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Original article

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Please cite this article as: Duan W, Cen Y, Lin C, *et al*. Inflammatory epithelial cytokines post *in vitro* respiratory syncytial viral infection are associated with reduced lung function. *ERJ Open Res* 2021; in press (https://doi.org/10.1183/23120541.00365-2021).

Inflammatory Epithelial Cytokines Post *in vitro* Respiratory Syncytial Viral Infection Are Associated with Reduced Lung Function

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Key words: Respiratory Syncytial Virus, Cystic Fibrosis, Pattern Recognition Receptors, cytokines, lung function

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 (HBE) and primary human nasal epithelial cells (PNECs) from individuals with CF and healthy control donors were infected with RSV. RT-PCR, plaque assay, cytokine detection, immunofluorescence and Western blot analyses were performed.

RSV replicated to higher level 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 CFTR PNECs produced more cytokines after RSV infection than control cells. Moreover, IL8 and TNF-α production post RSV negatively correlated with lung function (% predicted forced expired volume in 1 second or FEV_1) 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 supplement.

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 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 to 16 yrs and 50% of them were males. The age range of the healthy controls was between 28 to 63 yrs and 25% of them were males.

Nasal brushing and Air-Liquid interface (ALI) culture

Nasal brushing and ALI culture were performed as previously described [12-18].

Human bronchial epithelial (HBE) cell culture

CF-HBE (CFF-16HBEge CFTR p.Phe508del V470) were obtained from Cystic Fibrosis Foundation Therapeutics (Lexington, MA) and Wild-type HBE (WT-HBE = 16HBE14o) were obtained from Drs. D. Gruenert and B. Illek (UCSF).

RSV propagation and purification

RSV-A2 strain production was performed as previously described [15, 19, 20]. The recombinant strain of RSV expressing GFP rgRSV224 (RSV-GFP) was a gift from Dr. M.E. Peeples

(Children's Research Institute, Columbus, OH) and Dr. P.L. Collins (National Institutes of Health, Bethesda, MD) [21].

RSV infection and Transepithelial resistance (TER) 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 100µL RSV in PBS at 0.5 MOI in duplicates for each insert. Mock infection was performed with PBS addition to cells. TER was measured with an ohmmeter (World Precision Instruments, Sarasota, FL) following the manufacturer's instructions. HBE cells were treated with RSV-GFP in the same way as nasal cells.

Real-time PCR (qRT-PCR)

Buffer RLT was added onto the nasal cells and incubated at room temperature for 5min. 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 ºC as previously described [19, 20, 22].

Immunofluorescence (IF)

Immunofluorescence was conducted as described previously [16, 23, 24].

Western blots

Primary nasal epithelial cells were lysed in 150 µl 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 8000g for 15min and then the supernatant was collected and stored at -80 °C.

Cytokine measurements

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

Statistical analysis

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

Results

RSV titers in HBE

RSV viral load has been correlated to disease severity in infancy [25, 26]. 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 titers 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 (PNECs)

Immortalized cell lines may not represent the *in vivo* situation. With this in mind, we examined RSV titers in primary human epithelial cells obtained from both healthy controls and participants homozygous for p.Phe508del CFTR. These cells are grown in air liquid interface (ALI) culture and are thought to more faithfully reflect the *in vivo* response to RSV when compared to cell lines [27-30]. 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 titers 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 (Figure 2 and Figure 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 significant difference in TER (Figure 3C).

The gene expression of Pattern Recognition Receptors (PRRs) in PNECs

Due 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 PRRs mRNA expressions in the cells at 72hpi (Figure 4). 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 air liquid interface was collected at 72hpi and 36 cytokines were measured (Figure 5 and Figure S1). IL2, IL5, IL13, IL22, IL26, GM-CSF, Basic FGF and MIP-1α were not detected. The cells from p.Phe508del CFTR patients produced significantly more IL8, IL9, IL10, IL12p70, IL15, IL17A, TNF-α, VEGF and G-CSF compared to cells from healthy controls at 72 hours post RSV infection. At baseline, p.Phe508del CFTR cells produced more IL12p70 and IL15 than control cells (Figure 5). Importantly, we did not see reduced production of innate antiviral cytokines (IL28A, IL29) in p.Phe508del cells (Figure 6).

