In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo

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Respiratory syncytial virus (RSV) is the major viral cause of severe pulmonary disease in young infants worldwide. However, the mechanisms by which RSV causes disease in humans remain poorly understood. To help bridge this gap, we developed an ex vivo/in vitro model of RSV infection based on well-differentiated primary pediatric bronchial epithelial cells (WD-PBECs), the primary targets of infection in vivo. Our RSV/WD-PBEC model demonstrated remarkable similarities to hallmarks of RSV infection in infant lungs. These hallmarks included restriction of infection to noncontiguous or small clumps of apical ciliated and occasional nonciliated epithelial cells, apoptosis and sloughing of apical epithelial cells, occasional syncytium formation, goblet cell hyperplasia/metaplasia, and mucus hypersecretion. RSV was shed exclusively from the apical surface at titers consistent with those in airway aspirates from hospitalized infants. Furthermore, secretion of proinflammatory chemokines such as CXCL10, CCL5, IL-6, and CXCL8 reflected those chemokines present in airway aspirates. Interestingly, a recent RSV clinical isolate induced more cytokine-generation than the prototypic A2 strain. Our findings indicate that this RSV/WD-PBEC model provides an authentic surrogate for RSV infection of airway epithelium in vivo. As such, this model may provide insights into RSV pathogenesis in humans that ultimately lead to successful RSV vaccines or therapeutics.

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6. Gross and microscopic pathology from fatal cases of RSV infection are characterized by edema; small airway necrosis and sloughing; small airway plugs consisting of mucus, neutrophils, and
the prototypic strain RSV A2 or a recent clinical isolate, RSV BT2a. Although the consequences of infection were qualitatively similar for the two strains, the intensity of the responses often was higher following RSV BT2a infection. Our data suggest that the RSV strain used to study RSV pathogenesis in human tissues is important. We conclude that our model provides an authentic surrogate with which to elucidate mechanisms of RSV pathogenesis in pediatric airways.

**Results**

Both RSV A2 and BT2a productively infected WD-PBEC cultures and had similar growth kinetics (Fig. 1A). Virus titers peaked at $5 \log_{10}$ median tissue-culture infective dose (TCID$_{50}$/mL) at 96 h postinfection (hpi) and decreased slightly thereafter. However, on average, 4.5 times more cells (range 1.53–9.63) were infected with RSV BT2a than with RSV A2 at 144 hpi (Fig. 1B and Table S1).

No infectious viruses were detected in the basolateral medium, indicating that virus progeny release was polarized to the apical surface. Daily monitoring of infected WD-PBECs revealed no evidence of gross cytopathic effects (CPE) or culture deterioration over 6 dpi (Fig. 2A). Both RSV A2 and BT2a were restricted to the apical layer of WD-PBECs (Fig. 2B). Compared with uninfected controls, there was no obvious deterioration in the cultures following RSV infection in terms of the number of cell layers. Moreover,ZO-1 staining suggested that tight junctions remained intact at 144 hpi (Fig. 2C). Both viruses primarily infected ciliated and, occasionally, nonciliated epithelial cells (Fig. 2D and Movie S1). Furthermore, RSV most commonly infected noncontiguous cells; not all ciliated cells were infected. However, clumps of infected cells were observed also. In contrast, goblet cells were not infected by RSV (Fig. 2E and Movie S2).

Although gross CPE was not evident, readily identifiable cytopathogenesis was. Syncytia, hallmarks of RSV CPE in cell line monolayers in vitro and occasionally reported in autopsy lung tissue from fatal RSV cases (7, 13), were detected invariably after infection with both RSV strains (Fig. 3A–C and Movie S3), albeit at low frequencies ($\sim 1.18$ and $2.38$ per 1,000 infected cells for RSV BT2a and A2, respectively, at 144 hpi). Lumen epithelial cell sloughing is common in lung necropsy of fatal RSV cases (7–9, 11, 13), and apical washes of RSV-infected WD-PBECs contained considerably more nuclei than mock-infected controls, indicating sloughing (Fig. 3D). RSV BT2a induced more cell sloughing than RSV A2 (Fig. 3E). Many detached cells were TUNEL-positive, indicating apoptosis (Fig. 3F). More TUNEL-positive cells were evident in cytopsin of BT2a- than A2-infected cultures; very few cells in the uninfected controls were TUNEL-positive.

