

Inflammatory epithelial cytokines after *in vitro* respiratory syncytial viral infection are associated with reduced lung function

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This work demonstrates an association between epithelial inflammatory cytokines after *in vitro* viral infection and lung function in cystic fibrosis, and reinforces the importance of studying innate immune epithelial cell function in cystic fibrosis https://bit.ly/3gDNwwo

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Abstract

Respiratory syncytial virus (RSV) infections in early life predispose children with cystic fibrosis (CF) to more severe lung function decline in later life. The mechanisms explaining the associations between RSV and progression of CF lung disease are not clear.

In this study, a human bronchial epithelial cell line and primary human nasal epithelial cells (PNECs) from individuals with CF and healthy control donors were infected with RSV. Real-time PCR, plaque assay, cytokine detection, immunofluorescence and Western blot analyses were performed.

RSV is replicated to a higher degree in CF epithelial cells as compared to control cells; however, no defects in innate immune pathways were identified in CF cells. Rather, primary p.Phe508del cystic fibrosis transmembrane conductance regulator PNECs produced more cytokines after RSV infection than control cells. Moreover, interleukin-8 and tumour necrosis factor- α production post RSV negatively correlated with lung function (% predicted forced expiratory volume in 1 s) in the individuals who donated the cells.

These data suggest that CF epithelium has a dysfunctional response to RSV allowing for enhanced viral replication and an exaggerated inflammatory response that ultimately may predispose to greater airway inflammation and reduced lung function.

Introduction

Respiratory syncytial virus (RSV) is a leading cause of upper and lower respiratory tract infections among infants throughout the world resulting in significant morbidity and mortality [1–3]. While RSV readily infects healthy infants with no obvious risk factors, there are also a number of conditions that place infants at higher risk for more severe disease [4, 5]. Cystic fibrosis (CF) is one such condition, where some evidence suggests that infants with CF may fare worse with RSV than infants without CF [2, 6–8]. There are also some data to suggest that infants with CF who have a severe RSV infection are at risk for more significant subsequent lung disease [6, 7, 9].





It is not clear why severe RSV infection is associated with progression of CF lung disease, but one hypothesis is that inflammation associated with any infection may lead to lung damage. Consistent with this suggestion are reports from the AREST CF group that early life lung inflammation (at 3 months of age) in infants with CF is associated with the development of bronchiectasis at age 3 years [10]. In that work, neutrophil elastase levels at 3 months of age, a marker of lung inflammation, were a predictive factor

for subsequent bronchiectasis. It is known that neutrophils, the primary source of elastase in the airway, are recruited to the airway in acute RSV infection [11].

The respiratory epithelial cell is the primary target for RSV infection, and thus we were interested in examining the epithelial response to RSV in the setting of CF to better understand the link between RSV infection and subsequent progression of CF lung disease.

Materials and methods

For all sections, further details are available in the online supplementary material.

Participants

All participants were recruited and studied at SickKids in Toronto under local Research Ethics Board approved protocols. Participants or their guardians signed informed consent prior to participating. Twelve patients homozygous for p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) and 12 non-CF healthy controls were recruited and donated nasal cells for primary cell culture (REB 1000044783). The age range of the homozygous p.Phe508del patients was between 12 and 16 years, and 50% of them were males. The age range of the healthy controls was between 28 and 63 years, and 25% of them were males.

Nasal brushing and air-liquid interface culture

Nasal brushing and air-liquid interface (ALI) culture were performed as previously described [12-18].

Human bronchial epithelial cell culture

Human bronchial epithelial (HBE) cells CF-HBE (CFF-16HBEge CFTR p.Phe508del V470) were obtained from Cystic Fibrosis Foundation Therapeutics (Lexington, MA, USA) and wild-type HBE (WT-HBE=16HBE140) were obtained from Drs D. Gruenert and B. Illek (University of California, San Francisco, CA, USA).

RSV propagation and purification

RSV-A2 strain production was performed as previously described [15, 19, 20]. The recombinant strain of RSV expressing green fluorescent protein rgRSV224 (RSV-GFP) was a gift from Dr M.E. Peeples (Children's Research Institute, Columbus, OH, USA) and Dr P.L. Collins (National Institutes of Health, Bethesda, MD, USA) [21].

