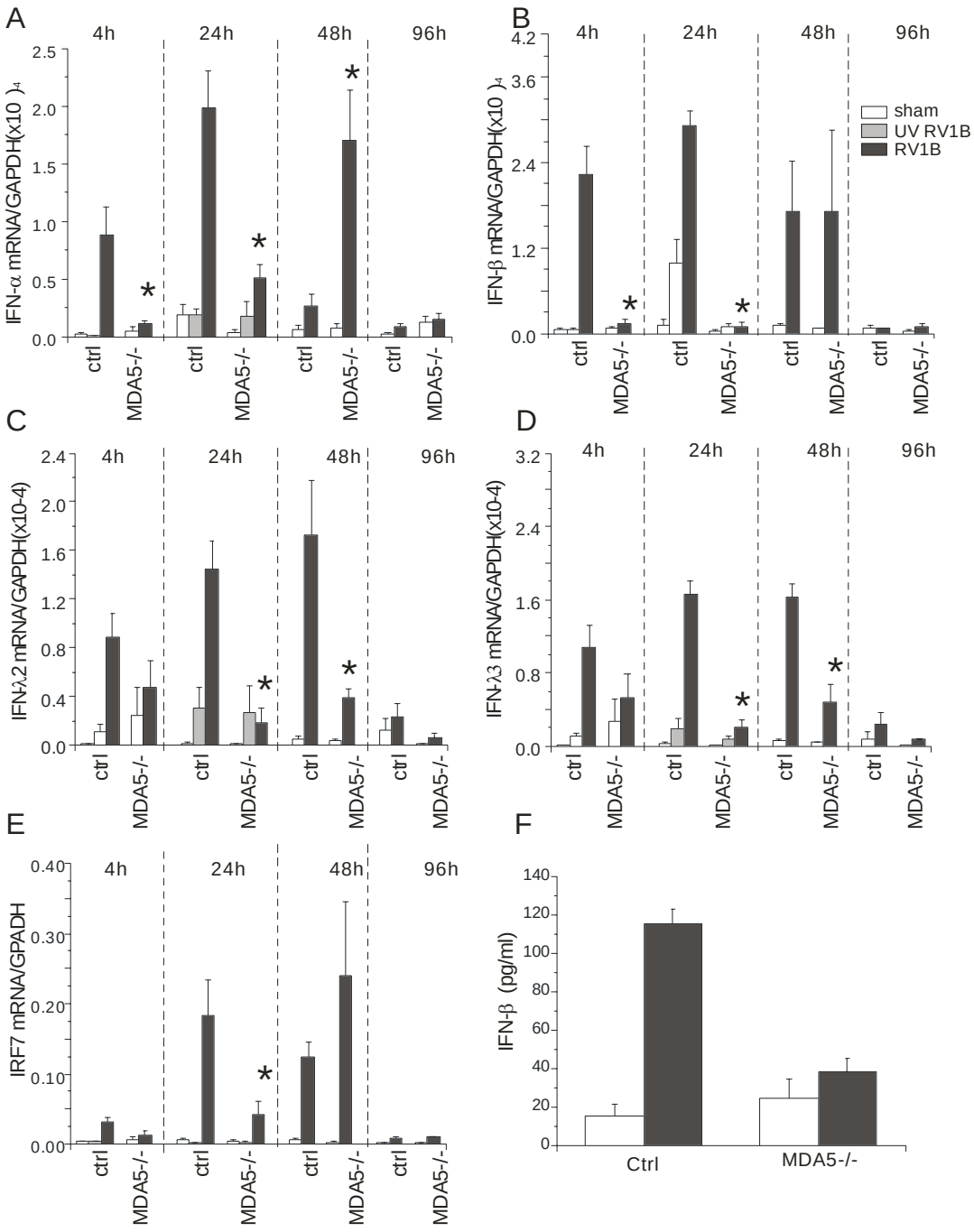


**Figure 3-5.** ***RV1B-induced expression of IFNs and interferon-stimulated genes in MDA5-/- mice.*** MDA5-/- and their control mice were inoculated with sham, UV-irradiated RV1B (UV RV1B), or intact RV1B. Lungs were harvested at 4, 24, 48, and 96 h post infection. A-E. The expression of IFN-α, IFN-β, IFN-λ2, IFN-λ3, and IRF7 at each time point was determined by qPCR. F. IFN-β protein production was measured by ELISA at 24 h post-infection. The expression of each target gene was normalized to GAPDH. Data represent mean±SEM for 3-7 mice (*$p<0.05$, one-way ANOVA).

*MDA5 regulates viral titer change.*

We determined lung viral titer and copy number by plaque assay and qPCR, respectively. There was a modest but statistically significant increase in viral titer and copy number in MDA5-/- mice compared to that of control mice 24 h post infection (Figures 3-6a, b). Viral titer and copy number were indistinguishable between the two groups at 48 h. Together, these data suggest that the delayed type I IFN response in MDA5 -/- mice is associated with a transient increase in viral titer.

*MDA5 signaling is required for RV1B-induced inflammatory responses.*

To determine whether MDA5 also plays a role in mediating RV1B-induced inflammatory responses, we measured the expression of pro-inflammatory cytokines in control and MDA5-/- mice. RV1B-infected MDA5-/- mice displayed significantly decreased expression of the neutrophil chemo-attractants CXCL1/KC and CXCL2/MIP-2 at both mRNA and protein levels compared to that of control mice (Figures 3-7a, b and h). To varying degrees, CCL2/MCP-1, CXCL10/IP-10, IL-6 and IFN-γ were also decreased 24 hr post infection (Figure 3-7c-f). There was no induction of CCL11/eotaxin-1 mRNA after RV1B infection in control or MDA5-/- mice (Figure 3-7g). These data suggest that MDA5 is required for RV1B-induced inflammatory responses *in vivo*. Accordingly, we also found significantly fewer neutrophils in the lungs of RV1B-infected MDA5 -/- mice compared to control mice 24 hr after infection (Figure 3-8b). This was reflected in H&E-stained lung sections (Figure 3-8a).

*MDA5 is required for RV1B-induced airway hyperresponsiveness*

We examined the airway cholinergic responsiveness of RV1B-infected control and MDA5 -/- mice 24 h after infection. RV1B-infected MDA5-/- mice displayed significantly lower airway responses to methacholine (Figure 3-8c). There was no difference in responsiveness between RV1B-infected MDA5-/- mice and sham-infected mice. Thus, like TLR3, MDA5 signaling is required for maximal RV1B-induced airway responsiveness.

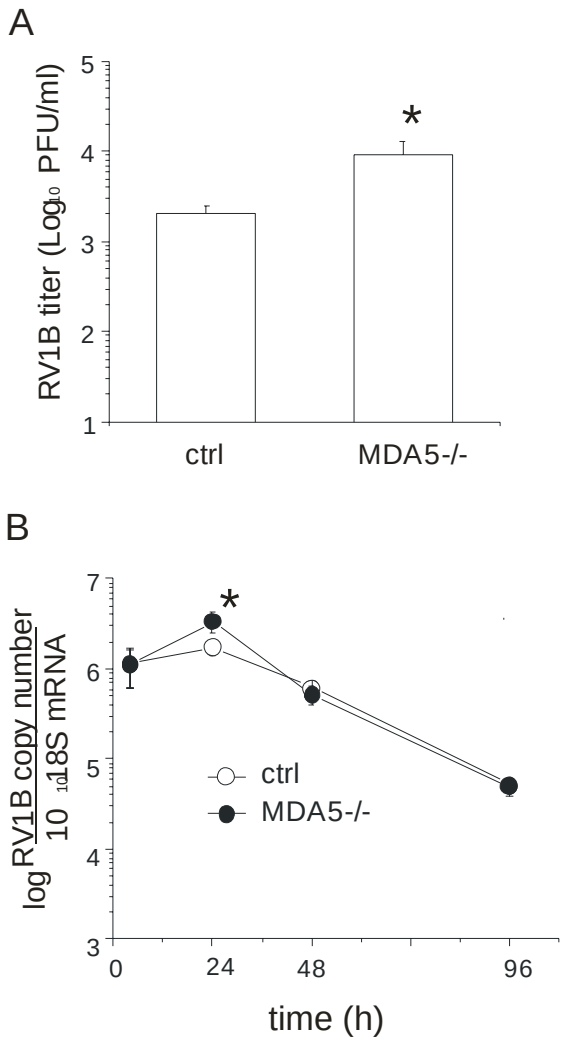


**Figure 3-6.** ***RV1B titer and copy number in MDA5-/- mice.*** MDA5-/- and their control mice were infected with RV1B. Lungs were harvested at 4, 24, 48, and 96 h after infection. A. Lung titer at 24 h post-infection was determined by plaque assay. B. RV1B copy number at each time point was determined by qPCR. RV copy number was normalized to 18S rRNA. Data represent mean±SEM for 3-7 mice (*$p<0.05$, one-wayANOVA).

