

# Tonic and early interferons defend against respiratory viruses in primary human lung organoid-derived air-liquid interface cultures

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**ABSTRACT** Innate defenses of the respiratory epithelium are the first barrier against incoming respiratory viruses. To understand the contribution of both basal (tonic) and induced interferon (IFN) to antiviral defenses in a physiologically relevant system, we established air-liquid interface (ALI) cultures of primary human bronchial epithelium (HBE) and small airway epithelium (HSE). Via an organoid intermediate stage, the limited healthy donor material was expanded while preserving stemness, and subsequently differentiated. Characterization by immunofluorescent profiling and transcriptomic analyses showed that the cellular diversity and architecture of our ALI cultures were comparable to native human lung epithelium. Infection with human rhinovirus (HRV16) induced a strong and early IFN response, whereas human coronavirus (HCoV-229E and HCoV-NL63) infection caused a more subdued and delayed response. HRV16, but not HCoV-229E, infection was eventually cleared from the cultures after more than 30 days. Depletion of tonic or early type I/III IFNs using neutralizing antibodies or scavengers increased infectious HRV production by ~10- or ~1,000-fold, respectively, suggesting a role of IFNs in clearance. Taken together, we present a method for generating primary lung epithelial air-liquid interface cultures that retain effective IFN responses, demonstrate clearance of HRV by innate defenses, and highlight the importance of tonic and early IFN.

**IMPORTANCE** Mild respiratory viral infections, for example, with human common cold coronaviruses or rhinoviruses, are a massive cause of human morbidity. The respiratory tract is the primary entry route for these viruses and also the contact site for initial innate immune defenses. Here, we show that primary human lung epithelial cell-derived air-liquid interface cultures mimic the architecture and cell composition of native human lung epithelium, and retain both induced and tonic interferon (IFN) responses. Notably, our data show that the model's innate immune defense, characterized by rapid and robust IFN responses, are sufficient to clear human rhinovirus (HRV) infections but not human coronavirus 229E. Finally, depletion of induced or tonic IFNs led to a marked increase in HRV infection. Thus, our data suggest that tonic low levels of IFNs contribute to the epithelial defense against viruses, maintaining the tissue's immune readiness.

**KEYWORDS** air-liquid interface models, coronavirus, rhinovirus, tonic interferon, lung epithelium, virus

Infections of the respiratory tract are a significant burden to human health, and consistently ranked among the top 10 leading causes of death worldwide over the last few decades (1). Endemic respiratory viruses, such as the human rhinoviruses (HRVs)

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and the human coronaviruses (HCoV-229E and HCoV-NL63), are major contributors to seasonal colds. Together, these viruses represent the most common cause of respiratory infections, making them among the most frequent viral encounters (2). While they mainly cause mild infection in healthy adults; children, immunocompromised, and elderly individuals may develop a severe course of the disease (3–6). The entry site for these respiratory pathogens is the airway epithelium, a thin continuous layer of specialized epithelial cells that line the respiratory tract. The majority of HRV strains infect cells via the intercellular adhesion molecule-1 (ICAM-1); a small group also uses the low-density lipoprotein receptor (LDLR) or the cadherin-like family member 3 (CDHR3) (7–10). Common cold coronaviruses infect cells via angiotensin-converting enzyme 2 (ACE2) (e.g., HCoV-NL63) or aminopeptidase N (e.g., HCoV-229E) as entry receptors that are expressed throughout the airway epithelium (11, 12).

Besides the upper conducting airways, the lower respiratory epithelium serves as a first barrier of immune defense against invading viruses, ideally enabling their rapid clearance with minimal impact on the host (13). It is mainly composed of ciliated cells, secretory cells, goblet cells, basal cells, club cells, and minor populations of pulmonary neuroendocrine cells, pulmonary ionocytes, hillock cells, and tuft cells (14). Ciliated cells are the most abundant and are responsible for the movement of mucus and trapped particles via coordinated ciliary motion. Secretory cells and goblet cells, interspersed among ciliated cells, produce mucus, which forms a protective layer that traps dust, pathogens, and other particulate matter. While the mucus layer serves as a physical barrier, the ciliary motion promotes mechanical clearance of mucus and trapped particles from the respiratory tract. Basal cells serve as stem cell progenitors, capable of differentiating into other epithelial cell types to maintain tissue integrity and regeneration (14–16). Additionally, epithelial cells secrete antimicrobial peptides, such as defensins and lysozyme. The epithelial cells also express pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs), leading to the release of pro-inflammatory cytokines, such as interferons (IFNs). Secretion of IFNs induces the expression of IFN-stimulated genes (ISGs), many of them with well-known anti-viral functions, setting the cells in an anti-viral state (17–19). Of note, whereas type I and II IFNs (e.g., IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ) are associated with systemic anti-viral responses, type III IFNs (IFN- $\lambda$ ) were proposed to have a more specialized role in protecting mucosal surfaces (20–23).

Recent studies have highlighted tonic or basal IFN as a means to maintain “immune readiness” in the absence of overt infection, shaping the basal innate immune defenses and inflammatory state of the airway epithelium (24). These tonic interferons, primarily type I IFNs, such as IFN- $\alpha$  and IFN- $\beta$ , maintain a low-level transcriptional program that primes cells for rapid antiviral responses while also contributing to immune homeostasis (25–28). Decreased levels of tonic IFNs have been associated with increased susceptibility to respiratory viruses, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and dysregulation of tonic IFNs has been implicated in autoimmune diseases, chronic inflammation, and tumor immune evasion (29–31). Despite their importance for defense, the impact of tonic IFNs on anti-viral defenses at the airway epithelium remains poorly understood. A major limitation is that most studies rely on immortalized cell lines or 2D-cultured primary bronchial epithelial cells, which do not accurately recapitulate the defense responses of the human airway epithelium. In recent years, human primary airway epithelial cells cultured at the air–liquid interface (ALI) have emerged as a powerful tool for studying respiratory host–pathogen interaction (32–34). Even though some challenges, such as donor availability, variability, and a short lifespan, come along with these models, they closely mimic the physiology of the human airways, providing complex and human-focused *in vitro* models for development of novel therapeutic approaches (35–37). To overcome these bottlenecks with existing techniques, we established here a protocol to generate ALI cultures from normal, commercially available human primary lung epithelial cells (37, 38). To overcome the limitations of cell and donor availability, we generated lung organoids that retain stemness and differentiated

them at ALI, making them similar to native human bronchial epithelium regarding cell composition and architecture. Using this model, we here show that HRV, which induces the highest level of initial IFNs, is cleared from the cultures after ~4 weeks. Furthermore, depletion of tonic type I and type III IFN reduces basal ISG levels and weakens epithelial defenses against HRV by more than 10-fold. Thus, our data show that tonic IFNs contribute to the defenses of the respiratory epithelium.

## RESULTS

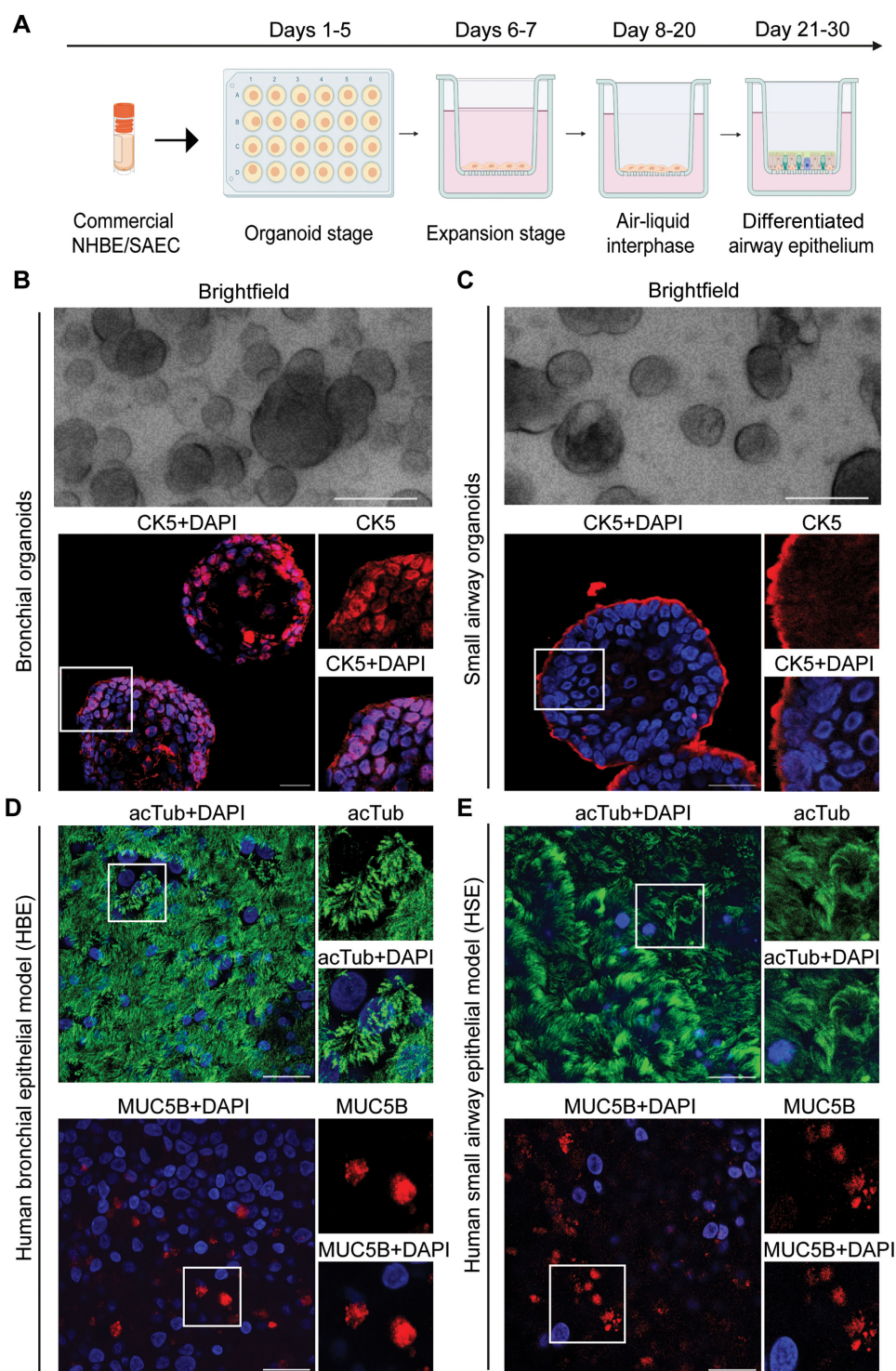
### Establishing an organoid-intermediate primary ALI culture lung model

