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Role of Double-Stranded RNA Pattern Recognition Receptors in Rhinovirus-Induced Airway Epithelial Cell Responses

Qiong Wang,* Deepi R. Nagarkar,* Emily R. Bowman,† Dina Schneider,† Babina Gosangi,† Jing Lei,† Ying Zhao,† Christina L. McHenry,† Richai V. Burgens,† David J. Miller,‡ Umadevi Sajjan,† and Marc B. Hershenson2*†

Rhinovirus (RV), a ssRNA virus of the picornavirus family, is a major cause of the common cold as well as asthma and chronic obstructive pulmonary disease exacerbations. Viral dsRNA produced during replication may be recognized by the host pattern recognition receptors 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 dsRNA. To examine this, BEAS-2B human bronchial epithelial cells were infected with intact RV-1B or replication-deficient UV-irradiated virus, and IFN and IFN-stimulated gene expression was determined by quantitative PCR. The separate requirements of RIG-1, MDA5, and IFN response factor (IRF)-3 were determined using their respective small interfering RNAs (siRNA). The requirement of TLR3 was determined using siRNA against the TLR3 adapter molecule Toll/IL-1R homologous region-domain-containing adapter-inducing IFN-β (TRIF). Intact RV-1B, but not UV-irradiated RV, induced IRF3 phosphorylation and dimerization, as well as mRNA expression of IFN-β, IFN-α1, IFN-α2/3, IFN-γ, RIG-1, MDA5, 10-kDa IFN-γ-inducible protein/CXCL10, IL-8/CXCL8, and GM-CSF. siRNA against IRF3, MDA5, and TRIF, but not RIG-1, decreased RV-1B-induced expression of IFN-β, IFN-α1, IFN-α2/3, IFN-γ, RIG-1, MDA5, and inflammatory protein-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-1, are required for maximal sensing of RV dsRNA and that TLR3 and MDA5 signal through a common downstream signaling intermediate, IRF3. The Journal of Immunology, 2009, 183: 0000–0000.
after infection with EMCV in fibroblasts and conventional dendritic cells (DC), plasmacytoid DCs use the TLR system for viral detection (9).

Little is known about the variations 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 polyinosinic-polycytidylic acid (poly(IC)) elicits a strong proinflammatory response in these cells (10, 11). In 16HBE14o− human bronchial epithelial cells, TLR3 is primarily localized in the endosomes but not 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 this 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 SV40-transformed airway bronchial epithelial cell line, were purchased from American Type Culture Collection (ATCC). Cells were grown on collagen-coated (5 μg/cm2) plates in bronchial epithelial growth medium (BEGM; Lonza) containing epidermal growth factor (25 ng/ml), bovine pituitary extract (65 ng/ml), epidermal growth factor (25 ng/ml), transferrin (10 μg/ml), hydrocortisone (0.5 μg/ml), triiodothyronine (6.5 ng/ml), gentamicin (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 × 10⁻⁸ M), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), gentamicin (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 × 10⁻⁸ M), and BSA (1.5 μg/ml).

RV infection

RV1B and RV39 were obtained from ATCC. 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 titrated as previously described (14, 15). RV1B was irradiated with UV light at 100 μJ/cm² for 10 min on ice, using a CL-1000 cross-linker (UVP).

Quantitative real-time PCR (qPCR)

Total RNA was extracted using the RNeasy kit (Qiagen) and then transcribed to first-strand cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems). First-strand cDNA is then used to quantify the expression of IFN-α, IFN-β, IFN-α1, IFN-α2/3, IRF-7, IP-10/CXCL-10, IL-2/3, IRF7, RIG-I, MDA5, and TLR3 (Fig. 1). The peak level of mRNA expression of several IFN-stimulated genes (ISG) including IRF7, RIG-I, MDA5, and TLR3, but not RIG-I, are required for RV-induced responses in human airway epithelial cells.

Viral dsRNA generated during replication may be detected by the PRRs RIG-I and MDA5, and/or TLR3. To determine which PRR is

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 33°C. Cellular total RNA was extracted from lysates to measure the gene expression at 1, 8, 18, 24, 48, and 72 h postinfection by quantitative PCR. Compared with replication-deficient UV-irradiated virus, intact RV1B increased the mRNA expression of IFN-β, IFN-α1, and IFN-α2/3, as well as the expression of several IFN-stimulated genes (ISG) including IRF7, RIG-I, MDA5, and TLR3 (Fig. 1). The peak level of mRNA expression was 24 h postinfection, except in the case of IFN-β, which was 48 h postinfection The fold-induction varied widely, from 4-fold (TLR3) to ~27,000-fold (IFN-α2/3). 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 I). RV1B infection also increased the protein expression of IFN-α1, IP-10/CXCL-10 and IL-8/CXCL-8 (Fig. 2, A–C). However, there was no induction of IRF7 protein expression (Fig. 2D).