IL8 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 IL8 and TNF-α produced from the p.Phe508del CFTR cells were significantly correlated to FEV_1 of the patients who donated these cells; more IL8 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 allows RSV to replicate to a higher degree than seen in CFTR competent 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 [25, 26]. 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 titers 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 [31]. Our observations suggest that to adequately address viral infection risk in CF, optimizing mucociliary clearance alone may not be adequate.

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 [32-35]. However, there are also data to suggest the innate immune response in CF cells is not impaired [36] and work that was published demonstrating an impaired interferon response has been subsequently retracted [37]. 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 recognized by PRRs to trigger an innate immune response culminating in the expression of interferons (IFNs), other cytokines, and IFN-stimulated genes (ISGs) [38-40]. Relevant PRRs include cell surface TLR2 and TLR4 as well as TLR3, 7, 8 and 9 located in endosomes. TLR4 recognizes RSV F protein but it is unclear how TLR2 detects RSV. TLR3 senses double stranded RNA whereas TLR7, 8, 9 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 controls 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 signaling 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 [28]. However, the principle and most relevant antiviral cytokine family in this model are the type 3 IFNs [29, 41]. Most significantly, despite observing higher viral titers, 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*. IL8 is a secondary cytokine that is stimulated by autocrine actions of the primary cytokines TNF-α and IL1β, which are induced post RSV infection [42, 43]. In our study, IL8 positively correlated with IL1β supporting this previous work (Figure S2). IL8 has previously been identified as a modifier gene influencing lung function in CF [44, 45]. Indeed, IL8 may have a broader role in lung health as links have been found between IL8 and lung function measures in COPD [46], wheezing in infancy [47], and asthma in childhood [48]. It is biologically plausible that more IL8 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 [49-51]. 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 IL8 production, that leads to 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 was linked with bronchiectasis at age 3 years [10].

One caveat to the line of reasoning suggesting a causal link between primary epithelial IL8 production and secondary reduced lung function is that it is also possible that the reverse relationship exists. Thus, it is feasible that elevated IL8 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 IL8 (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 [52]. 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 → inflammation alters cells to be pro-inflammatory post infection → virus induces inflammation). These possibilities remain a focus of future investigations.

Fourth, we observed a number of cytokines (including IL9, IL10, IL12, IL15, IL17A 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 IL9 mRNA levels than control infants; elevated IL9 production was thought to increase the inflammatory response and lung disease severity [53]. IL10 is a known

anti-inflammatory cytokine, however, its impacts on the antiviral immune response is complex. Thus, broadly in the setting of viral infection, IL10 can inhibit viral replication but has also been reported to promote viral persistence [54, 55]. This is also a pattern that has also been reported for IL12p70 and IL15 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) [56-58]. IL17A is thought to promote RSV pathogenesis as in a mouse model, anti-IL17A treatment reduced both inflammation and viral load significantly [59]. TNF-α is a pro-inflammatory cytokine and is known to be elevated in infants post RSV infection [60]. Both TNF-α and IL17A are known to induce G-CSF, an important mediator of neutrophil function [61]. In our study, IL17A, TNF-α and G-CSF all showed higher levels in CF cells.

Finally, our work highlights the importance of examining more than just CFTR current 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* IL8 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 for 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 analyze elastase activity. Future studies could address these limitations.

Acknowledgements

CF-HBE were obtained from Cystic Fibrosis Foundation Therapeutics and Wild-type HBE were obtained from Drs. D Gruenert and B Illek at UCSF. The recombinant strain of RSV expressing GFP rgRSV224 (RSV-GFP) was obtained from Dr. M.E. Peeples at the Children's Research Institute in Columbus and Dr. P.L. Collins at the National Institutes of Health. We would like to thank all members of CFIT for supporting this work.