Current primary cell-derived ALI cultures of lung epithelium are limited by the availability of donor material, and cells beyond passage three rarely differentiate (39). To address this limitation, we developed a method to amplify primary cells while retaining their stemness via an organoid intermediate state (37). Cells derived from the organoids could either be cryopreserved for long-term use, passaged up to 12 times, or directly seeded onto transwell inserts for ALI culture generation (Fig. 1A and Fig. S1A). In brief, commercially available primary normal human bronchial epithelial cells or small human airway epithelial cells were grown from an early passage with BME type II and organoid growth medium. On the fifth day, the cells displayed an organoid-like, rounded morphology and stained positive for cytokeratin 5 (CK5), identifying them as basal cells (Fig. 1B and C). To assess the potential of these continuously passaged basal cells to differentiate into ALI cultures, the organoids were dissociated into single cells and seeded onto transwell inserts, allowing them to expand and form a monolayer (Fig. 1A). Afterward, the apical medium was removed to create an air-liquid interface. The cultures derived from NHBE and SAEC organoids differentiated into a human bronchial epithelium (HBE) or human small airway epithelium (HSE), respectively. Whole mount staining of acetylated tubulin (acTub) shows kinocilia present on the ciliated cells across the apical surface, and MUC5B staining indicated the presence of secretory cells (Fig. 1D and E). Trans-epithelial electrical resistance (TEER) measurements from ALI cultures generated from low and highly passaged organoids confirm their capacity to form an intact barrier. The TEER values ranged from 500 to 900  $\Omega\cdot\text{cm}^2$ , which is within physiological levels (Fig. S1B). Lung organoids up to passage 12 still showed a mucociliary phenotype, suggesting that they differentiated (Fig. S1A). Live-cell imaging demonstrated the presence of functional cilia, characterized by the typical synchronized movement of Dynabeads Protein G particles (Video S1).

Taken together, these data demonstrate that ALI cultures were successfully differentiated from human primary bronchial epithelial cells or small airway epithelial cells via organoid-intermediate state, which allows easy amplification of the source material without diminishing its differentiation potential.

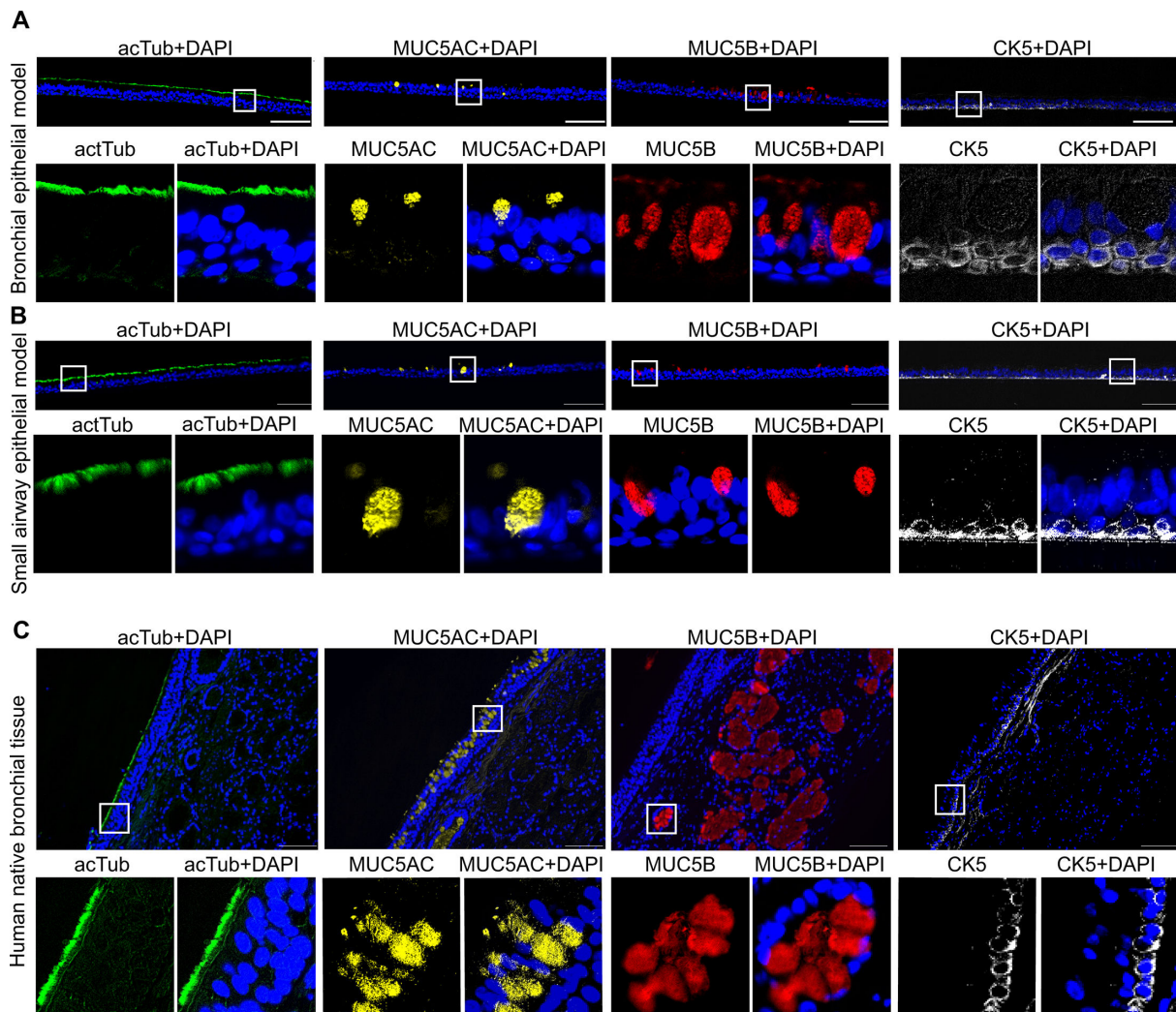
### Organoid-intermediate ALI cultures resemble the architecture and composition of native human lung epithelium

The respiratory epithelium is a pseudostratified columnar epithelium, with the columnar ciliated cells framed on the apical side, interspaced by mucus-producing goblet and secretory cells, with a final basolateral layer formed by basal cells (32). Z-axis sections of formalin-fixed paraffin-embedded (FFPE) of HBE ALI cultures confirmed the presence of kinocilia on the ciliated cells (acTub) on the apical surface. The major gel-forming mucins are MUC5AC and MUC5B secreted by goblet (MUC5AC) and secretory cells (MUC5B) (40). In HBE, the MUC5AC<sup>+</sup> and MUC5B<sup>+</sup> cells cluster toward the apical side (Fig. 2A). Finally, CK5<sup>+</sup> basal cells were exclusively found on the basal side of the epithelium (Fig. 2A). A similar polarized epithelial architecture was evident for the HSE ALI cultures, where ciliated cells, goblet, secretory, and basal cells were stained using their respective cell markers (Fig. 2B). To determine how closely our models resemble native human lung epithelium, we compared the presence and spatial organization of cellular markers detected in ALI cultures to native human bronchial tissue sections. The tissue was



**FIG 1** Generation and characterization of human airway epithelial models from primary human lung epithelial cells. (A) Schematic representation of the workflow of human airway epithelial model generation via organoid intermediate using human primary lung epithelial cells. (B–C) Representative immunofluorescence and brightfield images of bronchial and small airway organoids in brightfield microscopy or confocal scanning microscopy of formalin-fixed paraffin-embedded (FFPE) 5- $\mu$ m sections stained for basal cells (CK5+, red). Nuclei, DAPI (blue). Scale bars, 100  $\mu$ m (brightfield microscopy) and 25  $\mu$ m (confocal microscopy). (D–E) Representative laser scanning confocal microscopy images of differentiated human bronchial and small airway epithelial models stained for ciliated cells (acTub+, green) and secretory cells (MUC5B+, red). Nuclei, DAPI (blue). Scale bars, 25  $\mu$ m.





