RSV-induced expanded ciliated cells contribute to bronchial wall thickening

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ABSTRACT

Viral infection, particularly respiratory syncytial virus (RSV), causes inflammation in the bronchiolar airways (bronchial wall thickening, also known as bronchiolitis). This bronchial wall thickening is a common pathological feature in RSV infection, but it causes more fatalities in infants than in children and adults. However, the molecular mechanism of RSV-induced bronchial wall thickening remains unknown, particularly in healthy adults. Using highly differentiated pseudostatified airway epithelium generated from primary human bronchial epithelial cells, we revealed RSV-infests primarily ciliated cells. The infected ciliated cells expanded substantially without compromising epithelial membrane integrity and ciliary functions and contributed to the increased height of the airway epithelium. Furthermore, we identified multiple factors, e.g., cytoskeletal (ARP2/3-complex-driven actin polymerization), immunological (IP10/CXCL10), and viral (NS2), contributing to RSV-induced uneven epithelium height increase in vitro. Thus, RSV-infected expanded cells contribute to a noncanonical inflammatory phenotype, which contributes to bronchial wall thickening in the airway, and is termed cytoskeletal inflammation.

1. Introduction

Respiratory syncytial virus (RSV), a single-strand non-segmented negative-sense RNA virus, causes an enormous health burden on the healthcare system worldwide, as it increases morbidity and mortality in certain high-risk groups, including premature infants, infants with underlying medical conditions (such as chronic lung disease of prematurity and hemodynamically significant congenital heart disease), older adults, adults with chronic heart or lung disease, and adults with weakened immune systems (Ackerson et al., 2019; Falsey and Walsh, 2000; Hall et al., 2013; Sommer et al., 2011). Despite decades of research, no approved vaccines or cost-effective therapeutics against RSV are currently available.

RSV pathogenesis starts in the upper respiratory system via infection of airway epithelial cells (AECs) after RSV enters through the nostrils. The virus moves to the lower respiratory system and reaches the bronchioles, where viral replication induces pathophysiological mechanisms. RSV antigen is detected in both upper and lower respiratory track systems (Atherne et al., 1970; Carvajal et al., 2019; Welliver et al., 2007). RSV infection in the lower respiratory tract causes bronchial wall thickening in individuals of all ages (Darras et al., 2015; Kim et al., 2016; Osborne, 1978); however, the molecular mechanism of RSV-induced bronchial wall thickening, particularly in adults, remains unexplored. Bronchial wall thickening (bronchiolitis) is known as the inflammation of bronchioles (Colby,
1998). However, the definition and diagnosis of bronchiolitis remain obscure due to a diverse group of etiologically, clinically, and pathologically dissimilar lesions (Ryu et al., 2003). Based on the microscopic pattern, bronchiolitis is diverse and can be broadly separated into acute and chronic categories (Colby, 1998). While acute viral bronchiolitis may lead to severe bronchiolar epithelium necrosis (Hardy et al., 1988; Visscher and Myers, 2006), common features of RSV bronchiolitis are edema, airflow obstruction, and mucus over-secretion (Hall, 2001). Higher bronchial wall thickness is associated with multiple respiratory complications, including coughing and wheezing (Grydeland et al., 2010). RSV bronchiolitis can increase the risk of developing asthma in children in their later stages of childhood (Openshaw et al., 2003). In addition, RSV infection can be persistent and deteriorate lung function of COPD adults (Sikkel et al., 2008; Wilkinson et al., 2006). Therefore, it is worth to elucidate the mechanism behind RSV-induced bronchial wall thickening.

RSV infection is responsible for virus-induced pathogenesis, which starts with virus entry. Two surface glycoproteins: glycoprotein (G) and fusion (F), play a significant role in RSV entry by host cell receptor binding and fusion, respectively (Hendricks et al., 1987; Walsh and Hruska, 1983). RSV structural proteins, including nucleoprotein (N), phosphoprotein (P), RNA-dependent RNA polymerase (L), and viral polymerase cofactor (M2-1), are involved in viral replication and transcription as well as its regulation, whereas M protein is mainly involved in viral assembly (Talukdar SN, 2022). In contrast, RSV nonstructural proteins, including NS1 and NS2, are not the component of virion but play a significant role in viral pathogenesis by inhibiting Interferon (IFN)-I and III and inducing multiple cytokines and chemokines including RANTES, IL-8, and TNFα (Spann et al., 2004, 2005). The host actin cytoskeleton contributes to the different stages of RSV life cycles, including entry, transcription, replication, assembly, and budding (Paluck et al., 2021). RSV proteins interact with the host actin cytoskeleton and actin-associated or actin-regulatory proteins for its survival. RSV utilizes its ribonucleoprotein complex for replication in the host cytoplasm and a cytoplasmic structure named inclusion bodies formed by multiple viral proteins including N, P, L, and M2-1 (Garcia et al., 1993). The obvious virus-induced modulation of ARF2/3-complex driven actin polymerization was evident in the filopodia-driven RSV cell-to-cell spread (Meherdi et al., 2016, 2017b). However, RSV-induced cytoskeleton changes were evident in the in vitro 2D cell culture model. Thus, it is prudent to determine whether RSV-induced cytoskeleton modulation can be recapitulated in the more relevant 3D cell culture, e.g., air-liquid interface culture.

The aim of this study was to determine the molecular mechanism of RSV-induced bronchial wall thickening. Fort that, we used in vitro adult airway epithelium (3D cell culture), which resembles the epithelial airways in vivo with regard to morphology and functions, including mucus production, ciliary function, and membrane barrier integrity, but lacked immune cells (e.g., dendritic cells, macrophages, and T cells) (Pawlina, 2016; Rayner et al., 2019; Upadhyay and Palmberg, 2018; Villenave et al., 2012; Zhang et al., 2002). We demonstrated that RSV infection expands the actin cytoskeleton and increases the airway epithelial cell layer height in the epithelium. RSV-infected epithelium shows resiliency, despite cytoskeletal expansion; in addition, RSV-induced epithelial height increases through a process termed cytoskeletal inflammation and indicate the existence of a noncanonical epithelial response to RSV infection. Our results suggest that RSV-induced cytoskeletal expansion is a novel mechanism of bronchial wall thickening.

2. Results

2.1. RSV infects ciliated cells, expands actin cytoskeleton, and increases height of the epithelium

To determine the mechanism of RSV-induced bronchial wall thickening in the adult airway epithelium in vitro, we first differentiated NHBE cells of three healthy adults independently for four weeks to form a well-differentiated pseudostatified mucociliary airway epithelium, which contained all three main cell types of the respiratory epithelium (Ozan et al., 2022; Pawlina, 2016; Rayner et al., 2019): epithelial cells extending to the surface with cilia on the apical side, goblet cells containing mucinogen granules, and basal cells, which were confined to the basal portions of the epithelial layers on collagen-coated Transwells (Fig. S1A&B). To confirm whether the passaging of NHBE cells up to four times did substantially change in the transcriptome profile, we collected total RNA from each passage cell and determined the whole-genome transcriptome profile by whole genome RNAseq analysis. We found a few changes in the transcriptome profile passage to passage (Fig. S1C); likewise, the cells retained normal epithelial phenotypic characteristics (Fig. S1B) (Rayner et al., 2019). Additionally, the airway epithelium typically contains adherens, tight, and tricellular junctions (Holgate, 2007; Kojima et al., 2013) that form an impermeable barrier; the presence of these junctions in our airway epithelial model was confirmed by detection of the epithelial intercellular junction protein E-cadherin, the peripheral membrane protein zonula occludens (ZO)-1, and the tricellular junction protein tricellulin (also known as MARVELD2), respectively (Fig. S1D). We then infected this airway epithelium with RSV wild-type (RSV-WT) at a multiplicity of infection (MOI) of 4 for six days, which seems to be a standard duration for research on RSV infection in the airway epithelium (Liesman et al., 2014; Villenave et al., 2012). We compared the RSV-WT-infected airway epithelium with the uninfected (mock-infected) control epithelium under transmission electron microscopy (Yao et al., 2014) and found no evidence of gross cytopathic effects (CPEs) or culture deterioration (Fig. S2A). However, compared to the mock-infected respiratory epithelium, the RSV-infected respiratory epithelium showed a few phenotypic differences, e.g., disorganization of granules in goblet cells, the appearance of higher interdigitation (cell membrane folding between cells), and an increased density of microvilli (Fig. S2A). We confirmed RSV infects primarily ciliated cells by detecting RSV N mRNA in the infected but not in the goblet or basal cells (Fig. S2B). We detected RSV virions on the apical sides of the infected ciliated cells, which suggests that RSV virions bud out from such cells. It appeared that RSV filamentous virion is too large to bud out from a cilia (Fig. S2A, magnified). The possibility that RSV budding independent of cilia from the infected-ciliated cell is supported by a previous report of RSV budding from surface membrane microdomains in the nasal epithelium (Jumat et al., 2015). Therefore, we hypothesized that RSV shedding is independent of cilia in the bronchial epithelium. To confirm RSV shedding independent of cilia from the infected ciliated cells, the RSV-WT-infected (MOI = 0.5) airway epithelium was fixed, permeabilized, and stained for the RSV fusion (F) protein and the ciliated cell marker acetyl-α-tubulin at 3 DPI. Indeed, we found that filamentous RSV virions buds out from the apical sides of the infected ciliated cells and independent of cilia (Fig. S3A). No infectious RSV virions were detected in the basolateral medium by RSV immunoplaque assay, which suggests that virions are released from the apical surface of the airway epithelium; this finding is in line with those in previous reports (Villenave et al., 2012; Zhang et al., 2002). We also confirmed RSV primarily infected ciliated cells (Villenave et al., 2012; Zhang et al., 2002) similar to human metapneumovirus (HMPV) (de Graaf et al., 2013) but in contrast to influenza A virus, which infected both ciliated and goblet cells (Matrosovich et al., 2004) (Figs. S2B and S2C). Expectedly, RSV-infected cell monolayers infected with a variety of different coronaviruses, e.g., severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Middle East respiratory syndrome (MERS), as both viruses infected preferentially goblet cells (Fig. S3D) (Lee et al., 2014, 2012; Ozan et al., 2022). One of the striking differences we found was that RSV-infected cells were substantially enlarged, evident by the expanded actin cytoskeleton (Figs. 1A and S3C). For better visualization of the RSV-induced expanded cytoskeleton, we infected the epithelium with recombinant RSV expressing green fluorescence protein (RSV-GFP)
Fig. 1. RSV-induced expanded-cytoskeleton increases bronchial wall thickening. (A) The airway epithelium was mock-infected or infected with different respiratory viruses (RSV-WT, HMPV, or influenza A virus PR8) independently at an MOI of 0.5 for 3 days. RSV F, HMPV N, or influenza A virus PR8 N were detected by immunofluorescence. F-actin (red) was detected by rhodamine phalloidin staining. The arrowhead indicates expanded actin cytoskeleton in the RSV-infected cell. The scale bar is 5 µm. (B-C) Cell areas and cell perimeters values were determined based on the assessment of rhodamine phalloidin in multiple random areas to cover most cells in the image (at least 36 random cells). The data represent at least two independent experiments. The error bars represent the SEMs. Statistical significance was determined by a two-tailed unpaired t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; NS, Not Significant. (D-F) Epithelium height was measured by confocal Z-stack imaging of mock- or RSV-GFP (MOI = 4)-infected epitheliums at 6 DPI. The data were obtained from 3 donors (Donor 1, 2 and 3). (D-F, Top and Middle) Representative images of mock- and RSV-GFP-infected (MOI = 4) epithelium at 6 DPI obtained from 3 donors. F-actin (red) and nuclei (blue) were stained with rhodamine phalloidin and DAPI, respectively. The scale bar is 15 µm. Yellow capped line and yellow arrow indicate the distance from basal to apical part of epithelium and RSV (RSV-GFP)-infected cell, respectively. (D-F, Bottom) To determine the epithelium height of Donor 1 and Donor 2, data was obtained from two independent experiments and the epithelium height of Donor 3 was determined from one independent experiment. The error bars represent the SEMs. Statistical significance was determined by a two-tailed unpaired t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; NS, Not Significant.
2.2. RSV-infected thickened-airway epithelium is resilient