Author contributions: WD conceived of, performed, and planned all experiments, analyzed the data and wrote the paper. YC, CL, HO, KD, AK and BW performed experiments and edited the manuscript. JA performed the nasal brushing and edited the manuscript. HG provided guidance and edited the paper. TJM conceived of experiments, analyzed data, wrote and edited the paper, supervised, and provided support for the project.

Funding/Support: TJM was supported by CF Canada-SickKids Program for Individualized CF Therapy (CFIT) and Emily's Entourage.

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Fig 1.

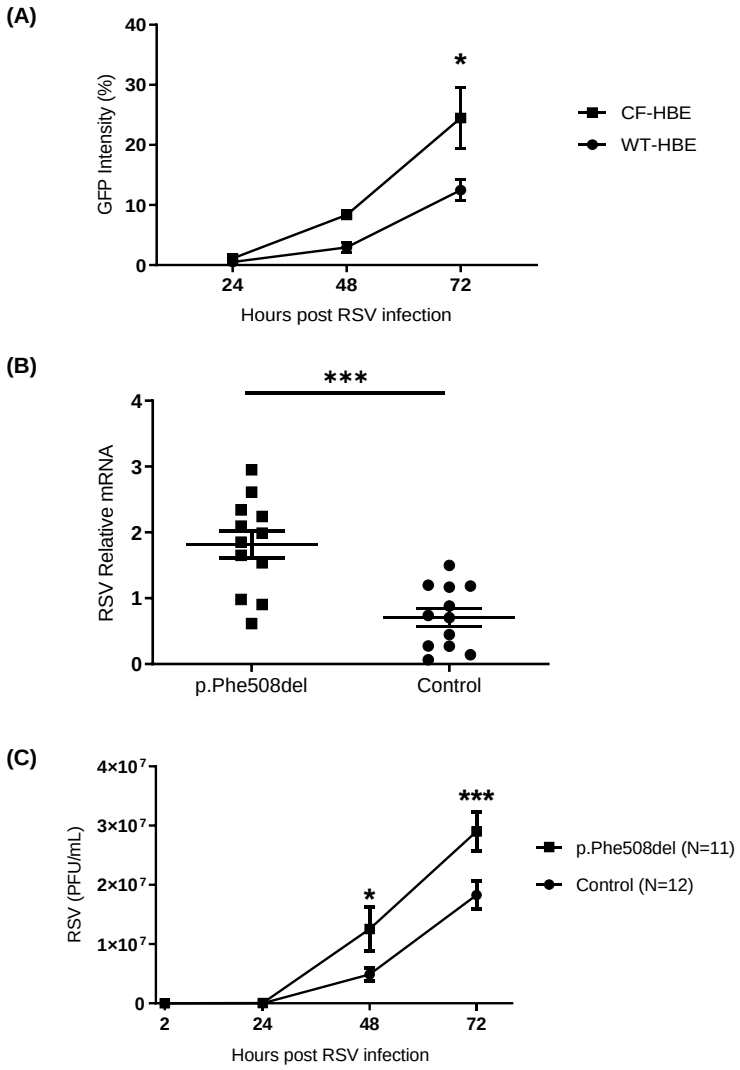
(A)
GFP Intensity (%)
40
30
20
10
0
*
24
48
72
Hours post RSV infection
CF-HBE
WT-HBE
(B)
***
RSV Relative mRNA
4
3
2
1
0
p.Phe508del
Control
(C)
RSV (PFU/mL)
4×10⁷
3×10⁷
2×10⁷
1×10⁷
0
*
***
2
24
48
72
Hours post RSV infection
p.Phe508del (N=11)
Control (N=12)

Figure 1. RSV titers are higher in CF HBE and PNECs. (A) RSV-GFP intensity (% of total nuclei) is higher in CF-HBE than in WT-HBE at 72 hpi. Data are representative of 2 biological replicates with 3 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 CFTR cells than in control cells (n = 12), by Mann-Whitney test. (C) Plaque assay shows higher RSV titers 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. * $p < 0.05$; *** $p < 0.001$.

Fig 2.