Ciliated cells from the airway epithelium are found in luminal cell debris during RSV disease, consistent with their role as primary targets for RSV infection (9). To determine whether our model also displayed a loss of ciliated cells following RSV infection, cultures were trypsinized at 144 hpi, collected by cytospin, and stained for β-tubulin (ciliated cells) (Fig. 4A). The percentage of ciliated cells in whole cultures was reduced by three times in the RSV BT2a- and A2-infected cultures compared with controls (Fig. 4B). To determine directly if ciliated cells were released from the apical surfaces of WD-PBECs following infection, cytopsinps of apical washes were stained with DAPI and anti-β-tubulin. At 144 hpi, more ciliated cells were present in apical rines from the BT2a- than from the A2-infected cultures, and few were evident from uninfected controls (Fig. 4C). These data confirmed that RSV infection of WD-PBECs caused a loss of ciliated cells, as described in fatal cases of RSV (9).

Enhanced mucous production is a hallmark of RSV bronchiolitis (9). We therefore investigated the modulation of goblet cell content following infection. Anti-Muc5Ac staining of apical wash cytopsinps from infected or control WD-PBECs (144 hpi) demonstrated a large increase in mucus secretion following infection (Fig. 5A). Furthermore, increased goblet cell numbers were evident at 144 hpi in RSV-infected as compared with control cultures, suggesting goblet cell hyperplasia/metaplasia (Fig. 5B and C). Interestingly, RSV BT2a induced more goblet cell hyperplasia/metaplasia than RSV A2. These data are consistent with clinical hallmarks of RSV infection.

To study components of the innate immune response to RSV in our transwell-cultured WD-PBECs, we analyzed the release of CCL5, CXCL8, CXCL10, IL-6, and TNF-related apoptosis-inducing ligand (TRAIL) to the basolateral medium (24, 96, 120, and 144 hpi) and apically (24 and 120 hpi) (n = 5) (Fig. 6 and Fig. S1). We also analyzed type I IFNs to determine if our RSV/WD-PBEC model reflected the very poor type I IFN responses evident following RSV infection of infants (Fig. S2) (24–25). Because the basolateral medium was changed and apical surfaces were rinsed daily, concentrations measured corresponded to the amount of cytokines/chemokines released within the preceding 24-h period. CXCL10 (A2 and BT2a infections) and IL-6 (BT2a infection) were the only chemokines significantly up-regulated in the basolateral medium at 24 hpi compared with controls. By 96 hpi, basolateral secretions of CXCL10, TRAIL, CCL5, IL-6, and CXCL8 were significantly higher in infected than in uninfected cultures. Expression of all chemokines except CXCL8 peaked between 24 and 96 or 120 hpi and maintained high levels through the 144 hpi time point. CXCL10 was the most strongly up-regulated of the chemokines tested, particularly in RSV BT2a-infected cultures. This trend continued through 144 hpi. Similarly, TRAIL at 96 hpi and CCL5 at both 96 and 120 hpi were significantly higher in the RSV BT2a-infected cultures. Indeed, where significant differences in analyte concentrations were evident between these viruses, concentrations always were higher in RSV BT2a infections. IL-6 and CXCL8 secretion levels were similar for both viruses at all times. CXCL8 was produced at high levels following RSV infection, peaking at 120 hpi, although uninfected controls also produced large amounts. With the exception of CXCL8, similar trends in chemokine secretions were evident in apical rines, albeit with higher baseline levels evident in mock-infected controls (Fig. S1). In contrast to the basolateral medium, there was no increase in CXCL8 concentrations in the apical washes at 120 hpi. Although direct comparisons are
difficult because of different harvesting protocols, absolute mean concentrations were higher in basolateral medium for CXCL10 and CXCL8; IL-6 and TRAIL concentrations were higher in apical washes; and CCL5 levels were similar in basolateral medium and apical washes. Finally, type I IFNs were not detected in basolateral medium tested at 24 or 96 hpi (Fig. S2).