RSV infection and transepithelial resistance measurement

At day 21 of ALI culture, nasal epithelial cells and HBE cells were infected with RSV-A2 and RSV-GFP. Nasal epithelial cells were infected apically with 100 μ L RSV in PBS at 0.5 MOI in duplicates for each insert. Mock infection was performed with PBS addition to cells. Transepithelial resistance (TER) was measured with an ohmmeter (World Precision Instruments, Sarasota, FL, USA) following the manufacturer's instructions. HBE cells were treated with RSV-GFP in the same way as nasal cells.

Quantitative real-time PCR

Buffer RLT was added onto the nasal cells and incubated at room temperature for 5 min. Then cells were scraped and stored at -80°C. mRNA purification was conducted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols.

Plaque assay

PBS washes of the apical surface of cells were diluted and added onto HEp-2 cells in 6-well plates. DMEM-F12/agarose was overlaid onto the cells and incubated for 6 days at 37°C as previously described [19, 20, 22].

Immunofluorescence

Immunofluorescence was conducted as described previously [16, 17, 23].

Western blots

Primary nasal epithelial cells (PNECs) were lysed in 150 μ L radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl PH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 0.2% SDS, and 0.1% Triton X-100, Roche complete protease inhibitor cocktail) on ice for 10 min. The cells were then scraped from the membrane and transferred to Eppendorf tubes. The cell lysates were spun down at 8000 g for 15 min and then the supernatant was collected and stored at -80° C.

Cytokine measurements

Basolateral medium was collected 72 h post infection (hpi) and cytokine expression was measured by Luminex bead assay (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed with SAS Version 9.4 (SAS Institute, Cary, NC, USA) and GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney, matched Wilcoxon and two-way ANOVA tests were applied to analyse the data. Spearman test was used to analyse the correlation. The values below the limit of detection (LOD) for cytokine measurements were calculated using LOD/SQRT(2). All data were represented as mean±sem. p<0.05 was considered as significant.

Results

RSV titres in HBE

RSV viral load has been correlated to disease severity in infancy [24, 25]. Thus we initially studied modified HBE cells expressing p.Phe508del CFTR (CF-HBE) and compared them to HBE cells expressing wild-type CFTR (WT-HBE). After infection with RSV-GFP, RSV titres were higher at day 3 post infection in CF-HBE cells when compared to WT-HBE cells (figure 1a).

Viral load in primary nasal epithelial cells

Immortalised cell lines may not represent the *in vivo* situation. With this in mind, we examined RSV titres in primary human epithelial cells obtained from both healthy controls and participants homozygous for p.Phe508del CFTR. These cells are grown in ALI culture and are thought to reflect more faithfully the *in vivo* response to RSV when compared to cell lines [26–29]. Consistent with the HBE data, by both plaque assay over 3 days post infection and qPCR at day 3 post infection, cells from p.Phe508del CFTR individuals consistently displayed higher amounts of RSV (figure 1b, c).

Morphology, Western blots and TER

We wondered if the higher RSV titres were associated with signs of epithelial barrier dysfunction. At day 3 post infection, immunofluorescence and Western blots were performed with β -tubulin, E-cadherin, β -catenin and ZO-1 antibodies in the PNECs in ALI (figures 2 and 3a, b). These data did not reveal any significant differences between homozygous p.Phe508del and control cells. Just before adding RSV and at 24, 48 and 72 hpi, TER was measured with an ohmmeter according to manufacturer's instructions. With RSV infection, p.Phe508del and control cells did not show a significant difference in TER (figure 3c).