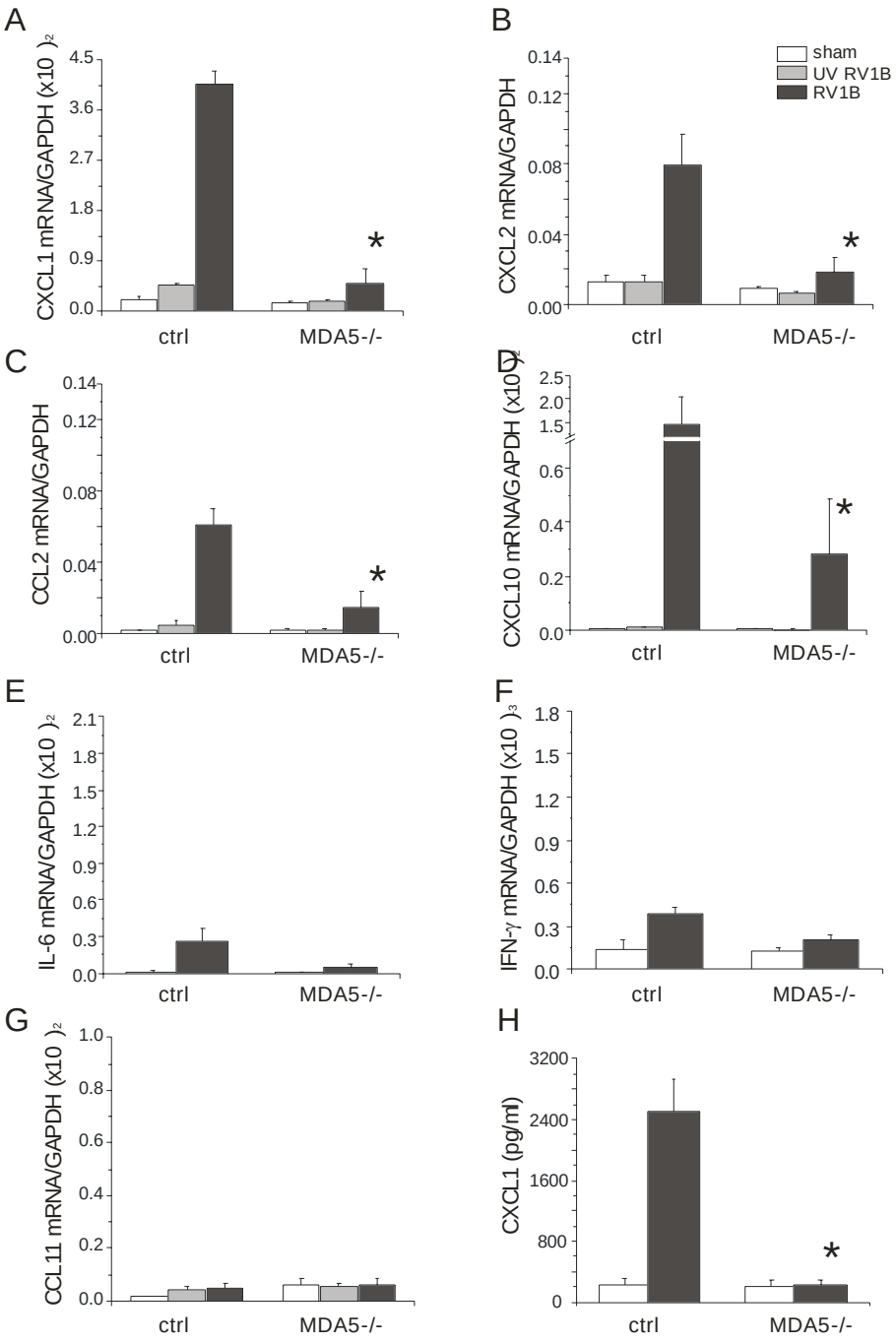


**Figure 3-7.** ***RV1B-induced pro-inflammatory cytokine expression in MDA5-/- mice.*** MDA5-/- and their control mice were inoculated with sham, UV-irradiated RV1B (UV RV1B) or RV1B. Lungs were harvested 24 after infection. A-G. The expression of CXCL1/KC, CXCL2/MIP-2, CCL2/MCP-1, CXCL10/ IP-10 and CCL11/eotaxin-1 was determined by qPCR. H. Protein production of CXCL1/KC was measured by bioplex assay. The expression of each target gene was normalized to GAPDH. Data represent mean±SEM for 3-7 mice ($p<0.05$, one-way ANOVA).

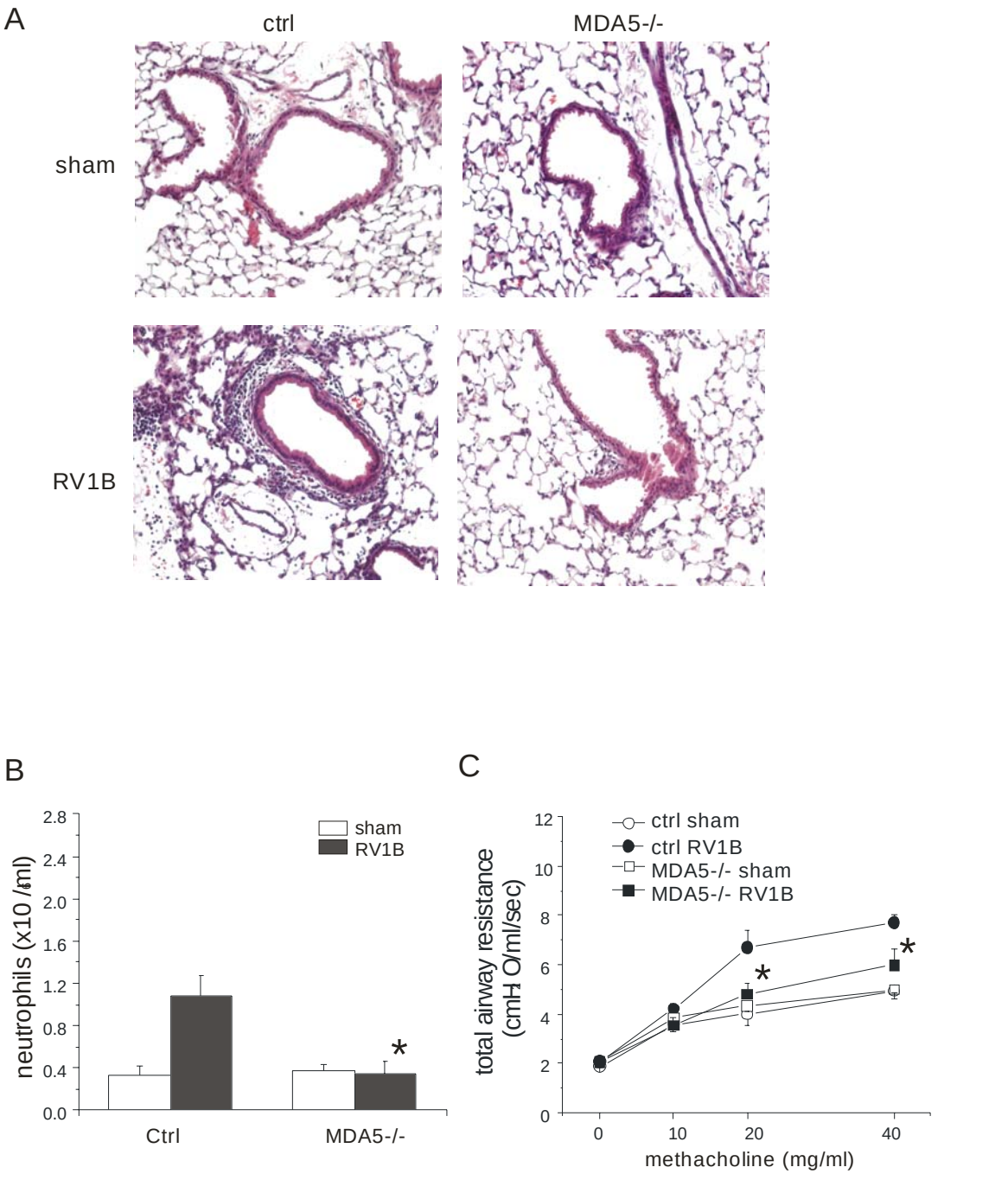


**Figure 3-8.** ***Airway inflammation and responsiveness in RV1B-infected MDA5-/- and control mice.*** MDA5-/- and their control mice were infected with RV1B. A. Twenty-four h after infection, lungs were fixed and stained with hematoxylin and eosin. B. RV1B-induced neutrophil infiltration was determined. Data represent mean±SEM for 3-7 mice, *p<0.05, one-way ANOVA). C. Total respiratory system resistance was determined by plethysmography. Data represent mean±SEM for 3-7 mice (*p<0.05, two-way ANOVA).