**FIG 2** Cellular marker comparison of human airway epithelial models to native human bronchial tissue. (A) Representative laser scanning confocal microscopy images of 5-μm FFPE z-sections of the human bronchial epithelial model. Stained for ciliated cells (acTub+, green), goblet cells (MUC5AC+, yellow), secretory cells (MUC5B+, red), and basal cells (CK5+, gray). Nuclei, DAPI (blue). Scale bars, 100 μm. (B) Representative laser scanning confocal microscopy images of 5-μm FFPE sections of human small airway epithelial models stained with acTub+ (green), MUC5AC+ (yellow), MUC5B+ (red), and CK5+ (gray). Nuclei, DAPI (blue). Scale bars, 100 μm. (C) Representative laser scanning confocal microscopy images of 5-μm sections of native human bronchial tissue stained with acTub (green), MUC5AC (yellow), MUC5B (red), and CK5 (gray). Nuclei, DAPI (blue). Scale bars, 100 μm.

obtained from a 48-year-old patient who underwent a partial lung resection due to pulmonary metastasis of a colorectal carcinoma. The section not only comprises the epithelial layer but also the underlying connective tissue, blood vessels, and submucosal glands, thus appearing thicker. Similar to the ALI cultures, the *ex vivo* tissue contains all the expected major cell types of the bronchial epithelium in largely similar spatial arrangement: acTub-positive kinocilia present in ciliated cells, MUC5AC-positive goblet cells, MUC5B-expressing secretory cells, and CK5-positive basal cells (Fig. 2C). However, the native tissue shows site-specific mucin expression: MUC5AC-producing cells are found in the superficial epithelium, whereas MUC5B-producing cells are identified in the submucosal glands (41, 42). In ALI models, these cell populations are both present in the apical compartment, since glands cannot be mimicked in a transwell-based setup. Qualitative analysis further suggested that the native tissue had a higher number of mucus-secreting cells compared with the ALI models. This is likely due to the fact that cells used to generate ALI models were obtained from healthy donors, and the native

tissue was taken from a tumor patient, which is often characterized by excessive mucus secretion due to inflammation (41).

Nonetheless, these data indicate that the primary architecture of both the HBE and the HSE models is largely similar to that of the native human lung.

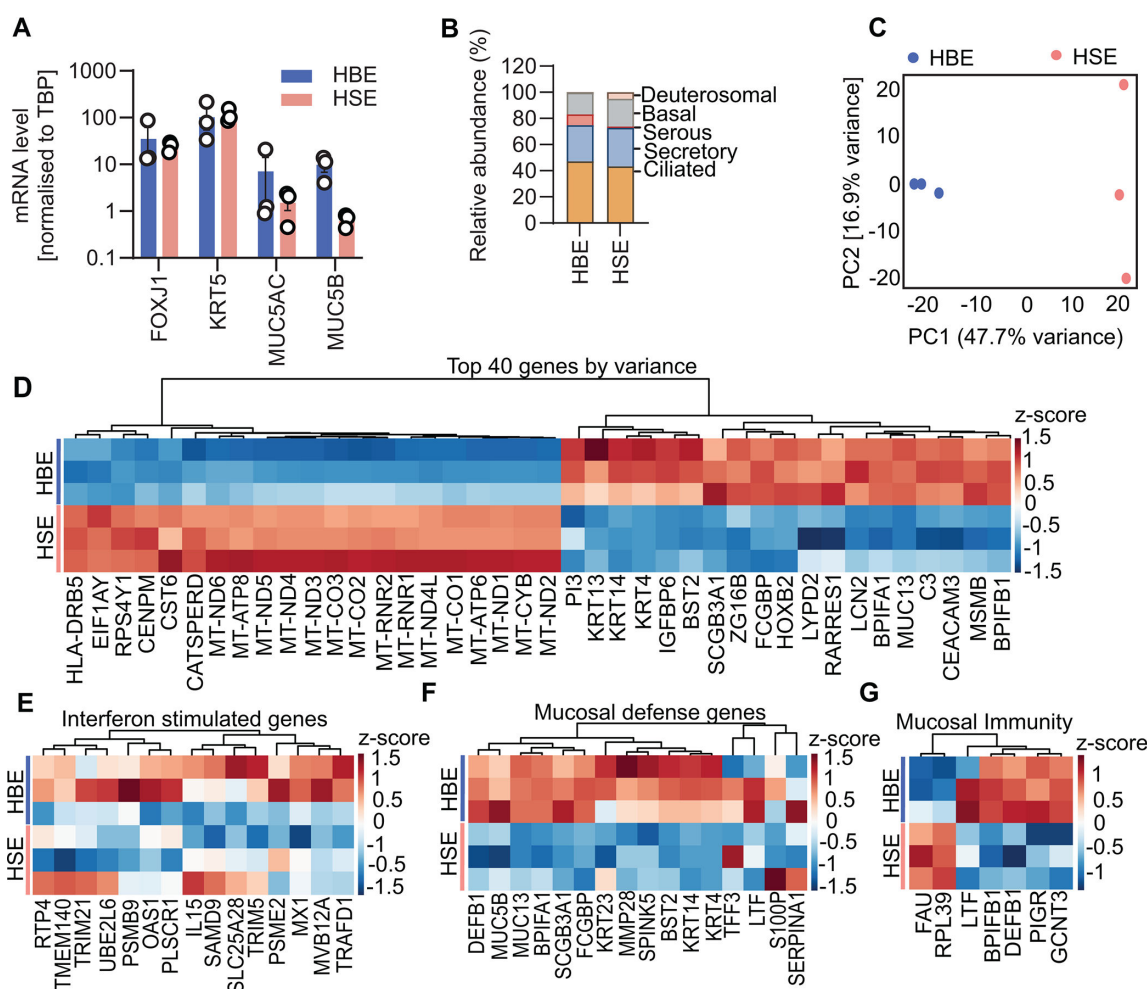
### HSE and HBE models express respiratory epithelium-associated genes

Different cell types of the human airway epithelium are characterized by specific gene expression patterns (14). Expression of *FOXJ1* is characteristic of ciliated cells, whereas *KRT5* expression marks basal cells. *MUC5AC* and *MUC5B* are expressed by goblet and secretory cells, respectively. Both the HBE and HSE models show marker gene expression assessed by qPCR, indicating successful differentiation (Fig. 3A). To comprehensively evaluate the expression patterns of cell-type-specific and respiratory epithelium-associated genes in the HSE and HBE models, we mapped bulk RNAseq across three individual inserts to the healthy lung atlas (Data S1) (14, 43). Cibersort-assisted analyses indicated the presence of transcriptional patterns from various cell types, including ciliated, basal, goblet, and secretory cells (Fig. 3B, Fig. S2A and Data S2). The majority of cells were ciliated, followed by secretory and basal cells. Additional cell types, such as serous cells found in submucosal glands, and intermediate cell populations, such as deuterosomal cells, reported as precursors of ciliated cells, which are otherwise difficult to distinguish using immunofluorescence, were also detected using RNAseq. By selectively clustering ciliated, secretory, serous, deuterosomal, and basal cell types, the ratios of the major cell types in the models were determined (Fig. 3B and Data S3), indicating that overall cell composition was similar between both models. However, principal component analysis of the data suggested that the gene expression profile still differs significantly between HBE and HSE models (Fig. 3C). This is also illustrated by the top 40 genes by variance (Fig. 3D and Data S4). Gene ontology analyses revealed that genes highly expressed in the HSE model are largely part of the mitochondrial respiratory chain, whereas those associated with the HBE model are innate immune and antimicrobial defense genes (Fig. S2B and Data S5). In line with visualization of basal-level expression of ISGs, mucosal defense, and immunity-associated genes, the HBE model shows higher expression of several prominent defense genes, including defensins, well-known anti-viral ISGs (TRIM5, tetherin/BST-2), and mucins (Fig. 3E through G and Data S5).

Taken together, the transcriptome analyses confirm successful differentiation of both models and indicate that the HBE exhibited elevated expression of defense factors, suggesting a more activated state than the small airway epithelium.