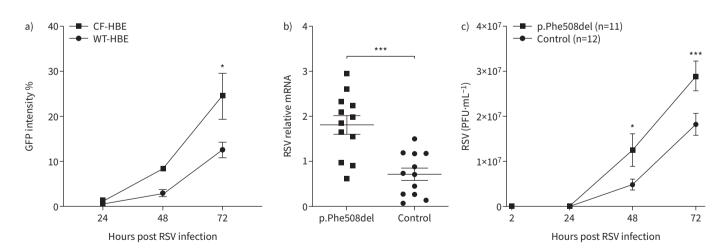


FIGURE 1 Respiratory syncytial virus (RSV) titres are higher in cystic fibrosis (CF) human bronchial epithelial cell line (HBE) and primary human nasal epithelial cells (PNECs). a) RSV-GFP intensity (% of total nuclei) is higher in CF-HBE than in wild-type HBE (WT-HBE) at 72 hpi. Data are representative of two biological replicates with three technical replicates in each cell line, with two-way ANOVA analysis followed by *post hoc* Bonferroni multiple comparisons. b) RSV mRNA expression at 72 hpi is higher in homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) cells than in control cells (n=12), by Mann–Whitney test. c) Plaque assay shows higher RSV titres in homozygous p.Phe508del CFTR cells when compared to control cells at 48 and 72 hpi, by two-way ANOVA analysis followed by *post hoc* Bonferroni multiple comparisons. *p<0.05; ***p<0.001. PFU: plaque-forming unit.

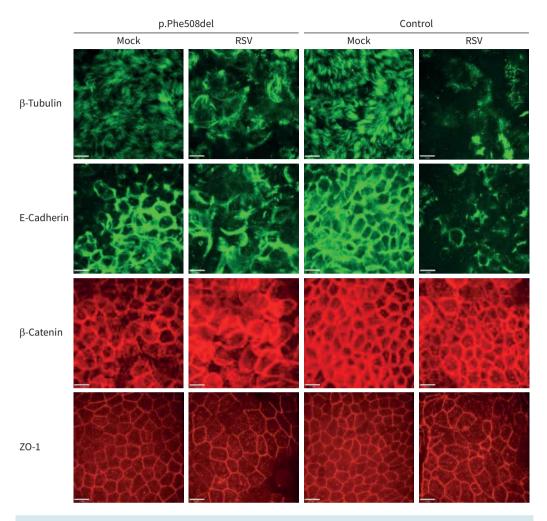


FIGURE 2 Morphology of epithelial cells post respiratory syncytial virus (RSV) infection in cystic fibrosis and control cells. Homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) and control primary human nasal epithelial cells (PNECs) in mock and RSV infection were stained at 72 h post infection. Representative immunofluorescence images demonstrate β -tubulin staining in epithelial cilia, E-cadherin and β -catenin staining in cell–cell adherens junctions and ZO-1 staining in tight junctions. Scale bar=10 μm.

The gene expression of pattern recognition receptors in PNECs

Owing to the difference seen in the viral load between p.Phe508del CFTR cells and control cells, we wondered if there was a defect in innate immune pathways. We measured pattern recognition receptors' (PRRs) mRNA expressions in the cells at 72 hpi (figure 4). Toll-like receptor-2 (TLR2), TLR3, TLR4, TLR7 and TLR9 levels were not significantly different between p.Phe508del CFTR cells and control cells, whereas RIG-I and MDA-5 levels were higher in p.Phe508del CFTR cells than in control cells post RSV infection. TLR8 was undetectable in PNECs. In addition, ISG56 mRNA expression was higher in p. Phe508del CFTR cells than in control cells post RSV infection.

Cytokine measurements

The basolateral medium from the primary human epithelial cells grown at ALI was collected at 72 hpi, and 36 cytokines were measured (figure 5 and supplementary figure S1). Interleukin (IL)-2, IL-5, IL-13, IL-22, IL-26, granulocyte–macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor and macrophage inflammatory protein- 1α were not detected. The cells from p.Phe508del CFTR patients produced significantly more IL-8, IL-9, IL-10, IL-12p70, IL-15, IL-17A, tumour necrosis factor- α (TNF- α), vascular endothelial growth factor and granulocyte colony-stimulating factor (G-CSF) compared to cells from healthy controls at 72 h post RSV infection. At baseline, p.Phe508del CFTR cells produced more IL-12p70 and IL-15 than control cells (figure 5). Importantly, we did not see reduced production of innate antiviral cytokines (IL-28A, IL-29) in p.Phe508del cells (figure 6).

