

# Rhinovirus Disrupts the Barrier Function of Polarized Airway Epithelial Cells

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**Rationale:** Secondary bacterial infection following rhinovirus (RV) infection has been recognized in chronic obstructive pulmonary disease. **Objectives:** We sought to understand mechanisms by which RV infection facilitates secondary bacterial infection.

**Methods:** Primary human airway epithelial cells grown at air-liquid interface and human bronchial epithelial (16HBE14o-) cells grown as polarized monolayers were infected apically with RV. Transmigration of bacteria (nontypeable *Haemophilus influenzae* and others) was assessed by colony counting and transmission electron microscopy. Transepithelial resistance ( $R_T$ ) was measured by using a voltmeter. The distribution of zona occludens (ZO)-1 was determined by immunohistochemistry and immunoblotting.

**Measurements and Main Results:** Epithelial cells infected with RV showed 2-log more bound bacteria than sham-infected cultures, and bacteria were recovered from the basolateral media of RV- but not sham-infected cells. Infection of polarized airway epithelial cell cultures with RV for 24 hours caused a significant decrease in  $R_T$  without causing cell death or apoptosis. Ultraviolet-treated RV did not decrease  $R_T$ , suggesting a requirement for viral replication. Reduced  $R_T$  was associated with increased paracellular permeability, as determined by flux of fluorescein isothiocyanate (FITC)-inulin. Neutralizing antibodies to tumor necrosis factor (TNF)- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$  reversed corresponding cytokine-induced reductions in  $R_T$  but not that induced by RV, indicating that the RV effect is independent of these proinflammatory cytokines. Confocal microscopy and immunoblotting revealed the loss of ZO-1 from tight junction complexes in RV-infected cells. Intranasal inoculation of mice with RV1B also caused the loss of ZO-1 from the bronchial epithelium tight junctions *in vivo*.

**Conclusions:** RV facilitates binding, translocation, and persistence of bacteria by disrupting airway epithelial barrier function.

**Keywords:** COPD; exacerbation; *Haemophilus influenzae*; tight junction; ZO-1

The coinfection of viruses and bacteria have been recognized in several respiratory diseases including pneumonia, otitis media (1–3), chronic obstructive pulmonary disease (COPD) (4–7) and cystic fibrosis (CF) (8–11). Viruses predispose the host to bacterial infection by various mechanisms including destruction of the respiratory epithelium, modulation of innate defenses, and alteration of cell membranes, which in turn facilitates bacterial adherence. Influenza virus damages both ciliated and nonciliated respiratory epithelial cells, leading to necrosis of the tracheo-

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

The mechanisms by which rhinovirus infection leads to secondary bacterial infection in patients with underlying respiratory diseases are not well understood.

### What This Study Adds to the Field

Rhinovirus infection facilitates transmigration of bacteria across polarized airway epithelia by dissociating zona occludens-1 from the tight junction complex, thereby impairing barrier function of the epithelium.

bronchial airway epithelium (12, 13). Respiratory syncytial virus (RSV) can also cause injury to ciliated cells, leading to ciliostasis, loss of cilia, and impairment of mucociliary clearance (14). Glycoproteins elaborated by influenza virus and RSV during replication can integrate into the host cell surface and serve as receptors for bacteria (15–17). Viruses can also expose or up-regulate the expression of native receptors for bacteria on the host cell surface, thereby increasing the bacterial adherence and colonization (18–21).

Rhinoviruses (RVs), members of the *Picornaviridae* family, are divided into two groups based on receptor utilization. The major group of RVs, greater than 90% of the serotypes, bind to intercellular adhesion molecule (ICAM)-1, whereas the remaining minor group serotypes bind to low density lipoprotein receptor and related proteins. RVs are single-stranded RNA viruses responsible for the majority of upper respiratory tract infections and their complications, including otitis media, sinusitis, and bronchitis. By a sensitive detection method (polymerase chain reaction), RV was detected in 25.6% of viral-induced acute otitis media (1), and up to one-half of all viral-associated COPD and CF exacerbations (4–7, 9). In each of these conditions, coinfection of RV and bacteria are thought to play an important pathogenetic role. Acute otitis media in children has been attributed to coinfections with RV or RSV and bacteria, and RV infection positively correlated with the presence of *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (1, 22). In COPD patients, exacerbations associated with both RV and nontypeable *H. influenzae* had greater bacterial loads, serum interleukin (IL)-6 levels, symptom counts and falls in FEV<sub>1</sub> than exacerbations without both pathogens (7).

Mechanisms by which viral infections facilitate bacterial growth or acquisition of new bacteria are not well understood. One of the mechanisms by which RV may promote bacterial infections is by increasing adherence to and/or invasion of epithelial cells by bacteria. *In vitro* studies have shown that RV increases the adherence of *S. pneumoniae* to bronchial epithelial cells by up-regulating the expression of platelet-activating factor receptor (19). RV pretreatment also promotes internalization of

*Staphylococcus aureus* into normally nonpermissive A549 respiratory epithelial cells, in part through increased expression of intercellular adhesion molecule (ICAM)-1 (20). Taken together, these observations suggest that RV infection can promote opportunistic bacterial infections in the airways, although the underlying mechanisms are not well understood.

In addition to providing a physical barrier, the airway epithelium represents a dynamic system for innate host defense. Tight junctions located at the apicolateral borders of adjacent airway epithelial cells contribute significantly to epithelial barrier function. Tight junctions regulate the selective passage of ions and solutes through the paracellular space and prevent paracellular migration of pathogens and their products from lumen to interstitium. Thus, perturbation of the tight junction barrier function may increase paracellular permeability, facilitate translocation of pathogens and their soluble products, and expose basolateral receptors. We hypothesized that, in addition to upregulating receptors for bacteria, RV also promotes bacterial transmigration across the epithelial barrier. We found that infection of polarized epithelium with major and minor group RV promotes the paracellular migration of nontypeable *H. influenzae* (NTHi), an opportunistic pathogen in COPD patients, by disrupting epithelial barrier function. This effect appears to be specific to RV, as it was not observed with either RSV or influenza A virus.

Some of the results of these studies have been previously reported in the form of an abstract (23).

## METHODS

See the online data supplement for additional details.

### Virus

RV serotypes 16, 39, and 1B and tissue culture-adapted influenza virus A (H1N1) were purchased from American Type Culture Collection (ATCC, Manassas, VA). RV and influenza stocks were generated by infecting HeLa cells and MDCK cells, respectively. RSV was provided by N. Lukacs (University of Michigan, Ann Arbor, MI). HeLa supernatants from uninfected cells served as controls (sham infection).

### Bacteria and Growth Conditions

NTHi isolates (6P5H, 5P19H1, 45P9H1) were obtained from COPD patients (from T. Murphy, University of Buffalo, Buffalo, NY). *Pseudomonas aeruginosa* strain PA01 is a laboratory isolate. *S. aureus* was purchased from ATCC. For infection assays, bacteria were subcultured on chocolate, nutrient, or brain–heart infusion medium, scraped off the plate, and suspended in cell culture medium.

### Cell Culture

Human airway epithelial cells, obtained from tracheal trimmings of anonymous donor lungs at transplantation, were grown at air–liquid interface for mucociliary differentiation, as previously described (24). Use of donor lungs was reviewed by the Institutional Review Board of the University of Michigan. 16HBE14o- human bronchial epithelial cells (25) and Calu-3 lung adenocarcinoma cells (ATCC) were grown in collagen-coated Transwells (Corning Life Sciences, Lowell, MA).

### Infection of Cell Cultures and Measurement of Transepithelial Resistance

Differentiated primary airway epithelial cells or polarized 16HBE14o- or Calu-3 cells were infected apically with RV, RSV, influenza A, or an equivalent volume of sham and incubated for 24 hours.  $R_T$  was measured by EVOM voltmeter (World Precision Instruments, Sarasota, FL).

### Paracellular Permeability

16HBE14o- or Calu-3 cells were infected with RV39 at a multiplicity of infection (MOI) of 1 for 24 hours and paracellular permeability of FITC-inulin (1 mg/ml) was determined (24, 26). In selected experiments,

cultures were infected with bacteria and transmigration assessed by colony counting.

### Nonidet P-40-soluble and P-40-insoluble Cell Extracts

After treatment, cells were rinsed with phosphate buffered saline (PBS) and incubated with NP-40 solubilization buffer (15 min on ice). NP-40-insoluble material was pelleted, the supernatant was saved, and Laemmli reducing buffer added to the pellet, which was then heated (10 min, 100°C).