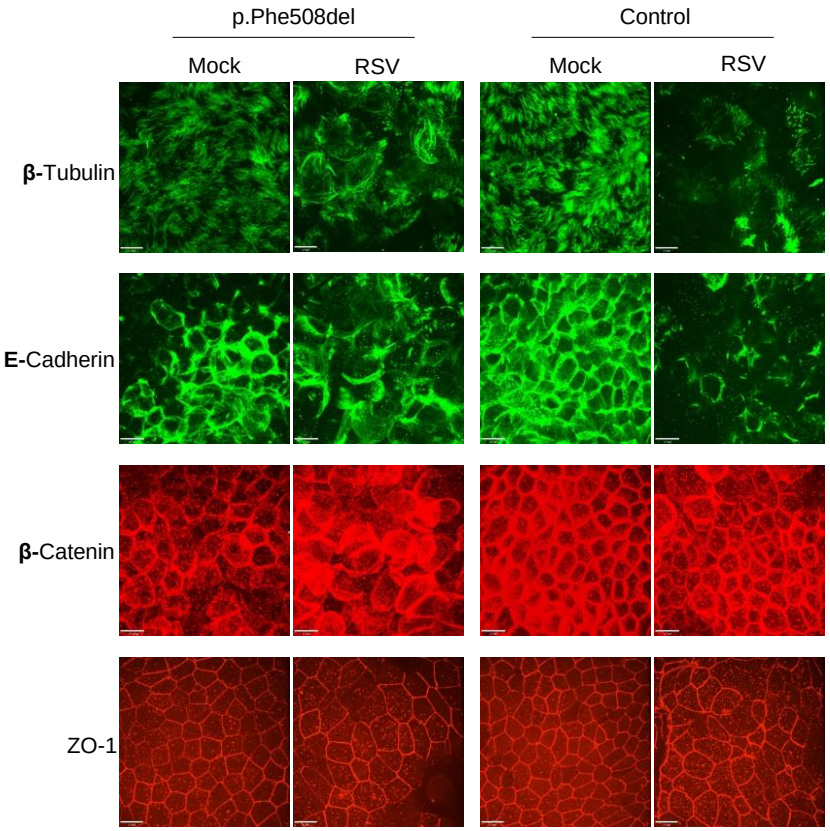
p.Phe508del
Control
Mock
RSV
Mock
RSV
β-Tubulin
E-Cadherin
β-Catenin
ZO-1

Figure 2. Morphology of epithelial cells post RSV infection in CF and control cells.

Homozygous p.Phe508del CFTR and control PNECs in mock and RSV infection were stained at 72 hours 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 = 10μm).

Fig 3.