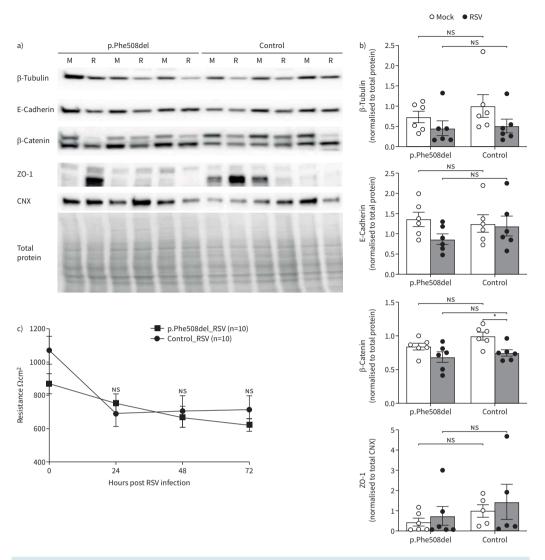


FIGURE 3 Protein expression and transepithelial resistance (TER) post respiratory syncytial virus (RSV) infection in cystic fibrosis and control cells. Protein was harvested from homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) and control primary human nasal epithelial cells in mock and RSV infection at 72 h post infection. Western blots were performed. a) Epithelial cilia marker β-tubulin, cell-cell adherens markers E-cadherin and β-catenin and tight junctions marker ZO-1. b) Quantification of Western blots demonstrates that expressions of the proteins are not significant between p.Phe508del CFTR and control cells (n=5-6), by Mann-Whitney test. c) The TER between homozygous p.Phe508del CFTR and control cells is not significant at 24, 48 and 72 hpi. The data were analysed with two-way ANOVA analysis followed by *post hoc* Bonferroni multiple comparisons. *p<0.05; Ns: not significant; M: Mock; R: RSV; CNX: calnexin.

IL-8 and TNF- α production in p.Phe508del CFTR cells correlate with lung function

Given the link between lung inflammation and subsequent lung dysfunction, we examined if pro-inflammatory cytokines produced in our cell model were associated with lung function in the individuals who donated the cells. Interestingly, we did find that the amount of IL-8 and TNF- α produced from the p.Phe508del CFTR cells was significantly correlated to forced expiratory volume in 1 s (FEV₁) of the patients who donated these cells; more IL-8 and TNF- α were produced in cells after RSV infection from individuals with lower lung function (figure 7a, b).

Discussion

The airway epithelium is a primary target of respiratory viruses. The epithelial response to RSV in CF is an important consideration given that CF results in epithelial cell dysfunction and RSV may be associated with enhanced morbidity in the setting of CF. In the current work, we provide evidence that CF epithelium

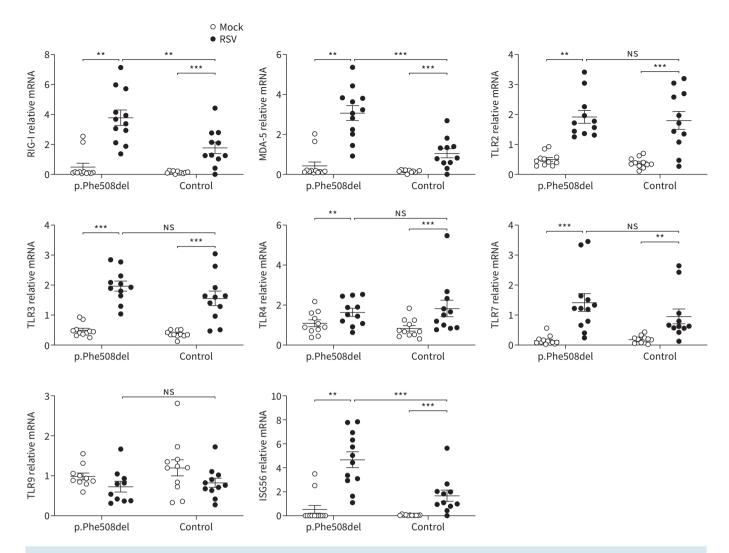


FIGURE 4 Pattern recognition receptors gene expression is increased in cystic fibrosis (CF) and control cells post respiratory syncytial virus (RSV). RSV increases RIG-I, MDA-5, TLR2, TLR3, TLR4 and TLR7 mRNA expression. TLR9 mRNA is unchanged and TLR8 mRNA is undetectable using Qiagen SBH0265788-200 TLR8 primers. The relative mRNA levels of RIG-I and MDA-5 are significantly higher in homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) cells compared to control cells post RSV infection. No significant differences are seen in TLR genes post RSV between CF and control cells. Interferon-stimulated gene ISG56 shows more mRNA expression in homozygous p.Phe508del CFTR cells. Non-paired data were analysed by Mann–Whitney test, and paired data were analysed by matched Wilcoxon test (n=11-12). **p<0.01; ***p<0.001; Ns=not significant.