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 used siRNA against RIG-I, MDA5, and the TLR3 adaptor protein TRIF/TIR domain-containing adapter molecule. Forty-eight h later, cells were infected with RV1B, UV-irradiated RV1B, 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% after treatment with RIG-I siRNA (Fig. 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 homolog protein, MDA5 (Fig. 4B). However, RIG-I siRNA failed to decrease RV-induced expression of IFNs or ISGs compared with nontargeting siRNA, suggesting that RIG-I is not required for sensing RV dsRNA (Fig. 5).

Next, we knocked down MDA5 expression using MDA5 siRNA (Fig. 4C). Again, an 80–90% knockdown efficiency was achieved. MDA5 protein expression was also induced by RV1B infection. MDA5 siRNA had no effect on RIG-I protein expression (Fig. 4D). After transfection with MDA5 siRNA, there were significant decreases in RV1B-induced IFN-β, IFN-α1, and IFN-α2/3 mRNA expression and IFN-α1 protein expression compared with cells transfected with nontargeting siRNA (Fig. 6). MDA5 siRNA also decreased RV1B-induced mRNA expression of the ISGs IRF7 and IP-10/CXCL10. Unlike IFNs, MDA5 siRNA treatment was associated with a paradoxic increase in RV1B-induced expression of IL-8 and GM-CSF. Together, these data suggest that MDA5, but not RIG-I, is required for maximal RV-induced IFN responses, but not for RV-induced cytokine responses.

To examine whether MDA5 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 nontargeting 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 (Fig. 7). There was a significant decrease in mRNA expression of IFN-β, IFN-α1, and IFN-α2/3 in cells transfected with MDA5 siRNA compared with cells transfected with nontargeting siRNA. MDA5 siRNA also decreased RV39-induced mRNA expression of the ISGs IRF7 and IP-10/CXCL10.

We then sought to determine whether MDA5 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 nontargeting

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

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RV1B</th>
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<tbody>
<tr>
<td></td>
<td>Ct, average</td>
<td>SE</td>
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<tr>
<td>IFN-β</td>
<td>39.47</td>
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</tr>
<tr>
<td>IFN-α1</td>
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<td>0.16</td>
</tr>
<tr>
<td>IFN-α2/3</td>
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<td>0.33</td>
</tr>
<tr>
<td>IRF7</td>
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</tr>
<tr>
<td>RIG-I</td>
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<td>0.29</td>
</tr>
<tr>
<td>MDA5</td>
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<td>0.20</td>
</tr>
<tr>
<td>TLR3</td>
<td>25.47</td>
<td>0.16</td>
</tr>
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siRNA. After 48 h, cells were infected with RV1B or UV-irradiated RV1B. Cellular mRNA was extracted 24 h after infection to determine mRNA expression by qPCR. Compared with nontargeting siRNA, MDA5 siRNA decreased RV1B-induced expression of IFN-β/H9252, IFN-α1/H9261, IFN-α2/3, IRF7, and IP-10/CXCL10 (Fig. 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/TIR domain-containing adapter molecule, the TLR3 adaptor molecule. Again, a high knockdown efficiency was verified by immunoblotting (Fig. 9). Like MDA5 siRNA, TRIF siRNA abolished RV1B-induced expression of IFN-β, IFN-α1, IFN-α2/3, IRF7, and IP-10/CXCL10 (Fig. 8). These data confirm that MDA5 is required for sensing RV dsRNA and induction of the subsequent IFN response in primary cells.

IRF3 is required for RV1B-induced IFN responses

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

Table II. Effect of RV1B infection on mean Ct values of IFN and ISG mRNA expression in primary cells

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RV1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-β</td>
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<td>32.75</td>
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<tr>
<td>IFN-α1</td>
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<tr>
<td>IFN-α2/3</td>
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<td>28.47</td>
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<tr>
<td>IRF7</td>
<td>29.94</td>
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</tr>
<tr>
<td>IP-10</td>
<td>30.78</td>
<td>28.04</td>
</tr>
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</table>

**FIGURE 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 24 h after infection, and the expression of IFN-β, IFN-α1, IFN-α2/3, IRF7, IP-10, IL-8, and GM-CSF was 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. Symbols represent the results of three different experiments. B–G, Time course of RV1B-induced responses in primary cells.