To determine whether the presence of substantially enlarged infected cells impacted the biophysical properties of the respiratory epithelium, we first assessed the membrane integrity by evaluating the expression/abundance of three junction proteins (E-cadherin, ZO-1, and tricellulin) between the mock-infected and RSV-GFP-infected epithelia at 6 DPI. RSV infection did not reduce the expression of these proteins at 6 DPI (Figs. 2A and S5A–C), indicating that membrane integrity was maintained. To assess whether the membrane permeability of the infected epithelia was stable throughout the infection process, we measured transepithelial electrical resistance (TEER) daily up to 6 DPI. TEER measurements, while very effective and powerful, are subject to variability. To obtain reliable impedance measurements, we subtracted the resistance measurement of a blank. We found that RSV infection did not reduce the membrane permeability of the infected epithelia relative to that of the mock-infected epithelia (Fig. 2B). We then determined whether RSV infection disrupts ciliary motion at the apical surface of the epithelium. Any disruption of ciliary beating may indicate compromised ciliary function and respiratory disease progression. To quantify ciliary beat frequency (CBF), we used high-speed video microscopy with a Leica DMi8 epifluorescence microscope attached to an environmentally controlled chamber and analyzed the video files with Sisson-Ammons Video Analysis (SAVA) software V.2.1.15 (Ammons Engineering, MI, USA). We measured the CBF before and after infection of each Transwell without adding any medium to the apical side of the epithelium. We found that RSV-GFP infection in the airway epithelium did not reduce CBF over time (tested up to 6 DPI), except for a slight decrease at 6 DPI (Fig. 2C). This reduction at 6 DPI probably due to deteriorating culture condition rather than viral increase over time, as RSV infection has been found to persist for longer than 6 days in a human airway epithelium model without obvious cytopathogenesis (Zhang et al., 2002).

To determine whether RSV infection, particularly the presence of substantially enlarged infected cells impacts the biophysical properties of the respiratory epithelium, we assessed the overall viability of the infected epithelium by live-cell imaging from 5 DPI to 6 DPI. No apparent epithelial damage, cell sloughing, or substantial loss of ciliated cells was observed in the RSV-infected airway epithelium. Interestingly, a few RSV-infected (GFP+) cells disappeared from the infected epithelium, which indicates infection resolved or infected-cell damage (Movies S1 and 2). It appeared that RSV-infected cells can survive longer, but the cell-to-cell virus spread is an inefficient process in the respiratory epithelium (Movies S1 and 2). To confirm an increase RSV replication over time in the epithelium for an indication of productive infection, we collected apical wash from the RSV-GFP-infected epithelium, and 10 µg of total protein was subjected to detect RSV structural proteins, such as nucleoprotein (N) and phosphoprotein (P), by Western blotting. We detected both N and P from 4 DPI onward and found that the levels of both proteins were higher at 6 DPI than at 4 DPI (Fig. 2D). The increase in viral protein level in the apical wash of the infected airway epithelium confirmed that RSV replicated in this airway epithelium. Elevated mucus secretion is a hallmark of RSV bronchiolitis (Aherne et al., 1970) that can be recapitulated in vitro airway models (Villenave et al., 2012). We therefore determined mucus production by measuring MUC5B protein levels in apical wash following infection by Western blotting. We found that mucus secretion increased over the course of RSV infection up to 6 DPI (Fig. 2E). We then investigated whether RSV infection induces cytotoxicity in the airway epithelium by quantifying lactate dehydrogenase (LDH) from both apical wash and basal medium every day following infection. We found that LDH release into the basal medium during early infection for the infected samples was comparable to that for the mock-infected control samples, except for a slight increase later during the infection (Fig. 2F). When we measured LDH release in the apical wash, the release was similar throughout the experimental period between the RSV-GFP-infected group and the mock-infected control group (Fig. 2G). Based on the LDH release data, RSV did not induce substantial cytotoxicity in the infected respiratory epithelium. To confirm whether airway epithelium intact after RSV infection, we stained formalin-fixed paraffin embedded RSV-WT infected airway epithelium (at 6 dpi) for RSV N mRNA using a N specific mRNA probe. Indeed, we found that the RSV-infected ciliated cells were sustained without an obvious damage of the epithelium, which is in line with the previous results described above (Figs. 2H and S2B). Overall, these results indicate that the adult airway epithelium shows substantial resilience in response to RSV infection.