allows RSV to replicate to a higher degree than seen in CFTR competent T-cells. However, this increased replication does not appear to be related to a defect in innate immunity. Infection also resulted in an exaggerated inflammatory response that was clinically relevant as the levels of cytokines produced *in vitro* were negatively associated with lung function in the subjects who donated the cells. Our findings raise a few points for discussion and suggest ongoing future investigations and experiments.

First, our work demonstrates that CF epithelial cells have an impaired ability to clear virus when compared to healthy control cells. This is a relevant observation as higher viral loads measured *in vivo* in acute illness are associated with a more severe illness [24, 25]. Importantly, it has been observed that some infants with CF have more severe acute RSV infection [2, 6–8]. Intuitively, more severe respiratory viral disease in CF has been attributed in part to impaired mucociliary clearance; our results suggest there are additional explanations independent of mucociliary dysfunction that can lead to higher viral titres in the setting of CF. It has also been observed that $CFTR^{-/-}$ mice demonstrate an impaired ability to clear RSV and an exaggerated inflammatory response to the virus [30]. Our observations suggest that to adequately address viral infection risk in CF, optimising mucociliary clearance alone may not be adequate.

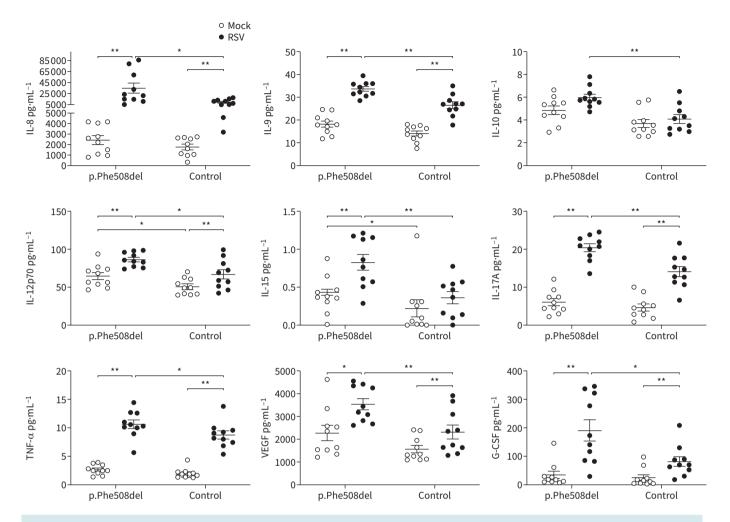


FIGURE 5 Cystic fibrosis cells produce higher amounts of cytokines post respiratory syncytial virus (RSV) infection as compared to control cells. The production of cytokines was measured by Luminex in basal media at 72 hpi in primary human nasal epithelial cells. Among the 36 cytokines measured, the levels of interleukin (IL)-8, IL-9, IL-10, IL-12p70, IL-15, IL-17A, tumour necrosis factor- α (TNF- α), vascular endothelial growth factor (VEGF) and granulocyte colony-stimulating factor (G-CSF) are significantly higher in homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) cells than in control cells. Non-paired data were analysed by Mann-Whitney test, and paired data were analysed by matched Wilcoxon test (n=10). *p<0.05; **p<0.01.