### Western Blot Analysis

NP-40-soluble and NP-40-insoluble fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and proteins transferred to polyvinylidene difluoride membranes. Membranes were incubated with antibodies to zona occludins (ZO)-1 (BD Biosciences, San Jose, CA) or  $\beta$ -actin (Invitrogen, Carlsbad, CA).

### Confocal Indirect Immunofluorescence

Cultures were fixed in methanol and incubated with antibody to ZO-1 (1  $\mu$ g/ml) or antibody to heat-killed NTHi (from G. Krasan, University of Michigan, Ann Arbor, MI).

### Transmission Electron Microscopy

Polarized monolayers of 16HBE14o- cells were infected with RV39 (MOI, 1). After 24 h, cells were superinfected with bacteria and fixed and processed for electron microscopy. Sections were viewed on a Philips CM100 at 60 kV.

### Detection of Apoptosis

Polarized 16HBE14o- cells were treated with thapsigargin (2–5  $\mu$ M, 24 h) and apoptotic, nonnecrotic cells detected by flow cytometry with FITC-conjugated annexin V and propidium iodide (Sigma-Aldrich, St. Louis, MO).

### Inoculation of Mice

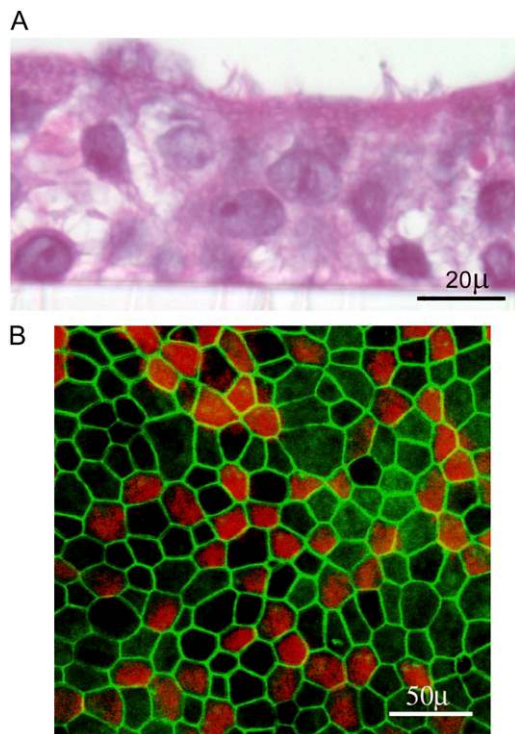
C57BL/6 mice were inoculated intranasally with RV1B (TCID<sub>50</sub>,  $5 \times 10^7$ /mouse) or equal volume of sham, as previously described (27). After 24 hours, mice were killed and lungs were perfused with ethylenediamine tetraacetic acid (EDTA), inflated and fixed in formalin, and embedded in paraffin.

## RESULTS

### RV Increases Bacterial Binding to and Transmigration across Well-differentiated Airway Epithelial Cells

Normal primary human airway epithelial cells, grown at air–liquid interface, polarize, form tight junctions, and differentiate into a mucociliary phenotype (Figures 1A and 1B). Differentiated cell cultures were infected apically with RV39 or RV1B (MOI of 1) or equivalent volume of sham, and incubated for 24 hours. NTHi (isolate 6P5H) were then added to the apical surface and incubated for another 24 hours. An aliquot of basolateral medium was plated to determine the number of bacteria transmigration across the epithelium. To determine the number of bound bacteria, cells were washed to remove unassociated bacteria, lysed, and serial dilutions of lysates were plated. Epithelial cells infected with RV showed 2 log greater bound bacteria than the sham-infected or media-treated cell cultures (Table 1). In addition, bacteria were recovered from the basolateral media of RV-infected but not from sham-infected or medium-treated cell cultures. These results indicate that RV not only increases bacterial binding to epithelial cells but also facilitates bacterial transmigration across differentiated airway epithelia.

To facilitate mechanistic studies, we also tested whether RV promotes transmigration of bacteria across polarized 16HBE14o-human airway epithelial cells. These cells are readily available and form tight junctions similar to primary epithelial cells when grown on a semipermeable membrane (25). 16HBE14o- cells



**Figure 1.** Morphologic features of primary airway epithelial cells differentiated into mucociliary phenotype. (A) Primary airway epithelial cells were grown in transwells at an air/liquid interface. Cells were fixed in buffered formalin, embedded in agarose-paraffin, and sections were stained with hematoxylin and eosin. (B) Primary airway epithelial cells grown at air/liquid interface were fixed in methanol and stained with zona occludins ZO-1 (green) and  $\beta$ -tubulin (red).

grown on semipermeable membranes were infected apically with RV39 (MOI of 1 for 1 h) and incubated for an additional 24 hours. NTHi were added to the apical chamber and the basolateral media were sampled at different time intervals to detect bacteria. Uninfected cultures and sham-infected cultures were used as negative controls. Cells treated with occludin peptide for 30 minutes were used as a positive control. Occludin peptide treatment induced a 44–48% reduction in  $R_T$  compared with medium-treated controls, consistent with earlier studies (28, 29). For negative controls (cells treated with either medium or sham), bacteria were not recovered from the basolateral media until 12 hours after

**TABLE 1. NONTYPEABLE HAEMOPHILUS INFLUENZAE BINDING TO AND TRANSMIGRATION ACROSS WELL-DIFFERENTIATED PRIMARY AIRWAY EPITHELIAL CELLS ARE INCREASED BY RHINOVIRUS INFECTION**

Infection	Bacteria Associated with Cells at 24 Hours Postinfection (cfu/well)	Bacteria Recovered from Basolateral Chamber (cfu/well)	
		12 h	24 h
Medium control	450 $\pm$ 100	0	0
Sham	360 $\pm$ 25	0	0
RV39	32000 $\pm$ 2600	400 $\pm$ 83	7600 $\pm$ 564
RV1B	24000 $\pm$ 4528	280 $\pm$ 52	4200 $\pm$ 672

*Definition of abbreviations:* NTHi = Nontypeable *Haemophilus influenzae*; RV = rhinovirus.

Data represent mean  $\pm$  SEM. Six independent experiments were performed in duplicate. Differences between medium- or sham-treated and RV-infected cells were statistically significant ( $P < 0.05$ , one-way analysis of variance).

adding the bacteria (Table 2). Cultures treated with occludin peptide for 30 minutes, or infected with RV for 24 hours, showed bacteria in the basolateral chamber within 1 hour of incubation. Basolateral chamber bacterial load increased with the time of incubation. Bacteria were not recovered from the basolateral chamber of cells infected with UV-irradiated RV39, indicating that replicating virus is required for increasing bacterial transmigration. These results indicate that infection with replicative RV facilitates transmigration of bacteria across polarized 16HBE14o-cells, as in primary cells. To determine whether the rate of migration depends on bacterial species, we then examined the effect of RV infection on the transmigration of other strains of NTHi and other bacterial species, such as *P. aeruginosa* and *S. aureus*. NTHi isolates 5P19H1, and 45P9H1 and *S. aureus* each showed a similar rate of transmigration, comparable to that of NTHi 6P5H (Table 3). *P. aeruginosa*, which can cause cell damage, transmigrated across both sham- and RV-infected cells, though at a higher rate in RV-infected cells. Together, these observations suggest that RV-facilitated transmigration of bacteria across polarized cell monolayers is not specific to one species of bacteria.

### Rhinovirus Reduces $R_T$

RV could facilitate transmigration of bacteria either by causing cell death or by disrupting tight junctions. To examine the capacity of RV to disrupt tight junctions, we measured  $R_T$  in both primary human mucociliary differentiated airway epithelial cells and 16HBE14o- cultures after RV infection. Primary airway epithelial cells differentiated into a mucociliary phenotype, with an  $R_T$  of 600–800  $\Omega \cdot \text{cm}^2$ , were infected apically with RV (MOI of 1) or an equal of volume of sham. After 5 hours, the infection medium was replaced with fresh medium and incubated further for 16 hours. The basolateral media were collected for detection of lactose dehydrogenase (LDH) activity (an indicator of cytotoxicity), and the  $R_T$  of cell cultures measured using a voltmeter. Cells treated with medium alone served as control. Media-treated cells showed minimal LDH activity. Cell lysate, which was used as a positive control for LDH activity, showed sevenfold greater activity than the medium control. Both sham- and RV-infected cells showed LDH levels similar to medium-treated controls, indicating that RV did not induce cytotoxic effects in well-differentiated cells (Figure 2A). On the contrary, epithelial cells infected with all three serotypes of RV showed moderate but statistically significant decreases in  $R_T$  compared with sham-treated controls (Figure 2B). Replication-deficient UV-treated virus had no effect on  $R_T$ , indicating that viral replication is required for the reduction in  $R_T$  (Figure 2C). We ultimately repeated the experiments with cells obtained from eight donors and in all cases we observed a similar reduction in  $R_T$  in response to RV39 infection (data not shown).