2.3. RSV modulates actin signaling pathways in the airway epithelium

To determine the molecular mechanisms mediating the expansion of the actin cytoskeleton in the thickened RSV-infected adult airway epithelium, we determined the global (genome-wide) transcriptional response of the RSV-WT-infected epithelium by RNA-seq analysis at 6 DPI. Briefly, we compared the resulting transcriptome profile with that of the mock-infected epithelia. First, we confirmed the validity of the transcriptome analysis by principal component analysis (PCA) of the transcriptome data, which showed that there was substantial variation between the mock-infected and RSV-infected transcriptome profiles, while there was much less variation within each group (Fig. S6A). Second, we determined and ranked a list of differentially expressed genes. The differential gene expression analysis showed that approximately 3200 genes were upregulated and that a similar number of genes were downregulated in the RSV-infected epithelia compared to the mock-infected epithelia (Fig. 3A and Table S1). Third, functional enrichment analyzes were performed for the differentially expressed genes using clusterProfiler and the signaling pathway impact analysis (SPIA) package from Bioconductor. Gene enrichment analyzes performed using STRING (a protein-protein interaction network database) against the Gene Ontology (GO) dataset for biological processes showed that most of the top 26 biological processes were significantly associated with cytoskeletal regulation except for three, which were l-kappaB kinase signaling that are known to be associated with nuclear factor kB (NF-kB)-driven inflammation (Fig. 3B and Table S2) (Hayden and Ghosh, 2014). Fourth, we generated an enrichment map to identify functional modules. Enrichment maps generally organize enriched terms into networks with edges connecting overlapping gene sets, which tend to cluster together (Khafaf, 2020). Based on the enrichment map, we found a major cluster linked to actin filament organization (Fig. 3C). We further evaluated the RSV-induced modulation of actin cytoskeleton regulation in the respiratory epithelium. We found that several genes of
Fig. 2. Resilience of the RSV-infected respiratory epithelium. (A) At 6 DPI, mock- and RSV-GFP-infected (MOI = 4) epithelium were fixed, permeabilized, and stained with primary Abs against E-cadherin, ZO-1 and tricellulin followed by the respective secondary Abs. The images represent at least two independent experiments. The scale bar is 5 µm. Yellow arrow indicates the expression of E-cadherin, ZO-1 and tricellulin with and without RSV infection (B) The TEER values of mock- and RSV-infected epithelium were measured immediately before infection and at 1, 2, 3, 4, 5 and 6 DPI with a volt-ohm meter. The graph represents data combined from two independent experiments. For each experiment, the data were obtained by combining three independent Transwell reads, and each Transwell read was an average of three independent replicate reads. The green bar represent average read from twelve independent Transwells before infection. The error bars represent the SEMs. (C) The CBFs of mock- and RSV-infected epithelium was measured by high-speed video microscopy followed by quantification using a SAVA system before infection and at 1, 2, 3, 4, 5 and 6 DPI. The graph represents data combined from two independent experiments. For each experiment, the data were obtained by combining three independent Transwell reads, and each Transwell read was an average of reads from six random points. The green bar represent average read from twelve independent Transwells before infection. The error bars represent the SEMs. (D) Apical washes were collected at 2 HPI and at 2, 4, and 6 DPI from RSV-GFP-infected (MOI = 4) epithelium, and RSV N and P protein levels were detected by Western blotting using specific Abs against N and P. (E) Mucin (MUC5B) production was detected by Western blotting (with reducing gels and heat treatment) of apical washes at 2 h post infection (HPI) and at 2, 4, and 6 DPI for mock-infected or RSV-GFP-infected epithelium. (F and G) Basal medium (F) and apical washes (G) from mock- and RSV-GFP-infected (MOI = 4) epithelium was tested for cytotoxicity by LDH assay. (H) RSV N mRNA was detected using RNAscope 2.5 RED assay and anti-RSV N probe. Scale bar is 25 µm.
the actin-regulatory network were substantially upregulated, contributing to the upregulation of actin polymerization (Figs. 3D and S6B-C and Table S3). Thus, RSV preferentially modulates actin signaling pathways, particularly actin-related protein ARP2/3 complex driven actin polymerization, which is in line with previous reports (Mehedi et al., 2017a, 2016).

2.4. ARP2/3 complex-driven actin polymerization contributes to RSV-induced cell expansion

The ARP2/3 complex is one of the three known actin nucleators in eukaryotes, which has a unique capability to organize filaments into branched actin network (Goley and Welch, 2006) for cell expansion (Garcia-Gonzalez et al., 2020) and its activity can be differentially regulated in cells (Gournier et al., 2001). RSV-driven modulation of ARP2/3 complex has already been shown in human lung epithelial A549 cells (Mehedi et al., 2016). Here, we hypothesized that RSV-induced infected cell expansion depends on the ARP2/3 complex-driven actin polymerization. To determine RSV-induced enhanced actin polymerization, we quantified total F-actin / G-actin ratio between mock-infected and RSV-infected airway epithelium at 6 DPI. We found a substantial increase in F-actin levels compared to G-actin levels in the RSV-infected epithelium, and the F-actin/G-actin ratio was higher in the infected epithelium than in the mock-infected epithelium, indicating that higher F-actin levels were correlated with actin cytoskeleton expansion in the RSV-infected epithelium (Fig. 4A and B). The increased actin polymerization mediated by ARP2/3 complex may correlate with the levels of F-actin, which plays a major role in organization of the apical actin cytoskeleton in polarized epithelium (Ivanov, 2008).

Among the actin-regulatory genes, ACTR2 encodes actin-related protein 2 (ARP2), a major constituent of the ARP2/3 complex, which drives branched actin polymerization (Goley and Welch, 2006). In
addition to playing a physiological role in actin polymerization, ARP2 has also been shown to favor RSV spread in vitro by contributing to at least two processes: virus shedding and filopodia-driven cell-to-cell spread (Mehedi et al., 2016). Therefore, we hypothesized that depleting ARP2 would reduce RSV shedding from infected cells. Due to a lack of success in depleting ARP2 in the airway epithelium model, we chose to deplete ARP2 in primary epithelial cells. We found that siRNA mediated ARP2 depletion in NHBE cells, which were the cells used to establish the respiratory epithelium model, was stable and nontoxic (Fig 4C and D). RSV-WT exerted substantial CPEs in the infected NHBE cell monolayer. We optimized an RSV-WT multi-step replicative cycle in NHBE cells by infecting cells with an MOI = 0.5 for three days. The NHBE cell monolayer was treated with either siARP2 or siControl for 48-, 72-, 96- and 120-hours post transfection (HPT). (D) NHBE monolayer cells were transfected with either siARP2 or siControl for 72 h. An alamarBlue assay was performed to determine cell viability at 24, 48, and 72 HPT. (E and F) NHBE monolayer cells were transfected with either siARP2 or siControl for 48 h and then infected with RSV-WT (MOI = 0.5) for three days. The extracellular infectious RSV level was determined by titration of the supernatants from the infected cells. The total RSV level was determined by titration of both the supernatants and cells together. Virus titration was performed using an immunoplaque assay (Mehedi et al., 2016). The data represent three independent experiments, each performed in triplicate. Titrations were performed on each sample in duplicate. The error bars represent the SEM.

Fig. 4. RSV-induced ARP2/3 complex-driven actin polymerization. (A and B) F-actin/ G-actin ratios was determined from the mock- or RSV-GFP infected (MOI = 4) airway epithelium at 6 DPI. (A) Various amounts of G-actin (10, 50, and 100 ng) were loaded for control. G-actin from the pellets (depolymerized F-actin) and supernatants of mock- and RSV-GFP-infected (MOI = 4) epithelium were loaded. G-actin was detected by Western blotting using a G-actin-specific Ab. (B) Bar chart shows the difference of F-actin/ G-actin ratio between mock- and RSV-GFP-infected (MOI = 4) 6DPI. The data were obtained from three independent experiments. The error bars represent the SEMs. Statistical significance was determined by a two-tailed unpaired t-test. *, p < 0.05; **, p < 0.01, ***, p < 0.001; ****, p < 0.0001; NS, Not Significant. (C) NHBE cell monolayer was transfected with either siARP2 or siControl for up to 120 h. Western blotting for ARP2 detection was performed at 48-, 72-, 96- and 120-hours post transfection (HPT). (D) NHBE monolayer cells were transfected with either siARP2 or siControl for 72 h. An alamarBlue assay was performed to determine cell viability at 24, 48, and 72 HPT. (E and F) NHBE monolayer cells were transfected with either siARP2 or siControl for 48 h and then infected with RSV-WT (MOI = 0.5) for three days. The extracellular infectious RSV level was determined by titration of the supernatants from the infected cells. The total RSV level was determined by titration of both (E) the supernatants and (F) supernatants and cells together. Virus titration was performed using an immunoplaque assay (Mehedi et al., 2016). The data represent three independent experiments, each performed in triplicate. Titrations were performed on each sample in duplicate. The error bars represent the SEM.
titration of extracellular RSV obtained from the supernatants of the infected NHBE cells over time (Fig. 4E). However, the total viral titers for both supernatants and cells combined were not decreased by ARP2 depletion (Fig. 4F). These results suggest that the contribution of ARP2 to RSV shedding in the NHBE cell monolayer, which was in line with the result in the previous report of RSV infected A549 cells (Mehedi et al., 2016).

2.5. Robust induction of both cytokines and chemokines during RSV-induced cell expansion

To determine RSV-induced cytokines and chemokines, we collected both apical wash and basal medium separately from mock-infected or RSV-GFP-infected (MOI = 4) epithelium at 2-, 6-, 12- and 24 h post infection (HPI) by repeated sample collections from the same Transwells. Then, we used a commercially available multiplex assay with a LegendPlex Human Anti-Virus Response Panel (13plex combination of cytokines and chemokines) (BioLegend) (Laing et al., 2020). We detected RSV-induced secretion of multiple cytokines and chemokines in both apical wash and basal medium and the higher expression as observed at 24 HPI (Fig. 5A and B). We repeatedly found IL-8 values for both mock and RSV-infection were way beyond the detection scale of the assay (shown in white, Fig. 5A and B). We observed RSV-induced higher secretion of type III IFN lamda 1(IFN-L1), which is well known for its role in antiviral immune activity (Fig. 5A and B) (Hamana et al., 2017). We found that IFN-L1 was induced as early as 6 HPI in the apical sup, where it took 24 HPI to detect in the basal medium (Fig. 5C). The observed delay in the detection of IFN-L1 in the basal medium might

Fig. 5. RSV induces substantial secretion of cytokines and chemokine. A LegendPlex Human Anti-Virus Response Panel was used to analyze both the apical and basal parts of mock- and RSV-GFP-infected (MOI=4) epithelium at early times post infection. The levels of cytokines and chemokines were determined in the apical supernatant (A) and basal medium (B) at 1 DPI. The data represent one independent experiments, each performed in triplicate. The levels of two cytokines IFN lamda-1 (C) and TNF-α (D) and one chemokine IP10/CXCL10 (E) were quantified at early times post infection. The data represent two independent experiments, each performed in triplicate. The error bars represent the SEM.
have correlated with the time required for viral protein production. The apparent low levels of cytokine detection by the assay were more likely due to successive samples (described above) were collected from the same Transwell than to limitations of detection by the kit.