Second, the observed reduction in viral clearance was not related to a measured defect in innate immunity. Previous work in other cell types and with different stimuli (e.g. Pseudomonas bacterial infection) has suggested an impaired innate antiviral response in CF cells [31-34]. However, there are also data to suggest the innate immune response in CF cells is not impaired [35], and work that was published demonstrating an impaired interferon (IFN) response has been subsequently retracted [36]. Discrepancies in the field may be related to different model systems used and a bias to not publishing negative results. In epithelial cells, RSV is recognised by PRRs to trigger an innate immune response culminating in the expression of IFNs, other cytokines and IFN-stimulated genes [37-39]. Relevant PRRs include cell surface TLR2 and TLR4 as well as TLR3, TLR7, TLR8 and TLR9 located in endosomes. TLR4 recognises RSV F protein, but it is unclear how TLR2 detects RSV. TLR3 senses double-stranded RNA, whereas TLR7, TLR8 and TLR9 sense single-stranded RNA. In our cell model, we did not detect significant amounts of TLR8 mRNA; however, there were no differences seen in any of the TLRs that were expressed between CF and control cells after RSV infection. We also examined the cytosolic viral PRRs RIG-I and MDA-5, both members of retinoic acid-inducible gene-I-like receptors (RLRs) family. In viral infection, RIG-I and MDA-5 signalling activates IFN and other pro-inflammatory cytokines. We did not find a defect in RIG-I and MDA-5. In our primary PNEC model, we did not detect Type I IFNs (IFN- α/β) release in response to RSV consistent with previous publications [27]. However, the principal and most relevant antiviral cytokine family in this model is the Type 3 IFNs [28, 40]. Most significantly, despite observing higher

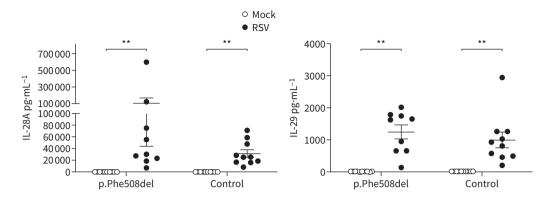


FIGURE 6 The production of Type III interferons (IFNs) is not reduced in cystic fibrosis cells. The production of Type III IFNs interleukin (IL)-28A and IL-29 were measured. IL-28A and IL-29 are not different between respiratory syncytial virus (RSV)-infected p.Phe508del cystic fibrosis transmembrane conductance regulator cells and control cells. The production of IL-28A and IL-29 in mock cells were below the limit of detection. Non-paired data were analysed by Mann-Whitney test, and paired data were analysed by matched Wilcoxon test (n=9-10). **p<0.01.

viral titres, we did not see a defect in Type 3 IFN production in CF cells. Thus, additional mechanisms must underlie the observation of reduced viral clearance in CF cells. One focus for our group is related directly to ion channel activity. It is known that viral infections can inhibit epithelial ion channel activity, and we have shown that modulation of epithelial ion channels can alter viral infection [15]. This remains an area of active investigation.

Third, we found a link between pro-inflammatory cytokine expression *in vitro* and lung function *in vivo*. IL-8 is a secondary cytokine that is stimulated by autocrine actions of the primary cytokines TNF- α and IL-1 β , which are induced post RSV infection [41, 42]. In our study, IL-8 positively correlated with IL-1 β supporting this previous work (supplementary figure S2). IL-8 has previously been identified as a modifier gene influencing lung function in CF [43, 44]. Indeed, IL-8 may have a broader role in lung health as links have been found between IL-8 and lung function measures in COPD [45], wheezing in infancy [46] and asthma in childhood [47]. It is biologically plausible that more IL-8 leads to enhanced recruitment of activated neutrophils with resultant release of more elastase causing progressive lung destruction. Indeed, in the setting of RSV infection, neutrophils are an important contributor to airway inflammation [48–50]. While more work will be needed to connect epithelial *in vitro* outcomes with *in vivo* lung function, our data do support a paradigm linking inflammatory stimuli (infections) with IL-8 production, which leads to

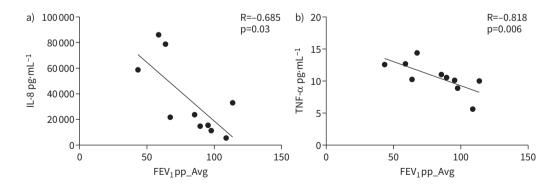


FIGURE 7 Interleukin (IL)-8 and tumour necrosis factor- α (TNF- α) production *in vitro* is associated with lung function. a) IL-8 production at 72 h post respiratory syncytial virus (RSV) infection in homozygous p.Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) cells negatively correlates with forced expiratory volume in 1 s (FEV₁ % pred), R=–0.685, p=0.03. b) TNF- α production at 72 h post RSV infection in homozygous p.Phe508del CFTR cells negatively correlates with FEV₁ (% pred), R=–0.818, p=0.006. Spearman correlation test was used to analyse the data.

neutrophil recruitment with more elastase production and then subsequent lung damage and dysfunction. This work is consistent with data from the AREST CF group where bronchoalveolar lavage elastase levels at age 3 months were linked with bronchiectasis at age 3 years [10].