To confirm the results obtained with primary cultures, and to examine the feasibility of using polarized 16HBE14o- cells in subsequent experiments, we infected monolayers of 16HBE14o-cells with  $R_T$  of 800–1000  $\Omega \cdot \text{cm}^2$  with RV39 or RV1B at MOIs ranging from 0.2 to 5 for 1 hour. The infection medium was removed, incubation continued for another 24 hours, and  $R_T$  was measured. RV39 and RV1B each decreased  $R_T$  in a dose-dependent manner (Figures 3A and 3B). Effects on  $R_T$  were greater in 16HBE14o- compared with primary cells. The 16HBE14o- cell line, a clonal isolate that likely represents one cell type, in contrast to heterogeneous primary cultures, does not produce mucus, which may interfere with viral infection. At the same time, none of the RV-infected cell cultures, except those infected with RV1B at 5 MOI, showed detectable amounts of LDH in the basolateral medium (Figure 3C). Based on these results, we chose to use RV at an MOI of 1.0 for subsequent experiments, as this concentration reduced  $R_T$  without inducing cytotoxic effects.

**TABLE 2. RHINOVIRUS FACILITATES MIGRATION OF NONTYPEABLE HAEMOPHILUS INFLUENZAE FROM THE APICAL TO THE BASOLATERAL SURFACE OF POLARIZED 16HBE140- CELLS**

Infection	Bacteria Recovered from Basolateral Chamber (log cfu/well)					
	0.5 h	1 h	2 h	3 h	6 h	12 h
Medium	0	0	0	0	0	1.34 ± 1.65
Sham	0	0	0	0	0	2.25 ± 1.84
RV39	0.6 ± 1.2	0.8 ± 1.6	1.89 ± 2.37	2.93 ± 2.48	5.15 ± 0.96	9.30 ± 0.51
UV-RV39	0	0	0	0	0.43 ± 1.25	2.67 ± 2.31
Occludin peptide	0	1.37 ± 1.71	2.91 ± 2.44	4.23 ± 2.47	5.86 ± 1.25	8.95 ± 0.46

Definition of abbreviations: RV = rhinovirus; UV = ultraviolet.

Data represent mean ± SEM. Experiments were repeated three times in six replicates.

To determine the kinetics of RV-induced reductions in  $R_T$ , polarized 16HBE140- cells were infected with RV39 (MOI of 1 for 1 h) and  $R_T$  monitored at different time intervals up to 24 hours. A reduction in  $R_T$  was not observed until 12 hours (Figure 3D). Next, we infected polarized cells with UV-irradiated RV39 (1 or 5 MOI) or equivalent volumes of sham. UV-irradiated RV did not affect the  $R_T$  of polarized cells even at 5 MOI (data not shown). Taken together with results from primary cells, these data suggest that replication rather than initial binding or endocytosis of RV is required to cause significant reductions in  $R_T$ .

#### RV-induced Reductions in $R_T$ Are Not Mediated by Proinflammatory Cytokines

The proinflammatory cytokine IL-1 $\beta$  and a combination of TNF- $\alpha$  and IFN- $\gamma$  have been shown to decrease the  $R_T$  of differentiated airway epithelial cells (26). To examine whether cytokines released in response to RV infection mediate the observed reduction in  $R_T$ , we infected 16HBE140- airway epithelial cells with RV in the presence or absence of neutralizing antibodies to IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  alone or in combination. Normal IgG was used as a negative control, and recombinant IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  were used as positive controls. Polarized 16HBE140- cells incubated with IL-1 $\beta$  or a combination of TNF- $\alpha$  and IFN- $\gamma$  demonstrated a 20% reduction in  $R_T$ . These reductions were completely reversed by their respective neutralizing antibodies (Figure 4A). Normal IgG did not affect  $R_T$ . In contrast, none of the neutralizing antibodies tested reversed the effect of RV on  $R_T$  (Figure 4B), suggesting that the cytokines released in response to RV infection do not mediate the observed reduction in  $R_T$ .

#### Apoptosis Does Not Contribute to RV-induced Reductions in $R_T$

Though RV infection is notable for the lack of cytotoxicity relative to other respiratory viruses, such as RSV and influenza (14, 30, 31), RV has been shown to induce apoptosis by activating

caspase 3 and caspase 9 and poly(ADP-ribose) polymerase activation in nonpolarized airway epithelial cells (32). To examine whether RV causes apoptosis in primary airway epithelial cells differentiated into mucociliary phenotype and in polarized 16HBE140- cells, we infected these cultures with RV39 (MOI of 1) or sham, as described above, and determined the population of apoptotic cells by staining with annexin V and propidium iodide followed by flow cytometric analysis. As a positive control we treated cells with thepsigargin for 16 hours. For both primary airway epithelial cells and polarized 16HBE140- cells, control, sham-infected and RV-infected cells showed approximately 10% apoptotic cells, and there was no significant increase in the apoptotic cell population following RV infection (Figures 5A and 5B). On the other hand, thapsigargin-treated cell cultures showed 24.1 and 29.3% apoptotic cells in primary and 16HBE140- cells, respectively. These results suggest that RV does not cause significant apoptosis in differentiated or polarized airway epithelial cells, and hence, the observed reductions in  $R_T$  in RV-infected cells cannot be attributed to apoptosis.

#### RV Increases Paracellular Permeability of Polarized Epithelial Cells

To correlate RV-induced reductions in  $R_T$  with the barrier function of tight junctions, inulin permeability was measured after RV39, RV1B, or sham infection in polarized 16HBE140- cells. Uninfected cultures were used as negative controls. Both RV39 and RV1B caused significant increases in permeability, as expressed by the permeability coefficient  $P_{app}$ , compared with uninfected or sham-infected cultures (Figure 6). These results indicate that RV-induced reductions in  $R_T$  are associated with a functional increase in permeability to solute. This may occur as a result of disruption in the function of tight junctions.

#### RV Causes Redistribution of ZO-1

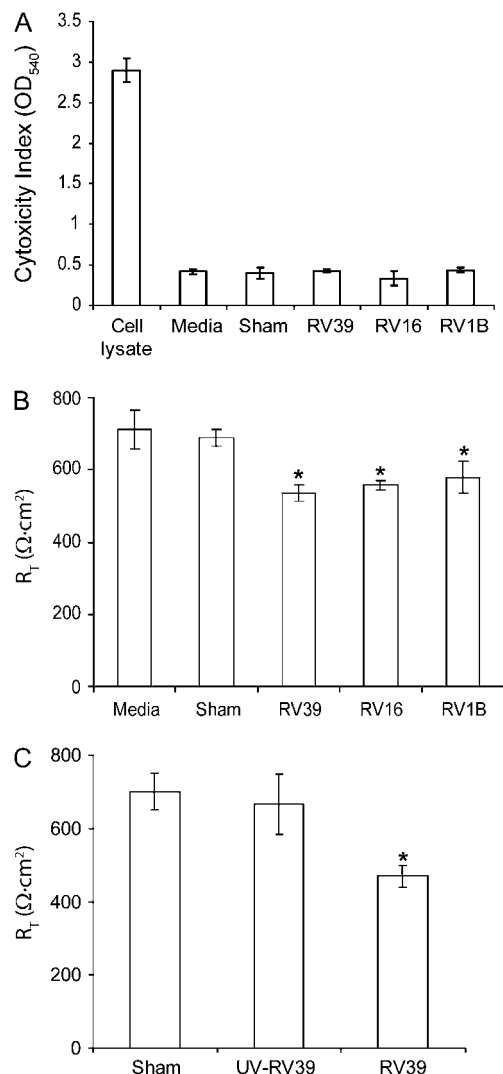
Based on the effects of RV on  $R_T$  and  $P_{app}$ , we hypothesized that RV redistributes or degrades tight junction proteins. Well-

**TABLE 3. RHINOVIRUS FACILITATES MIGRATION OF BACTERIA FROM THE APICAL TO THE BASOLATERAL SURFACE OF POLARIZED 16HBE140- CELLS**

Bacteria	Bacteria Recovered from Basolateral Chamber (log cfu/ml)					
	Sham			RV39		
	1 h	3 h	6 h	1 h	3 h	6 h
NTHi 5P19H1	0	0	0	1.56 ± 1.28	2.54 ± 2.08	5.28 ± 0.66
NTHi 45P9H1	0	0	0	0.92 ± 1.12	2.78 ± 1.53	5.49 ± 0.94
<i>P. aeruginosa</i>	0	0.98 ± 1.20	3.78 ± 0.39	3.89 ± 0.59	5.15 ± 0.74	7.11 ± 0.89
<i>S. aureus</i>	0	0	0	1.06 ± 1.31	2.74 ± 1.43	5.35 ± 0.75

Definition of abbreviations: NTHi = Nontypeable Haemophilus influenzae; *P. aeruginosa* = Pseudomonas aeruginosa; RV = rhinovirus; *S. aureus* = Staphylococcus aureus.