Discussion

In the current study, we show that RSV infection of WD-PBECs demonstrates several characteristics that are remarkably similar to histological changes in lung tissues of fatal cases—the only RSV cases for which tissues are available—and to the pathophysiology of RSV-induced bronchiolitis. The restriction of RSV infection to apical-layer ciliated and occasional nonciliated cells is consistent with studies in adult-derived HAE cultures and histology of RSV-infected pediatric lungs (19, 26, 27). Infection of noncontiguous cells and occasional cell clumps corresponds with previous reports on lung tissues from fatal RSV and an ex vivo model of RSV infection based on human fetal tracheal ring cultures (10, 14, 28). The considerable cell sloughing from RSV-infected WD-PBECs is consistent with the fibrin-mucocellular airway occlusions that are hallmarks of RSV histopathology in infants (9). Interestingly, Fig. 2. RSV infection does not cause gross CPE but is restricted to apical ciliated and occasional nonciliated cells. WD-PBECs were infected as indicated in Fig. 1. (A) At 144 hpi, RSV- and mock-infected cultures were observed by phase-contrast microscopy for evidence of CPE. The white-cloud appearance is caused by mucus secretion. (Magnification: 10×.) (B) Confocal orthogonal sections of WD-PBECs, fixed at 144 hpi and stained for RSV F protein (green); nuclei were counterstained with DAPI (blue). (Magnification: 40×.) (C) En face and orthogonal (Insets) confocal images of RSV-infected WD-PBECs at 144 hpi, fixed and stained for RSV F protein (green) and ZO-1 (red). White arrowheads show tight junctions. (Magnification: 63×.) At 144 hpi, WD-PBECs were fixed, permeabilized, and stained for (D) RSV F protein (green) and β-tubulin (red) or (E) RSV F (green) and Muc5Ac (red). (Magnification: 63×.) Lower panels show orthogonal sections. Images are representative of five different donors.

Fig. 3. RSV causes syncytia formation, apical cell sloughing, and apoptosis. WD-PBEC cultures were infected as indicated in Fig. 1. (A) At 144 hpi, cultures were fixed and stained with DAPI and anti-RSV F mAb to visualize nuclei (blue) and RSV-infected cells (green), respectively. Confocal data from RSV A2-infected (A) and BT2a-infected (B) cultures revealed syncytia formation after RSV infection (white arrows). Two different planes (main panel and Inset) are presented for BT2a infection. (Magnification: 63×.) (C) Large syncytium following BT2a infection. The orthogonal section (Lower) shows the fusion (white arrow) between two adjacent syncytia. (Magnification: 63×.) Images are representative of five different donors. (D) Cytospins of apical washes were performed 72 hpi, and slides were stained for DAPI to visualize apical cell sloughing. RSV infection induced considerable sloughing of cells compared with noninfected control slides, with BT2a > A2 >> uninfected cultures. (Magnification: 63×.) (E) Quantification of nuclei following cytospins of apical washes of RSV A2-, BT2a-, and mock-infected cultures (n = 3 donors). Values are means ± SEM. Areas under the curve were calculated for each donor and compared. **P < 0.01. (F) Cytospins were performed at 72 hpi and stained for evidence of apoptosis using the TUNEL assay. BT2a-infected > A2-infected >> uninfected cultures in terms of apoptosis induction. (Magnification: 63×.)
apoptosis evident within the sloughed cells reflects recent observations in histology slides of RSV-infected infant lung (10). The reduction of ciliated epithelial cells within infected WD-PBEC cultures and the concomitant increase in ciliated cells among sloughed cells are consistent with characteristics of RSV bronchiolitis (9). Syncytia observed in our RSV/WD-PBEC model were remarkably similar to syncytia described recently in conducting airway tissues in RSV-infected infant lungs (7). The detection of goblet cell hyperplasia/metaplasia following RSV infection is consistent with excessive mucus production in RSV bronchiolitis (2, 12). Finally, secretions of a panel of chemokines from RSV-infected WD-PBECs are similar to levels observed in RSV-infected infants (5, 6, 10).