Along with cytoskeletal regulatory signaling, I-kappaB kinase signaling was one of the major biological processes modulated by RSV infection in the airway epithelium model (Fig. 3B). The canonical activation of NF-kb depends primarily on I-kappaB kinase signaling (Liu et al., 2017). To confirm the involvement of NF-kB signaling, we further analyzed our transcriptome data for gene enrichment via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and found that the tumor necrosis factor (TNF) signaling pathway was one of the top pathways modulated by RSV infection (Table S4). Activation of NF-kB by TNF-family cytokines in viral infection is well established (Hayden and Ghosh, 2014; Osborn et al., 1989). We found that several genes of the TNF signaling network were upregulated in our transcriptome data (Fig. S7 and Table S5). Indeed, we found that TNF-α secretion was higher in both the apical wash and basal medium from the RSV-infected epithelium than in those from the mock-infected control epithelium at the early time point (Fig. 5D). However, the detection of TNF-α in both apical wash and basal medium from the mock-infected control epithelium indicated the presence of nonspecific or inherent inductions.

NF-kB activation induces not only proinflammatory cytokines, e.g., TNF-α, but also proinflammatory chemokines, e.g., IP-10 (CXCL10) (Fig. S7) (Liu et al., 2017). We hypothesized that proinflammatory chemokines also contributed to the observed thickening of the RSV-infected airway epithelium. Indeed, we found substantial and robust IP-10 induction in the infected epithelium at the early time point of infection (Fig. 5E). This robust IP-10 induction was also evident when quantified using a different multiplex assay with a LegendPlex Human Proinflammatory Chemokine Panel (BioLegend). The observed delay in the detection of IP-10 in the Basal medium might have correlated with the time required for viral protein production/replication-dependent modulation of IP-10 signaling (Fig. 5E, bottom). Importantly, the RNA-seq data indicated that the RSV-induced IP-10 secretion was sustained at the later time of infection (Table S1). These results suggest that RSV infection induced both proinflammatory cytokines (IFN-L1 and Fig. 6. Neutralizing IP-10 reduces RSV infection. RSV-GFP-infected (MOI = 4) airway epithelia were mock-treated or treated with an IP-10 Ab (5 µg/ml) in the basal medium for 6 days. For the control, mock-infected cells were also treated with the IP-10 Ab (5 µg/ml) for six days. (A) IP-10 neutralization in the airway epithelium. IP-10 was detected in the cells by IP-10 specific mouse mAb by Western blotting. For a loading control, Alpha (A)-tubulin was detected using A-tubulin specific mouse mAb. (B) Reduction of RSV-infected cells. RSV-GFP-infected (GFP +) cells were quantified from Transwells by epifluorescence imaging at 6 DPI. The graph was generated by combining the total counts from two independent Transwells per experiment (two independent experiment). The error bars represent the SDs. (C) RSV phosphoprotein (P) was detected by P specific mouse mAb at 6 DPI. For a loading control, Alpha (A)-tubulin was detected. (D) The western blotting signaling for P was quantified by normalization with A-tubulin. The data obtained from two independent experiments. The error bar shows SD. (E) ARP2 was detected by using ARP2 specific rabbit mAb by Western blotting. For loading control, A-tubulin was detected. (F) ARP2 signals were quantified by normalization with A-tubulin. The data obtained from two independent experiments.
TNF-α) and chemokine (IP-10), whether these cytokines and chemokine contribute to the RSV-induced modulation of actin polymerization for the observed cell expansion remains to be determined.

2.6. IP-10 neutralization reduces RSV infection

It has been known that IP-10 is selectively produced in the human airways upon respiratory virus infection. IP10 and its receptor CXCR3 are crucial for actin reorganization in respiratory epithelial cells (Kelsen et al., 2004). The IP-10 role in actin reorganization in the context of viral infection has also been described (Bonacchi et al., 2001; Poggi et al., 2007). Thus, we wanted to determine whether RSV-driven IP-10 induction contributes to the bronchial wall thickening. We hypothesized that neutralizing IP-10 function with an IP-10-specific antibody (Ab) would modulate actin regulation, thus affecting RSV replication in the airway epithelium. To determine the contribution of IP-10, we neutralized IP-10 function in the airway epithelium model with the IP-10-specific Ab (5 µg/ml in the basal medium) (Kim et al., 2011) immediately after RSV-GFP infection (MOI = 4). First, we confirmed IP-10 neutralization in the airway epithelium by detecting low or undetectable IP-10 in the IP-10 Ab-treated airway epithelium at 6-day post-treatment (Fig. 6A). Second, we confirmed a significant IP-10 induction in the RSV-infected airway epithelium at 6 DPI. Third, we found that IP-10 Ab treatment also reduced IP-10 induction (or neutralization) in the RSV-infected airway epithelium after IP-10 Ab treatment (Fig. 6A). Second, we confirmed a substantial IP-10 induction in the RSV-infected airway epithelium at 6 DPI. Third, we found that IP-10 Ab treatment also reduced IP-10 induction (or neutralized) in the RSV-infected airway epithelium after IP-10 Ab treatment (Fig. 6A). We then quantified RSV-infected cells with or without IP-10 Ab treatment. We found a reduction of RSV-infected cells due to the IP-10 Ab treatment (Fig. 6B). In line with the decrease in RSV infection, RSV phosphoprotein (P) was also reduced (Fig. 6C and D). Next, we sought to determine whether neutralizing IP-10 affected processes related to actin regulation, particularly ARP2 expression. IP-10 neutralization reduced ARP2 levels only in the RSV-infected epithelium (Fig. 6E and F). This reduction might have been related to a decrease in RSV-GFP infection, as IP-10 neutralization reduced RSV spread in the airway epithelium. Thus, IP-10 neutralization reduced RSV infection, which resulted in the reduction of virus-induced ARP2 induction. When we determined the IP-10 neutralization effect directly on different biophysical properties of the epithelium, e.g., ciliary function and membrane integrity, we found IP-10 neutralization increased CBF (Fig. S8A). Expectedly, RSV infection also increased CBF, as shown in Fig. 2C. Interestingly, RSV infection did not modulate IP-10 neutralization-induced increased CBF (Fig. S8A). Similarly, IP-10 neutralization also increased epithelial membrane integrity (Fig. S8B). Expectedly, RSV infection did not change IP-10 neutralization-induced increased TEER (Fig. S8B). These results suggested that IP-10 neutralization increased ciliary function and membrane integrity; however, the mechanism and consequences remain to be determined. Overall, IP-10 neutralization reduced RSV infection in the airway epithelium.

2.7. Robust expression of NS2 during RSV-induced cell expansion

To determine the contributions of viral proteins to the thickening of the RSV-infected airway epithelium, we analyzed RNA-seq data to assess all RSV genes for differential expression, which are available through the GEO under the accession number GSE146795. To quantify RSV gene specific transcripts, the reads from RNA-seq were aligned to the human RSV genome (GenBank accession KT992094) with STAR v2.7.1a (Dobin et al., 2013). We found that most of the genes encoding for structural and nonstructural proteins were substantially upregulated except M2-2 and L (Fig. 7A). Importantly, NS2 transcript was the highest among all the viral transcripts. RSV nonstructural protein 2 (NS2) has already been implicated for RSV-induced pathophysiology (Liesman et al., 2014).
functions, which are in line with previous publications (Pawlina, 2016; Rayner et al., 2019). Previous studies have shown that both early-passage and later-passage primary NHBE cells from different donors can form airway epithelium with similar phenotypic characteristics (Rayner et al., 2019; Villenave et al., 2012; Zhang et al., 2002). In this study, we confirmed that passing NHBE cells up to four times did not substantially change the whole-genome transcriptome profile (Fig. S1C); likewise, the cells retained normal epithelial phenotypic characteristics (Rayner et al., 2019). Additionally, we found that neither passing NHBE cells nor differentiating them into an airway epithelium required either ROCK inhibitors or feeder cells (Osan et al., 2020). In separate studies, we also showed establishing an in vitro airway epithelium by using P4 NHBE cells of a chronic obstructive lung disease (COPD) patient without using of a Rock inhibitor or feeder cells (Osan et al., 2022).

We confirmed RSV infects primary ciliated cells in the adult airway epithelium without any obvious cytopathogenicity, which is in line with previous study (Zhang et al., 2002). We found that filamentous RSV virus budding in the absence of cilia on the surfaces of the infected ciliated cells, in agreement with the observations of other researchers (Coulats et al., 2019). The most striking difference we found was that RSV infection, in contrast to HMPV or influenza virus A infection, expanded the actin cytoskeleton of the infected cells. While virus-induced modulation of actin has been well established in vitro (Chan et al., 2020; Taylor et al., 2011; Walsh and Naghavi, 2019), a functional cytoskeletal system may be dispensable for viral replication (Matthews et al., 2013). Specifically, the involvement of actin in RSV entry (Krzyzaniak et al., 2013), replication (Kallewaard et al., 2005), and spread (Mehedi et al., 2016) in vitro has already been established. RSV-induced changes in the infected cell morphology were shown in the human airway culture of tracheobronchial specimen from a lung transplant patient (Liesman et al., 2014). Here, we show that RSV infection in adult airway epithelium expands the actin cytoskeleton, which consequently increases the epithelial height and may provide a novel explanation of the mechanism of bronchial wall thickening in vivo. Bronchial wall thickening (airway inflammation) is a common finding in lower respiratory tract infections caused by RSV in infants, children, and adults (Kim et al., 2016; Osborne, 1978). RSV-induced epithelial height increment is not always uniform, and it is probably due to a random spatial distribution of the infected ciliated cells and the degree of infection. As the human airway contains a higher percentage of ciliated cells than other types of cells, e.g., goblet cells, RSV-infection-induced cytoskeletal expansion and its contribution to bronchial wall thickening are primarily related to the degree and persistent of infection. Further research is necessary to determine how RSV infection’s spatial and temporal distribution in the bronchiolar airway epithelium contributes to bronchiolitis. SARS-CoV-2 infection apparently does not cause bronchiolitis (Van Bruselen et al., 2021). Whether disparity in cell tropism cause different pathophysiology between SARS-CoV-2 and RSV infections yet to be determined.