### Rhinovirus activates strong and early cytokine defenses in HBE models

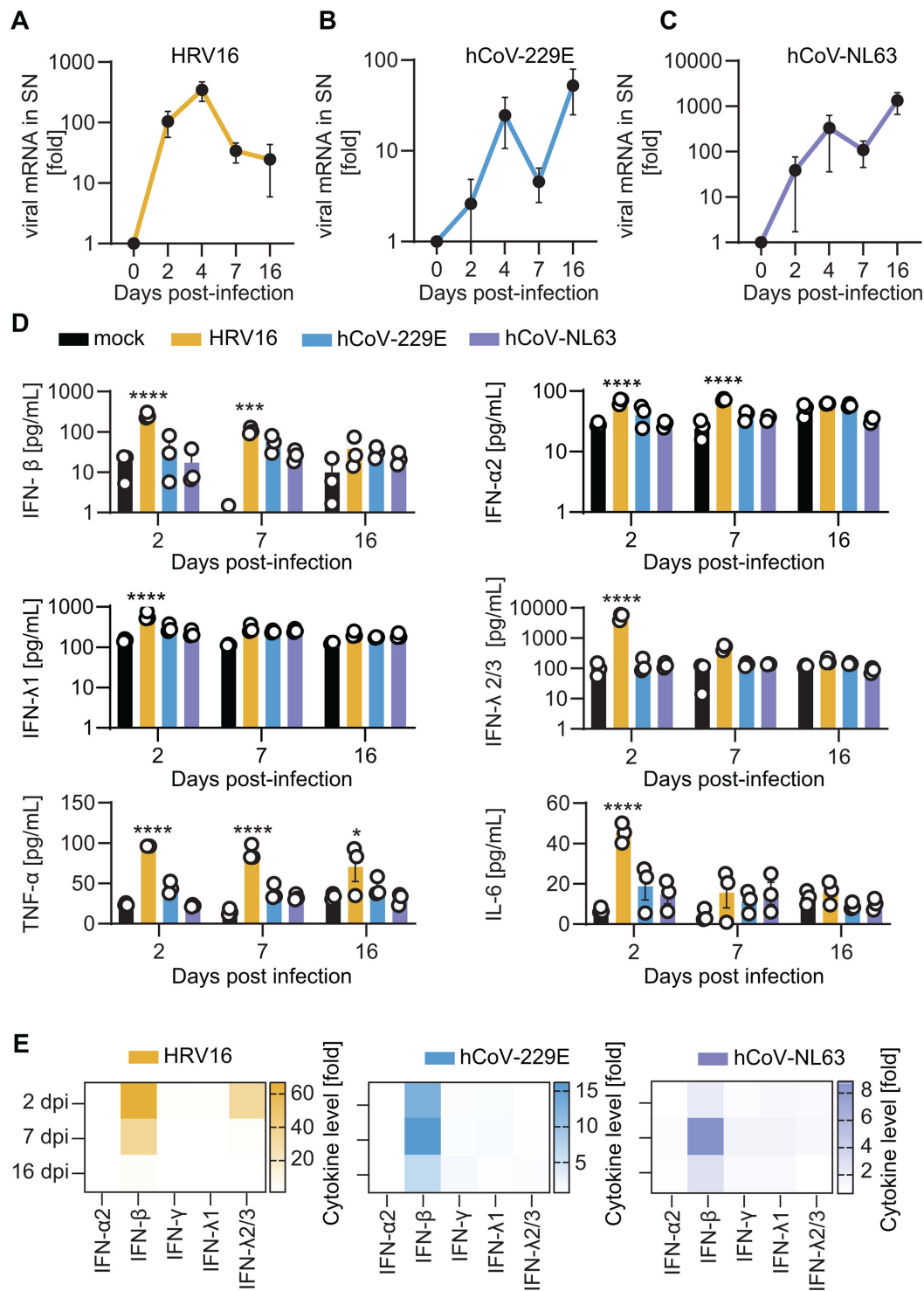
To analyze the interplay between respiratory virus infections and the innate immune system of the lung epithelium, we infected the HBE model with three clinically prevalent respiratory viruses: human rhinovirus A (HRV16) and the human coronaviruses HCoV-229E and HCoV-NL63. The HBE model was chosen because it represents the entry tissue for these three viruses. Quantification of viral RNA in apical supernatants showed that all three viruses productively infected the models (Fig. 4A through C). Next, HRV16-infected cultures were treated with rupintrivir (Rup 3  $\mu$ M), and 229E- and NL63-infected cultures were treated with remdesivir (Rem 10  $\mu$ M) or camostat mesylate (CM 20  $\mu$ M) starting 4 h post-infection. Rupintrivir significantly reduced HRV16 RNA levels by ~200-fold at day 4 (Fig. S3A). Both remdesivir and camostat mesylate inhibited 229E by ~20-fold and NL63 by ~300-fold (Fig. S3B and S3C). Of note, HRV16 RNA in the supernatant peaked around day 4 and declined thereafter until 16 dpi (Fig. 4A). In contrast, 229E and NL63 RNA remained at high levels up to day 16 post-infection, suggesting that an equilibrium was reached resembling a quasi-persistent infection (Fig. 2B and C). None of the infections were accompanied by large-scale cell death or visible ciliary cessation (Videos S2 through S4). To analyze temporal differences in cytokine release during infection, we used a multiplex cytokine assay. HRV16, but not the two coronaviruses, induced a strong cytokine response, including significantly higher levels of IFN- $\alpha$ , IFN- $\beta$ ,



**FIG 3** Characterization of human airway epithelial models at RNA level. (A) mRNA levels of major epithelial marker RNAs, as indicated, assessed by qPCR. Ciliated cells (FOXJ1), basal cells (CK5), goblet cells (MUC5AC), and secretory cells (MUC5B). Normalized to housekeeping gene TBP. The bars represent the mean of  $n = 3 \pm \text{SEM}$  (independent experiments). (B) Transcriptomic data of human airway models mapped to the human healthy airway atlas to identify cell types. The data show the relative abundance of individual cell types from three independent human bronchial and small airway models aggregated from the CIBERSORTx output (Data 2). (C) Principal component analysis (PCA) of the data in panel B. (D) Heatmap representation (colored by z-score) of the top 40 genes by variance showing distinct gene expression patterns between the human bronchial and small airway epithelial models. (E–G) Heatmap representation (colored by z-score) of representative basal type I IFN-stimulated genes (E), mucosal defense genes (F), or mucosal immunity genes (G) in the expression cluster in the human airway models. Levels of the individual biological triplicates are shown.

IFN- $\lambda$ 2/3, IFN- $\lambda$ 1, IL-8, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IP-10 especially at early timepoints (Fig. 4D and Fig. S4). Cytokine responses against both coronaviruses were generally much lower. For example, whereas IFN- $\beta$  was induced ~60-fold upon HRV infection 2 days post-infection, HCoV-229E and HCoV-NL63 induced only 12-fold or 3-fold, respectively. To analyze the temporal induction of cytokines, we calculate fold inductions relative to the respective mock controls. The cytokine responses against HRV16 peaked on day two and then declined. In contrast, the HCoV infections induced not only a weaker but also a more delayed response, peaking at day 7 post-infection (Fig. 4E).

Taken together, HRV16 infection is accompanied by a strong and early cytokine response, whereas the innate immune response against the human coronaviruses was much more subdued.



**FIG 4** Human bronchial epithelial models support common cold respiratory viral replication and enable long-term monitoring of infection kinetics. (A–C) Quantification of the viral RNA levels by qPCR of HBE models infected from the apical side (MOI 0.01, indicated viruses) for 3 h. At indicated days post-infection, apical washes were collected. Dots represent the mean of  $n = 3 \pm \text{SEM}$  (independent experiments). (D) Absolute cytokine levels as assessed by cytokine bead array (Legendplex) of the models in panel A. Bars represent the mean of  $n = 3 \pm \text{SEM}$  (independent experiments). Two-way ANOVA with Tukey's multiple comparisons test. \*\*\*\* $P < 0.0001$ , \*\*\* $P = 0.0003$ , \* $P = 0.03$ . (E) Heatmap depiction of type I, II, and III IFN data in panel D separated by virus and calculated as fold-change relative to control. The data are shown as mean of three independent experiments.



## Human bronchial epithelial models self-clear HRV16 in long-term cultures

In humans, early and strong IFN responses are considered protective against pathogens, whereas late and weaker responses are associated with more severe disease and may even contribute to pathological symptoms (44, 45). To understand the consequences of the differential innate immune response against HRV and HCoV-229E, we followed their replication over 35 days. Curiously, long-term follow-up of the HCoV-229E showed that it entered a quasi-persistent state, continuously replicating in the model (Fig. 5A). In contrast, long-term analyses of HRV16 replication in the HBE model revealed that the RT-qPCR cycle levels of the viral RNA in the supernatant (SN) reached background levels within 14–35 days, depending on the donor (Fig. 5B). To verify that the infected HBE cultures fully recovered and did not release infectious virus, we inoculated H1-Hela cells with the apical SN from human bronchial epithelial models after 31 days post HRV16 infection. The H1-Hela cells showed no cytopathic effect after 3 days, indicating the absence of infectious virus particles. As a positive control, we used supernatant from HRV16-infected models from earlier days (Fig. 5C). This clearance was accompanied by pronounced MUC5B secretion; however, the ciliated cell layer (acTub) remained intact (Fig. S5A). Overall, the integrity of the models was not compromised, as shown by the FITC-dextran membrane permeability assay and TEER measurements at 35 days post-infection (Fig. S5B).

