Respiratory Syncytial Virus Induces TLR3 Protein and Protein Kinase R, Leading to Increased Double-Stranded RNA Responsiveness in Airway Epithelial Cells

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Respiratory Syncytial Virus Induces TLR3 Protein and Protein Kinase R, Leading to Increased Double-Stranded RNA Responsiveness in Airway Epithelial Cells

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Respiratory syncytial virus (RSV) preferentially infects airway epithelial cells, causing bronchiolitis, upper respiratory infections, asthma exacerbations, chronic obstructive pulmonary disease exacerbations, and pneumonia in immunocompromised hosts. A replication intermediate of RSV is dsRNA. This is an important ligand for both the innate immune receptor, TLR3, and protein kinase R (PKR). One known effect of RSV infection is the increased responsiveness of airway epithelial cells to subsequent bacterial ligands (i.e., LPS). In this study, we examined a possible role for RSV infection in increasing amounts and responsiveness of another TLR, TLR3. These studies demonstrate that RSV infection of A549 and human tracheobronchial epithelial cells increases the amounts of TLR3 and PKR in a time-dependent manner. This leads to increased NF-κB activity and production of the inflammatory cytokine IL-8 following a later exposure to dsRNA. Importantly, TLR3 was not detected on the cell surface at baseline but was detected on the cell surface after RSV infection. The data demonstrate that RSV, via an effect on TLR3 and PKR, sensitizes airway epithelial cells to subsequent dsRNA exposure. These findings are consistent with the hypothesis that RSV infection sensitizes the airway epithelium to subsequent viral and bacterial exposures by up-regulating TLRs and increasing their membrane localization. The Journal of Immunology, 2006, 176: 1733–1740.

R espiratory syncytial virus (RSV) is a ubiquitous pathogen causing upper respiratory infections in healthy adults, bronchiolitis and pneumonia in young children, exacerbations in patients with obstructive lung disease, and life-threatening pneumonia in immunosuppressed patients. RSV is a member of the Paramyxoviridae family and consists of a negative-strand RNA genome in a nucleocapsid surrounded by an envelope. Entry into the host respiratory epithelium is by cell surface fusion, and infection leads to viral replication and subsequent host inflammatory response.

Toll-like receptors (TLRs) are type I integral membrane proteins that are central to early host defense. They detect bacterial, viral, and environmental exposures and activate both innate and adaptive immune responses (1, 2). TLRs recognize pathogen-associated molecular patterns expressed by infectious agents, and they mediate production of antimicrobial peptides and cytokines needed for host defense. The ligand for TLR3 is dsRNA (3) of both viral and nonviral origin (4). TLR3 is known to be constitutively expressed in respiratory epithelial cells (5). In this study, we evaluated the effect of RSV on TLR3 expression.

As well as variations in total amounts of TLR3, location also may contribute to TLR3’s inflammatory potential. Studies in immature dendritic cells localized the site of the TLR3 receptor to an intracellular vesicle, where it would be ideally positioned to detect the intracellular dsRNA RSV replication intermediate (6, 7). One study in epithelial cells also supported an intracellular location of TLR3 (5). Another study in human fibroblasts found TLR3 located on the cell surface (8). The advantage of a membrane-localized TLR3 is that, along with intracellular replication intermediates, it also could recognize extracellular dsRNA, contributing to a heightened inflammatory response.

IFN-inducible RNA-dependent protein kinase R (PKR) is a cytoplasmic serine-threonine kinase. PKR can be activated by dsRNA of viral or synthetic origin (9). Recently, a study by Vijay-Kumar et al. (10) indicated that intestinal epithelial cell detection of dsRNA is dependent on PKR. Upon binding of dsRNA, PKR dimerizes and is autophosphorylated at multiple sites (11, 12). Activated PKR has a number of demonstrated effects, including activation of NF-κB and MAPKs (13–18). In this study, we hypothesized that PKR also plays a central role in RSV-induced inflammation.