Increases in cell size and number along with other factors, e.g., recruitment and activation of immune cells, have been shown to be contributing factors for bronchial wall thickening in chronic lung disease, such as asthma (Bara et al., 2010). Viral infection is known to increase airway inflammation (in acute exacerbation or chronic inflammation), and immune cells are key players in this process. Here, we showed that how epithelial cells particularly RSV-infected ciliated cells, contributed to the height increment of airway epithelium and suggesting an existence of a noncanonical inflammatory phenotype in the airway epithelium. This noncanonical inflammatory phenotype can be explained by RSV-induced epithelial inflammatory phenotype by virus-infected ciliated cells without presence of any classical immune cells (monocyte, dendritic cells and macrophages). Thus, the inflammatory phenotype driven by RSV-induced cytoskeletal expansion without the presence of classical immune cells can be termed cytoskeletal inflammation, which may contribute to bronchial wall thickening – a pathophysiological feature of RSV infection in the lungs (Kim et al., 2016). Thus, RSV-induced cytoskeletal inflammation may shed light on the mechanism of bronchiolitis in infants with immature immune responses (Florin et al., 2017).

The expanded actin cytoskeleton in the RSV-infected airway epithelium model showed strong resilience, as increased viral replication over time did not impact membrane integrity, ciliary functions, or cell sloughing. Although these fatal RSV pathophysiological features can be recapitulated in the pediatric bronchial epithelium (Villenave et al., 2012), they were not recapitulated in the adult airway epithelium, except for elevated mucus secretion. MUC5B expression was significantly higher during RSV infection in this study which also observed in different in vivo and in vitro studies (Fonseca et al., 2009; Lee et al., 2017; Li et al., 2016). We believe that this discrepancy could be due to differences between the pediatric and adult airway epithelium, and it suggests that the airway epithelium in adults combats RSV infection better than those in infants and children. Alternatively, the virus strain, the MOI of the virus inoculum, or the duration of infection (up to six days) may have contributed to the observed discrepancy. However, RSV infection in the airway epithelium can be sustained for longer than six days without any obvious CPEs (Zhang et al., 2002).

We identified potential cytoskeletal, immunological, and viral factors contributing to the cytoskeletal inflammation in response to RSV-induced bronchiolitis. With regard to cytoskeletal factors, we have shown that RSV modulates numerous genes relevant to cytoskeletal signaling, particularly ARPK2/3, a complex of which drives actin polymerization. The higher F-actin in the RSV infected epithelium compared to mock infection confirmed RSV-induced modulation of ARPK2/3 complex dependent actin cytoskeleton signaling. RSV-induced modulation of ARPK2/3 causes infected cell expansion in the airway epithelium, which is different from RSV-induced filopodia induction in A549 cells (Mehedi et al., 2016). Neither synctia formation nor cell-to-cell spreads was evident in the RSV infected airway epithelium, but both are common in the RSV infection in A549 cells (Mehedi et al., 2016). Although it is obvious that RSV modulates ARPK2/3 complex-driven actin cytoskeleton signaling in both 2D (A549 cells) and 3D (airway epithelium) culture models, the observed phenotypical differences are probably due to the generic difference between the models. No or less synctia in the RSV-infected airway epithelium resembles clinical feature of RSV infection (Johnson et al., 2007). Importantly, ARPK2/3 complex driven actin polymerization requires in RSV replication has been evident both lung epithelial A549 cells and primary NHBE cells. Thus, our result provides further evidence in support of the role of ARPK2 in RSV replication cycle (Mehedi et al., 2016). With regard to immunological factors, we found that RSV infection induces robust secretion of both cytokines (particularly, IFN-L1 and TNF-α) and chemokine (particularly, IP10/CXCL10). RSV-induced higher IFN-L1 level was also observed in previous studies (Groves et al., 2019; Hillyer et al., 2015). We also found a substantial induction of proinflammatory cytokine TNF-α, which is part of the NF-kB signaling pathway and is considered the central activator of inflammation and innate immunity in response to RSV infection (Haeberle et al., 2002; Liu et al., 2017; Yoboua et al., 2010). In our studies, the most reliable and robust chemokine induction was IP10/CXCL10 and IL8 (above the saturation of detection limit), which we quantified by using two independent commercially available panels: a Human Anti-Virus Response Panel and a Human Proinflammatory Chemokine Panel. The results were consistent with previous reports of in vivo and clinical studies (McNamara et al., 2005; Osahony et al., 2010; Villenave et al., 2012). In addition, multiple studies reported IP-10 and IL8 are the most abundant chemokines caused by RSV infection (Machado et al., 2017). Our data were also in line with previously reported upregulation of IP-10 and IL8 due to RSV infection in vitro (Coulter et al., 2017). It has been known that IP-10 can selectively be produced in the airway epithelium after respiratory viral infections (Alves et al., 2013). Rhinovirus infection induces IP-10, a biomarker for virus-induced asthma exacerbation (Wark et al., 2007). Our results
aligned with previous clinical findings that IP-10 induction in RSV infection (Luo et al., 2012; McNamara et al., 2005). We found that RSV directly induces IP-10 in the infected airway epithelium. We also found that RSV increased ARP2/3-driven actin polymerization. However, our results do not explain how RSV-induced IP-10 contributes to the higher ARP2/3-driven actin polymerization, resulting in the thickening of the infected airway epithelium. We are currently investigating to find a direct interaction between IP-10 and actin rearrangement, specifically ARP2/3-driven actin polymerization, to elucidate the mechanism of RSV-induced cytoskeletal inflammation, a proposed noncanonical mechanism of RSV bronchiolitis. However, neutralization of IP-10 reduced RSV spread in the infected airway epithelium. Thus, further studies must confirm whether IP-10 neutralization can effectively treat reduced RSV spread in the infected airway epithelium. We are currently investigating to find a ARP2/3-driven actin polymerization, resulting in the thickening of the infected airway epithelium. With regard to viral factors, we found that NS2 is predominantly expressed in the RSV-infected epithelium, which is in line with a previous report of NS2 contribution to cell rounding phenotype (Liesman et al., 2014). Nonstructural protein-mediated actin rearrangement during RSV infection is not uncommon, rather several other bacterial and viral non-structural proteins demonstrated their interaction with actin cytoskeleton and their stimulating role for host cellular actin remodeling (Armer et al., 2008; Bamia et al., 2020; Furnon et al., 2019; Liu et al., 2022; Skoble et al., 2009; Zeng et al., 2022). RSV-infection induces actin polymerization for increasing epithelial layer as we hypothesized whereas most of the abovementioned viruses have negative impact on actin polymerization for their growth. Our result demonstrated RSV infection stimulates higher TNFα secretion and higher F-actin/G-actin ratio indicating substantial actin polymerization which is identical with the previous study showing the induction of TNFα during airway inflammation was associated with the increase of total actin concentration and F-actin/G-actin ratio (Dogan et al., 2017). NS2-deleted re-combinant RSV infection showed reduced NF-κB activation and lower TNFα production compared to wild-type RSV infection (Spann et al., 2005). The possibility of NS2’s contribution to increase actin polymerization can be predicted because Listeria ActA protein driven ARP2/3 complex activation has already been characterized (Skoble et al., 2000). NS2’s role in the RSV-induced ARP2/3-complex-driven actin polymerization remains to be determined. Additionally, we found that RSV structural proteins, particularly N, P, M, and F, are essential for viral replication and are highly expressed. M- or F-induced F-actin interactions or modulation, respectively can be established in vitro, suggesting that these proteins may play a role in RSV-induced cytoskeletal expansion (Mehedi et al., 2016; Shahriri et al., 2018).

In conclusion, we found that RSV infects primarily ciliated cells in the adult airway epithelium and showed a noncanonical inflammatory phenotype which resembles bronchial wall thickening. This RSV-induced cytoskeletal inflammation is due to upregulation of ARP2/3 complex driven actin polymerization, and robust induction of proinflammatory cytokines (IFN-1 and TNF-α) and chemokine (IP10/ CXCL10), and robust secretion of viral proteins, particularly NS2. The thickened airway epithelium shows resilience, which supports the existence of a noncanonical mechanism of bronchial wall thickening due to RSV infections in adults.