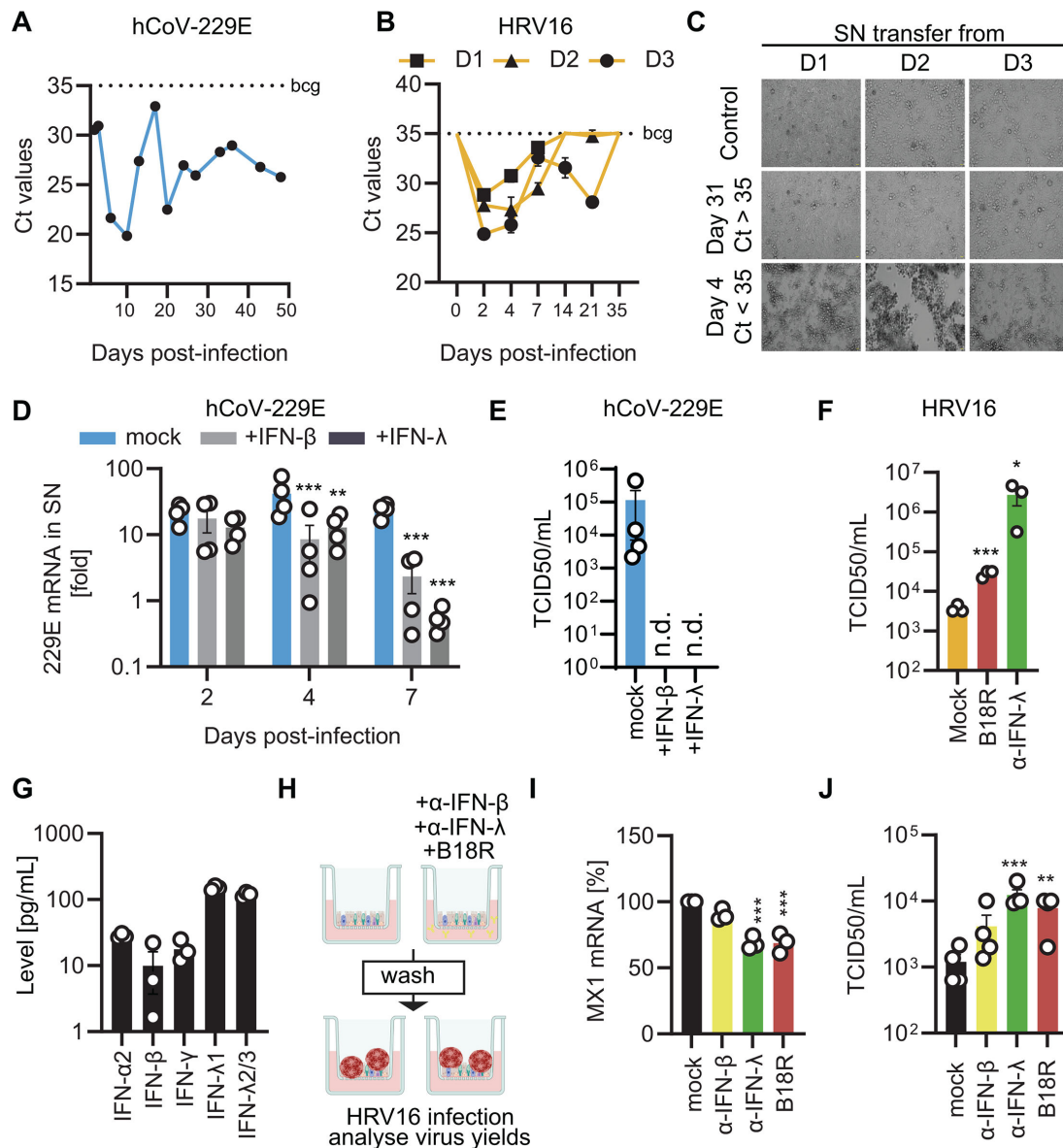
To test whether early and strong IFN would lead to the clearance of HCoV-229E-GFP-infected models, we added either exogenous IFN- $\beta$  or IFN- $\lambda$ 1/2/3/4 (Fig. S5C). IFN treatment led to a more than 10-fold reduction of viral RNA in the supernatant after 7 days (Fig. 5D), with GFP expression from the virus almost undetectable in the cultures (Fig. S5D). TCID<sub>50</sub> assays of the supernatant confirmed that no infectious particles were released 14 days post the initial infection anymore (Fig. 5E). Vice versa, to see whether dampening of the early and strong IFN response in HRV16-infected models would promote their replication, we scavenged type I IFN by B18R or used neutralizing antibodies against IFN- $\lambda$ s (Fig. S5E). Depletion of the type I or type III IFN responses caused a 10- or 1,000-fold increase in HRV16 growth in ALI cultures, respectively (Fig. 5F).

These data suggest that clearance of HRV in ALI cultures, but failure to clear HCoV-229E, is due to differential IFN responses.

## Tonic interferons in the lung epithelium serve as a major barrier against HRV16

Our data showed that the HBE model may be in a pre-activated state, with prominent IFN-stimulated genes highly expressed at basal levels (Fig. 3). Indeed, in the basolateral compartment of the uninfected models, various IFNs were readily detectable. On average, the HBE model secreted ~28 pg/mL of IFN- $\alpha$ 2, ~10 pg/mL of IFN- $\beta$ , ~18 pg/mL of IFN- $\gamma$ , ~150 pg/mL of IFN- $\lambda$ 1, and ~120 pg/mL of IFN- $\lambda$ 2/3 (Fig. 5G). To determine the impact of the tonic IFN on HRV16, we depleted them by pre-treating the HBEs with neutralizing antibodies against IFN- $\beta$ , IFN- $\lambda$ s, or B18R, a Type I IFN scavenger, for 2 days prior to the infection (46). Afterward, the treatments were removed, and the cultures infected (Fig. 5H). Depletion of the tonic IFN was successful, as shown by ~31% reduced basal MX1 levels in both B18R and anti-IFN  $\lambda$ -treated HBE cultures, and ~10% reduction in MX1 levels in anti-IFN- $\beta$  (Fig. 5I). Together, the anti-IFN- $\beta$  and anti-IFN- $\lambda$  treatments resulted in ~2-fold higher intracellular viral RNA, and B18R treatment resulted in ~5-fold higher HRV16 RNA content compared with untreated controls (Fig. S5F). Importantly, infectious virus yields in the apical supernatants assessed after 24 h post-infection increased markedly in IFN-depleted cultures (Fig. 5I). On average, depletion of type I IFN by neutralizing antibody, depletion of type III, or scavenging of IFN by B18R increased infectious virus yields by ~3-, 10-, and 7-fold, respectively (Fig. 5J).

Summarizing these findings indicates that tonic IFN signaling contributes to protective antiviral defense in the human bronchial epithelium against HRV16.



**FIG 5** Lung epithelial tonic interferon serves as a major barrier against respiratory virus invasion. (A) HBEs were infected with 229E (MOI 0.01), and on the indicated days post-infection, apical washes were collected. Viral RNA levels were determined by qPCR. Shown are the raw Ct values obtained from one donor sample over the course of 48 days post-infection. Dots represent the mean  $n = 1$  (donors). Bcg, background (gray line). (B) Quantification of the viral RNA levels by qPCR of HBE models, infected with HRV16 (MOI 0.01). On the indicated days post-infection, apical washes were collected. Shown are the raw Ct values obtained. Dots represent the mean  $n = 3 \pm$  SEM (donors). Bcg, background (gray line). (C) Representative bright-field microscopic images of H1-HeLa cells inoculated with apical washes collected at 31 dpi from indicated donors. SN from 4 dpi served as positive controls. Scale bar, 100  $\mu$ m. (D) Quantification of HCoV-229E viral RNA by qPCR from HBEs infected with HCoV-229E (MOI 0.01) stimulated with IFN- $\beta$  (0.5 nM) or IFN- $\lambda$ s cocktail (2 nM each of IFN- $\lambda$ 1/2/3/4) in the media compartment at 2 and 7 dpi, untreated samples served as mock control. Bars represent the mean of  $n = 4 \pm$  SEM (biological replicates). (E) Infectious HCoV-229E released from HBEs shown in panel C, after 14 dpi assessed by TCID<sub>50</sub>. Bars represent the mean of  $n = 4 \pm$  SEM (biological replicates). (F) HBEs were infected apically with HRV16 (MOI 0.01) and were treated with B18R (200 ng/mL) or anti-IFN- $\lambda$  cocktail (1  $\mu$ g/mL each of  $\alpha$ -IFN- $\lambda$ 1, 2, and 3) on 1 and 3 dpi. The infectious HRV16 released apically 6 dpi as determined by TCID<sub>50</sub>; untreated samples served as control. Bars represent the mean of  $n = 3 \pm$  SEM (biological replicates). Lognormal Welch's t test, \*\*\* $P = 0.004$ , \* $P = 0.0164$ . (G) Absolute levels of various IFNs, as detected via multiplex ELISA, in the basal supernatant of uninfected HBE models. Bars represent the mean of  $n = 3 \pm$  SEM (independent experiments). (H) Schematic representation of the workflow of tonic interferon depletion using neutralizing antibodies or type I IFN scavenger. (I) Quantification of the MX1 mRNA levels 48 h post-treatment of the HBE models as assessed by qPCR. Normalized to housekeeping gene TBP. Bars represent the mean of  $n = 3 \pm$  SEM (biological triplicates). One-way ANOVA with Dunnett's multiple comparisons test. \*\*\* $P = 0.0002$ . (J) Infectious HRV16 yields in the apical supernatants 1 dpi from HRV16 (MOI 0.01) infected HBEs assessed by TCID<sub>50</sub>. Bars represent the mean of  $n = 4 \pm$  SEM (independent experiments).

## DISCUSSION

Understanding host-pathogen interactions at the innate immune interface is crucial to understand the molecular pathogenesis but may also inspire preventive and cure strategies. To assess the molecular details, physiologically relevant models are key. Primary ALI cultures of the human lung epithelial cells are considered to reflect the natural respiratory interface. A major limitation of previous protocols was the limited availability of primary donor cells and the short virus growth times, ~7 days (34, 47, 48). Here, we introduced a method that tackled both these issues. Our data show that we can amplify and expand the primary lung epithelial cells for up to 12 passages without losing their differentiation potential via an organoid-intermediate state (Fig. 1, Fig. S1A and B). Furthermore, we showed that our primary ALI models supported long-term infection studies for up to 35 days (Fig. 4D). This suggests that our system could also be used to evaluate antiviral toxicity, define the dosing required for viral clearance, and follow cellular fate after the infection has been cleared.