*MDA5 is required for maximal RV1B-induced type I and III IFN responses in mice with allergic airways disease.*

Since RV is the most frequent pathogen associated with asthma exacerbations, we combined RV infection with a commonly-used model of allergic airways disease, ovalbumin-sensitization and challenge. Wild-type and MDA5 -/- mice were injected intraperitoneally with PBS or a solution of alum and OVA, and then challenged intranasally with PBS or OVA. Mice were infected with RV1B immediately following the last OVA or PBS treatment. Lung IFN and inflammatory responses were measured as previously described in naïve mice.

Twenty-four h after infection, RV1B-infected OVA-treated wild-type mice displayed significantly increased levels of IFN-β, IFN-λ2 and IFN-λ3 expression compared to sham-inoculated OVA-treated mice. IFN responses were severely diminished in RV1B-infected OVA-treated MDA5-/- mice (Figures 3-9a-c). To determine the effect of reduced IFN expression on RV titer, we measured lung titers. Consistent with our previous results (21), after ovalbumin sensitization and challenge, both control and MDA5-/- mice showed significantly lower viral titers compared to naïve mice, indicating indicating either enhanced viral clearance or perhaps a failure of the virus to establish infection (Figure 3-9d). There was no difference observed between OVA-treated control or MDA5-/- mice. Thus, in the context of allergic inflammation, MDA5-deficient mice showed normal viral clearance, despite an abnormal IFN response.

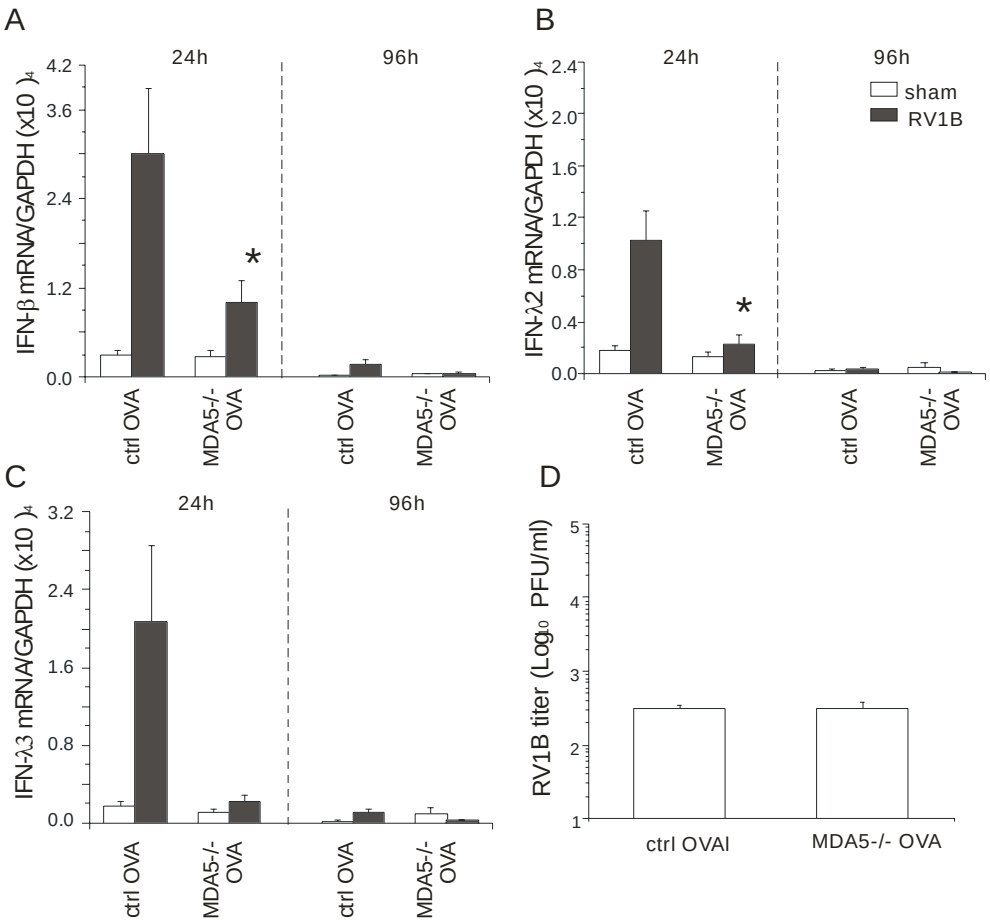


**Figure 3-9.** ***RV1B-induced IFN responses and viral clearance in OVA-treated MDA5-/- mice.*** OVA-treated MDA5-/- mice and control mice were infected with sham, or RV1B. Total lungs were harvested at 24 post infection. A-C The expression of IFN-β, IFN-λ2, IFN-λ3 was determined by qPCR. D. Total lung titer at 24h post infection was determined by plaque assay. The expression of each target gene was normalized to GAPDH. Data represent mean ± SEM for five-seven mice.

*MDA5 is required for maximal RV1B-induced airways inflammation and hyper-responsiveness in mice with allergic airways disease.*

We have previously shown that RV1B infection and OVA sensitization and challenge have additive effects on lung inflammation in control mice one day after infection (21). Thus, as expected, sham-infected OVA treated wild-type mice showed more lung inflammation than sham-infected or RV1B-infected naïve wild-type mice. Baseline levels of cytokines (IL-6, CCL2/MCP-1, IFN-γ and CCL11/eotaxin-1) were increased in the lungs of sham-infected OVA-treated mice. Twenty-four h after RV1B infection, CXCL1/KC, CXCL2/MIP-2, IL-6, CCL2/MCP-1, and IFN-γ expression in wild-type mice increased 3-5 fold (Figures 3-10a-e). MDA5 deficiency significantly reduced the induction of CXCL1/KC, CXCL2/MIP-2, IL-6, CCL2/MCP-1 and IFN-γ. The expression of CCL11/eotaxin-1 mRNA was not significantly elevated 24 h after infection and was not different between control and MDA5-/- mice (Figure 3-10f). Neutrophil infiltration in the MDA5 -/- mice was significantly lower than wild-type mice (Figures 3-11a). There was no significant difference observed in the number of macrophages, eosinophils or lymphocytes (Figures 3-11b-d). H&E staining showed less inflammation around the airways of RV1B-infected OVA-treated MDA5-/- mice compared to wild-type mice (Figures 3-12a). Consistent with the greater amount of inflammation present in the lung, RV1B-infected, OVA-treated control mice displayed the highest airways responsiveness compared to any other treatment. Airways responsiveness was decreased in RV1B-infected MDA5 -/- mice (Figure 3-12b).