Data represent mean ± SEM. Experiments were repeated three times in triplicate.



**Figure 2.** Rhinovirus (RV) decreases the transepithelial resistance ( $R_T$ ) of well-differentiated primary airway epithelial cells. Primary cultures grown at air/liquid interface were infected apically with (A) RV or (C) ultraviolet-treated RV at one multiplicity of infection and incubated for 5 hours. Infection medium was then removed, and the cells further incubated for 24 hours. Lactate dehydrogenase activity in the basolateral medium (A) and the cell culture  $R_T$  (B and C) were measured. Cell lysate used in (A) was generated from representative media-treated cells. Results represent the mean of three independent experiments performed in triplicate; bars represent SEM. Statistical differences between experimental groups were determined by one-way analysis of variance ( $*P < 0.05$ ).

differentiated primary human airway epithelial cell cultures and 16HBE14o- cells were infected with RV39 or sham and immunostained with antibody to ZO-1, a protein of the tight junction complex, or normal IgG. In sham-infected cell cultures, ZO-1 was observed on the cell periphery (Figures 7A and 7C). In contrast, cultures infected with RV for 24 hours showed patches of cells with diffuse ZO-1 staining in the cytoplasm but no ZO-1 around the periphery, indicating dissociation of ZO-1 from the tight junction complex (Figures 7B and 7D). Similar results were observed when cells were infected with RV1B instead of RV39 (not shown). Cell cultures incubated with normal IgG instead of ZO-1 did not show signal, indicating specificity of the ZO-1 antibody.

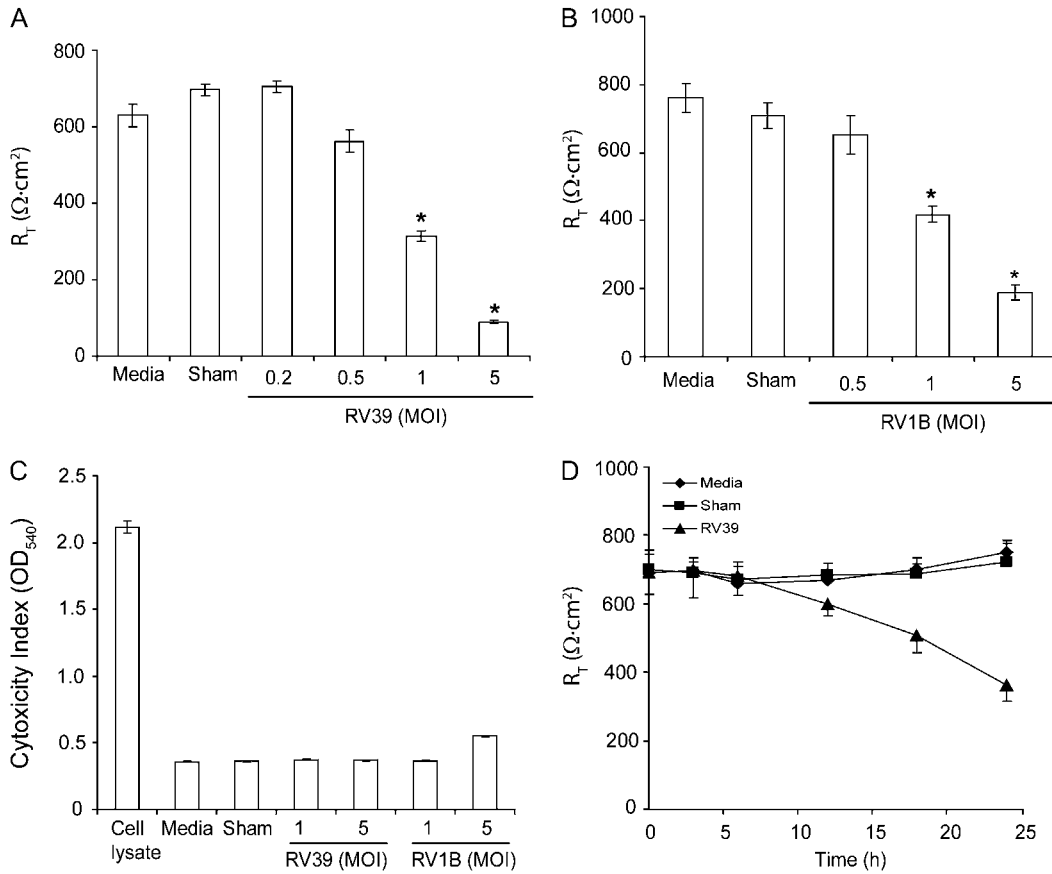
To confirm the dissociation of ZO-1 from the tight junction complex, NP-40 soluble (cytoplasm) and NP-40 insoluble (cytoskeleton) fractions were prepared from 16HBE14o- cultures infected with RV39 or RV1B, and proteins were subjected to Western blot analysis with antibody to ZO-1. The ZO-1 bands were quantitated by densitometry. Uninfected and sham-infected cultures showed intense ZO-1 bands in the NP-40 insoluble fraction, corresponding to approximately 70% of the total (Figure 7E, and 7F). In contrast, RV-infected cultures showed light bands corresponding to approximately 20% of the total ZO-1 in the NP-40 insoluble fraction and relatively intense bands in the NP-40 soluble fraction. These results confirmed the dissociation of ZO-1 from cytoskeletal tight junction complexes to the cell cytoplasm following RV infection.

### RV Causes Transmigration of Bacteria by the Paracellular Route

As noted above, our infection assays indicated that RV increased transmigration across well-differentiated airway epithelial cells. Because RV infection increased inulin permeability and altered tight junction complexes, we reasoned that bacteria transmigrate in RV-infected cultures by the paracellular route. To examine this, transmission electron microscopy was performed on 16HBE14o- cells infected with RV39 or sham for 24 hours, followed by incubation with NTHi 6P5H for 3 hours. Sham-infected cells appeared to have intact tight junctions, with bacteria mainly on the apical surface (Figure 8A). In contrast, RV-infected cells showed bacteria between the cells where tight junctions appeared to be disrupted (Figure 8B). To confirm these results, confocal fluorescence microscopy was conducted on cultures immunostained with antibodies against both ZO-1 and NTHi. Apical views of sham-infected cultures showed a normal distribution of ZO-1, and bacteria was found mainly on the apical surface (Figure 8C). Z-sections, created from serial optical sections taken through the whole thickness of the culture, also showed bacteria on the apical surface (Figure 8E). As noted previously, RV-infected cells showed altered ZO-1 expression (Figure 8D). Further, the Z sections showed bacteria located at the culture basolateral surface in an area devoid of ZO-1 staining (Figure 8F). These results indicate that RV infection facilitates transmigration of bacteria by the paracellular route.