The infection of noncontiguous or clumps of cells with RSV and the overall extent of infection in our WD-PBEC model is consistent with previous reports with adult WD-HAE cultures, including those derived from primary adenoid and nasal epithelial cells (27). As we do, Zhang et al. (19) described restriction of infection in their WD-HAE cultures to apical ciliated cells but also more extensive infection and higher replication with recombinant RSV A2-expressing GFP [rgRSV(224)] than we observed. The epithelial cell origins (adult with/without cystic fibrosis vs. infant), specific culture conditions, and/or the virus strains used might explain these differences. Indeed, the rgRSV(224) genome contains several introduced mutations compared with wild-type RSV A2 (29).

The lack of gross WD-PBEC CPE following infection with either RSV strain is consistent with previous reports (19, 26) and suggests that culture regeneration compensates for the loss of cells through sloughing. Lung histology data from RSV-infected infants revealed areas of epithelium that were RSV positive and either relatively intact or destroyed (7, 10). Our data suggest that RSV infection alone is unlikely to be the principal cause of epithelium destruction in infants. Immune cells responding to the infection may be responsible for damage to the airway epithelium in vivo. This suggestion is consistent with the concept that much of the disease associated with RSV may be immune mediated. Future research, in which specific immune cells will be included in our RSV/WD-PBEC model, will address this possibility.

We also sought to address whether the RSV strain influenced experimental outcomes. We found that the consequences of infection of WD-PBECs were qualitatively similar for both RSV strains. However, where quantitative differences were evident, they were generally in favor of the clinical isolate. This finding may be explained, at least in part, by the increased number of cells infected by RSV BT2a relative to A2. These data contrasted with our work in monolayer PBECs, in which RSV A2 caused substantially more CPE and had higher growth kinetics than RSV BT2a (30). The cumulative data from both studies suggest that the differentiation status of the PBECs and the RSV strain used are important considerations in studying RSV pathogenesis. The striking similarities between our RSV/WD-PBEC model and aspects of the histopathology of RSV in infants suggest that this model is physiologically more authentic than the monolayer PBEC/RSV model.

Intriguingly, some ciliated cells were not infected by RSV in our model, even by 7 dpi. This result suggests that the RSV receptor is not present on all ciliated cells and/or some of them are innately or become refractory to infection. Heparan sulfate, which functions as the RSV receptor in continuous cell lines (31), is absent from the apical surface of WD-HAE cultures (18) and therefore is unlikely to function as such in WD-PBECs or in vivo. Thus, the “real” RSV receptor remains to be identified before we can address the hypothesis of receptor-associated infection restriction in some ciliated cells. Alternatively, the induction of an antiviral state in neighboring cells, as suggested by high CXCL10 secretion, may explain the limited virus spread (32). Interestingly, our data suggest that type I IFNs are not responsible for inducing such an
antiviral state in RSV-infected WD-PBECs. Intriguingly, recent work suggests that type III IFNs may be implicated instead (33).

The detection of apoptosis among sloughed cells from RSV-infected WD-PBECs is consistent with a previous report of strong caspase 3 staining in bronchial epithelial cells from infants that died of RSV (10). As a potent inducer of apoptosis (34), the high level of TRAIL induced by infection suggests that it is implicated in RSV-induced apoptosis and corresponds with its putative role in epithelial injury during severe RSV disease (35). Further work is necessary to elucidate the role of apoptosis and TRAIL in RSV pathogenesis.

Enhanced mucus secretion, another characteristic of RSV bronchiolitis (13), was reproduced faithfully in our model. The increased numbers of goblet cells following RSV infection suggests that goblet cell hyperplasia/metaplasia might explain the increase in mucus production. Interestingly, Zhang et al. (18) also reported increased goblet cells following infection of HAE cultures with HPIV3, suggesting that this phenomenon may be common after infection with viruses from the Paramyxoviridae family. The mechanisms of RSV-induced goblet cell hyperplasia/metaplasia remain to be elucidated. However, EGF-EGF receptor interactions and exogenous IL-13 were shown recently to stimulate goblet cell hyperplasia in WD-PBEC and animal models (36–39).