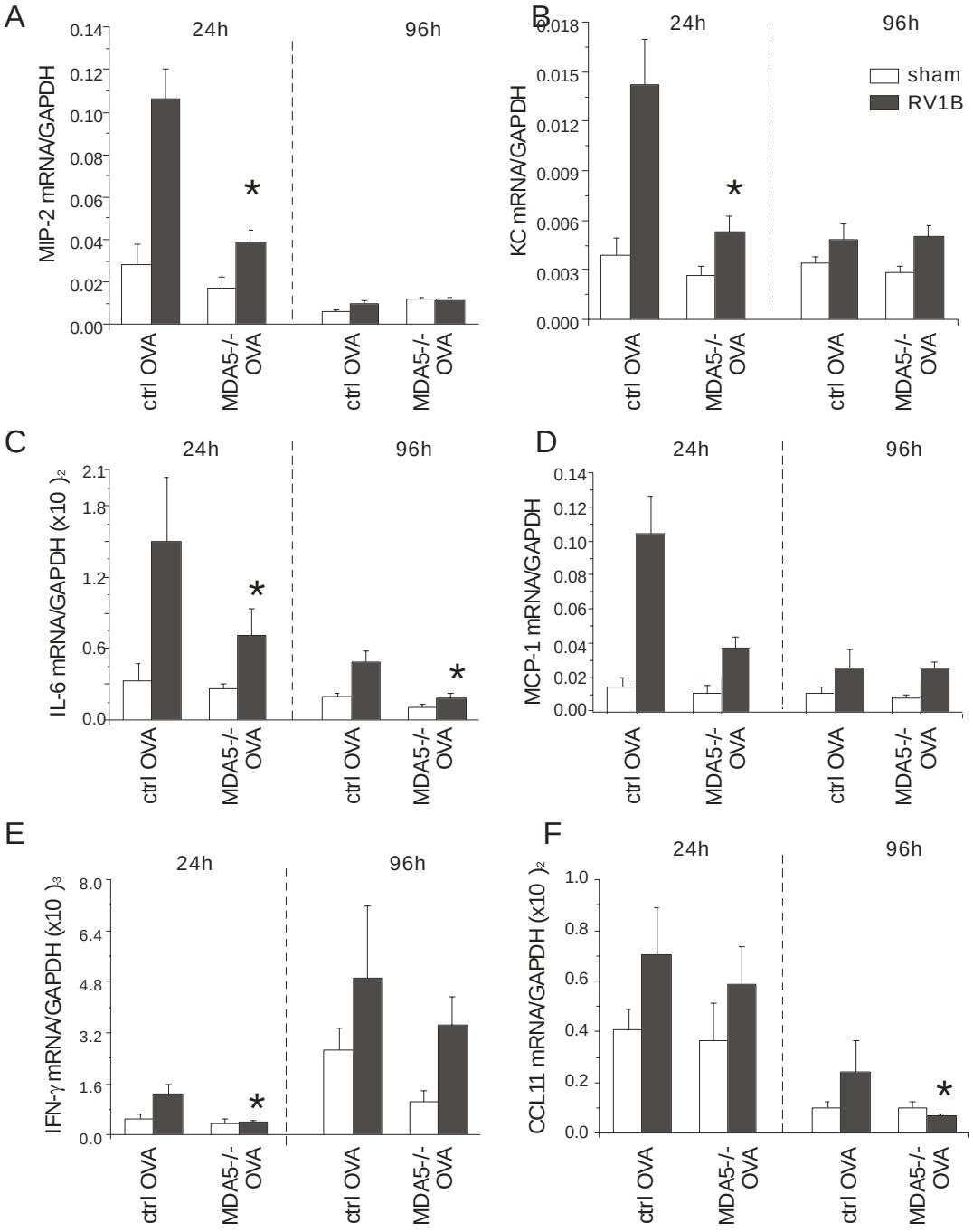


**Figure 3-10.** ***RV1B-induced pro-inflammatory cytokine expression in OVA-treated MDA5-/- mice***. OVA-sensitized and -challenged MDA5-/- and control mice were inoculated with sham or RV1B. Lungs were harvested at 24 and 96 h after infection. A-F. The expression of CXCL1/KC, CXCL2/MIP-2, IL-6, CCL2/MCP-1, IFN-γ and CCL11/eotaxin-1 was determined by qPCR. The expression of each target gene was normalized to GAPDH. Data represent mean±SEM for 4-7 mice (*$p<0.05$, one-way ANOVA).

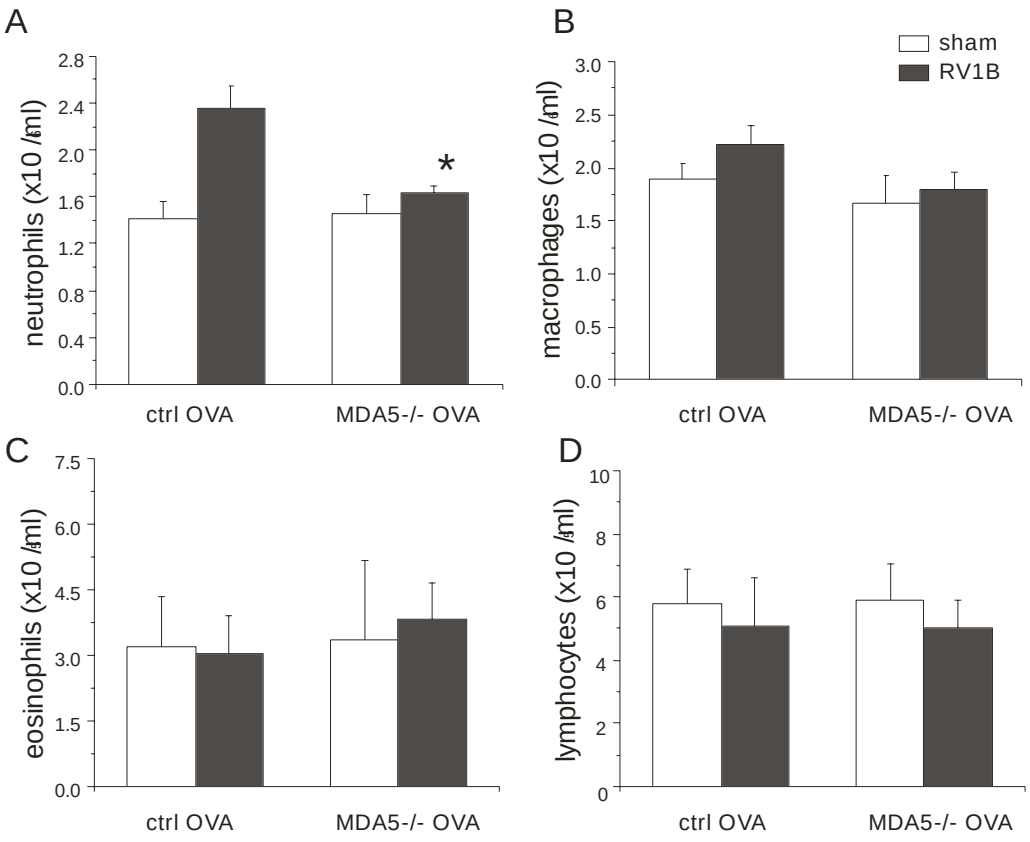


**Figure 3-11.** ***Early lung inflammation in OVA-treated RV1B-infected MDA5-/- mice cell counts.*** MDA5-/- and their control mice were OVA-sensitized and -challenged and then infected with RV1B. Twenty-four h after infection, lungs were digested by collagenase. A-D. The number of infiltrated neutrophils, macrophages, eosinophils and lymphocytes were counted. Data represent mean±SEM for 6 mice (*$p<0.05$, one-way ANOVA).