### RV Decreases $R_T$ in Calu-3 Cells

To test the effects of RV in another respiratory epithelial cell line, we cultured Calu-3 lung adenocarcinoma cells in transwells as polarized cell monolayers. Calu-3 cells with  $R_T$  of 1500–1900  $\Omega \cdot \text{cm}^2$  were apically infected with RV39 or UV-irradiated RV39 at MOI of 1 or equal volume of sham for 16 to 18 hours, as described for 16HBE14o- cells, and measured  $R_T$  at the end of the incubation period. RV-infected cells showed a reduction in  $R_T$ , but not cells infected with UV-irradiated RV or sham (Figure 9A). RV did not cause a significant reduction in  $R_T$  until 12 hours after infection, consistent with the notion that viral replication is necessary for this effect (Figure 9B). Neutralizing antibodies to neither IL-1 $\beta$  nor a combination of TNF- $\alpha$  and IFN- $\gamma$  restored RV-induced reductions in  $R_T$ , indicating that the effect is not mediated by the release of these proinflammatory cytokines in response to RV infection (Figure 9C). Increased transmigration of NTHi isolate 6P5H was observed in RV-infected cultures relative to sham-infected cells (Figure 9D). Confocal microscopy revealed that, whereas sham-infected cultures showed the presence of ZO-1 around the periphery of most cells, RV-infected cells showed a loss of ZO-1 in scattered patches, although nuclei were visible (Figure 9E and 9F). Taken together, these results indicate that, as in bronchial epithelial cells, RV infection disrupts



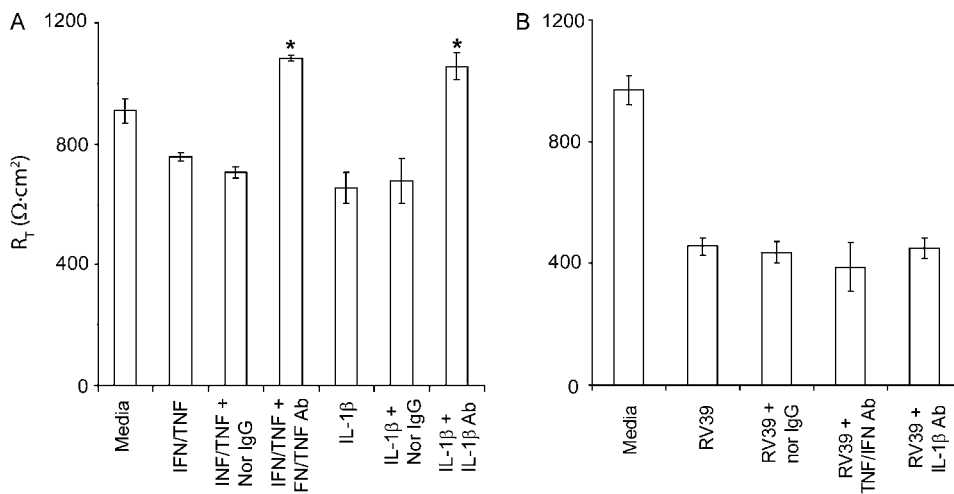
**Figure 3.** RV decreases the  $R_T$  of polarized 16HBE14o- cells in a dose- and time-dependent manner. Cells grown in transwells were infected apically with (A) RV39 or (B) RV1B at doses ranging from 0.25 to 5 MOI and incubated for 1 hour. Infection medium was removed, incubation continued for another 23 hours and  $R_T$  was measured. Cytotoxic effect was determined by measuring LDH levels in basolateral media (C). The time course of RV-induced reductions in  $R_T$  was determined by infecting polarized cell monolayers with RV39 at MOI of 1 for 1 hour, replacing the infection media with fresh cell culture media, and measuring  $R_T$  measured at the indicated times (D). Data represent the mean and standard error of four independent experiments carried out in triplicate ( $P < 0.05$ , analysis of variance).

the barrier function of Calu-3 cells by dissociating ZO-1 from the tight junction complex.

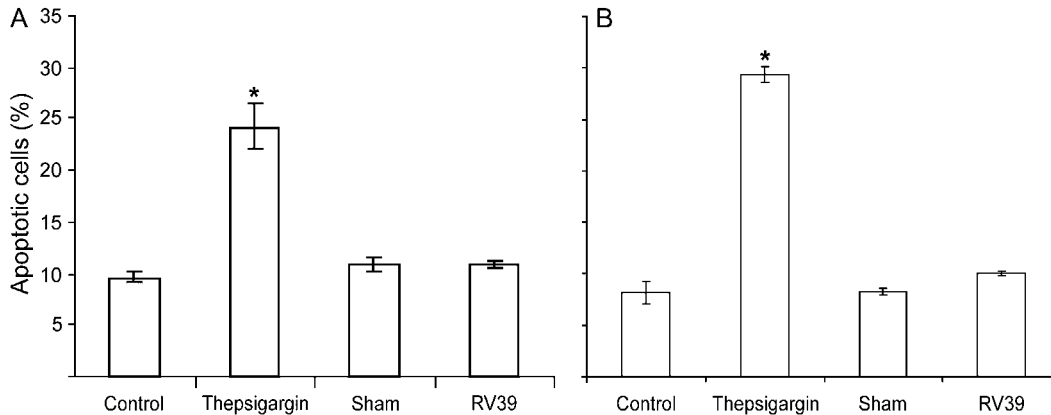
**Effects of RSV and Influenza A Virus on Tight Junction Function**

To examine whether other respiratory viruses disrupt the function of tight junctions similar to RV, we infected 16HBE14o- cells apically with RSV or influenza virus for 24 hours and determined  $R_T$ , LDH activity, and ZO-1 distribution. RSV had no effect on either  $R_T$  or LDH activity (Figure 10A and 10B). Corroborating the  $R_T$  measurements, RSV-infected cells showed a distribution of ZO-1 similar to sham-infected cultures, indicating that RSV did not dissociate ZO-1 from tight junction complex (Figure 10C). In contrast, influenza A virus decreased  $R_T$  by 39% and

increased LDH activity nearly threefold compared with sham-infected cells. Confocal microscopy revealed not only the loss of ZO-1 in affected areas, but also the absence of cell nuclei (Figure 10D). These results indicate that influenza A virus reduces  $R_T$  by inducing cell death rather than disrupting tight junctions. We then measured the transmigration of *S. aureus* in sham-, RSV- or influenza A-infected cell monolayers. Similar to sham-infected cells, no bacteria were recovered from the basolateral chamber of RSV-infected cell cultures up to 6 hours after bacterial inoculation. In contrast,  $3.5 \times 10^5 \pm 1.2 \times 10^5$  cfu/ml were recovered 30 minutes after adding bacteria to influenza A-infected cultures. Such a high rate of bacterial migration from apical to basolateral surface occurs following exposure of the bare membrane after the loss of cells.



**Figure 4.** RV-induced reductions in  $R_T$  are not mediated by proinflammatory cytokines. Polarized 16HBE14o- human bronchial epithelial cells were either treated with proinflammatory cytokines (A) or infected with RV for 24 hours (B) in the presence or absence of neutralizing antibodies to IFN- $\gamma$  and TNF- $\alpha$ , neutralizing antibody to IL-1 $\beta$ , or normal IgG.  $R_T$  was measured as described above. Results represent the mean of three independent experiments carried out in triplicate; bars represent SEM ( $P < 0.05$ , analysis of variance).



**Figure 5.** Apoptosis does not contribute to rhinovirus (RV)-induced reductions in transepithelial resistance ( $R_T$ ). (A) Primary differentiated airway epithelial cells or (B) polarized 16HBE14o- human bronchial epithelial cells were infected with RV39 or sham or treated with 5  $\mu$ M thepsigargin for 1 hour. Media-treated cells were used as negative controls. Cells were stained with mixture of FITC-annexin V and propidium iodide and analyzed by flow cytometry. Results represent the mean of three independent experiments performed in triplicate; bars represent mean  $\pm$  SEM ( $P < 0.05$ , analysis of variance).

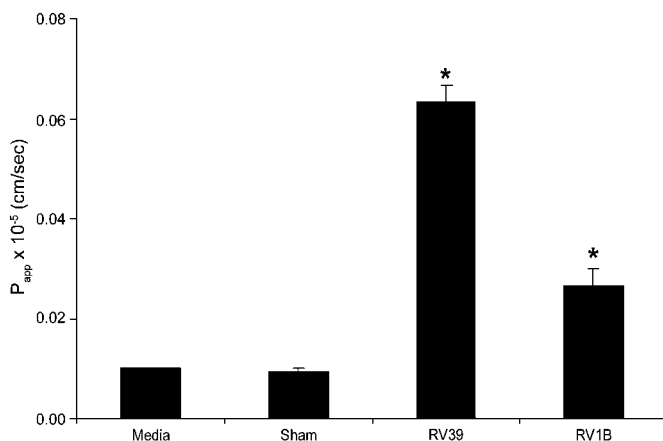
### RV Disrupts ZO-1 Distribution *In vivo*

We developed a mouse model of RV exposure using RV1B, a minor serotype RV. Intranasal inoculation of C57BL/6 mice with RV1B causes airways inflammation and hyperresponsiveness (27). We inoculated mice with RV1B or sham by the intranasal route ( $1 \times 10^9$  TCID<sub>50</sub>/ml) and lungs were harvested 24 hours later. Paraffin sections were immunostained with ZO-1 antibody and analyzed by confocal microscopy. In sham-inoculated animals, there was uniform distribution of ZO-1 in both large and small airways (Figures 11A and 11C). In contrast, RV1B-infected mice showed discontinuity in the distribution of ZO-1 (Figures 11B and 11D). Taken together, these results suggest that RV disrupts airway epithelial cell barrier function by dissociating ZO-1 from tight junctions.