This study demonstrates RSV up-regulation of both TLR3 and PKR, leading to a priming of lung epithelial cells for subsequent exposure to extracellular dsRNA. The increased epithelial cell responsiveness is due to activation of the inflammation-related transcription factor NF-κB and increased release of the chemokine IL-8. This is the first description of RSV infection contributing to an altered inflammatory response to a subsequent viral ligand in airway epithelial cells.

Materials and Methods

Materials

Chemicals were obtained from Sigma-Aldrich and Calbiochem. Protease inhibitors were obtained from Roche Applied Science. Polynosinic-polycytidylic acid causing upper respiratory infections in healthy adults, bronchiolitis and pneumonia in young children, exacerbations in patients with obstructive lung disease, and life-threatening pneumonia in immunosuppressed patients. RSV is a member of the Paramyxoviridae family and consists of a negative-strand RNA genome in a nucleocapsid surrounded by an envelope. Entry into the host respiratory epithelium is by cell surface fusion, and infection leads to viral replication and subsequent host inflammatory response.

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acid (poly(I:C)) was purchased from InvivoGen. TLR7/TLR8 ligand (ss-PolyU/Lyovec catalog no. tlr7-lpu) was obtained from InvivoGen. TLR9 ligand (Cp-G, ODN1826) was provided by Dr. J. Kline (University of Iowa, Iowa City, IA). IFN-α, IFN-β, and TNF-α were from PBL Biomedical Laboratories. Chloroquine and 2-aminoquinine were from Sigma-Aldrich. Monoclonal mouse IgG1 Ab to TLR3 (IMG-315A) was obtained from Imgenex, and polyclonal rabbit IgG Ab to p65 (sc-109) was obtained from Santa Cruz Biotechnology. Purified mouse IgG1 Ab to TLR3 (IMG-315A) was obtained from Immunofluorescent secondary Ab was a goat anti-mouse IgG Ab, Alexa Fluor 568, purchased from Molecular Probes. Polyclonal rabbit IgG Ab to total- PKR (no. 3072) was purchased from Cell Signaling Technology.

**Epithelial cell culture**

A549 lung epithelial cells were obtained from the American Type Culture Collection and cultured in MEM (Invitrogen Life Technologies) supplemented with 10% FBS (JRH Biosciences) and gentamicin. For most viral infections, cells at ~80% confluence were treated with RSV strain A-2 (multiplicity of infection (MOI) of 2). Viral stocks were obtained from Advanced Biotechnologies. The initial stock (1 × 10^7 50% tissue culture infective dose) was aliquoted and kept frozen at -70°C. A549 and hTBE cells were plated in a slide chamber and grown to ~80% confluence. A549 bronchoalveolar carcinoma cells were exposed to poly(I:C) alone at the above concentrations for 6 h, RSV (MOI of 2) for 16 h, or RSV for 16 h followed by poly(I:C) for 6 h. Supernatants were collected, and ELISA for IL-8 was performed. The graph reflects the mean and SE of three experiments. Student’s t test indicates that a statistically significant difference exists between RSV plus poly(I:C) (10 μg/ml) and RSV alone (p < 0.05) and between RSV plus poly(I:C) (1 μg/ml) and RSV alone (p < 0.05). Right panel, A549 cells were exposed to poly(I:C) at 1 μg/ml for 3 h, RSV (MOI of 2) for 16 h, or RSV for 16 h followed by poly(I:C) for 3 h. Real-time PCR was used to quantify IL-8 mRNA. The graph reflects mean and SE of three experiments. Student’s unpaired t test indicates that a statistically significant difference exists between RSV plus poly(I:C) (1 μg/ml) and RSV alone (p < 0.01).

P-40, with added protease and phosphatase inhibitors: 1 protease minitab (Roche Applied Science)1/10 ml and 100 μl of 100× phosphatase inhibitor mixture (no. 524625; Calbiochem)1/10 ml. Lysates were sonicated for 20 s, kept at 4°C for 30 min, spun at 15,000 × g for 10 min, and the supernatant was saved. Protein concentrations were determined using a commercial kit (Protein Assay; Bio-Rad). Cell lysates were stored at −70°C until use.