Massive lung infiltration of neutrophils, monocytes, lymphocytes, and, to a much lesser extent, eosinophils is characteristic of severe RSV disease in infants (2, 7, 40, 41). Chemokines released by RSV-infected epithelial cells are undoubtedly important in this infiltration. Indeed, increased expression of IL-6, CCL5, CXCL8, and CXCL10 was evident in bronchoalveolar or nasopharyngeal lavage of infants suffering from RSV infections, compared with age-matched controls (5, 6, 10). Our data on chemokine secretion are consistent with these clinical data. Interestingly, although absolute concentrations differed, and some discrepancies were evident, the 24-h chemokine secretion trends for CCL5, CXCL8, and CXCL10 were similar to those reported by Oshansky et al. (23) at the same time point using primary normal human bronchial epithelial cells from a single donor grown at ALI.

Fig. 6. Basolateral chemokine secretion induced following RSV infection. WD-PBECs were infected as indicated in Fig. 1. Chemokine secretions in the basolateral medium of RSV- and mock-infected cultures harvested at 24, 96, 120, and 144 hpi were measured (n = 5 donors). Because the medium was replaced every day, the data correspond to chemokine secretions within the preceding 24 h. Values are means + SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
In addition to infected cells, these authors demonstrated that exogenously added RSV F and G glycoproteins induced early chemokine responses. The much higher secretions evident from 96 hpi in the present study suggest that virus replication is the principal driver of chemokine expression, although the F and G glycoproteins produced during infection could be responsible.

In summary, our RSV/WD-PBEC model mirrors several characteristics of RSV infection in vivo. Our data also suggest that the RSV strain used to study pathogenesis may influence experimental outcomes and, thereby, our understanding of RSV pathogenesis. In conclusion, our model represents an exciting experimental outcomes and, thereby, our understanding of RSV collagen-coated particulate driver of chemokine expression, although the F and G glycoproteins produced during infection could be responsible.

Materials and Methods

Generation and Infection of WD-PBECs. PBECs were obtained from healthy children undergoing elective surgery (n = 5) (42). The generation and characterization of WD-PBEC cultures were described previously and are outlined in ref. 21. Briefly, primary cells were expanded in collagen-coated flasks until almost confluent and then transferred onto collagen-coated semipermeable (0.4-μm pore size, 12-mm diameter) membrane supports. When confluent, apical medium was removed to create an air–liquid interface (ALI). At 21 d post-ALI, when cultures formed a pseudostratified epithelium, the apical surface was rinsed six times with 500 μL PBS. After the final rinse, and every 24 h thereafter, the apical surface was rinsed with 500 μL PBS for 10 min at 37 °C, and the rinse was harvested, snap frozen, and stored in liquid nitrogen. In parallel, basal medium (500 μL) was harvested and stored at −80 °C until used. The harvested medium was replaced with 500 μL fresh culture medium. Apical rinses and basal medium were used to determine virus growth kinetics and cytokine/chemokine responses. All cultures were monitored daily for CPE by light microscopy.