**FIGURE 4.** RIG-I and MDA5 siRNA knockdown efficiencies. A and B, RIG-I or nontargeting 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 Abs against RIG-I (A) or MDA5 (B). Note the inductions in RIG-I and MDA5 expression with intact RV, as well as the apparent degradation of MDA5 following viral infection. C and D, MDA5 or nontargeting siRNA was transfected into BEAS-2B cells. After transfection, cells were infected with RV1B, UV-irradiated RV1B (UV-RV1B), or sham infected. After infection, cell lysates were probed with Abs against either MDA5 (C) or RIG-I (D). Blots are representative of three separate experiments.
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 (Fig. 11). IRF3 siRNA nearly abolished RV1B-induced expression of IFNs-1, 2, 3; IRF7; and IP-10/CXCL10. However, there was no effect on IL-8 or GM-CSF expression. Taken together, these data suggest 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.

IRF3 functions downstream of MDA5 signaling

RIG-I/MDA5 and TLR3 signaling pathways converge on a common TNF receptor-associated factor 3 adapter complex, which then activates two IRF3 kinases, TANK-binding kinase 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 nontargeting siRNA and then infected with RV1B, UV-irradiated RV1B, or sham HeLa cell lysate. IRF3 dimerization was resolved by native PAGE (Fig. 12). We found that MDA5 and TRIF siRNA each caused a partial reduction in RV-induced IRF3 dimerization compared with nontargeting siRNA-transfected cells, confirming in airway epithelial cells the general notion that IRF3 functions downstream of MDA5 and TRIF.

Discussion

Host pathogen recognition, as reflected by the induction of type I IFNs, is mediated by activation of PRRs. The membrane dsRNA receptor, TLR3, and the recently identified cytoplasmic dsRNA receptors, RIG-I and MDA5, are responsible for sensing viral dsRNA (2–4). 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 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 DCs, plasmacytoid DCs use the TLR system for viral detection (9). Although 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 article, 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 after 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-α1, IP-10/CXCL-10, IFN-β/H9252, IFN-α1/2/3, IRF7, IP-10, IL-8, and GM-CSF 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 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 nontargeting siRNA, significantly inhibited RV1B-induced expression of a type I IFN, IFN-α1/H9252, as well as a number of ISGs. Knockdown of MDA5 also attenuated expression of the type III IFNs IFN-α1 and IFN-α2/3, which functionally resemble type I IFNs (17) and are also induced by RV infection (18). These data are in agreement with previous data suggesting that MDA5 is required for sensing picornavirus dsRNA (9). Alternatively, 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 respiratory syncytial virus, a paramyxovirus.

FIGURE 7. siRNA against MDA5 blocks RV39-induced IFN and ISG expression. A–G, Total RNA was extracted, and the expression of IFN-β, IFN-α1, IFN-α2/3, IRF7, IP-10, IL-8, and GM-CSF was determined by qPCR. Expression levels are represented as the fold increase vs sham-infected, nontargeting siRNA-transfected cells. The y-axis has been broken to show the effects of siRNA on both basal and maximal gene expression. Bars, means ± SEM for three experiments; numbers over bars indicate the fold increase (×) compared with sham-infected sample within its own siRNA group. * p < 0.05 vs MDA5 siRNA-transfected RV-infected sample. ANOVA. siNT, Non-targeting siRNA; siMDA-5, siRNA against MDA-5.

FIGURE 8. siRNA against MDA5 blocks RV1B-induced IFN responses in primary tracheal epithelial cells. A, MDA5 siRNA or nontargeting siRNA was transfected into primary tracheal epithelial cells. After transfection, cells were infected with RV1B or UV-irradiated RV1B. After infection, cell lysates were probed with anti-MDA5 Ab. Blot is typical for three experiments. B–H, Total RNA was extracted and the expression of IFN-β, IFN-α1, IFN-α2/3, IRF7, IP-10, IL-8, and GM-CSF was determined by qPCR. Expression levels are represented as the fold increase vs cells treated with UV-irradiated virus and nontargeting siRNA. The y-axis has been broken to show the effects of siRNA on both basal and maximal gene expression. Bars, means ± SEM for three experiments; numbers over bars indicate the fold increase (×) compared with sham-infected sample within its own siRNA group. * p < 0.05 vs MDA5 siRNA-transfected RV-infected sample. ANOVA. siNT, Non-targeting siRNA; siMDA-5, siRNA against MDA-5.
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, although 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 up-regulation of proinflammatory cytokine expression following MDA5 and IRF3 knockdown may represent a compensatory mechanism by which the airway epithelial cells increase immunosurveillance 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 this study, we could not verify TLR3 knockdown by immunoblotting or flow cytometry in BEAS-2B cells (not shown), leading us to use siRNA against TRIF. In contrast to MDA5 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 MDA5 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 RIP-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 Akt phosphorylation and IL-8 expression in cultured 16HBE14o− human bronchial 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 RVs 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-α 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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Disclosures

The authors have no financial conflict of interest.

References