**Western blot analysis**

Forty micrograms of protein was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromphenol blue, and 1.25 M Tris (pH 6.8)) and separated using SDS-PAGE. Cell proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Equal loading of proteins was evaluated using Ponceau S dye staining (Sigma-Aldrich). The polyvinylidene difluoride membrane was saturated with methanol, washed, and then incubated with primary Ab. Blots were washed four times and incubated with HRP-conjugated anti-IgG Ab (1/5,000–1/20,000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Biosciences), and were detected by autoradiography. Protein levels were quantified using densitometry via a FluorS scanner (Bio-Rad) and Quantity One software (Bio-Rad) for analysis. Densitometry is expressed as fold increase (experimental value/control value).

**Cytokine measurements**

A549 lung epithelial cells and primary hTBE cells were plated at ~80% confluence. Supernatants were collected and frozen at −70°C. Human IL-8 concentrations in cell culture supernatants were determined using DuoSet ELISA kits (R&D Systems).

**EMSA**

A549 cells were infected with RSV, 1 μg/mpl poly(I:C), or both, and nuclear protein was isolated as described previously (20). NF-κB consensus sequence oligonucleotides (Promega) were end-labeled with [γ-32P]ATP according to the manufacturer’s instructions. The DNA binding reaction (EMSA) was done at room temperature in a mixture containing 5 μg of nuclear proteins, 1 μg of poly(dI:dC), and 20,000 cpm of 32P-labeled double-stranded oligonucleotide probe for 30 min. Protein-DNA complexes were separated on a 5% polyacrylamide gel in 1× TBE (6.05 g/L Tris base, 3.06 g/L boric acid, 0.37 g/L EDTA-Na2-H2O). The gel was dried, and results were determined with the Personal Fx Imager (Bio-Rad).

**Immunofluorescence**

A549 and hTBE cells were plated in a slide chamber and grown to ~80% confluence. A549 and hTBE cells were stimulated with RSV using MOIs...
of 2 and 10, respectively. After 24 h, cells were washed with PBS and fixed with ice-cold methanol. Cells were then washed with PBS twice, followed by a 0.1% Triton X wash. Cells were blocked with 10% normal goat serum for 1 h. Following three PBS washes, primary IgG1 mouse isotype control Ab and TLR3 Ab were added, and the cells were incubated. After three more PBS washes, the secondary anti-mouse Alexa Fluor 568 Ab was added. Cells were again washed in PBS. The cell chamber was removed, and the cells were fixed with Vectashield (Vector Laboratories). Slides were observed using a Leitz Diaplan fluorescent microscope (Easter Scientific Instrument).

Flow cytometry analysis
A549 cells were grown to 70% confluence in six-well tissue culture dishes, RSV was added at a MOI of 2, and cells were incubated for an additional 20 h. Cells were incubated with 10 mM EDTA/PBS, 2 ml/well for 15 min at 4°C, harvested, and spun at 1000 rpm for 5 min. The cells were then resuspended in 1 ml of FACS buffer (3% FCS, 0.1% sodium azide in PBS). Cells were stained with PE-labeled Ab against TLR3 (eBioscience) and FITC-labeled goat polyclonal Ab against RSV (BioDesign). PE- and FITC-labeled isotype control Abs were used to set up compensation. Cytometric analysis was performed using a FACScan flow cytometer (BD Immunocytometry Systems).

Statistical analysis
Statistical analysis was performed on ELISA results and real-time PCR data. Significance was determined by Student’s t-test (GraphPad statistical analysis software).