4. Materials and methods

4.1. Primary cells, cell line, and virus

Primary NHBE cells (from 3 healthy nonsmoker adult donors) (deidentified) were provided by Dr. Kristina Bailey, at the University of Nebraska Medical Center (UNMC), Omaha, NE under an approved material transfer agreement (MTA). Vero cells (an African green monkey kidney epithelial cell line, ATCC-1586) and the viruses RSV-WT (A2 strain, GeneBank ID 2992094) and RSV-GFP (in which the GFP gene was inserted between the P and M genes of RSV-WT) were obtained from Dr. Peter Collins at NIH. Both viruses were grown separately in Vero cells and sucrose-purified using density-gradient ultracentrifugation. Virus stocks were stored at -80 °C and titrated by immunoplaque assay using a cocktail of three monoclonal Abs specific to RSV F (Le Nouen et al., 2009; Mehedi et al., 2016). HMPV was obtained from Dr. Peter Collins at NIH. The influenza A virus PR8 strain was obtained from Dr. Nadeem Khan at UND.

4.2. Primary cells subculture

NHBE cell monolayer subculturing was performed in a 100 mm culture dish (Corning, Inc.). Briefly, a culture dish was coated with PureCol (Advanced Biometries) before seeding of cryopreserved (-80 °C) passage-one (P1) NHBE cells (approximately 10⁶ cells), which were thawed in a 37 °C water bath. The cells were maintained in complete AEC growth medium (PromoCell) containing AEC supplement (PromoCell), 2% penicillin/streptomycin (Thermo Fisher Scientific) and 1% amphotericin B (Thermo Fisher Scientific) at 37 °C in a 5% CO₂ incubator.

4.3. Air-liquid interface culture

Transwells (6.5 mm) with 0.4 µm-pore polyester membrane inserts (Corning Inc.) were coated with PureCol for 20 min before cell seeding. NHBE cells (5 x 10⁴) suspended in 200 µl of complete AEC medium were seeded in the apical part of each Transwell. Then, 500 µl of complete AEC medium was added to the basal part of the Transwell. When the cells formed a confluent layer on the Transwell insert, the AEC medium was removed from the apical insert, and PneumaCult-ALI basal medium (Stemcell Technologies) with the required supplements (Stemcell Technologies), 2% penicillin/streptomycin and 1% amphotericin B was added to the basal chamber. The ALI medium in the basal chamber was changed every other day. The apical surface was washed with 1x Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) once per week initially but more frequently when more mucus was observed on later days (when it was difficult to see the apical cells, it was determined that the difficulty was probably due to a thick layer of mucus). All cells were differentiated for up to four weeks (at 37 °C with 5% CO₂ until the desired cellular and physiological properties of an epithelial layer were obtained, such as a CBF greater than 6 Hz and a TEER greater than 500 ohm/cm².

4.4. Virus infection

The four-week-cultured highly differentiated pseudostratified airway epithelia were washed with 200 µl of 1x DPBS to remove mucus and infected on the apical side with sucrose-purified RSV-WT, HMPV, or influenza virus (A/Puerto Rico/8/1934(H1N1)) at an MOI of 0.5 in 1x DPBS for two hours (at 37 °C with 5% CO₂). The viral inoculum was then removed, and the epithelia were washed twice with 200 µl of 1x DPBS. Fresh ALI medium (500 µl) with supplements was added to the basal part of each Transwell, and the apical part was kept empty. Mock-infected (1X DPBS without virus) and RSV-infected Transwells were incubated for three days at 37 °C in an incubator. A similar approach was used to infect the epithelia with RSV-GFP or RSV-WT at an MOI of 4 for six days. SARS-CoV-2 or MERS infection in the airway epithelium was described previously (Osin et al., 2022).

4.5. Confocal microscopy

The airway epithelium (apical side) was washed with 1xPBS, and
both the apical and basal parts were fixed with 4% paraformaldehyde (PFA) (Polysciences, Inc.) in 1x PBS for 30 min at room temperature (RT). The epithelium was then washed twice with 1x PBS and blocked with a 10% goat serum solution in immunofluorescence (IF) washing buffer (130 mM NaCl, 7 mM Na$_2$HPO$_4$, 3.5 mM NaH$_2$PO$_4$, 7.7 mM Na$_2$SO$_4$, 0.1% BSA, 0.2% Triton-X 100 and 0.05% Tween-20) for 1 h at RT. After 2 washes with 1xPBS, the Transwell inserts were incubated with one of the following primary Abs in IF wash buffer overnight at 4 °C: anti-ZO-1 rabbit polyclonal (1:200) (3195S, Cell Signaling Technologies), anti-MUC5B rabbit monoclonal (1:500) (HPA008246, Atlas Antibodies), anti-MARVELD2/ Tricellulin rabbit polyclonal (1:100) (48-8400, Thermo Fisher Scientific), anti-acetyl-$\alpha$-tubulin rabbit monoclonal (1:500) (5335, Cell Signaling Technologies), anti-MUC5AC mouse polyclonal (1:100) (H00004586-A01, Abnova), anti-Respiratory Syncytial Virus F mouse monoclonal (1:100) (ab43812, Abcam), anti-Metapneumovirus N mouse monoclonal (1:25) (MCA4674, Bio-Rad), or anti-Influenza A nucleoprotein (NP) mouse monoclonal (1:30) (MCA400, Bio-Rad). The next day, after washing with the wash buffer, the inserts were incubated with an anti-rabbit Alexa Fluor 647-conjugated secondary Ab (1:200) (Thermo Fisher Scientific) and anti-mouse Alexa Fluor 488-conjugated secondary Ab (1:200) (Thermo Fisher Scientific) in IF wash buffer for 3 h in dark at 4 °C. The cells were washed 2 times with 1xPBS and then incubated with rhodamine phalloidin (PHDR1; 1:500) (Cytoskeleton Inc.) in IF wash buffer for 30 min at RT in the dark. After 2 washes with 1xPBS, the cell nuclei were stained with NucBlue Fixed Cell Stain ReadyProbes Reagent (4’,6-diamidino-2-phenylindole, DAPI) (Thermo Fisher Scientific) for 30 min in the dark at RT. The Transwell inserts were washed with 1xPBS, and then the entire membrane was removed from each well and placed on a microscope slide (TechMed services). ProLong Gold antifade reagent (Thermo Fisher Scientific) was used to mount coverslips on the slides. Images were captured using a confocal laser scanning microscope (Olympus FV3000) with a 60x objective. The 405-nm laser was used to excite DAPI for nucleus detection; the 488 nm laser was used to excite Alexa Fluor 488 (RSP F staining) or GFP for RSV detection; the 561 nm laser was used to excite rhodamine phallolidin for F-actin detection; and the 640 nm laser was used to excite Alexa Fluor 647 for MUC5B, acetyl-$\alpha$-tubulin, E-cadherin, ZO-1, or tricellulin detection. At least three independent fields were chosen for imaging from each experiment. Slides prepared from more than two independent experiments were imaged. Imaris software version 9.5.1 (Oxford Instruments Group) was used for the conversion of Z-stack images (.oir format) to .tiff format and for additional image postprocessing.

### 4.6. Paraformaldehyde (PFA)-fixation, paraffin embedding, and RNAscope

The airway epithelium (apical side) was washed with 1xPBS, and both the apical and basal parts were fixed with 4% paraformaldehyde (PFA). The PFA-fixed airway epithelium was then paraffin-embedded and sectioned at 5 µm (Osan et al., 2022, 2021). 5 µm section slides were first deparaffinized by incubating them following solutions in a coplin jar: 1. Histo-Clear for 5 min, x2; 2. 100% ethanol for 5 min, x3, 5% ethanol for 5 min, x1; 4. 70% ethanol for 5 min, x1; and 5. distilled water for 5 min, until the next step. The deparaffinized slides were immediately incubated in 0.5% TritonX-100 solution in 1x PBS for 30 min in a coplin jar. The slides were washed three times with 1x PBST (1x PBS with Tween 20) for 5 min. A hydrophobic barrier was drawn around the 5 µm section on the slides by using an Immedge Hydrophobic Barrier Pen. To reduce nonspecific antibody binding, the section was blocked 10% goat serum solution (Vector Laboratories) in 1x PBST for 2 h at room temperature. The slides were then incubated with anti-RSV N mRNA probe (V-RSV-NP-01) and RNAscope 2.5HD Detection kit (RED) according to manufacturer’s instruction (Advanced Cell Diagnostics). The section slide was undergone H&E staining, followed by mounting it on a Tech-Med microscope slide using prolong gold mounting medium (Thomas Scientific).

### 4.7. Transmission electron microscopy

Airway epithelium cultured on 6.5 mm Transwell membranes were fixed for 1 h at RT in 2% PFA /2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The membranes were then removed from the inserts, rinsed with 0.1 M cacodylate buffer, postfixed on ice for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 1.5% potassium ferrocyanide, dehydrated in a graded series of ethanol solutions (50, 70, 95, and 100%), embedded in EMBed (Electron Microscopy Solutions, Inc.) and sectioned at 80-nm thickness in a direction parallel to the Transwell membrane. Thin sections were stained with 2% alcoholic uranyl acetate and 0.2% lead citrate and imaged using a Hitachi H-7500 transmission electron microscope.