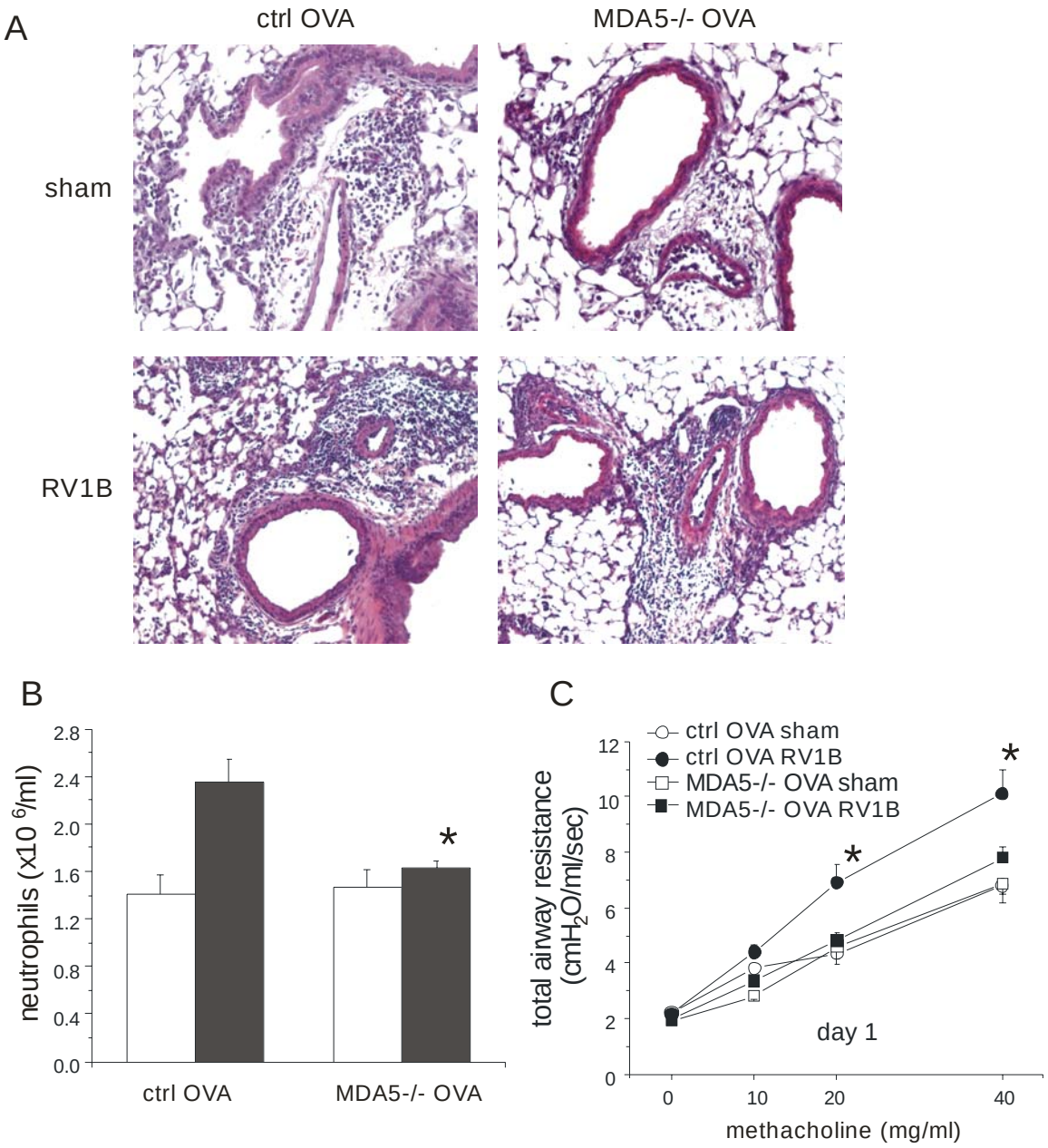


**Figure 3-12.** ***Early lung inflammation in OVA-treated RV1B-infected MDA5-/- mice: histology and airways responsiveness.*** MDA5-/- mice and their control mice were sensitized and challenged with OVA and then infected with RV1B. A. Twenty-four h after infection, lungs were fixed and stained with hematoxylin and eosin. B. Total respiratory system resistance was determined by plethysmography. Data represent mean±SEM for 6 mice (*$p<0.05$, two-way ANOVA).

Ninety-six h after infection, RV1B-induced airways hyperresponsiveness is dependent on CCL11/ eotaxin-1-mediated eosinophilic airway inflammation (21). At this time point, in contrast to the other cytokines, IFN-γ mRNA expression increased in both control and MDA5-/- mice (Figures 3-13a-f). Also, compared to control mice, RV1B-induced CCL11/eotaxin-1 and IL-6 levels were significantly decreased in MDA5-/- mice. No difference in neutrophil infiltration between wild-type and MDA5 null mice was observed. However, eosinophil and macrophage infiltration was significantly lower in MDA5-/- mice (Figures 3-13a). Finally, the airways responsiveness of RV1B-infected OVA-treated MDA5-/- mice was decreased at 96 h post-infection.

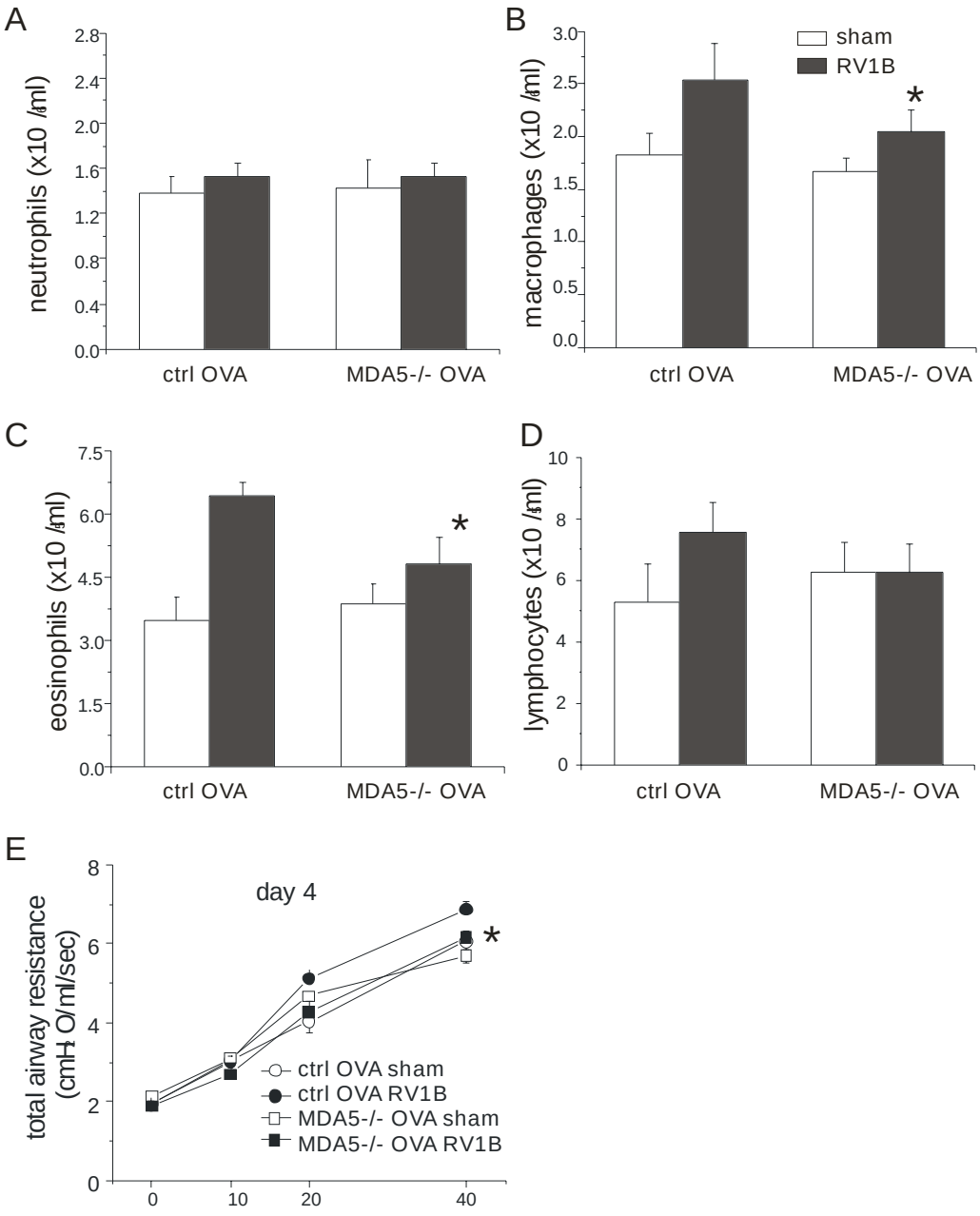


**Figure 3-13.** ***Late lung inflammation in OVA-treated RV1B-infected MDA5-/- mice.*** MDA5-/- and their control mice were sensitized and challenged with OVA and then infected with RV1B. A. Ninety-six hour after infection, the number of infiltrated neutrophils, macrophages, eosinophils, and lymphocytes was counted. Data represent mean±SEM for 4-7 mice (*p<0.05, one-way ANOVA). B. Total respiratory system resistance was determined by plethysmography. Data represent mean±SEM for 4-7 mice (*p<0.05, two way ANOVA).

## Discussion

Pattern recognition receptors regulate multiple effector molecules, including type I IFNs and other pro-inflammatory cytokines (23-25). The innate immune response to viral infection is specific to cell type/organ as well as to invading pathogen (26, 27). The ultimate host response is likely to be an integration of both IFN and pro-inflammatory responses. Because viral infections, in particular RV, are the most common cause of asthma exacerbations, we examined the roles of the pattern recognition receptors TLR3 and MDA5 in the response to RV infection. We found that both TLR3 and MDA5 were required for RV1B-induced maximal inflammatory responses and airways cholinergic hyper-responsiveness *in vivo.* TLR3 null mice showed more-or-less normal IFN responses and normal viral titer. MDA5 mice showed a delayed type I IFN and attenuated type III IFN response to RV1B infection, leading to a transient defect in viral titer. Further, MDA5 null mice with allergic airways disease showed enhanced viral clearance despite deficient IFN responses, as well as decreased airway inflammatory and contractile responses. Together, these results suggest that TLR3 and MDA5 individually initiate pro-inflammatory signaling pathways leading to airways inflammation and cholinergic hyper-responsiveness.