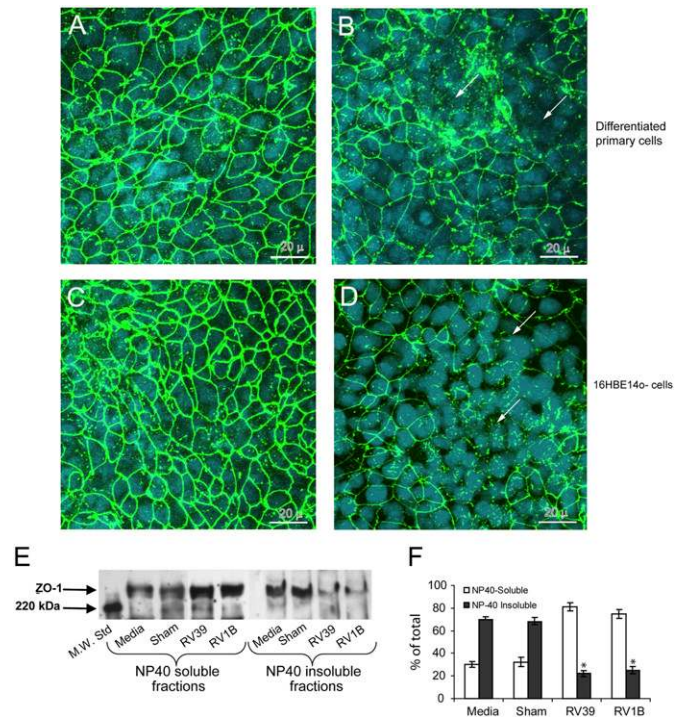
### DISCUSSION

Our results suggest that RV, which causes a majority of common colds, facilitates the transmigration of bacteria across the airway epithelium. Increased transmigration was accompanied by re-

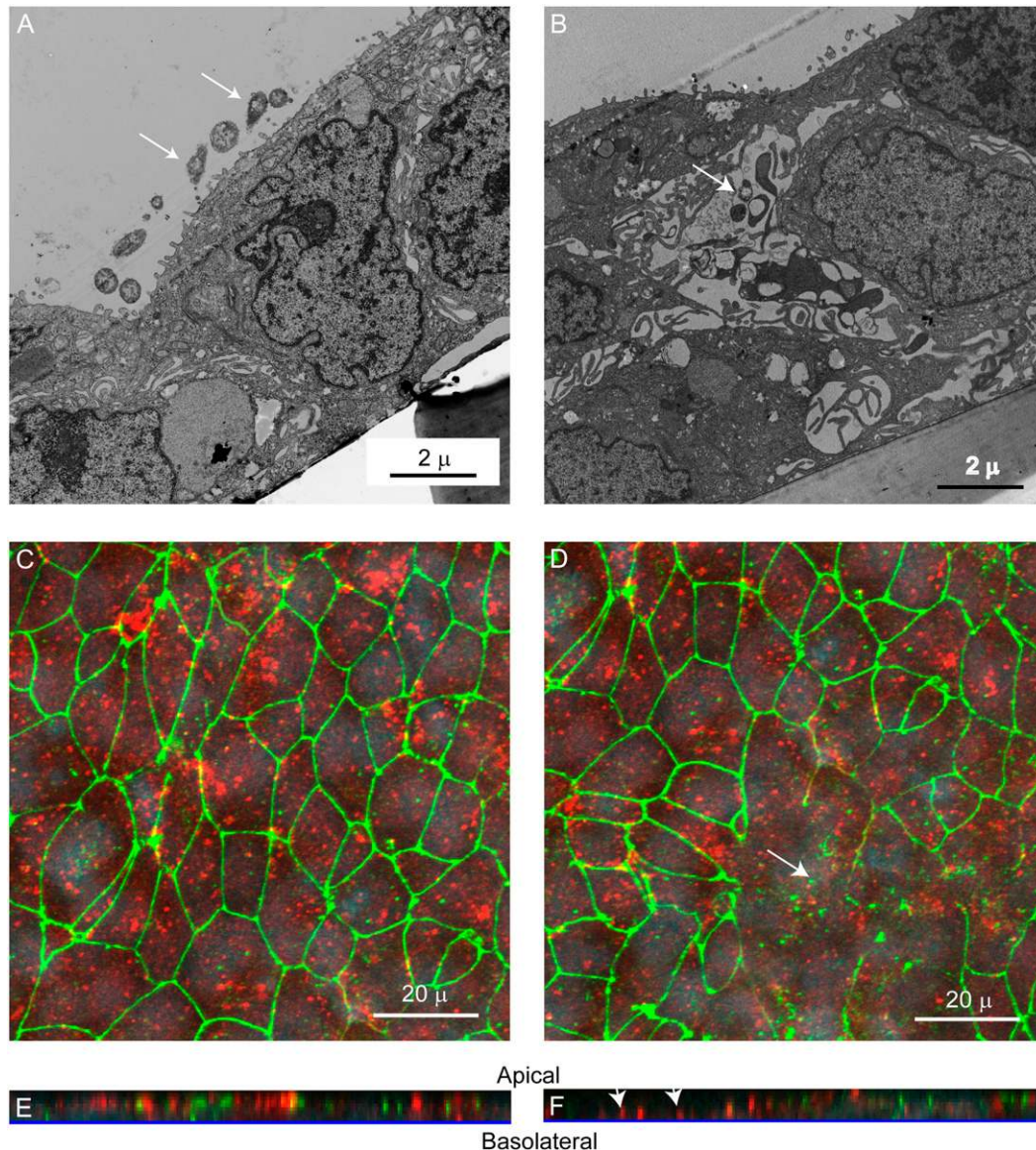
duced  $R_T$  and increased permeability to inulin. RV infection did not cause cytotoxicity or apoptosis. Exposure to sham HeLa cell supernatants or UV-irradiated RV did not compromise barrier



**Figure 6.** Effect of RV on the paracellular permeability of polarized epithelial cells to inulin. Polarized 16HBE14o- cells were infected with RV39 or RV1B at MOI of 1 for 24 hours. FITC labeled-inulin was added to the apical chamber, the basolateral chamber was sampled at different time intervals for fluorescence, and  $P_{app}$  was calculated. Results represent the mean of three independent experiments carried out in quadruplicate; bars represent SEM ( $P < 0.05$ , analysis of variance).



**Figure 7.** Rhinovirus (RV) infection reduces transepithelial resistance by dissociating zona occludins (ZO)-1 from tight junction complex. Well-differentiated (A and B) primary or (C and D) polarized 16HBE14o- human bronchial epithelial cells were infected with either (A and C) sham or (B and D) RV39 and incubated for 24 hours as described above. Cells were fixed in cold methanol and immunostained with antibody to ZO-1 (green). Nuclei were stained with DAPI (blue) (Sigma-Aldrich, St. Louis, MO). Arrows represent dissociation of ZO-1 from the tight junction complex. NP40 soluble and insoluble fractions from medium-treated, sham, RV39, or RV1B-infected polarized 16HBE14o- cells were subjected to Western blot analysis with (E) antibody to ZO-1. (F) A representative blot from three independent experiments is presented. Group mean data from three independent experiments (bars represent mean  $\pm$  SEM;  $P < 0.05$ , analysis of variance).