Cell area and cell perimeter measurements: The cell area and perimeter were determined for both RSV-GFP- and mock-infected cells via Fiji (Schindelin et al., 2012) with the plugin PaCeQuant (Moller et al., 2017) with a slight modification. Briefly, confocal images of the mock-infected cells were input into the Fiji program and converted into 8-bit grayscale images. The images were then input into the PaCeQuant plugin from the MiToBo plugin set, which segmented the images into cells and calculated the cell area and perimeter in multiple random areas, covering most cells of the image (at least 36 random cells). The RSV-GFP-infected cells were subjected to the same process as the mock-infected cells, with some differences. Whereas the mock-infected cell data were generated directly from the PaCeQuant plugin, the RSV-GFP-infected cells were analyzed only using the segmentation feature of PaCeQuant for successful demarcation of infected cells. The data for the RSV-infected cells, which were defined as those exhibiting GFP fluorescence, were then extracted from the segmented images of GFP-positive cells via the Magic Wand tool and the Region of Interest. The data represent at least two independent experiments.

### 4.8. Epithelial layer thickness measurement

The airway epithelial layer thickness was evaluated using Imaris software. Confocal images (Z-stack, 60x oil objective) of the mock-infected or RSV-GFP-infected epithelium was evaluated for thickness using the 3D View tab in the IMARIS software program. The visible apical surface was stained for F-actin with rhodamine phallloidin (Texas Red), and the visible basal surface (bottom) was stained with DAPI. Height was then measured from the merged image (F-actin and DAPI) using the Magic Wand option. Briefly, an Ortho Slicer tab was created and set to the XZ plane. After rotating the slice into view and selecting a desired slice in the Ortho Slicer tab, a Measurement Points tab was created. In the Edit tab, the “intersects with” option was changed to “surface of object”, and in the setting tab, the line mode was changed to “pairs.” After switching back to the Edit tab of Measurement Points, two points were placed by holding the shift button on the keyboard and selecting a point on the apical layer and a point on the basal layer that were presumed to be in line on the X plane. To ensure that the two selected points were directly in line on the X plane, the XYZ position that appeared when one of the points was selected was altered, if necessary, to match the other point’s X position. The line between the two points automatically calculated a distance in microns. This generated 5 Z thickness values for each image. At least two images were quantified per treatment from each experiment. The data were then plotted in GraphPad Prism to compare epithelial height between mock-infected and RSV-infected epithelium. To determine the thickness of only GFP-infected cells, the above method was repeated for only the RSV-GFP-infected images, but rather than choosing random slices and apical and basal points, the measurements were intentionally chosen to include only infected epithelial areas. To do this, the FITC channel, which represented the RSV-GFP-infected cells, was turned on along with the DAPI.
and Texas Red channel. The slices and apical and basal points were selected to directly measure the epithelial height of the tissues at the locations of the RSV-GFP-infected cells.

4.9. F-actin/G-actin ratio determination

Protein samples from ALI cultures were collected at 6 DPI and infected with RSV-GFP at an MOI of 4. Briefly, the apical part of a Transwell was washed twice with 1xPBS, and then all the cells were scraped out and transferred into a 1.5 ml tube. The cells were pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C after removal of the supernatant, 110 µl of LAS2 buffer was prepared according to the protocol of a G-actin/F-actin in vivo assay Biochem Kit (Cytoskeleton, Inc.) and mixed with the pellet. The mixture was transferred into a QIAshredder tube (Qiagen) for centrifugation at 15,000 rpm for 3 min at 4 °C in a tabletop centrifuge and then incubated at 37 °C for 10 min. Then, 100 µl of the eluate was transferred into a Beckman 11 mm ultracentrifuge tube (Beckman Coulter) for ultracentrifugation in an Optima TLX ultracentrifuge (Beckman Coulter) at 60,000 rpm for 1 h (37 °C). 100 µl of supernatant was transferred to a new tube, and 100 µl of F-actin depolymerization buffer was added and mixed with the pellet. Both tubes were kept on ice for 1 h, and then 25 µl of 5x SDS was added to each tube. Equal amounts of supernatant and pellet samples (15 µl) and G-actin protein standards (10, 50, and 100 µg) were separated on 4–12% Bis-tris SDS polyacrylamide gels, and the separated proteins were transferred by dry blotting onto polyvinylidene fluoride (PVDF) membranes according to the manufacturer’s instructions (Thermo Fisher Scientific). After transfer, the membranes were blocked in TBST/5% nonfat milk (10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.01% Tween 20/5% nonfat milk) for 30 min at RT and then washed in TBST 3 times for 10 min each per wash at RT. After blocking and washing, the membranes were incubated with the primary Ab (anti-rabbit antibody polyclonal Ab) supplied with the G-actin/F-actin in vivo assay Biochem Kit (diluted 1:500 in TBST/0.1% nonfat milk) for 1 h at RT and then washed 3 times in TBST for 10 min per wash at RT. The membranes were incubated with a secondary goat anti-rabbit HRP Ab (Sigma-Aldrich) diluted 1:5000 in TBST/0.1% nonfat milk 1 h at RT and then washed 5 times in TBST for 10 min per wash at RT. The proteins were detected with a chemiluminescent substrate according to the manufacturer’s instructions (GE Healthcare). The membranes were processed for chemiluminescence detection of actin (43 kDa) using an Azure C600 system (Azure Biosystems, USA).

4.10. Live-cell imaging

Airway epithelium cultured on 6.5 mm Transwell membranes was infected with RSV-GFP (MOI=4) at the apical side (described above). At 5 DPI, live-cell SiR-actin (Cytoskeleton, Inc.) was added to the complete medium at the basal side, and the epithelium was cultured for six hours before imaging using a 20x objective under a Leica DMi8 inverted fluorescence microscope equipped with an environmentally controlled chamber to maintain 37 °C and 5% CO2. Images were taken every 5 min to capture the epithelial tissue (phase-contrast), RSV-GFP (AF488), and F-actin (Cy5) for approximately 16 h. LASX software (Leica Microsystems) installed in the microscope was used to create a movie file.

4.11. Membrane permeability assay

The differentiated epithelial layer permeability was determined by measuring TEER. TEER was measured using an epithelial mult-ohm meter (EVOM2, World Precision Instruments, Inc.). The EVOM2 was calibrated according to the manufacturer’s instructions, and the STX2 electrode was sterilized with 70% ethanol before use. The internal electrode (smaller in size) was placed in the apical part of each Transwell (PBS was added during TEER reading), and the external electrode (larger in size) was placed in the basal part of the Transwell, which contained ALI basal medium, to measure the membrane voltage and resistance of the epithelial layer. An empty Transwell insert (filled with 1xPBS) containing no NHBE cells was used to correct for background resistance. Three readings were taken for each Transwell. The TEER value of each sample was calculated by subtracting the background value.

4.12. Ciliary function assay

Ciliary beating on the apical surfaces of cells in the differentiated epithelial layer was quantified by determining the CBF. Briefly, cilia were visualized in phase-contrast mode with a 20x objective on a Leica DMi8 microscope. For each Transwell, 6 different random fields were recorded for 2.1 s at 120 frames per second. The images were captured at 37 °C and analyzed using the SAVA system V.2.1.15 to determine the CBF in Hz.

4.13. Western blotting

Protein samples were collected from the airway epithelium infected with RSV-GFP at an MOI of 4 at 6 DPI. Briefly, the apical part of a Transwell was washed 2 times with 1xPBS, and then scraped to collect all the cells, which were transferred into a 1.5 ml tube. The cells were pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C. After removal of the supernatant, the cells were collected into a QIAshredder tube and incubated for 1 min at RT with 100 µl of gel-loading buffer made from 2.5 ml of 4x LDS loading buffer (Thermo Fisher Scientific), 1 proteinase inhibitor tablet and 7.5 ml of PBS. The cell mixture was centrifuged at 15,000 rpm for 3 min at 4 °C. The eluate from the QIAshredder tube was stored at –20 °C in a freezer. A similar approach was used to collect protein samples from NHBE monolayers in a 24-well plate. The protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific). To detect ARP2, total protein (10 µg) was separated on 4–12% Bis-tris SDS polyacrylamide gels (nonreducing), and the separated proteins were transferred by dry blotting onto PVDF membranes according to the manufacturer’s instructions (Life Technologies). Similarly, to detect NS2, we run 10 µl of apical wash of RSV-GFP-infected transwell on a 4–12% Bis-tris SDS polyacrylamide gels (nonreducing), and the separated proteins were transferred by dry blotting onto PVDF membranes according to the manufacturer’s instructions (Life Technologies). For E-cadherin, Tricellulin (MARVELD2), MUC5B, respiratory syncytial virus nucleoprotein, respiratory syncytial virus phosphoprotein detection, samples were denatured at 90 °C for 10 min with a 10x reducing agent (Thermo Fisher Scientific) before electrophoresis on 4–12% Bis-tris gels. α-tubulin and GAPDH were used as loading control. For all analyses, the PVDF membranes were incubated in Li-COR blocking buffer (1:1 in 1x PBS) (Li-COR Biosciences) for 1 h at RT and then overnight at 4 °C with a primary Ab (1:1000) in Li-COR blocking buffer. The membranes were washed 3 times for 5 min with PBS and then incubated with a secondary Ab (1:15,000) (Li-COR Biosciences) for 1 h at RT. After washing 3 times for 5 min with 1xPBS, fluorescence was analyzed using an Odyssey imaging system (Li-COR Biosciences). To detect IP-10, A-tubulin, RSV P, RSV NS2, ARP2, E-cadherin, and MUC5B, mouse monoclonal antibodies including anti-IP-10 (Cat# MAB-23,819, Thermo Fisher Scientific), anti-A-tubulin (Cat#T6199, Sigma-Aldrich), anti-P (Cat# ab94965, Abcam), rabbit monoclonal antibodies including anti-ARP2 (Cat# ab129018, Abcam), anti-E-cadherin (Cat# 3195S, Cell Signaling Technologies), anti-MUC5B (1:500) (Cat# HPA008246, Atlas Antibodies), anti-NS2 (rabbit mAb, custom made by ABclonal) were used, respectively followed by a goat anti-rabbit IRDye800-labeled secondary Ab (Cat# 926-32,211, Li-COR Biosciences). To detect GAPDH and Tricellulin (MARVELD2), rabbit polyclonal antibodies including anti-GAPDH (Cat# GP8,495, Sigma-Aldrich) and anti- MARVELD2 (Cat# 48-8400, Thermo Fisher Scientific), were used, respectively followed by a goat anti-rabbit IRDye800-labeled secondary Ab (Cat# 926-32,211, Li-COR Biosciences). To detect α-tubulin, respiratory syncytial virus nucleoprotein and respiratory syncytial virus phosphoprotein, mouse monoclonal antibodies including
anti-α tubulin (Cat# T6199, Sigma-Aldrich), anti-respiratory syncytial virus nucleoprotein (Cat# ab94806, Abcam) and anti-respiratory syncytial virus phosphoprotein (Cat# ab94965, Abcam) were used, respectively followed by a goat anti-mouse IRDye680-labeled secondary Ab (Cat# 926-68-070, LI-COR Biosciences). Image Studio 5.2 (LI-COR Biosciences) was used to quantify protein signal.