Previous studies by our group demonstrated that TLR3 is required for RV39-induced IL-8 expression in cultured airway epithelial cells (9) and that the TLR3 adaptor protein TRIF is required for both IFN and cytokine responses (8). In contrast, the present study showed that TLR3 was not required for RV1B-induced IFN responses or viral clearance *in vivo*. The differential effects of TLR3 deficiency on chemokine and IFN production

suggest that, in the context of RV infection, TLR3 preferentially activates NF-κB- rather than IRF3-dependent gene expression. This discrepancy may also reflect the cell-type specificity of pattern recognition receptors (26, 27). Furthermore, TLR3 is only one of three pattern recognition receptors capable of signaling in response to viral infection (the others being RIG-I and MDA5). Finally, TLR4 signals through TRIF to upregulate IRF3-dependent genes and has been implicated in the innate immune response to vesicular stomatitis virus (28, 29). These redundant signaling pathways may explain why TLR3 is dispensable for IFN production and protection from RV infection.

Mice lacking TLRs and key molecules of TLR signaling pathways have been shown to display diverse viral infectious phenotypes depending on the host gene-pathogen combination. Studies have shown that TLR3 plays a protective role against various viral infections *in vivo*. Mice lacking TLR3 display reduced IFN-α/β production and an increased viral load against mouse cytomegalovirus infection (30). In response to coxsackievirus B4 infection, TLR3-/- mice produce less pro-inflammatory mediators and are unable to control viral replication at the early stages of infection, resulting in severe cardiac damage and reduced survival (31). On the other hand, alternative studies have suggested that TLR3 plays a detrimental role upon viral infection. Despite an increased viral load, influenza A virus-infected TLR3-/- animals display significantly reduced pro-inflammatory mediators, suggesting that TLR3 critically contributes to a detrimental host inflammatory response (32). In addition, TLR3 signaling has also been reported to have a detrimental effect in phlebovirus and vaccinia infections (33, 34). The role of TLR3 in West Nile virus infections is controversial. In one study, TLR3-/- mice displayed impaired expression of inflammatory cytokines and IFNs and enhanced viral load in the

peripheral blood, but reduced viral load and inflammation in the brain (35). In a second study, the absence of TLR3 enhanced mortality and increased viral burden in brain neurons but had little effect on peripheral IFN expression or viral load (36). Our study demonstrated that, as with influenza infection, TLR3-/- mice displayed a decreased inflammatory response upon RV1B infection, resulting in a significantly lower airways cholinergic responsiveness, suggesting for the first time that TLR3 signaling is maladaptive following RV infection. These data agree with a recent report showing that TRIF-/- mice challenged with dsRNA display decreased airway responsiveness and pulmonary inflammation compared to control mice (37).

Unlike TLR3-/- mice, our studies showed that MDA5-/- mice displayed a reduction in type I IFN expression after RV1B infection, leading to a transient increase in viral titer and copy number 24 h after infection. Interestingly, MDA5-/- mice displayed increased IFN-α/β expression 48 h after RV1B infection, suggesting a delayed onset of IFN signaling. We speculate that this was due to the compensatory activation of another host pattern recognition receptor. On the other hand, MDA5 null mice showed a persistent defect in the expression of type III IFNs. These data suggest that type III IFN responses are a less critical host defense against RV1B infection and are regulated independently of type I IFN responses. In support of this concept, IFN-λ is not required for immunity to influenza in wild-type mice, though it protects influenza-infected IFN-α/β knockout mice (38). The IRF7 response seemed to be more closely associated to type I IFN rather than type III IFN responses in both TLR3 and MDA5 knockout mice.

Together, these data suggest that MDA5 rather than TLR3 is the primary receptor for containing RV replication, and that type I IFNs play a core role in this antiviral event.

In addition to decreased IFN responses, MDA5-/- mice also displayed reduced expression of pro-inflammatory cytokines such as CXCL1, CXCL2, CCL2 and IL-6. The coupling of IFN and pro-inflammatory responses has also been observed in other studies. Bone marrow-derived dendritic cells from MDA5 -/- mice display significantly lower expression of IFN-α, CCL2/MCP-1 and IL-6 against murine norovirus infection compared those from control mice (16). On the other hand, reduced viral clearance has ultimately led to enhanced inflammatory responses in some models. MDA5-/- mice show significantly decreased IFN mRNA expression profiles five days after Sendai virus infection but compensatory IL-6 mRNA expression, resulting in increased mortality and severe histopathological changes in the lower airways (39). Thus, host inflammatory responses in the absence of IFN expression are pathogen-specific: When challenged by pathogenic lethal viruses such as Sendai virus, the host initiates inflammatory responses to defend against viral invasion. However, when confronted with non-pathogenic viruses such as RV, it is advantageous for the host to terminate the inflammatory response, in order to avoid adverse effects.

Despite differences in their IFN responses, TLR3 and MDA5 knockout mice displayed reduced expression of the neutrophil chemoattractants CXCL1/KC and CXCL2/MIP-2 following RV infection, leading to decreased neutrophil infiltration into the airways. Decreased neutrophilic airways inflammation was associated with decreased airways responsiveness. This is consistent previous studies from our group showing that

CXCR2 ligands are required for RV1B-induced airway inflammation and hyperresponsiveness (40).

It has been proposed that asthmatics are susceptible to RV infection due to deficient IFN production. RV-infected airway epithelial cells from asthmatic subjects show impaired production of IFN-β and -λ (11, 12) and asthmatics experimentally infected with RV16 showed a reduced IFN-γ/IL-5 mRNA ratio in their sputum (13). To examine whether an allergic background alters the response of wild-type and MDA5 knockout mice to viral infection, we combined RV infection with a commonly-used model of allergic airways disease, OVA-sensitization and challenge. First, we found that, following OVA treatment, both wild-type and MDA5-/- mice demonstrated reduced viral titers following infection with RV. This is consistent with our previous data (21), as well as data from guinea pigs that were sensitized to OVA and subsequently infected with parainfluenza (41). The precise mechanism for increased viral titer in mice with allergic airways disease is unclear. Prior to RV infection, these mice showed increased baseline levels of neutrophils, macrophages, lymphocytes, eosinophils, IFN-γ, IL-6 and CCL11/eotaxin-1, each of which could have contributed to an antiviral response. Eosinophils are known to contain a number of granule proteins that can neutralize viruses, such as eosinophil cationic protein and eosinophil-derived neurotoxin, each of which possess strong ribonuclease activity (42). Eosinophils have been shown to neutralize respiratory syncytial virus in a concentration-dependent manner, and this effect could be completely reversed by a ribonuclease inhibitor (43, 44). Second, we found that RV1B-infected OVA-treated MDA5-/- mice showed significantly reduced IFN and cytokine

levels compared to wild-type mice. Reduced cytokine expression, in turn, led to persistent attenuations in airway inflammation and responsiveness. Thus, pro-inflammatory and IFN responses were strictly linked: Reduced IFN responses in MDA5 -/- mice were associated with less robust, not increased, inflammatory responses. These data are consistent with our recent findings in airway epithelial cells isolated from patients with COPD (45). These cells showed increases in both pro-inflammatory cytokines and IFNs.

Finally, we would like to add a few comments about our mouse model of human RV1B infection. We (15) and others (46) have found that a much higher viral titer is required to infect mice compared to humans. This is to be expected, as differences in the homology of viral receptors and intracellular signaling mechanisms are likely to restrict viral infection and replication in mice. This restriction in viral replication could have limited the effects of pattern recognition receptor knockout in our model. On the other hand, we have clearly shown that human RV1B replicates to some extent in mouse lungs, as evidenced by: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice; 2) transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells; 3) the induction of a robust lung interferon response; and 4) a modest increase in lung vRNA following infection(15). In addition, we have demonstrated a significant reduction in RV1B clearance in mice chronically treated with lipopolysaccharide and elastase, a model of chronic obstructive pulmonary disease in which IFN and IL-10 responses to RV1B are deficient (47).

In summary, to our knowledge this is the first study to examine the contribution of TLR3 and MDA5 to RV responses *in vivo*. Our results suggest that TLR3 and MDA5 individually initiate pro-inflammatory signaling pathways leading to airways inflammation and cholinergic hyper-responsiveness. Thus, TLR3- and MDA5-driven innate immune responses to RV, a relatively non-pathogenic virus, are maladaptive in this model. Therefore, antagonists against TLR3 and MDA5 could provide potential therapeutic agents in the treatment of virus-induced asthma exacerbations. Future studies focusing on the interactions and coordination between the two receptors would be useful in understanding the precise mechanism of RV-induced, pattern recognition receptor-mediated innate immune responses.