**Figure 8.** Rhinovirus (RV) promotes transmigration of bacteria by the paracellular route. 16HBE14o- human bronchial epithelial cells grown as polarized monolayers were infected apically with (A) sham or (B) RV39 for 24 hours. NTHi (0.1 ml of  $1 \times 10^9$  cfu/ml) was added to the apical surface and incubated for 3 hours. Cells were fixed and processed for transmission electron microscopy. Arrows point to bacteria. Cultures were immunostained with antibodies to zona occludins (ZO)1 (green) and nontypeable *Haemophilus influenzae* (NTHi) (red) and analyzed by confocal microscopy, taking sections every 0.5  $\mu$ m. (C and D) Apical view of sham- and RV- infected cells, respectively. (E and F) Z-sections of sham- and RV- infected cells. Arrows in D indicate areas devoid of ZO-1. Arrows in F indicate bacteria at the basolateral surface.

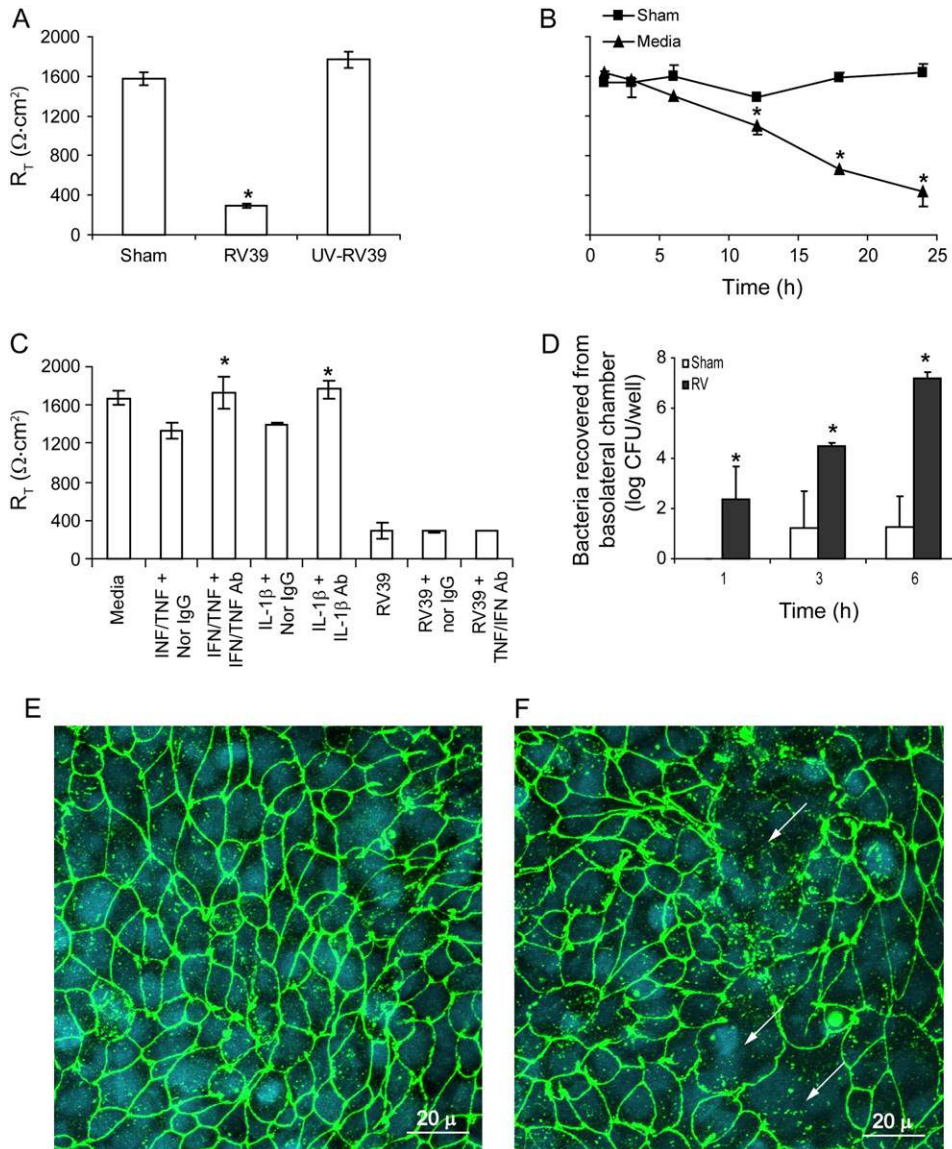
function, indicating that the observed effect is a specific effect of replicating virus, and not a contaminant in the virus stock. Finally, we demonstrated that RV induces dissociation of ZO-1 from the tight junction complex *in vitro* and *in vivo*. In contrast, influenza A virus increased permeability of polarized airway epithelial cells by inducing cytotoxic effects, whereas RSV had no effect on either  $R_T$  or ZO-1 distribution. These studies provide insight into a new mechanism by which RV may predispose the host to secondary bacterial infection, as occurs in otitis media (1, 12) and exacerbations of COPD (4–7).

RV has a limited capacity to cause lower airway infection, and stimulates a relatively mild inflammatory response in the upper respiratory tract. Unlike influenza virus and RSV, RV infection does not significantly damage the airway epithelium (14). Nevertheless, there is limited evidence suggesting that RV infection can increase binding of bacteria to and promote internalization of bacteria by epithelial cells. For example, RV infection increases the binding of *S. pneumoniae* to bronchial epithelial cells by up-regulating expression of the platelet-activating factor receptor (19). RV infection promotes internalization of *S. aureus* by A549 pulmonary epithelial cells, which are otherwise nonpermissive (20). RV increases the expression of ICAM-1 (20, 33), which

serves as a receptor for NTHi (34) as well as RV. Thus, it is conceivable that RV infection increases the adherence of NTHi to airway epithelial cells by increasing surface ICAM-1 expression. However, the effect of RV infection on the transmigration of NTHi across well-differentiated airway epithelial cells has not been observed previously.

Migration of bacteria from apical to basolateral surface in polarized epithelium can occur either by a transcellular or paracellular pathway. Polarized epithelial cells are not permeable unless they are damaged or their barrier function is disrupted. Hence, we examined the effect of RV on both  $R_T$  and inulin permeability, as measured by  $P_{app}$ .  $R_T$ , which correlates with the number of tight junction strands, does not necessarily correlate with paracellular permeability to noncharged solutes (26). On the other hand, increases in  $P_{app}$  represent a functional change in paracellular permeability and are always accompanied by reductions in  $R_T$ . We found that RV significantly decreased  $R_T$  and increased permeability to inulin 24 hours after infection. The observed reduction in  $R_T$  did not occur until 8 hours after infection. Moreover, replication-deficient, UV-treated RV had no effect on  $R_T$ . RV-induced changes in  $R_T$  were most evident when infected cells were incubated at 33°C, the optimal temperature for RV





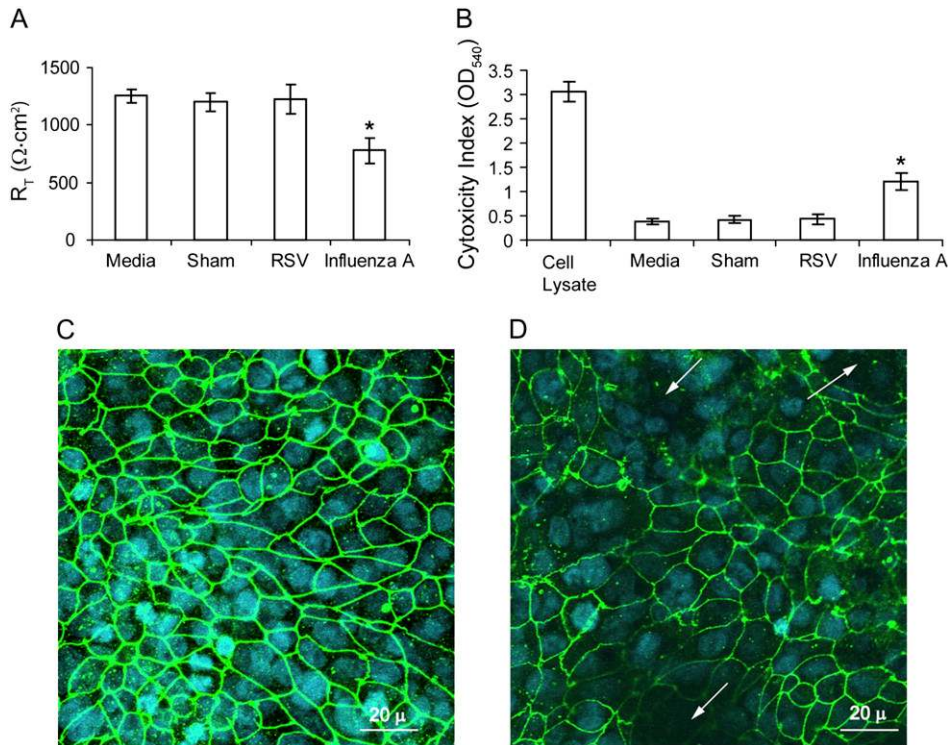
**Figure 9.** Rhinovirus (RV) disrupts the barrier function of Calu-3 cells. (A) Polarized monolayers of Calu-3 cells were infected with RV39, ultraviolet-irradiated RV39, or sham for 24 hours and transepithelial resistance ( $R_T$ ) was measured. (B) The time course of reductions in  $R_T$  in response to RV infection. (C) In some experiments, monolayers were either treated with proinflammatory cytokines or infected with RV for 24 hours in the presence or absence of neutralizing antibodies to IFN- $\gamma$  and tumor necrosis factor- $\alpha$ , neutralizing antibody to IL-1 $\beta$  or normal IgG. Calu-3 monolayers infected with either sham or RV39 for 24 hours were incubated apically with nontypeable *Haemophilus influenzae* isolate 6P5H for 6 hours. (D) Medium from the basolateral chamber was sampled for bacteria at 1, 3, or 6 hours. Monolayers infected with (E) sham and (F) RV39 for 24 hours were immunostained with antibody to zona occludins (ZO-1) (green) and counterstained with DAPI (blue). Results represent the mean of three independent experiments performed in triplicate; bars represent mean  $\pm$  SEM ( $P < 0.05$ , analysis of variance). Arrows in E and F point to the dissociation of ZO-1 from the periphery of cells.