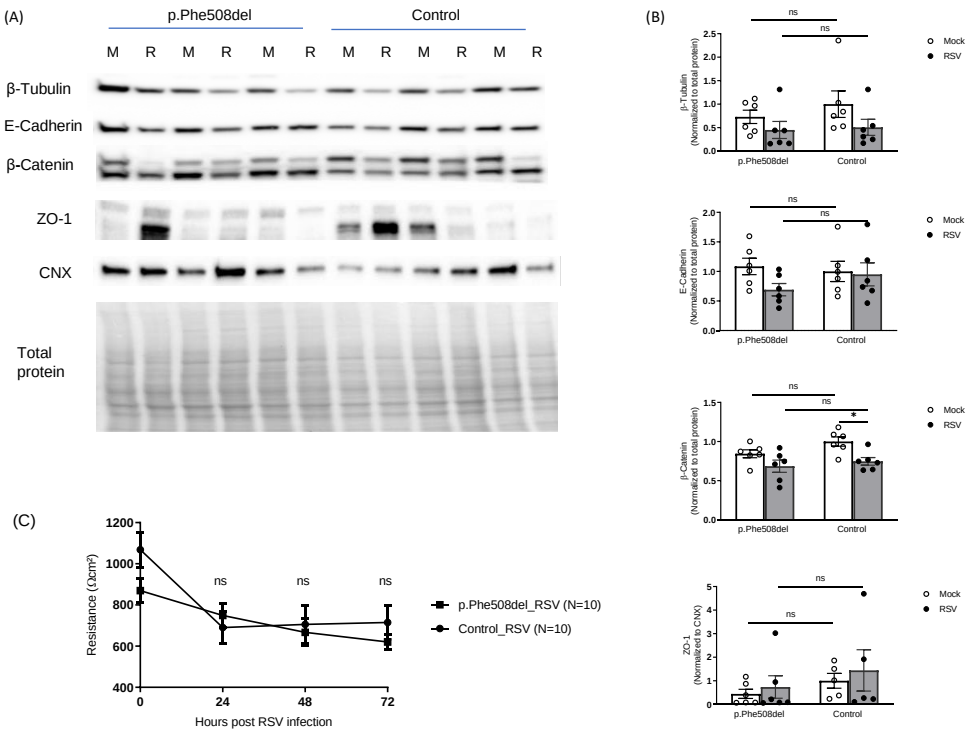
(A)
p.Phe508del
Control
M R M R M R M R M R M R
β-Tubulin
E-Cadherin
β-Catenin
ZO-1
CNX
Total protein
(B)
ns
Mock
RSV
β-Tubulin (Normalized to total protein)
E-Cadherin (Normalized to total protein)
β-Catenin (Normalized to total protein)
ZO-1 (Normalized to CNX)
p.Phe508del
Control
(C)
Resistance (Ωcm²)
Hours post RSV infection
p.Phe508del_RSV (N=10)
Control_RSV (N=10)

Figure 3. Protein expression and TER post RSV infection in CF and control cells.

Protein was harvested from homozygous p.Phe508del CFTR and control PNECs in mock and RSV infection at 72 hours 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 was analyzed with two-way ANOVA analysis followed by *post hoc* Bonferroni multiple comparisons. * $p < 0.05$; ns=not significant; M=Mock; R=RSV; CNX= Calnexin.

Fig 4.