Results
RSV primes airway epithelial cells for increased responsiveness to subsequent dsRNA exposure
Having found that RSV increased epithelial-cell LPS responsiveness, we were interested in seeing whether the same concept might apply to viral stimuli. We examined whether RSV would enhance inflammation upon subsequent exposure to dsRNA (poly(I:C)). To perform these studies, epithelial cells were infected with RSV, followed by a shorter exposure to poly(I:C). Supernatants were collected, and ELISA for IL-8 was performed. Results above reflect the mean and SE of three experiments.
RSV infection followed by poly(I:C) resulted in IL-8 release that was greater than the sum of the IL-8 protein produced by either RSV or poly(I:C) alone. The lowest effective dose of poly(I:C) was 1 μg/ml. We noted a similar increase in IL-8 mRNA in A549 cells exposed to RSV for 16 h followed by poly(I:C) at 1 μg/ml for 3 h (Fig. 1). This finding was confirmed in primary hTBEs (Table I). To confirm that live replicating RSV virus but not UV-inactivated RSV viral protein was enhancing inflammation, we repeated the experiment and compared IL-8 protein quantities when cells were treated with live RSV or with UV-inactivated RSV. We found that only when cells were treated with live RSV virus was there any significant IL-8 production (data not shown).

**RSV primes airway epithelial cells to subsequent poly(I:C) but not ssRNA or DNA exposure**

Next, we determined whether this synergistic effect of RSV and poly(I:C) was observed with other TLRs that respond to RNA and DNA; TLR7, TLR8, and TLR9. We treated cells with RSV for 16 h and subsequently added ssRNA, the TLR7/8 ligand, or Cp-G, the TLR9 ligand, at varying doses. In neither case did we observe the synergy seen with the TLR3 ligand (Fig. 2). RSV increased the IL-8 production, but secondary stimulation with TLR7/8 or TLR9 ligands led to no enhanced inflammatory effect as measured by IL-8.

**RSV increases TLR3 mRNA and protein**

The known receptor for poly(I:C) is TLR3. Next, we asked whether the increased poly(I:C) responsiveness could be explained by an increase in TLR3. We exposed A549 airway epithelial cells to RSV for 0, 6, and 16 h and then quantified TLR3 mRNA expression using real-time RT-PCR. There was an increase in TLR3 mRNA after 16 h of RSV exposure (Fig. 3A). To determine whether the increased TLR3 mRNA translated into increased amounts of TLR3 protein, we performed a Western blot analysis and found that TLR3 protein is increased after 24 h of RSV infection in A549 cells (Fig. 3A, inset). To confirm that this observation was not limited to a cell line, we examined the effect of RSV on TLR3 mRNA in primary hTBEs. Again, the results demonstrated that the amount of TLR3 mRNA was significantly increased in hTBE cells at 16 h (Fig. 3B). A recent study suggested that IFN-β, a cytokine produced by airway epithelial cells in response to RSV, leads to a modestly increased amount of TLR3 (21). To investigate whether the TLR3 increase was due to a soluble mediator, we exposed the cells to TNF-α, IFN-α, and IFN-β. In all cases, we did not see an increase in TLR3 and IL-8, suggesting a specific effect of RSV (data not shown). This finding is in agreement with a recent study in which anti-IFN-αβ receptor Abs were used to show that type I IFN signaling is not required for RSV-induced chemokine production (22).

**RSV primes airway epithelial cells for increased NF-κB activation after poly(I:C) exposure**

A549 epithelial cells were exposed to RSV, poly(I:C), or both. Cells were harvested, and nuclear protein was isolated. An electromobility gel-shift assay run with a probe specific for NF-κB showed that exposure of cells to RSV or poly(I:C) resulted in NF-κB activation and nuclear translocation, but this translocation is significantly increased in the cells exposed to RSV followed by poly(I:C) (Fig. 4). Gel-shift densitometry confirmed the findings. Nuclear protein was isolated as above, and Western blot analysis was performed with an Ab to NF-κB protein, p65. As shown in the Western blot, an increased amount of nuclear p65 was seen in the cells exposed to RSV followed by poly(I:C). Combined, these experiments suggest that one effect of the increased epithelial cell response to RSV with poly(I:C) is an increase in the nuclear localization of the inflammation relevant transcription factor, NF-κB.