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# Chapter 4

## Summary, Limitations, and Future Directions

### Summary

Most virus-induced asthma exacerbations in children and at least half of those in adults are caused by RV (1-2). While RV has traditionally been regarded as an upper respiratory pathogen, recent studies have revealed its presence in the lower airways, although the extent of replication remains unknown (3-6). Several studies have reported that RV infection can induce IFN and ISG expression in cultured airway epithelial cells (7-8). The expression of IFNs is an early event and a major component of the host innate immune response. However, the mechanism by which RV infection activates the IFN expression signaling pathway is not well understood.

Host PRRs have become a compelling research topic in the field of innate immune responses because they play an essential role in the recognition of the specific molecular patterns of different viruses (9). This thesis has three objectives: 1. to determine which dsRNA PRRs are required to sense RV dsRNA and trigger downstream antiviral signaling events in airway epithelium; 2. to determine the contribution of MDA5 and TLR3 in RV-induced innate immune responses *in vivo*; 3. to determine the effect of

antiviral IFN responses on airways that have a pre-existing allergic inflammation. To achieve these objectives, we first used a human airway epithelium cell line to examine the changes in the expression of IFNs and pro-inflammatory genes after RV1B infection; then, using our established model of RV infection, we examined the IFN responses and pro-inflammatory changes observed after RV inoculation in naïve mice; and finally, we extended this line of study to a mouse model of allergic airway disease that we developed recently in our laboratory. (10)

dsRNA produced during viral infection represents an important stimulus of the host innate immune response. It is recognized and engaged by three PRRs. TLR3, which is localized to the endosomal and plasma membranes, senses viral dsRNA released from dying cells and signals through its adaptor protein TRIF (11). RIG-I and MDA5, which are localized to the cytosol, sense viral dsRNA in the cytoplasm. (12-13)

Although all three PRRs recognize viral dsRNA, they appear to be specialized in their recognition of particular viruses. RIG-I and TLR3 are required for respiratory syncytial virus (RSV)-induced expression of IFN-β and IP-10 in airway epithelial cells (14). RIG-I-deficient mice fail to produce type I IFNs in response to infection with certain negative-sense ssRNA viruses – NDV, SeV, VSV, and influenza virus – and the positive-sense ssRNA Japanese encephalitis virus; whereas MDA5-deficient mice fail to produce IFNs in response to EMCV infection (9). The engagement of PRRs also appears to be cell type-specific. For example, in fibroblasts and cDCs, MDA5 is essential for the induction of type I IFNs against EMCV, a member of picornavirus family; whereas pDCs use the TLR system for viral detection (9). While these results are compelling, it seems

premature to conclude that all picornaviruses are sensed by MDA5 in all cell types, because, to date, studies have been limited to only a few members of the picornovirus family (15).

For the first time, we determined that MDA5 and TRIF, but not RIG-I, are required for maximal sensing of RV dsRNA in cultured human airway epithelial cells. Transfection of a human bronchial epithelial cell line (BEAS-2B cells) with siRNA against MDA5 and TRIF, but not RIG-I or non-targeting siRNA, significantly inhibited RV1B-induced expression of type I IFNs, and the IFN-stimulated gene IRF7, but not the pro-inflammatory genes GM-CSF or IL-8. Knockdown of MDA5 and TRIF also attenuated the expressions of the type III IFNs IFN-λ1 and -λ2/3, which functionally resemble type I IFNs and are also induced by RV infection (16-17). Further, we confirmed the role of MDA5 in primary tracheobronchial epithelial cells. These data are in agreement with previous data suggesting that MDA5 is required to sense picornavirus dsRNA. We also examined the contribution of IRF3 to RV-induced responses in airway epithelial cells. IRF3 siRNA nearly abolished RV-induced expressions of IFN and ISG. MDA5 and TRIF knockdown also decreased IRF3 dimerization. These data are consistent with the idea that TLR3 and MDA5 regulate IFN expression via the same downstream intermediate, IRF3.

Next, we examined the airway responses of TLR3- and MDA5-deficient mice to infection with RV1B, a minor group virus that replicates in mouse lungs (18). Compared to control mice, TLR3-/- mice showed essentially normal IFN responses and normal viral clearance. MDA5-/- mice displayed a delayed type I IFN response and an attenuated type

III IFN response to RV1B infection, leading to a transient increase in viral titer. Further, RV-infected TLR3 and MDA5 null mice both displayed reduced neutrophil infiltration in the lungs, reduced expression of pro-inflammatory chemokines (i.e., MCP-1, MIP-2, and KC), and reduced cholinergic responsiveness in the airway, suggesting that both TLR3 and MDA5 are required for RV-induced airway inflammation *in vivo.*

Finally, we tested the impact of MDA5 deficiency on RV-infected airways with pre-existing allergic inflammation induced by OVA sensitization and challenge. RV-infected MDA5-/- mice displayed higher inflammatory responses overall as a result of OVA treatment, leading to decreased viral titer despite a deficient IFN response. One day after RV infection, the airways of MDA5-/- mice exhibited attenuated contractile responses and decreased neutrophilic inflammation; 4 days post-infection, there was decreased eosinophilic inflammation. Together, these results suggest that, in the context of RV infection, TLR3 and MDA5 each initiate pro-inflammatory signaling pathways leading to airway inflammation and cholinergic hyper-responsiveness, implying that TLR3 and MDA5 signaling might be maladaptive.

Although some studies indicate that the susceptibility of asthmatics to RV may be due to a deficient IFN response in epithelial cells (17, 19), the evidence is controversial and has raised heated debate (20). Our study clearly shows that a deficient IFN response need not necessarily exacerbate an asthmatic situation; and may indeed be associated with a reduction in signs and symptoms.

## Limitations and Future Directions

*RV infection*

In these studies, we infected mice with human minor group serotype RV1B. Species differences in the major group virus ICAM-1 receptor present a challenge in developing an optimal mouse model for major group RV infection. In addition, we have been unable to detect sustained expression of negative-strand viral RNA in C57BL/6 or BALB/c mice infected with RV1B, a result that would more faithfully reproduce human infection. However, we have shown clearly that human RV1B replicates to some extent in mouse lungs: our previous studies revealed the presence of negative-strand viral RNA in the lungs of inoculated mice; we have also shown that lung homogenates from RV-infected mice, when overlayed on Hela cell monolayers, produce cytopathic effects on cultured HeLa cells; and we have shown that RV inoculation of naïve mice induces a strong IFN response that is dependent on double-stranded viral RNA (18). In addition, we have demonstrated a significant increase in RV1B copy number in mice chronically treated with lipopolysaccharide and elastase, a model of chronic obstructive pulmonary disease in which both IFN and IL-10 responses are deficient (21). Nevertheless, in future studies, we might make adjustments in our model to enhance viral replication. For example, we could attempt to adapt minor group RV to the mouse by repeatedly passing the virus through mouse cells, either *in vitro* or *in vivo*. Transgenic mice with an incomplete defect in antiviral response could also be employed.

*Limitations of the allergen sensitization and challenge protocol*

In our studies, we employed OVA, a chicken egg antigen, to generate an allergic response in murine lungs. We used aluminum hydroxide as an adjuvant, which helps to process and present the antigen. However, OVA is not a physiologic allergen in humans, and human allergen sensitization does not require an adjuvant or systemic administration. It is conceivable that the increase in viral clearance we observed after allergen sensitization and challenge is an artifact of our allergen sensitization and challenge protocol. Future studies should therefore consider other, more physiologic allergens, such as cockroach frass or dust-mite extract, which combine both an antigen and protease, obviating the need for an adjuvant. .

*Integration of immune responses in the whole animal*

Based on our previously presented data, it should be clear that the overall response of the airways to viral infection represents the integration of a number of overlapping and sometimes conflicting pathways. First, the response includes both antiviral pathways, driven by viral dsRNA and pattern recognition receptors, and NF-κB-mediated pro-inflammatory pathways. NF-κB may be activated by both early events in the viral life cycle (*i.e*., binding and endocytosis) and viral replication (22).

Second, the response is mediated by a number of different cell types. Until recently, the main target of RV infection in the lung was thought to be the airway epithelial cell. The epithelial surface is regarded as the first line of host defense. Research has shown that there is a prominent early activation of the IFN-signaling protein Stat1

when airway epithelial cells are infected with SeV (23). Infected Stat1-/- mice exhibited increased viral replication and neutrophilic inflammation in concert with overproduction of TNF-α and the neutrophil chemokine CXCL2. After reconstitution with wild-type bone marrow, Stat1-/- mice remain susceptible to infection with SeV. This suggests that the predominant IFNs originate from lung epithelium instead of hematopoietic cells, another potential source of IFN production. We therefore initially examined the pattern recognition receptors responsible for RV-induced IFN production in cultured airway epithelial cells. However, it has recently become apparent that RV may infect resident or infiltrating inflammatory cells. Recent immunohistochemical studies from our laboratory have shown that RV can infect lung macrophages *in vivo* (10). We (10) and others have also shown that monocytes and macrophages produce IFNs and other pro-inflammatory cytokines, including TNF-α and MCP-1 *in vitro* (24-25). Therefore, the IFN and inflammatory responses we observed in mice likely arise from both lung epithelial and immune cells, which may amplify the antiviral responses from epithelium alone.