Our data show a clear morphological similarity of our ALI model to the native tissue (Fig. 2). However, whereas the native tissue shows site-specific mucin expression, MUC5AC-producing cells in the superficial epithelium and MUC5B-producing cells in the submucosal glands, these cell populations are both present in the apical compartment of the ALI models. Of note, submucosal glands are not forming in transwell-based setups, as the ALI models largely mimic the epithelial layer (48, 49). Specialized cultures are required to recapitulate the architecture of submucosal glands *in vitro* (50). In addition, the density of mucus-secreting cells appeared to be increased in the native tissue (Fig. 2). Even though the native tissue sample obtained from a patient with pulmonary metastasis was considered healthy from a clinical and pathological aspect, cellular and molecular impairments in the patient's tissue cannot be excluded. Pulmonary metastasis could elicit tissue inflammation and associated goblet cell hyperplasia and mucus hypersecretion also in the vicinity of the metastasis, which could be a reason for the elevated mucin signals in the native bronchial tissue (51, 52). Thus, our models may represent a healthy lung epithelium.

While overall architecture and cell composition of the HBE and HSE models were similar, PCA analyses revealed differences in gene expression profiles. Interestingly, the HBE models expressed overall higher levels of mucosal defense genes compared with HSE (Fig. 3E through G). This difference may be attributed to structural and anatomical distinctions, as the bronchial epithelium is pseudostratified columnar, whereas the small airway epithelium is predominantly cuboidal (53). Previous studies using airway epithelial cells have also reported variation in responses towards treatment across the different anatomical sites of the airway (32, 54).

Cytokine profiling revealed a strong upregulation of host antiviral defenses during the early replication phase of HRV16. Consistent with our findings, both clinical and organotypic studies have also reported elevated early proinflammatory cytokine release during HRV16 infection (55–57). Notably, HRV16 could be cleared from the cultures in the absence of adaptive immune cells, suggesting that innate immune activation in the respiratory epithelium is sufficient to defend against HRV16 in healthy donors. In contrast, the HCoV-229E and HCoV-NL63 were able to antagonize or delay the induction of type I and III IFN responses similar to SARS-CoV-2 (46–48, 58, 59). Interestingly, external stimulation with IFN- $\beta$  and IFN- $\lambda$ s resulted in loss of HCoV-229E infectivity within 2 weeks post-infection compared with untreated control. This suggests that the virus is still sensitive to IFN-mediated clearance, but the amount induced by the infection itself is not sufficient (Fig. 5D). Hence, it is tempting to speculate that the robust and early IFN response likely facilitates clearance of HRV16, whereas evasion of this response enables prolonged infections by common cold coronaviruses (60).

Our data support that the early and robust IFN response of the models toward HRV16 may drive its clearance from the ALI models. Patients with defects in the type I IFN receptors (IFNAR1 or 2) or patients with type I IFN autoantibodies were not reported to show increased severity of HRV infections, despite being associated with increased viral

susceptibility e.g., against coronaviruses (61). At the mucosal interface, type III IFNs are considered the key antiviral cytokines (62). In line, our data show that depletion of type III IFN increased HRV16 replication by more than 1,000-fold (Fig. 5), whereas depletion of type I IFN only caused a 10-fold increase. In patients with defects specifically in the type I IFN system, the type III IFN defenses are still largely intact. Bypassing the mucosal route may also bypass the protective capacity of type III IFNs. For example, measles, mumps, and rubella (MMR) vaccination by subcutaneous infection was fatal in a child with non-functional IFNAR2 (63). It will be interesting to see in future studies whether type III IFN autoantibodies or defects in type III IFN signaling lead to more frequent and more severe HRV infections. However, current studies suggest that autoantibodies against type III IFNs are rarely neutralizing (64).

Our data further show that tonic IFNs, in addition to infection-induced IFNs, play an important role in the airway epithelium's antiviral defenses. Infectious virus yields were more than 10-fold higher upon depletion of tonic IFNs (Fig. 5). This suggests that tonic IFNs may pre-activate barriers in potentially vulnerable tissues at the viral entry site to be ready for defense. Whether the levels of IFN in our model correspond exactly to those locally in the human lung remains open. Data from lung organoids derived from primary patient tissue suggest detectable cytokine levels in human lungs (30). In mouse models, tonic IFN levels, including IFN- $\gamma$ , usually range from low pg/mL, similar to our detected IFN levels in the human HBE model (65, 66). Of note, tonic IFN levels have been studied more in another mucosal surface, the gut (67, 68). Here, they were also shown to contribute to innate defenses and immune homeostasis (68, 69) or even hematopoiesis (70).

Several studies have proposed that tonic IFNs are mainly driven by the microbiota, often detected via cGAS-STING DNA sensing pathways (24, 67, 68, 71). For example, stimulation of innate defenses by commensal microbiota through lung stroma or via dendritic cells may contribute to tonic IFN levels (24, 71) and anti-viral defense (24). However, our models were not colonized with bacteria, and they do not possess resident macrophages or dendritic cells but still exhibit antiviral tonic IFN activity, thus their source is likely to be intrinsic. It was shown that basal IFN- $\lambda$  helps shape the mucosal epithelial barrier even in the absence of pathogens in human epithelial cell models, driven by cGAS-STING-mediated mitochondrial DNA (69). Loss of USP22 in the respiratory epithelium enhances STING-dependent STAT1 activation, IFN- $\lambda$  secretion, and ISG expression even in the absence of an infection, leading to antiviral protection (72). Recent work also suggested that disruption of post-Golgi trafficking activates tonic cGAS-STING signaling in the absence of pathogenic stimuli, enhancing basal interferon signaling and conferring antiviral and antitumor protection (73). With the integration of single-cell and spatial transcriptomic and proteomic approaches, future studies may delineate the epithelial subsets responsible for tonic IFN production and map viral receptor distributions that influence disease severity using our model.

## MATERIALS AND METHODS

### Primary cells, cell lines, and media

Normal human bronchial epithelial cells (NHBE, Lonza, CC-2540, CC-2540S) and small airway epithelial cells (SAEC, Lonza, CC-2547) were cultivated in PneumaCult-Ex Plus complete media (StemCell Technologies, 05040) for expansion in 2D culture conditions. H1-HeLa cells (ATCC #CRL-1958) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41965039) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, A5256701), 2 mM GlutaMAX (Thermo, 35050061), and 6.5  $\mu$ g/mL gentamicin (PAN-Biotech, 15710-049). Huh-7 cells (kindly provided by Anna-Laura Kretz, Department for General and Visceral Surgery, Ulm University) were grown in DMEM supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin (PAN-Biotech, P06-07100), 2 mM L-glutamine (PAN-Biotech, P04-80100), and 10% heat-inactivated FBS. Caco-2 cells (kindly provided by Holger Barth, Institute of Experimental and Clinical Pharmacology,



Toxicology and Pharmacology of Natural Products, Ulm University Medical Center) were grown in DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Thermo, 11360039), 1× non-essential amino acids (Gibco, 11140050), and 20% heat-inactivated FBS. LLC-MK-2 cells (kindly provided by Lia van der Hoek, Laboratory of Experimental Virology, Department of Medical Microbiology, Amsterdam UMC, Location AMC, University of Amsterdam) were cultured in MEM (Sigma-Aldrich, M4655) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1× non-essential amino acids, and 8% FBS.

### Organoid generation and culture reagents

Epithelial cells were thawed and centrifuged at  $800 \times g$  for 3 min. After removing the supernatant, the pellet was resuspended in BME Type II (R&D Systems, 3533-010-02) and plated as 50 µL domes/well in a pre-warmed 24-well plate (Sarstedt, 60823644). The plate was incubated at 37°C for approximately 15 min to allow the polymerization of BME Type II. The domes were then overlaid with 500 µL of organoid growth medium supplemented with 10 µM Y27632 Rock-Inhibitor, only added freshly on the day of seeding (StemCell Technologies, 72304) per well. The organoid growth media were developed in-house based on previous research (37). It consists of Advanced DMEM-F12 (Thermo, 12634010) with the following factors: 1× R-spondin1-CM (produced in-house), 1× GlutaMax (Gibco, 35050-038), 10 mM HEPES (Lonza, 17-737E), 1× B27 supplement (Thermo, 12587-010), 5 mM Nicotinamide (Merck, N3376-100G), 1.25 mM N-acetylcysteine (Sigma, A9165-5G), 100 µg/mL Primocin (Invivogen, ant-pm-2), 100 ng/mL mNoggin (Peprotech, 250-38), 25 ng/mL FGF 7 (Peprotech, AF-100-19), 100 ng/mL FGF 10 (Peprotech, 100-26), 500 nM A 83-01 (Tocris, 2939), 10 µM Y27632 Rock-Inhibitor, only added freshly on the day of seeding, and 500 nM SB202190 (Selleckchem, S1077). For passaging, 500 µL of TrypLE Express (Invivogen, 12604013) was added to each dome. Dome structures were destroyed by pipetting up and down and transferred into a 15-mL reaction tube (Sarstedt, 62554502). Organoids were incubated with TrypLE for up to 15 min at 37°C in a water bath. Singularization was supported by mechanical forces, pipetting up and down at least 15 times. The reaction was stopped by adding resuspension media to reach a final volume of 10 mL, and cell number was determined. Upon centrifugation at  $800 \times g$  for 3 min, the cell pellet was resuspended in BME type II and plated as 50-µL domes in a 24-well plate containing approximately 75,000 to 100,000 cells/dome. After polymerization for 15 min at 37°C, 500 µL of medium supplemented with 10 µM Y27632 was added.