**RSV increases total PKR**

The kinase PKR can be activated downstream of TLR3 or can be activated by poly(I:C) itself. The downstream effects of PKR activation include a proinflammatory effect on NF-κB and MAPKs (13–18) and an antiviral effect on the translation initiation factor, eukaryotic initiation factor 2α (12). To test whether RSV infection was altering amounts of PKR, A549 cells were exposed to RSV for 0, 1, 3, 6, 16, or 24 h, and cells were harvested for protein. Western blot analysis showed a time-dependent increase in total PKR with RSV infection (Fig. 5A). We next asked whether the increased PKR played a role in the increased poly(I:C) responsiveness of epithelial cells following RSV infection.

**Inhibition of PKR decreases the poly(I:C)-induced IL-8**

A549 cells were stimulated with RSV followed by poly(I:C). In some cases, the PKR inhibitor 2-aminopurine was added at the
Cells were exposed to poly(I:C) (10 µg/ml) for 6 h, RSV (MOI of 2) for 16 h, or RSV for 16 h followed by poly(I:C) for 6 h. 2-Aminopurine was added at the time of RSV 16 h before harvest. Supernatants were collected and ELISA for IL-8 was performed. Student’s unpaired t test indicates a statistically significant decrease when cells were stimulated with just poly(I:C) and not RSV. This suggests that PKR does not play a role in primary RSV-induced inflammation, but does play a role in poly(I:C)-induced secondary inflammation. To confirm that the 2-aminopurine was affecting PKR signaling and not the induction of TLR3 by RSV, real-time PCR with TLR3 primers was run on the mRNA isolated from the cells described above. Fig. 5C demonstrates that A549 cells exposed to RSV for 16 h contained higher amounts of TLR3 protein, regardless of whether the PKR inhibitor was added. Thus, PKR does not directly affect expression of TLR3 but does affect the increased IL-8 production when poly(I:C) exposure follows RSV infection.

**RSV alters TLR3 cellular localization**

A number of studies have shown that TLR3 is localized on internal endosomal membranes (5–7). In the experiments shown here, we evaluated the localization of TLR3 after RSV infection. First, we performed immunofluorescent staining of epithelial cells. Immunofluorescent photomicrographs showed accumulation of signal on the cell surface and a paucity of signal within the cell after 24 h of RSV (Fig. 6A). The observation was replicated in primary hTBE cells (Fig. 6B). Note that the primary cells required a higher MOI (10) for efficient infection. Next, we performed flow cytometry. Cells were exposed to either RSV for 20 h or no RSV. Flow cytometry of nonpermeabilized cells for both RSV and TLR3 indicated that increased surface expression of TLR3 was evident primarily in the cells that also were positive for RSV (i.e., RSV infected). The composite graph indicates a shift of the curve with exposure of RSV. These figures are representative of three experiments (Fig. 6C). FACS analysis of hTBE cells also demonstrated that the surface TLR3 expression increased by 100% (data not shown). Finally, it has been shown in experiments with other TLRs located on the acidic intracellular endosome (TLR7 and TLR9) that adding chloroquine creates a less acidic environment and abrogates the TLR function (23). We added chloroquine to cells exposed to no RSV, poly(I:C) alone, RSV alone, or RSV plus poly(I:C) and found that there was no change in IL-8 protein expression (Fig. 6D). Cumulatively, these results support a plasma membrane location of TLR3 after RSV infection that contributes to the increased IL-8 response to extracellular poly(I:C).