One caveat to the line of reasoning suggesting a causal link between primary epithelial IL-8 production and secondary reduced lung function is that it is also possible that the reverse relationship exists. Thus, it is feasible that elevated IL-8 levels seen in CF cells are explained by epigenetic changes within those cells. Individuals with reduced lung function likely have been exposed to a different infectious and inflammatory milieu over their life course when compared to individuals with higher lung function. This different milieu may result in an altered epigenetic profile of epithelial cells which in turn results in a different IL-8 (inflammatory) response to RSV when studied *in vitro*. Outside of epithelial cells, there are data to suggest that neutrophilic inflammation predisposes the lung to more severe RSV infection [51]. Thus, it is conceivable that inflammation leads to worse infection as opposed to the reverse. It is also possible that a causal association exists in both directions leading to an auto-amplification loop (*i.e.* virus induces inflammation à inflammation alters cells to be pro-inflammatory post infection à virus induces inflammation). These possibilities remain a focus of future investigations.

Fourth, we observed a number of cytokines (including IL-9, IL-10, IL-12, IL-15, IL-17A and TNF- α) produced at higher levels in CF cells post RSV infection consistent with the idea of an exaggerated inflammatory response. In the past, it has been reported that infants with RSV bronchiolitis have greater IL-9 mRNA levels than control infants; elevated IL-9 production was thought to increase the inflammatory response and lung disease severity [52]. IL-10 is a known anti-inflammatory cytokine; however, its impact on the antiviral immune response is complex. Thus, broadly in the setting of viral infection, IL-10 can inhibit viral replication but has also been reported to promote viral persistence [53, 54]. This is a pattern that has also been reported for IL-12p70 and IL-15 where both pro-inflammatory or anti-inflammatory viral responses have been reported likely reflecting differences in model systems studied and when in the course of the infection the analyses were performed (*i.e.* early *versus* late in infections) [55–57]. IL-17A is thought to promote RSV pathogenesis as in a mouse model, anti-IL-17A treatment reduced both inflammation and viral load significantly [58]. TNF- α is a pro-inflammatory cytokine and is known to be elevated in infants post RSV infection [59]. Both TNF- α and IL-17A are known to induce G-CSF, an important mediator of neutrophil function [60]. In our study, IL-17A, TNF- α and G-CSF all showed higher levels in CF cells.

Finally, our work highlights the importance of examining more than just current CFTR as an *in vitro*CF epithelial outcome measure. Clearly, CF epithelial cells have a number of deficits that can be evaluated and may predispose to or be associated with lung disease. Viral clearance and cytokine production are such outcomes, and changes should be considered at baseline and post candidate interventions/therapies. With this in mind, a future direction of this work will be to examine the impact of current and novel CFTR modulator agents on *in vitro* viral clearance and *in vitro* cytokine production.

In summary we present data supporting an association between *in vitro* IL-8 production and lung function and also the notion that innate immune function is an important epithelial phenotype that should be studied in CF cells as we look towards improving clinical outcomes.

Limitations

There are a few limitations to this study. Only 12 CF patients and 12 controls donated nasal cells for the culture work; it is possible that a larger sample size may unveil significantly different findings. In addition, CF and control samples were not age or sex matched. Lastly, sputum was not collected in this study to analyse elastase activity. Future studies could address these limitations.

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Author contributions: W. Duan conceived of, performed and planned all experiments, analysed the data, and wrote the paper. Y. Chen, C. Lin, H. Ouyang, K. Du, A. Kumar and B. Wang performed experiments and edited the manuscript. J. Avolio performed the nasal brushing and edited the manuscript. H. Grasemann provided guidance

and edited the paper. T.J. Moraes conceived of experiments, analysed data, wrote and edited the paper, supervised, and provided support for the project.

Conflict of interest: None declared.

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