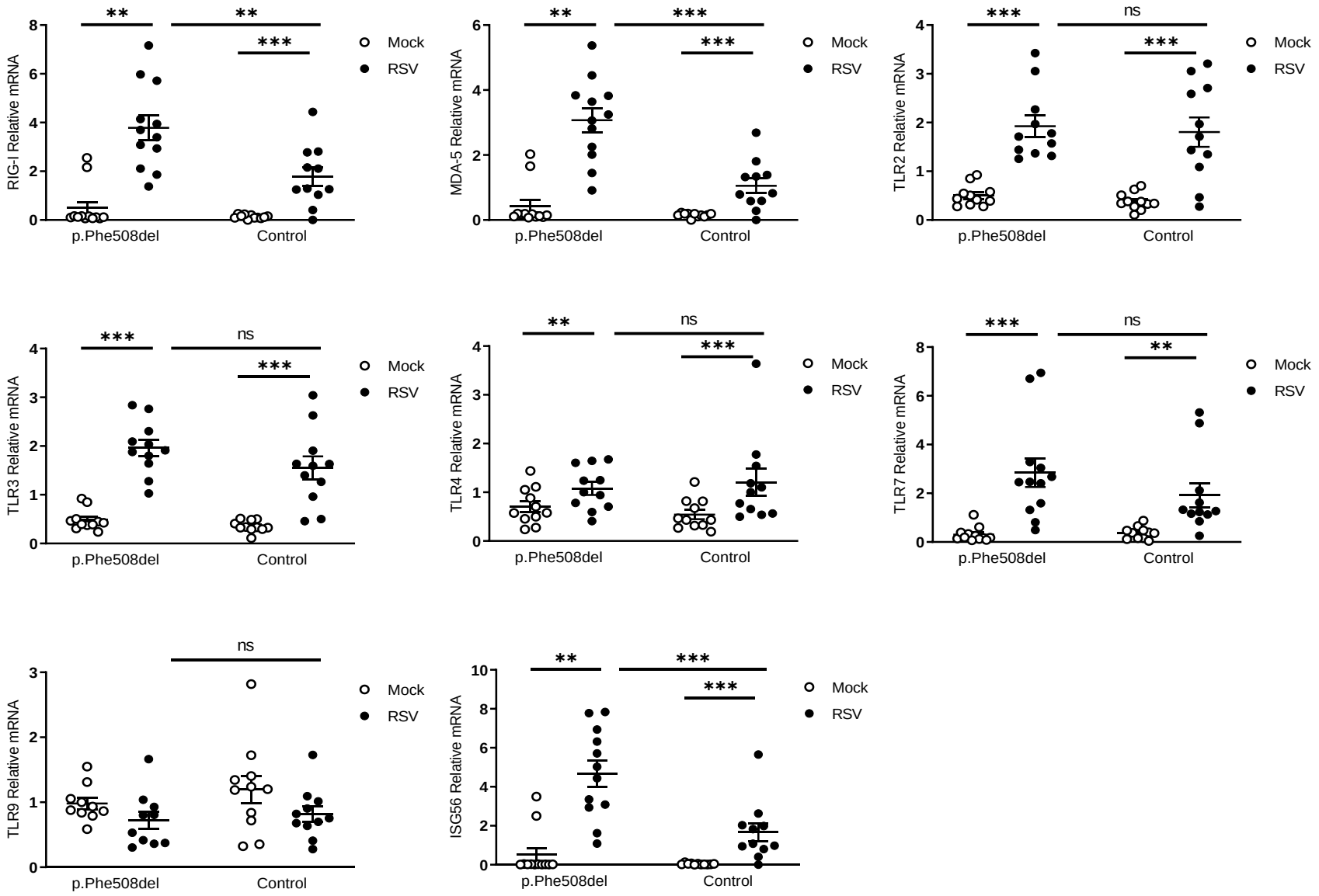
RIG-I Relative mRNA
MDA-5 Relative mRNA
TLR2 Relative mRNA
TLR3 Relative mRNA
TLR4 Relative mRNA
TLR7 Relative mRNA
TLR9 Relative mRNA
ISG56 Relative mRNA
Mock
RSV
p.Phe508del
Control
**
***
ns

Figure 4. PRRs gene expression is increased in CF and control cells post RSV. RSV increases RIG-I, MDA-5, TLR2, 3, 4 and 7 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 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 analyzed by Mann-Whitney test and paired data were analyzed by matched Wilcoxon test (n = 11 – 12). ** $p < 0.01$; *** $p < 0.001$; ns=not significant.

Fig 5.