**Discussion**

In this study, we demonstrate that RSV increases the expression of TLR3 on the surface of airway epithelial cells, and this is associated with increased sensitization to dsRNA. This is the first study to show significant up-regulation of TLR3 by RSV and the first to demonstrate altered localization of TLR3 subsequent to a viral infection. This observation is unique to the TLR3 receptor, because challenge with either ssRNA or Cp-G DNA after RSV infection did not have a synergistic effect on IL-8 production. One known effect of RSV signaling is up-regulation of the serine/threonine kinase PKR (24). In RSV-infected epithelial cells, PKR does not mediate IL-8 production by RSV alone, but it plays a role in the late sensitization of the cells to poly(I:C) because the enhanced inflammation mediated by RSV followed by poly(I:C) is decreased by 2-aminopurine, a PKR inhibitor. However, the 2-aminopurine did cause a statistically significant decrease when cells were stimulated with just poly(I:C) and not RSV. This suggests that PKR does not play a significant role in primary RSV-induced inflammation but does play a role in poly(I:C)-induced inflammation, both on initial exposure and when exposure follows RSV infection. In summary (Fig. 7), our data demonstrate that RSV infection enhances the immune response to subsequent dsRNA via an effect on TLR3 and the downstream kinase, PKR. These findings are consistent with the hypothesis that RSV alters epithelial responsiveness by increasing expression and plasma membrane localization of receptors involved in the innate immune system.
FIGURE 6. RSV infection results in TLR3 located on the cell surface. A, A549 cells were exposed to RSV (MOI of 2) for 24 h and fixed. TLR3 primary Ab was added, followed by a species-specific labeled secondary Ab. Immunofluorescent photos demonstrate increased staining on cell surface in cells exposed to RSV. B, hTBE cells were exposed to RSV (MOI of 10) for 24 h and fixed. TLR3 primary Ab was added followed by a species-specific labeled secondary Ab. Immunofluorescent photos demonstrate increased staining on cell surface in cells exposed to RSV. C, A549 cells were exposed to RSV (MOI of 2) for 20 h. Flow cytometry of nonpermeabilized cells was performed. The composite graph demonstrates the difference in geometric means between cells exposed to RSV and control cells. Graphs are representative of three experiments. D, A549 cells were exposed to poly(I:C) (1 μg/ml) for 6 h, RSV (MOI of 2) for 16 h, or RSV for 16 h followed by poly(I:C) for 6 h. Chloroquine was added with the RSV, 16 h before harvest. Supernatants were collected, and ELISA for IL-8 was performed. The graph reflects the mean and SE of three experiments. Student’s unpaired t test demonstrates no statistical significance between RSV-plus-poly(I:C) group with and without chloroquine.
A recent study demonstrated that RSV mediates an increase in the activity of TLR3 inflammatory pathways (22). Our study complements this previous work by showing that this enhanced inflammation is the result of increased TLR3 receptor expression on the cell surface.

It also has recently been shown that influenza A virus and poly(I:C) independently induce the expression of TLR3 by both mRNA real-time PCR measurement and by Western blot analysis (5). The study demonstrated the primary effects of poly(I:C) and influenza A on TLR3 mRNA and protein expression. Our study examining the effect of a prior viral infection (RSV) on a second exposure is consistent with the physiological role of TLR3 modulation in inflammation. Our study supports the conclusion that the RSV effect on TLR3 is not the result of a soluble mediator (Fig. 6C). In this figure, the FACS analysis demonstrates that only in the RSV-infected cells does TLR3 increase on the surface. If it were a soluble mediator altering TLR3 amounts and location, then both infected and uninfected cells should show increased TLR3.

Our study differs from the study by Guillot et al. (5) in that we examined the effect of a secondary stimulus (poly(I:C)) after the cells had been infected by RSV. We hypothesized that with this increased expression of TLR3 after RSV in A549 and hTBE cells, the cells were primed for subsequent exposure to synthetic dsRNA (poly(I:C)). Cells already exposed to RSV had increased expression of TLR3 and increased production of IL-8 mRNA and protein upon stimulation with poly(I:C). To our knowledge, this is a unique observation, and similar studies have not been published.