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Supporting Information

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SI Materials and Methods

Primary Cells, Cell Lines, Viruses, and Virus Growth Curves. Hep-2 cells were kindly provided by Ralph Tripp (University of Georgia, Athens, GA). The prototypic RSV A2 was kindly provided by Geraldine Taylor (Institute for Animal Health, Compton, UK). Our clinical respiratory syncytial virus (RSV) isolate, designated “RSV BT2a,” was isolated from an infant hospitalized with bronchiolitis (1). Cell and virus culture and virus growth curve generation were described previously (1). Primary pediatric bronchial epithelial cells (PBECs) were obtained from healthy children undergoing elective surgery (2). The generation and characterization of well-differentiated (WD)-PBEC cultures were described previously (3, 4). Briefly, the cells were seeded in collagen-coated 10-cm² flasks using Airway Epithelial Cell Basal Medium (C-21260) supplemented with Supplement Pack/Airway Epithelial Growth Medium (C-39160) (Promocell) at 37 °C in 5% CO₂ until confluent. Then the cells were passaged into collagen-coated 75-cm² flasks and expanded at 37 °C in 5% CO₂ for approximately 1 wk. Upon reaching ~80% confluence, the cells were trypsinized and seeded onto collagen-coated, semipermeable membrane supports (12-mm diameter; 0.4-μm pore size; Transwell-Col; Corning-Costar) at 10⁴ cells per well. When confluence was reached, the apical medium was removed, and an air–liquid interface (ALI) was established to trigger differentiation. Cultures were maintained at ALI for 21 d with the basolateral medium changed every second day. The apical side was rinsed with PBS every week to remove excess mucus production. Transepithelial electric resistance (TEER) was determined for each transwell using an epithelial volt ohmmeter (EVOM²) and Endohm chamber (World Precision Instruments), and cilia beating and mucus production were monitored by light microscopy (Nikon Eclipse TE-2000 U). The capacity of cilia to beat in our cultures is demonstrated in Movie S4. Cultures with TEERs ≥300 Ω·cm² and extensive coverage with beating cilia were retained for experimentation.

Infection of WD-PBECs. WD-PBEC cultures from each individual donor (n = 5) were infected in parallel with RSV A2 or RSV BT2a or were mock infected. All infections (multiplicity of infection ~4 in 800 μL) were undertaken in duplicate in WD-PBEC cultures 21 d post-ALI. The inocula or medium-only controls were added to the apical surface of the cultures and incubated for 2 h at 37 °C, 5% CO₂. Subsequently, the inoculum was removed, and the apical surface was rinsed six times with 500 μL PBS. After the final rinse, and even 24 h post infection (hpi). The apical surface was rinsed with 500 μL PBS for 10 min at 37 °C, and the rinse was harvested, snap frozen, and stored in liquid nitrogen. In parallel, 500 μL basal medium was harvested and stored at −80 °C until used. This medium was replaced with 500 μL fresh culture medium. These samples were used to determine virus growth kinetics and cytokine/chemokine responses, respectively. All cultures were monitored daily for cytopathic effects by light microscopy (Nikon Eclipse TE-2000 U). To quantify the number of syncytia per 1,000 infected cells, we calculated the surface area of a single 12-mm-diameter insert (113 mm²) and the surface area of five microscope fields (6.5 mm²). Knowing the average number of infected cells for RSV A2 and BT2a (presented in Table S1) in five microscope fields, we calculated the mean number of infected cells in a single transwell. Thus, on average, 4,207 cells were infected with RSV A2, and 14,360 cells were infected with RSV BT2a. We also determined the average number of syncytia in a single transwell for five individual donors infected with RSV A2 (n = 10) and BT2a (n = 17). Based on the extrapolated mean number of infected cells, an average of 1.18 and 2.38 syncytia per 1,000 infected cells were evident following RSV BT2a and A2 infection, respectively.

Cytospins, Immunofluorescence, and Apoptosis. To determine the consequence of RSV infection on epithelial cell sloughing, the apical surface of dedicated infected and control WD-PBEC cultures were rinsed with 500 μL PBS every 24 h post infection (hpi). Rinses were harvested and cytospins undertaken as previously described (3). Briefly, apical washes were centrifuged at 1,000 x g for 2 min, air-dried for 1 h at room temperature, fixed with 100% ice cold acetone, rinsed with PBS, and stored at −20 °C until used. Dedicated paraformaldehyde-fixed and permeabilized cultures were stained for β-tubulin (a ciliated cell marker), Muc5Ac (a goblet cell marker), and ZO-1 (a tight junction marker), as previously described (3). RSV-infected cells were detected using an anti-RSV F–specific mouse mAb (clone 133–1H conjugated with Alexa Fluor 488, diluted 1:200; Chemicon). Following treatment with DAPI-mounting medium (Vectorshield; Vector Laboratories), fluorescence was detected in the cultures by confocal laser-scanning microscopy (TCS SP5; Leica). Apoptosis was detected using the TUNEL system (Roche) according to the manufacturer’s instructions.