4.14. Cell cytotoxicity determination

40 µl of apical wash (1x PBS) and basal medium from airway epithelia mock-infected or infected with RSV-GFP (MOI = 4) at 6 DPI were collected and assessed for cell cytotoxicity with a commercially available Cell Cytotoxicity Detection Kit (LDH). 40 µl of sample was mixed with 40 µl of freshly prepared reaction mixture and incubated for 30 min at RT. PBS and basal medium were used for the low control and for high control the cells were treated with 2% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature, and the absorbance of the samples was measured at 490 nm by using a BioTek Synergy HT (BioTek Instruments). The cytotoxicity percentage was calculated by using the following equation: cytotoxicity = (experimental value – low control/ high control – low control) x 100.

4.15. RNA extraction

Airway epithelium cultured on 6.5 mm Transwell membranes was washed and treated for 1 min at RT with RLT buffer (Qiagen) with 0.01% β-mercaptoethanol (Sigma-Aldrich). The cells were scraped using a cell scraper, collected into a QIASHredder tube and centrifuged at 15,000 rpm for 3 min at 4 °C. The eluate was used to extract total RNA using a Total RNA/RNA Extraction Kit (Qiagen), and DNase I treatment was performed to remove DNA from the samples according to the manufacturer’s instructions. The RNA concentration was determined with an Epoch instrument (BioTek Instruments).

4.16. RNA-seq for transcriptome analysis

RNA was extracted (as described above) from ALI cultures at 6 DPI after mock infection or infection with RSV-WT at an MOI of 4. Preliminary quality control analysis of the FASTQ files was performed with FastQC v0.11.8 (Andrews, 2010). The adapters were trimmed using Trimmomatic v0.39 (Bolger et al., 2014). The reads were aligned to the human reference genome (hg19) with STAR v2.7.1a (Dobin et al., 2013). Gene expression was quantified using CuffNorm v2.2.1 (Trapnell et al., 2010). The read counts were summarized using featureCounts v1.4.6 (Liao et al., 2014). Differential expression analysis of RSV- vs mock-infected samples was performed using the R/Bioconductor package DESeq2 v1.24.0 (Love et al., 2014). A gene was considered differentially expressed if it had an FDR of 0.05 or less. GO and KEGG pathway enrichment analyses were performed using the R/Bioconductor package clusterProfiler v3.12.0 (Yu et al., 2012). A GO term or KEGG pathway was considered enriched if it had a p-value of 0.05 or less, a Benjamini-Hochberg adjusted p-value of 0.05 or less. GO and KEGG pathway enrichment analyses were performed using the R/Bioconductor package clusterProfiler v3.12.0 (Yu et al., 2012). A GO term or KEGG pathway was considered enriched if it had a p-value of 0.05 or less, a Benjamini-Hochberg adjusted p-value of 0.05 or less.

4.17. siRNA transfection and RSV infection

NHBE cells were transfected with siARP2 (s223082, Thermo Fisher Scientific) or a nonspecific sControl (Silencer Select Negative Control #2, Thermo Fisher Scientific). Briefly, 2 × 10⁵ cells were seeded in PureCol-coated 24-well plates and incubated overnight at 37 °C in a CO₂ incubator. The cells reached approximately 70% confluency the next day. The cells were transfected with an siRNA transfection reagent (Thermo Fisher Scientific), and 7.5 µl of siRNA (2 µM) for 48 h at 37 °C. Over 80% ARP2 knockdown was achieved, as confirmed by Western blotting. For RSV-WT infection, the medium was removed, and 100 µl of virus inoculum (prepared in complete AEC medium) (RSV-WT, MOI=0.5) was added to the NHBE monolayer. The mixture was incubated for one hour at 37 °C. The virus inoculum was then removed from the well, and the cells were washed twice with complete AEC medium and incubated in complete AEC medium at 37 °C in a CO₂ incubator for 72 h.

4.18. Cytokine and chemokine measurements

We collected samples from the apical and basal parts of mock-infected and RSV-GFP-infected (MOI = 4) respiratory epithelial cultures at 2, 6, 12 and 24 h post infection. Apical washes and basal medium were collected to determine chemokine induction in the epithelium. To analyze the concentrations of cytokines (such as TNF-α) and chemokines (such as IP10/CXCL10), a LegendPlex Human Anti-Virus Response Panel were used according to the manufacturer’s directions (BioLegend). Briefly, 20 µl of supernatant from each sample was added individually to a well of a 96-well V-bottom plate. 10 µl of analyte beads were added to each well, and the plate was incubated at RT with shaking at 800 rpm on a plate shaker for two hours while covered in aluminum foil. After incubation, the beads were washed twice for 5 min per wash by centrifugation at ~250x g at RT with the included wash buffer diluted to 1x in Milli-Q deionized water. After the second wash, the beads were resuspended by shaking at 800 rpm for 1 min, and then biotinylated Abs were added to each well. The plate was shaken at 800 rpm for one hour at RT while covered in aluminum foil. After one hour, 10 µl of SA-PE provided with the kit (BioLegend) was added to each well, and the plate was again shaken at 800 rpm while covered in aluminum foil for 30 min at RT. The beads were then washed twice with 1x wash buffer and run on a BD FACs Symphony flow cytometer (BD Biosciences) under the default settings by following the manufacturer’s instructions (BioLegend). The data were analyzed using LegendPlex software (BioLegend), and the concentration of IP10/CXCL10 was determined by comparing the mean fluorescence intensity of each sample supernatant to its respective standard curve, which was generated by following the manufacturer’s instructions (BioLegend). Data were analyzed using the LegendPlex data analysis software v.8 for Windows.

4.19. Cell viability assay

NHBE cell monolayer viability was evaluated using a resazurin-based alamarBlue assay (Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, a 10% volume of alamarBlue was added to the cell culture medium and incubated at 37 °C for 3-4 h. To evaluate cell viability, alamarBlue fluorescence, a marker for metabolic activity, was analyzed using a Synergy 2 Multi Mode microplate reader (BioTek).

4.20. RSV transcript quantification

To quantify transcript expression in RSV-infected cells, reads were aligned to the human RSV genome (GenBank accession KT992094) with STAR v2.7.1a (Dobin et al., 2013). The read counts were summarized using featureCounts v1.4.6 (Liao et al., 2014). The FPKM values were calculated using the fpkm function in DESeq2 v1.24.0 (Love et al., 2014).

4.21. IP-10 neutralization

The highly differentiated airway epithelium was mock-infected or infected with RSV-GFP (MOI=4) described earlier. For IP-10 neutralization, the epithelium was treated with the IP-10-specific Ab (5 µg/ml in the basal medium) immediately after RSV-GFP infection and maintained the epithelia for six days. The basal medium with IP-10 Ab was changed on alternate days. For the control group, we treated the mock-infected airway epithelium similarly. Both CBF and TEER were measured every day, as described earlier. In addition, IP-10, RSV P, and ARP2 were...
detected separately using Western blotting, as described earlier.

4.22. Statistical analysis

Parameters such as the number of replicates, number of independent experiments, mean +/- Standard error of mean (SEM), and statistical significance are reported in the figures and figure legends. A P-value less than 0.05 was considered to indicate significance. Where appropriate, the statistical tests and post hoc statistical analysis methods are noted in the figure legends or Methods section.

Author contributions

M.M. conceived the project and designed the experiments. K.B. provided the primary cells as well as training and guidance on primary cell differentiation. M.M., S.N.T., and J. O. performed the experiments. B.G. generated and analyzed the TEM images. S.N.T. and S.Y. performed cell differentiation. M.M., S.N.T., and J. O. performed the experiments. M.M. edited the paper. S.N. edited the paper.

Declaration of Competing Interest

The authors declare that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2023.199060.

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