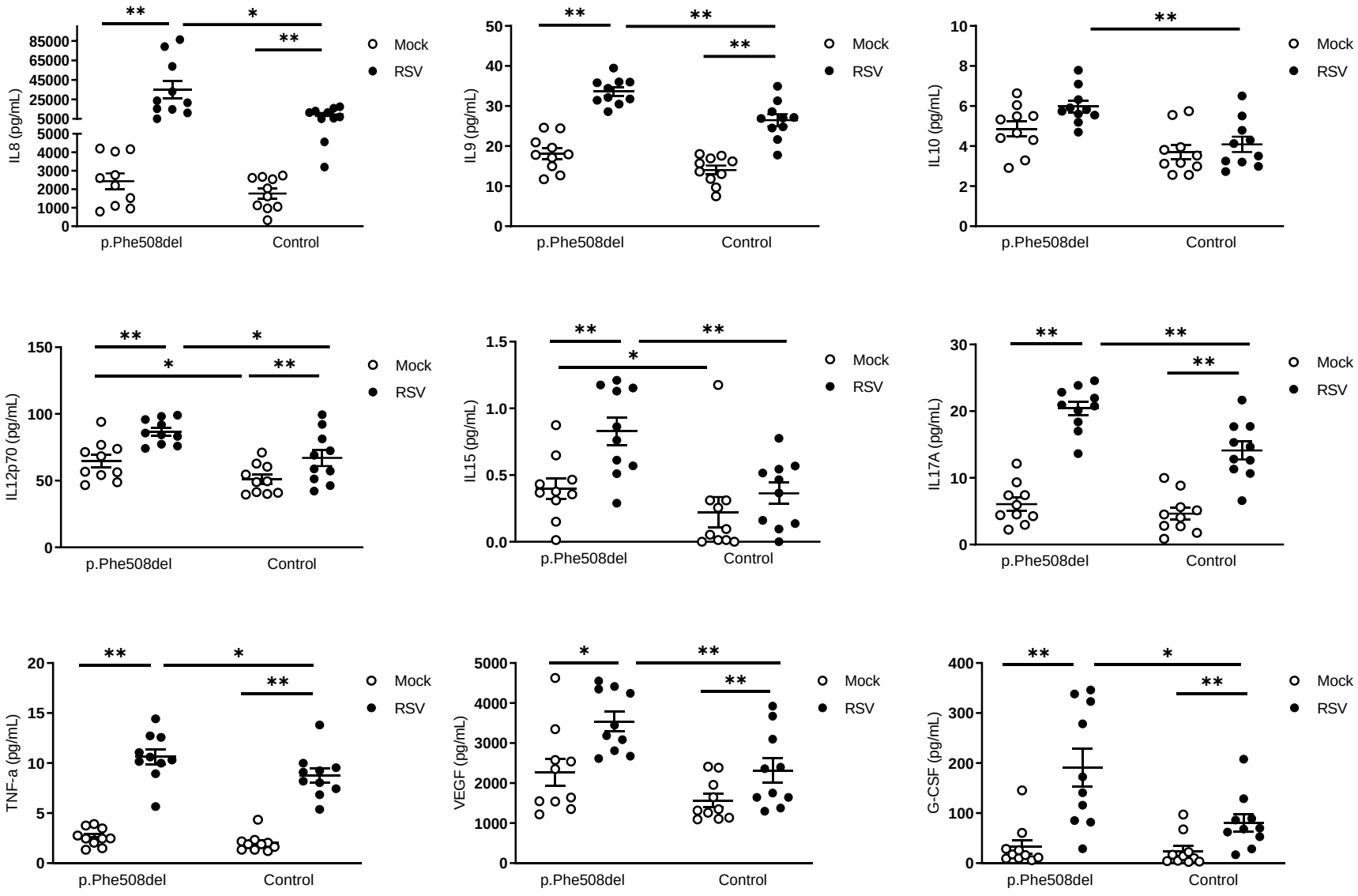
IL8 (pg/mL)
IL9 (pg/mL)
IL10 (pg/mL)
IL12p70 (pg/mL)
IL15 (pg/mL)
IL17A (pg/mL)
TNF-a (pg/mL)
VEGF (pg/mL)
G-CSF (pg/mL)
Mock
RSV
p.Phe508del
Control

Figure 5. CF cells produce higher amounts of cytokines post RSV infection as compared to control cells. The production of cytokines was measured by Luminex in basal media at 72hpi in PNECs. Among the 36 cytokines measured, the levels of IL8, IL9, IL10, IL12p70, IL15, IL17A, TNF-α, VEGF and G-CSF are significant higher in homozygous p.Phe508del CFTR cells than in control cells. Non-paired data were analyzed by Mann-Whitney test and paired data were analyzed by matched Wilcoxon test (n = 10). * $p < 0.05$; ** $p < 0.01$.

Fig 6.