### ALI model generation

Collagen (Stemcell Technologies, 4902) was diluted 1:50 in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, 14190-094), and 100 µL was added to 6.5-mm transwell with 0.4-µm pore polyester membrane inserts (Corning, 3470). The plates were incubated overnight with the lid open to dry the collagen and UV sterilized for 30 min on the following day. The plates with inserts were directly used for experiments or stored at 4°C until use. The surface of the inserts was washed with PBS before seeding the cells. For cell differentiation on transwell inserts, organoids were treated with TrypLE as described above, and 1,000,000 cells were resuspended in expansion media containing 10 µM Y27632. A total of 50,000 cells from dissociated organoids (NHBE and SAEC) were seeded on the inserts with PneumaCult-Ex Plus Medium (Stemcell Technologies, 05040) and cultured under submerged conditions (300-µL media on apical compartment and 700-µL media on the basal compartment) until they were confluent (3–4 days). Media from the apical compartment were removed, and the models were cultured under air-liquid interface (ALI) to induce cell differentiation with 600 µL of complete PneumaCult-ALI medium (Stemcell Technologies, 05001) on the basal compartment. Fresh media were replaced thrice a week for 20 days and washed apically with 100-µL warm PBS once a week until a muco-ciliary phenotype was observed (within 30 days after seeding).

## Viral strains and propagation

HCoV-229E-GFP (GFP gene in place of ORF4a, kindly provided by Volker Thiel, Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern) was propagated in Huh-7 cells, HCoV-NL63 (kindly provided by Lia van der Hoek) on LLC-MK2 cells, and HRV16 (ATCC #VR-283) on H1-HeLa cells. To this end, 80% confluent cells were inoculated with an MOI of 0.1 in medium supplemented with 2% FCS and incubated at 33°C. Cells were monitored daily at the light microscope until a strong cytopathic effect was visible (4 days for HCoV-229E-GFP, 5 days for HCoV-NL63, and 3 days for HRV16). Supernatant was harvested and clarified by centrifugation at 1,300 rpm for 5 min to remove cellular debris and stored at –80°C until use.

## TCID50 assay

Infectious titers of HCoV-229E-GFP, HCoV-NL63, and HRV16 were determined in 96-well format on Huh7 cells, Caco-2 cells, or H1-HeLa cells, respectively. A total of 25,000 cells (Huh7, Caco-2) or 20,000 cells (H1-HeLa) were seeded in 96-well plates in medium containing 2% FCS. The next day, cells were inoculated with a 10-fold serial dilution of the virus stock and incubated at 33°C. At 7 days post-infection, CPE was evaluated by light microscopy, and TCID50 was calculated according to the Reed-Muench method (74).

## Live-cell video microscopy

Dynabeads Protein G particles (Invitrogen, 1004D) were mixed into DPBS to a final concentration of 30 µg/mL and pipetted 50 µL onto the models after apically washing with warm DPBS. Short videos of the transport of the particles across the surface of the models were recorded using high-speed video microscopy (Leica DMIL S80 PH FLUO).

## Immunofluorescence staining

The ALI cultures were fixed using 4% PFA (600 µL at the bottom and 100 µL at the apical compartment) overnight at 4°C and replaced with PBS and stored at 4°C until use. The insert was inverted, and the membranes containing the epithelial layer were gently removed using a scalpel and were either used directly for whole mount staining or were paraffin-embedded to make 5-µm sections on glass slides. For immunostaining, the paraffin was removed by placing the slides at 60°C overnight, followed by washes with xylene (2× 10 min), 96% ethanol (2× 1 min), 70% ethanol (1 min), 50% ethanol (1 min), and distilled water (1 min). The sections were unmasked to expose the epitopes, post-embedding. The slides were immersed in sodium-citrate buffer (pH 6) or Tris-EDTA buffer (pH 9), depending on the antibody as recommended by the manufacturer, or optimized for each antibody and placed in a steam chamber for 10 min. The slides were retrieved and placed in deionised water for 3 min to cool before immunostaining. The FFPE sections or the whole epithelium for whole-mount staining were permeabilized and blocked with 5% BSA in 0.5% TritonX-100 (Sigma-Aldrich, T8787) in PBS for 1 h to reduce the non-specific binding of the antibodies. The primary antibodies were diluted in the same blocking buffer and pipetted over the sections, and incubated overnight at 4°C in a humidified chamber. The slides were washed three times in wash buffer (PBS containing 0.05% Tween-20 (Sigma-Aldrich, P9416)), followed by incubation with secondary antibodies and DAPI (Invitrogen, SBA010020) for 1 h at RT. The slides were rewashed three times, mounted, and covered with coverslips to prevent drying. The primary antibodies used were CK5 (1:200; Agilent, M7010), acetylated tubulin (1:1,000; Sigma-Aldrich, T8328), MUC5B (1:100; Sigma-Aldrich, HPA008246), MUC5AC (1:1,000; Thermo, MA138223). The secondary antibodies used were Goat anti-Mouse IgG2b, Alexa Fluor 488 (1:400; Thermo, A-21141), Goat anti-Rabbit IgG (H+L), Donkey anti-Rabbit IgG, Alexa Fluor (1:400; Thermo, A-31572), Alexa Fluor 647 (1:400; Thermo, A-21245). Negative controls (omission of primary antibodies) were performed in each experiment to monitor non-specific binding. The samples were imaged using Zeiss LSM 710 confocal laser

scanning microscope using the ZEN 2010 software, and Keyence BZ9000 E BIOREVO System, and image analysis was conducted with ImageJ (75).

## RNA isolation and RT-qPCR

The RNA was isolated from the supernatants using QIAamp Viral RNA Mini kit (Qiagen, 52906), and cellular RNA was isolated using RNeasy Plus Mini Kit according to the recommended protocol (Qiagen, 74136). To determine the HRV16 (Thermo, Vi99990017\_po), HCoV-229E (Thermo, Vi06439671\_s1), and HCoV-NL63 (Thermo, Vi06439673\_s1) levels, real-time quantitative PCR (RT-qPCR) was performed using TaqMan Fast Virus 1-Step Master Mix and on StepOnePlus Real-Time PCR System (96-well format, fast mode). qRT-PCR for cellular RNA was performed in one step using the Luna Universal Probe One-Step RT-qPCR (New England Biolabs Inc., E3006E) kit on a StepOne-Plus Real-Time PCR System according to the manufacturer's instructions. TaqMan probes for each individual gene (FOXJ1 [Hs00230964\_m1], CK5 [Hs00361185\_m1], MUC5AC [Hs01365616\_m1], MUC5B [Hs00861595\_m1], MX1 [Hs00895608\_m1]) and housekeeping gene TATA-box binding protein gene (*TBP*, Hs00427620\_m1) were acquired as premixed TaqMan Gene Expression Assays and added to the reaction (Table 1). Expression level for each target gene was calculated by normalizing against TBP using the  $\Delta$ CT method.

## NGS library preparation and RNA-seq analyses

RNA-seq libraries were prepared using the prime-seq protocol, a bulk RNA sequencing method that introduces barcodes during reverse transcription (RT) previously described (76). For each sample, 40 ng of total RNA was used as input for RT. After cDNA synthesis and amplification, sequencing libraries were constructed using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB #E6177). Libraries were sequenced on an Illumina NovaSeq X (25B flow cell) with paired-end 150 bp reads (Read 1: 150 bp, Read 2: 150 bp) and dual 8 bp indexes (Index 1: 8 bp, Index 2: 8 bp) targeting 5 million reads per sample. Raw reads were processed with the zUMIs pipeline (v2.9.7e) and aligned to the human reference genome GRCh38.p13 using STAR (v2.7.9a) with GENCODE v41 annotations (77, 78). The resulting count matrix was imported into R; read counts were filtered, normalized with DeSeq2, and variance-stabilized (vst) for downstream PCA and heatmap visualization. To estimate relative cell-type abundance from bulk RNA-seq, we used the CIBERSORTx web tool (79). A signature matrix was derived from single-cell RNA-seq data of healthy human airways and applied to impute cell-type fractions (14).

## Analyses of anti-viral response using LEGENDplex

The cytokines present in the basal compartment of ALI-cultures were quantified using the LEGENDplex Hu Anti-Virus Response Panel 1 (BioLegend, 741,270), and its LEGENDplex Data Analysis Software. The assay and measurement by flow cytometry (FACS-Canto II) were performed according to the manufacturer's instructions.