Determination of Cytokine/Chemokine Concentration. To study components of innate immune responses to RSV infection, we selected a limited panel of cytokines/chemokines that were chosen on the basis of strong associations with severe RSV disease in infants (CCL5, CXCL8, CXCL10, and IL-6) (5–9) or that had functions that might help explain, in part, the pathophysiology of RSV (TNF-related apoptosis-inducing ligand (TRAIL)) (10, 11). Apical rinses and basolateral samples were thawed and analyzed for chemokine concentrations using custom Bio-Plex assays (Bio-Rad) targeting CCL5, CXCL10, IL-6, CXCL8, and TRAIL according to the manufacturer’s instructions. Type I IFN concentrations in basolateral medium were measured by ELISA. Human pan–IFN-α (subtypes 1/13, 2, 4, 5, 6, 7, 8, 10, 14, 16, and 17) ELISA kits were purchased from Mabtech, and human IFN-β ELISA kits were purchased from R&D Systems. Type I IFNs were titered according to the manufacturer’s instructions. Limits of detection were 8 pg/mL for the human pan–IFN-α ELISA kit and 25 pg/mL for the human IFN-β ELISA kit.

Statistical Analysis. Data were described as mean ± SEM, and skewed data were log transformed before comparisons were made by Student’s paired t test using Graphpad Prism 5.0. P < 0.05 was considered statistically significant.

Ethics. This study was approved by the Office for Research Ethics Committees Northern Ireland. Written informed parental consent was obtained.


Fig. S1. Apical chemokine secretions induced following RSV infection. WD-PBECs were infected as indicated in Fig. 1. Chemokine secretions in the apical rinses of RSV- and mock-infected cultures harvested at 24 and 120 hpi were measured (n = 5 donors). Because the rinses were performed every day, the data correspond to chemokine secretions within the preceding 24 h. Values are means ± SEM. *P < 0.05, **P < 0.01.
Fig. S2. Type I IFN secretion in basal medium induced after RSV infection. WD-PBECs were infected as indicated in Fig. 1. (A) Pan–IFN-α and (B) IFN-β secretion in basolateral medium of RSV- and mock-infected cultures harvested at 24 and 96 hpi were measured (n = 5 donors). Values are means ± SEM. The limits of detection are indicated by dotted lines.

Table S1. Infected cell count

<table>
<thead>
<tr>
<th>Patient</th>
<th>A2</th>
<th>BT2a*</th>
<th>Ratio BT2a/A2</th>
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<td>519</td>
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<tr>
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Differential infectivity of RSV A2 and BT2a. WD-PBECs were infected and fixed at 144 hpi, as indicated in Fig. 1. They were stained with Alexa 488-conjugated anti-RSV F protein mAb (green), and nuclei were counterstained with DAPI (blue). Slides were observed under a fluorescent microscope, and infected cells were enumerated in five random fields of five cultures derived from five different donors infected with either RSV A2 or BT2a. The average of the five fields was determined for each RSV strain, and the BT2a/A2 ratio was calculated. The five fields represent an area of 6.5 mm².

*P < 0.05 BT2a vs. A2.

Movie S1. WD-PBECs were infected as indicated in Fig. 1 and were stained with DAPI (blue), RSV F protein (green), and β-tubulin (red). This movie shows that RSV A2 and BT2a infect primarily ciliated cells.

Movie S1
**Movie S2.** WD-PBECs were infected as indicated in Fig. 1 and were stained with DAPI (blue), RSV F protein (green), and MUC5AC (red). This movie shows that RSV A2 and BT2a do not infect goblet cells.

**Movie S3.** WD-PBECs were infected as indicated in Fig. 1 and were stained with DAPI (blue) and RSV F protein (green). This movie shows a 3D reconstruction of a syncytium.

**Movie S4.** Mucociliary activity of WD-PBECs. This movie shows the typical movement of mucus at the surface of the cultures caused by cilia beating. It was captured with a phase-contrast microscope (Nikon Eclipse TE-2000 U). (Magnification: 4×; speed: 2×.)