The increased IL-8 mRNA and protein was accompanied by increased NF-κB nuclear localization. We have demonstrated previously the important role of NF-κB in RSV-induced IL-8 (25). Another recent study also confirmed the role of NF-κB, IFN regulatory factor 3, and IFNs in the stimulation of proinflammatory cytokines by RSV (26). These results all suggest a transcriptional up-regulation of the IL-8 gene via the increased NF-κB. Another possibility is that the TLR3 signal is increasing the stability of the IL-8 mRNA. Sorting out the possible role of altered stability vs transcription will be the subject of further investigation.

In our studies, RSV increased the amount of TLR3 and PKR. Both TLR3 and PKR are known to stimulate NF-κB and result in the expression of inflammatory chemokines and cytokines (3, 13). In this setting, we show that the cells are sensitized to respond more to extracellular dsRNA. In contrast to its role in the RSV/poly(I:C) system, PKR does not appear to play a role in IL-8 production or NF-κB activation mediated by RSV alone. Its effect is apparent only after the cells express TLR3 on the cell membrane and are sensitized to respond to extracellular dsRNA.

The location of the TLR3 receptor has been a topic of other recent investigations. Two studies found a subcellular location of TLR3 in monocyte-derived immature dendritic cells, plasmacytoid blood dendritic cells, and HEK293 cells (6, 7). Another study using A549 cells and flow cytometry similar to our methods, found evidence supporting an intracellular location (5). The discrepancy between these other findings and those of our study can be explained in three ways. First, the prior studies evaluated TLR3 expression in baseline conditions. Without RSV infection, we did not detect TLR3 on the cell surface. Also, the TLR3 receptor location may be transient. It is well documented with a variety of receptors that they traffic from the cell surface to an intracellular location upon stimulation and then are cycled back to the cell surface again (27–30). It also is possible that this cell surface location of the TLR3 receptor may be unique to our system of RSV and airway epithelial cells.

Two other papers validate the presence of the TLR3 receptor on the epithelial cell surface using flow cytometry. Hewson et al. (31) recently showed that in BEAS-2B cells exposed to rhinovirus, the TLR3 receptor is expressed on the cell surface, and Ueta et al. (32) showed that TLR3 is expressed on the cell surface of human corneal epithelial cells. The function of TLR3 on the cell surface is a subject of intense study. TLR3 may augment the inflammatory response as our data suggest. Increased TLR3 receptor expressed on the cell surface may prime the cells for subsequent stimulation by dsRNA. The source of the dsRNA remains a question. It is widely believed that ssRNA viruses go through a dsRNA phase during replication (33, 34), and Martinez and Melero (35) demonstrated the formation of short intramolecular dsRNA segments during replication of human RSV. Our model requires that this dsRNA get into the extracellular space, and it might do this through RSV-induced cell destruction and necrosis. Very small quantities of dsRNA, as little as one molecule per cell, can have profound effects on the cellular physiology (9). The microenvironment surrounding the epithelial cell would presumably have these minute amounts of dsRNA that might transiently interact with the TLR3 receptor. The function of the TLR3 receptor is not entirely clear. Hewson et al. (31) demonstrated in a recent paper that the surface TLR3 receptor assists in antiviral activities within the cell entirely independent of poly(I:C) and inflammation. Our data suggest a further role for TLR3 in the inflammatory process induced by a combination of RSV and dsRNA (from either RSV or some other source).

RSV imposes a significant health burden, particularly to infants, patients with obstructive lung disease, and the immunosuppressed. Our study demonstrates that RSV induces increased TLR3 and PKR in airway epithelial cells, priming them for an enhanced inflammatory response when subsequently exposed to extracellular dsRNA. As a paradigm, these observations are consistent with RSV setting up airway epithelial cells for an increased inflammatory response to subsequent viral infections. These observations also suggest that TLR3 and PKR might be important targets for therapy in RSV infection.

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Disclosures
The authors have no financial conflict of interest.

References


25. Mastronarde, J. G., B. H. Monick, N. Mukaied, K. Matsuhashi, and G. W. Hunninghake. 1996. Induction of interleukin (IL)-8 gene expression by respiratory syncytial virus involves activation of nuclear factor (NF-κB) and NF-κB.