TABLE 1 Primer probes

| Target gene | Details       | Company                    |
|-------------|---------------|----------------------------|
| FOXJ1       | Hs00230964_m1 | Thermo                     |
| CK5         | Hs00361185_m1 | Thermo                     |
| MUC5B       | Hs00861595_m1 | Thermo                     |
| MUC5AC      | Hs01365616_m1 | Thermo                     |
| MX1         | Hs00895608_m1 | Thermo                     |
| TBP         | Hs00427620_m1 | Thermo                     |
| HRV16       | Vi99990017_po | Thermo (Microbe detection) |
| HCoV-229E   | Vi06439671_s1 | Thermo (Microbe detection) |
| HCoV-NL63   | Vi06439673_s1 | Thermo (Microbe detection) |

## Infections

The models were given fresh medium and washed apically with warm PBS (140  $\mu$ L) and infected with HCoV-229E, HCoV-NL63, and HRV16 (0.01 MOI). Then, 50  $\mu$ L of virus suspended in serum-free media was added to the apical compartment and incubated at 37 °C. After 3–4 h, the airway epithelium was washed with 2 $\times$  warm PBS and further cultured with or without respective inhibitors. Inhibitors were added at a concentration of 10  $\mu$ M remdesivir (Selleckchem, S8932), 20  $\mu$ M camostat mesylate (Merck, SML0057), and 3  $\mu$ M rupintrivir (Sigma-Aldrich, PZ0315) to respective wells. For short- (0, 2, 4 dpi), long-term (0, 2, 4, 7, and 16 dpi), and HRV16 clearance (0, 7, 14, 21, and 35 dpi) infection kinetics, the apical washes were collected, and after RNA isolation, viral RNA was quantified by RT-qPCR.

## Transepithelial electrical resistance (TEER)

The barrier integrity of the HBE and HSE models was assessed with TEER measurements. The models were given an apical wash with PBS and transferred to 600  $\mu$ L PBS-containing 24-well plates, and the apical compartment was filled with 300  $\mu$ L PBS. The measurements were made using Millicell ERS 3.0 Digital Volt-Ohm meter (Merck, MERS03000) in triplicates. The final TEER was expressed in  $\Omega$ -cm<sup>2</sup> by multiplying the values from the Ohm meter by 0.33 (area of transwell insert).

## FITC-dextran epithelial barrier integrity assay

Fluorescein isothiocyanate (FITC)-conjugated dextran (4 kDa; 46944-100MG-F, Sigma, Germany) flux across the apical to basal compartment was used as a readout for measuring paracellular transport and epithelial barrier integrity. The apical surface was washed with PBS to remove excess mucus, and 600  $\mu$ L of fresh medium was added to the basal compartment. Then, 100  $\mu$ L of 0.25 mg/mL FITC-dextran dissolved in Opti-MEM-reduced serum media (Gibco, 31985047), sterile filtered using 0.4- $\mu$ m filters, was incubated on the apical compartment for 30 min protected from light in the incubator. Then, 100  $\mu$ L of the medium was collected from the basal compartment into a 96-well flat-bottom plate in duplicates, and absorbance at 490 nm was measured using Fluostar Omega. The mean absorbance measured in three independent experiments ( $N = 3$ , in duplicates) was normalized to a cell-free transwell insert, and the permeability of the human airway epithelial model to FITC-dextran was displayed as percentage values.

## Tonic interferon depletion assays

The fully differentiated ALI cultures were washed apically with PBS and transferred onto fresh plates containing 600- $\mu$ L medium with or without neutralizing antibodies or inhibitors in biological triplicates. Two experimental time points were assessed: Day 0 to check for the basal ISG level and Day 1 to check for the viral RNA in each treatment group post 24 h of HRV16 infection. Each time point had four treatment groups, treated either with Human IFN- $\beta$  antibody (1  $\mu$ g/mL; R&D, MAB814), or human IFN- $\lambda$  cocktail made by combining IFN- $\lambda$  1 antibody (1  $\mu$ g/mL; R&D, MAB15981), IFN- $\lambda$  2 antibody (1  $\mu$ g/mL; R&D, MAB1587), and IFN- $\lambda$  3 antibody (1  $\mu$ g/mL; R&D, MAB5259), or B18R (200 ng/mL; type I Interferon inhibitor), and untreated samples served as control. The models were treated for 48 and 24 h with a fresh media change in between treatments and apically washed to remove most of the tonic interferons. The Day 0 samples were immediately lysed after treatments ( $\times 2$ ) and subjected to RNA isolation, and in parallel, the infection group was infected with 0.1 MOI HRV16 for 24 h, followed by cellular RNA isolation and apical supernatant collection for TCID50.

## Interferon depletion assays

The ALI cultures were washed apically with PBS and given a fresh media change followed by infection with HRV16 (0.01 MOI). Two experimental time points were assessed for IFN



depletion: 1 and 3 dpi. Each time point had four treatment groups, treated either with human IFN- $\lambda$  cocktail made by combining IFN- $\lambda$  1 antibody (1  $\mu$ g/mL; R&D, MAB15981), IFN- $\lambda$  2 antibody (1  $\mu$ g/mL; R&D, MAB1587), and IFN- $\lambda$  3 antibody (1  $\mu$ g/mL; R&D, MAB5259), or B18R (200 ng/mL; type I interferon inhibitor), and untreated samples served as control. The apical washes were collected for RNA isolation at 0 and 6 dpi, viral RNA was quantified by RT-qPCR, and at 6 dpi, TCID50 was performed.

## Interferon stimulation assays

The fully differentiated ALI cultures were washed apically with PBS and transferred onto fresh plates containing 600- $\mu$ L medium. Apically infected with HCoV-229E at an MOI of 0.01. Then, 50  $\mu$ L of virus suspended in serum-free media was added to the apical compartment and incubated at 37°C for 4 h. Followed by 2 $\times$  PBS wash and further cultured for 2 days. At 2 and 7 dpi, the ALIs were treated with IFN- $\beta$  (0.5 nM, PeproTech, 300-02B.C.BC-50UG) and IFN- $\lambda$  cocktail (2 nM, PeproTech, 300-02L-20UG; 2 nM, R & D systems, 8417-IL-025/CF; 2 nM, R & D Systems, 5259-IL-025/CF; 2 nM, R & D Systems, 9165-IF-025) to the basal compartment of respective wells in biological quadruplicates, untreated served as controls. The apical washes were collected for RNA isolation at 0, 2, 4, and 7 dpi, viral RNA was quantified by RT-qPCR, and at 14 dpi, TCID50 was performed.

## Software

Statistical analyses were performed using GraphPad PRISM 10 (version 10.5.0). Figures were assembled and arranged using Adobe Illustrator (version 30.1). Schematic representations were created with BioRender.com (80–83).

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Rinu Sivarajan, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Paul C. Kirchgatterer, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – review and editing | Jan Lawrenz, Data curation, Investigation, Resources, Writing – review and editing | Eszter Tanner-Matiz, Data curation, Investigation, Writing – review and editing | Jessica Lindenmayer, Investigation, Resources, Writing – review and editing | Verena Renz, Data curation, Investigation, Resources, Writing – review and editing | Tapan Joshi, Data curation, Investigation, Resources, Writing – review and editing | Heike Oberwinkler, Data curation, Investigation, Resources, Writing – review and editing | Thorsten Walles, Resources, Writing – review and editing | Giorgio Fois, Data curation, Formal analysis, Investigation, Resources, Supervision, Validation, Writing – review and editing | Alexander Kleger, Resources, Writing – review and editing | Manfred Frick, Investigation, Resources, Supervision, Validation | Jan Münch, Resources, Writing – review and editing | Moritz M. Gaidt, Data curation, Formal analysis, Investigation, Resources, Supervision, Writing – review and editing | Maria Steinke, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Writing – review and editing | Konstantin M. J. Sparrer, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review and editing

## DATA AVAILABILITY

RNAseq data were deposited to Gene Expression Omnibus (NCBI) under accession no. [GSE312700](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE312700).

## ETHICS APPROVAL

Organoids and ALI cultures were derived from fully anonymized, commercially available human primary epithelial cells (NHBE, Lonza, CC-2540, CC-2540S; SAEC, Lonza, CC-2547). Human bronchial tissue used for immunofluorescent staining was obtained from one donor (female, 48 years old) undergoing elective pulmonary resection for secondary pulmonary malignoma at the University Hospital Würzburg. Informed consent was obtained beforehand, and the study was approved by the institutional ethics committee on human research of the Julius-Maximilians-University Würzburg (vote 182/10).

## ADDITIONAL FILES

The following material is available [online](#).

### Supplemental Material

**Data Set S1 (JVI02104-25-S0001.xlsx).** Bulk RNAseq raw UMI counts.

**Data Set S2 (JVI02104-25-S0002.xlsx).** CIBERSORTx-assisted signature matrix of transcriptional patterns used for cell clustering.

**Data Set S3 (JVI02104-25-S0003.xlsx).** Relative cell type abundance calculated from CIBERSORTx cell type clustering.

**Data Set S4 (JVI02104-25-S0004.xlsx).** Top 40 genes by variance.

**Data Set S5 (JVI02104-25-S0005.xlsx).** Gene ontology analyses.

**Supplemental material (JVI02104-25-S0006.docx).** Fig. S1 to S5; legends for Videos S1 to S4.

**Video S1 (JVI02104-25-S0007.mp4).** Functional ciliary activity in HBE cultures.

**Video S2 (JVI02104-25-S0008.mp4).** Representative live-cell imaging of HRV16-infected HBE cultures at 16 dpi at an MOI of 0.01.

**Video S3 (JVI02104-25-S0009.mp4).** Representative live-cell imaging of 229E-infected HBE cultures at 16 dpi at an MOI of 0.01.

**Video S4 (JVI02104-25-S0010.mp4).** Representative live-cell imaging of NL63-infected HBE cultures at 16 dpi at an MOI of 0.01.

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