Third, there are temporal aspects of the response which may be carried out by different cell types, distinct pattern recognition receptors, or even distinct signaling pathways within the same cell. Compared to control mice, MDA5-/- mice displayed a delayed type I IFN response after RV infection. The precise mechanism of this compensatory response is not known. In the absence of MDA5, some other dsRNA PRR, such as TLR3, may be activated at a later point to induce type I IFN expression. Little is known about the relative contributions of these receptors or their cooperative effects on antiviral defense (26-27). One study showed that RIG-I and TLR3 mediate RSV in a

temporal manner, with RIG-I mediating an early response and TLR3 mediating a later response (28). In contrast, another study suggested opposite roles for TLR3 and RIG-I in the inflammatory response to dsRNA in a tumor cell line (29). Given the physiological location of these receptors and the insertion location of RV dsRNA, it is conceivable that the TLR3 system is activated only after lysis of infected cells, leading to free dsRNA for endocytosis. Future studies using double-knockout mice of MDA5 and TLR3 may provide valuable information about whether TLR3 is indeed the receptor that is activated in a delayed fashion in MDA5-/- mice following RV infection and about the cooperation of these receptors in a host defense system against RV infection. It is plausible that the double knockout mice will display a persistently decreased, instead of a merely delayed, type I IFN response after RV infection.

Fourth, there may be a spatial aspect of coordination among these receptors, given their different cellular locations. As noted above, early responses may require recognition of dsRNA in the cytoplasm by MDA-5, whereas later responses may require recognition of free dsRNA by plasma membrane/endosomal TLR3.

On a related note, since these receptors tend to share common downstream signaling pathways, the mechanism by which both MDA5 deficiency and TLR3 deficiency each attenuate RV-induced inflammation is unclear. Unlike RV-induced IFN responses, there does not seem to be a redundancy between MDA5 and TLR3 when it comes to chemokine responses. The two receptors do not appear to compensate for each other in regulating RV-induced inflammatory responses *in vivo*. The explanation for this is unclear, but it is conceivable that different cell types utilize the two different receptors

in vivo, and that each cell type is capable of producing a significant inflammatory response. It is also possible that the absence of TLR3/MDA5-mediated signaling causes dysregulation of normal innate immune system activation.

Downstream of PRRs are common kinases that are required for the expression of IFNs and cytokines in response to viral infection (30-31). The IκB kinases IKK-α and IKK-β and the IKK-related kinases TBK-1 and IKK-ε have essential roles in innate immunity through signal-induced activation of NF-κB, IRF3, and IRF7. These kinases could participate in compensatory responses following knockout of upstream PRRs. In fact, since these kinases are the converging points of upstream receptor signaling, their function could substitute for a double or triple knockout of upstream receptors. Over-expression of IKK-ε results in restoration of defective antiviral IFN signaling against hepatitis C virus infection (32). Conversely, it is plausible that the deficiency of these kinases may abolish the RV-induced innate immune response more thoroughly than each individual receptor. It has been shown that mice lacking IKK-ε produce normal amounts of IFN-β, but are hyper-susceptible to influenza virus infection because of a defect in the IFN signaling pathway (33). Future studies using IKK-ε knockout mice from the Saltiel laboratory at the University of Michigan may provide insight into the role of this kinase against RV infection. It is reasonable to speculate that the deficiency of IKK-ε may result in a complete shut-down of RV-induced IFN and inflammatory responses. However, given that the precise role of non-canonical IKKs is not completely understood, and the overlapping, cross-linking nature of the inflammatory pathways (34-35), the effects of IKK-ε knockout are difficult to predict.

Throughout the studies in this thesis, we have examined the two closely-linked arms of the innate immune response: virus-induced IFN and inflammatory responses. PRR receptor knockout mice provided a good model for studying the roles of TLR3 and MDA5 in the innate immune response. However, as these receptor proteins reside at a proximal point in the innate immune signaling cascade, their absence will inevitably affect both IFN and inflammatory responses. Even the IKK-ε knockout could have complex effects. To truly determine the specific role of RV-induced IFN production in the airway response, we could employ IFN α/β receptor knockout mice whose IFN responses are completely deficient, yet maintain intact inflammatory signaling. At first blush, one might expect these mice to have an increased inflammatory response due to an increase in viral load. However, given the modest pathogenicity of this virus and the small amount of viral replication which occurs in these mice (and, in particular, allergen-sensitized and -challenged mice), we speculate that the inflammatory response in IFN receptor knockout mice could be unchanged, depending on the strength of infection.

*Virus and immune invasion strategies*

In our studies we have focused on the host response to viral infection. However, many viruses have evolved the ability to either suppress the host immune system at various points along the antiviral signaling pathways. Hepatitis B and C virus have been shown to interfere with IFN-α/β defenses by down-regulating IPS-1 and inhibiting the activation of transcription factors IRF3 and NF-κB, respectively (32, 36-37). Paramyxoviruses can block the production of IFN-β by binding to MDA5 (38). Similarly, influenza A virus can inhibit IRF3 activation by binding to RIG-I (39). Picornavirus has

been shown to cleave RIG-I and MDA5 *in vitro* via the virus 3C protease and the caspase/proteasome pathways, respectively (40-41). RV1A has been shown to evade IFN responses by cleaving IPS-1 (42). However, most of these studies were conducted in susceptible cell lines such as HeLa cells, and none were conducted in airway epithelial cells. In our studies, we observed the cleavage product of MDA5 in BEAS-2B cells after RV infection; however, the degree of inhibition of the IFN response was negligible. Inhibition of the IFN response did not occur in our *in vivo* studies either. The virus-induced inhibitory effect may be specific to cell type and affected by dose of virus used. The significance of the cleaved product of MDA5 after RV infection could be explored further in future.

The role of apoptosis in the response to RV infection is not clear. It has been argued that the early induction of apoptosis in infected cells increases the release of viral particles (43). On the other hand, others have suggested that apoptosis protects against viral infection by reducing viral release (44). In our studies, we did not specifically evaluate RV-induced apoptotic events. Deszcz and colleagues showed that RV infection could induce typical apoptotic morphological alterations in both Hela and 16HBE14o-immortalized human bronchial epithelial cells (43). Bronchial epithelial cells of asthmatic humans showed an impaired apoptotic response to HRV infection (19). On the other hand, relative to other viruses such as RSV or influenza, RV-infected cells show minimal cytopathic effects. In fact, studies from our group have shown that infection of polarized airway epithelial cell cultures with RV for 24 hours causes a significant decrease in tight junction resistance without causing cell death or apoptosis (45).

**Physiological Relevance and Significance**

RV accounts for most virus-induced asthma exacerbations, although the precise mechanisms are not well understood (1). In the studies for this thesis, we focused on the role of PRRs in the innate immune response after RV infection in airway epithelium. Our studies using a mouse model indicate that TLR3 and MDA5 mediate increased pro-inflammatory responses and result in airway hyper-responsiveness in control mice compared to the knockout mice after RV infection. The same result is observed in OVA-sensitized and -challenged mice. Therefore, while the presence of MDA5 provides a strong IFN response to clear invading viruses, both MDA5 and TLR3 cause increased inflammation in the infected lungs. These results are provocative. Is this inflammation necessary? Does the inflammation provide some benefit to the host?

Our data suggest that the severity of airway disease is determined mostly by the degree of inflammation caused by infection; therefore, we can say with some confidence that, in mice, the increased inflammation generated by a weakly pathogenic virus, such as RV, is harmful to the host. In the OVA-sensitized and -challenged model, we found equal viral clearance ability between control and MDA5-/- mice, suggesting that the degree of inflammation generated by allergic sensitization and challenge is more than necessary to clear viral infection, even in the absence of MDA5. Therefore, MDA5 and TLR3 antagonists or inhibitors may provide potential therapeutic strategies for RV-induced airway diseases, and more importantly, asthma exacerbations. Alternatively, a second approach might be to infect airway epithelial cells with RV and screen chemical libraries for compounds which block the inflammatory response. These strategies, which focus on

the host response to virus rather than the virus itself, would represent a new approach to antiviral therapy.

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