replication (data not shown). It should be noted that, though 33°C is less than core body temperature, lower temperatures are commonly found in the proximal airways, especially when breathing cold air (as in winter) or at high levels of minute ventilation (as during exercise) (35). Finally, we showed that RV infection in mice leads to loss of ZO-1 in the proximal airway epithelium. Taken together, these data suggest that RV-induced reductions in  $R_T$  are dependent on viral replication and are biologically relevant.

Recently, transient binding of particular viruses to the apical cell surface has been shown to disrupt tight junctions, leading to an increase in epithelial permeability, which in turn allows migration of virus to the basolateral surface where the major viral receptors are located. Initial binding of coxsackievirus, a member of the *Picornoviridae* family, to decay-accelerating factor on the apical cell surface activates Abl kinase and triggers Rac-dependent actin rearrangement, thereby permitting virus migration across the tight junctions and interaction with its major receptor, coxsackievirus-adenovirus receptor (36–38). In contrast to our findings with RV, epithelial  $R_T$  decreased within 15 minutes of coxsackievirus infection and returned to normal by 1 hour, indicating that initial binding of virus is sufficient to cause the observed changes. In addition, the VP8 subunit of rotavirus

capsid protein VP4 transiently disrupts barrier function of the polarized intestinal epithelium, thus permitting the interaction of rotavirus with its basolateral integrin receptors, which is required for its entry into cells (38).

Based on RV-induced changes in  $R_T$  and  $P_{app}$ , we examined the redistribution of ZO-1, a tight junction cytoplasmic adaptor protein that acts as a bridge between integral tight junction membrane proteins such as occludins, junctional adherence molecule and cytoskeletal proteins, and is present in the periphery of polarized epithelial cells. Immunofluorescence staining and immunoblots from RV-infected airway epithelial cells showed translocation of ZO-1 from the membrane to the cytoplasm, indicating dissociation of ZO-1 from the tight junction complex. Further, fluorescent confocal microscopy of airways from RV1B-inoculated mice showed discontinuity in the distribution of ZO-1. Taken together, these data suggest that RV disrupts airway epithelial cell barrier function by dissociating ZO-1 from tight junctions. Viral infection of epithelial cells has been shown to induce cytotoxicity, apoptosis, and production of pro-inflammatory cytokines, each of which has been shown to induce structural changes in tight junctions (26, 39, 40). During apoptosis in polarized epithelial cells, cysteine protease family



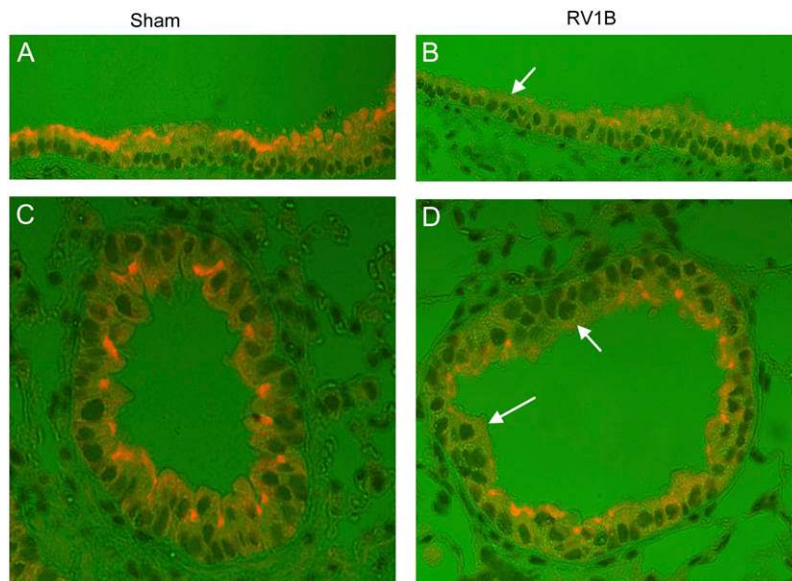
**Figure 10.** Influenza A, but not RSV, causes a cytopathic effect in polarized 16HBE14o- human bronchial epithelial cells. Cells grown in Transwells were infected apically with RSV or influenza A virus and incubated for 1 hour. Infection medium was removed, cells were further incubated for 23 hours and  $R_T$  of cell cultures (A) and LDH levels in basolateral medium (B) were measured. In some experiments, cells were infected with RSV (C) or influenza A virus (D) as described above, fixed in methanol, and immunostained with antibody to ZO-1. Results represent the mean of three independent experiments performed in triplicate; bars represent mean  $\pm$  SEM ( $P < 0.05$ , analysis of variance). Arrows in panel D show loss of both ZO-1 and nuclei.

caspsases are activated, leading to proteolytic cleavage of the tight junction proteins occludin, ZO-1 and ZO-2. However, under the experimental conditions used, there was no evidence of cytotoxicity, as assessed by trypan blue exclusion assay (see Figure E1 in the online supplement) and LDH release, except at a high viral doses (5 MOI) 24 hours after incubation. Neutralizing antibodies to proinflammatory cytokines IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$  had no effect on RV-induced disruption in barrier function. RV infection did not increase apoptosis, as assessed by annexin V staining. These results suggest that cytotoxic effects, pro-inflammatory cytokines, and apoptosis are not responsible for the observed tight junction disruption.

Intranasal challenge in human volunteers with rhinovirus has been shown to attenuate mucociliary clearance (41). Thus, it is

conceivable that, in addition to increasing paracellular permeability, RV facilitates secondary bacterial infection of the airway epithelium by attenuating mucociliary clearance. Although this cannot easily be tested in an *in vitro* model, we developed an animal model of RV infection in which mice show persistence of viral RNA up to 4 days, mild neutrophilic lung inflammation, lung interferon production, and airways cholinergic hyperresponsiveness (27).

In summary, we have shown that RV disrupts the barrier function of airway epithelium and induces dissociation of ZO-1 from the cell membrane both *in vitro* and *in vivo*. The effect on barrier function appears to depend on viral replication. This study provides insight into a mechanism by which RV can predispose the host to secondary bacterial infection.



**Figure 11.** Rhinovirus (RV)1B disrupts airway epithelial tight junctions *in vivo*. C57BL/6 mice were infected with RV1B ( $5 \times 10^7$  TCID<sub>50</sub>) or an equal volume of sham. Mice were killed at 24 hours post-infection, and paraffin sections prepared from the lungs were deparaffinized, rehydrated, and immunostained with antibody to ZO-1. The bound antibody was detected by anti-rabbit IgG conjugated with AlexaFluor 488. (A and C) Large and small airways, respectively, from sham-infected mice. (B and D) Large and small airways, respectively, from RV1B-infected mice. Arrows represent absence of dissociation of zona occludins-1 from the tight junction complex.

**Conflict of Interest Statement:** U.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Q.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.C.G. received royalties for licensing cells used in this study through the University of California, San Francisco. M.B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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