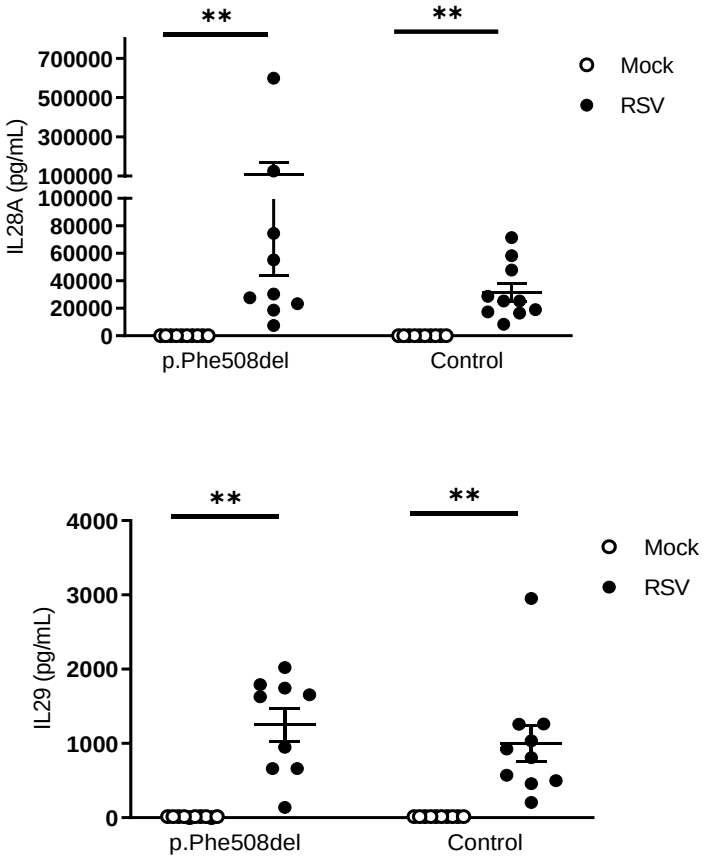
**
**
700000
500000
300000
100000
100000
80000
60000
40000
20000
0
IL28A (pg/mL)
Mock
RSV
p.Phe508del
Control
**
**
4000
3000
2000
1000
0
IL29 (pg/mL)
Mock
RSV
p.Phe508del
Control

Figure 6. The production of Type III IFNs is not reduced in CF cells. The production of Type III interferons (IFN) IL28A and IL29 were measured. IL28A and IL29 are not different between RSV infected p.Phe508del CFTR cells and control cells. The production of IL28A and IL29 in mock cells were below the limit of detection. Non-paired data were analyzed by Mann-Whitney test and paired data were analyzed by matched Wilcoxon test (n = 9 – 10). ** p <0.01.

Fig 7.

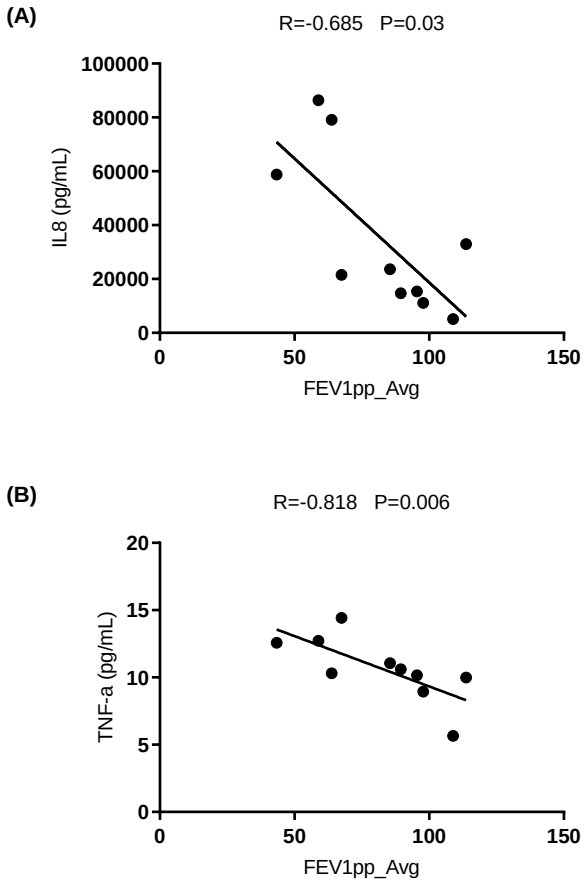
(A)
R=-0.685 P=0.03
IL8 (pg/mL)
100000
80000
60000
40000
20000
0
0
50
100
150
FEV1pp_Avg
(B)
R=-0.818 P=0.006
TNF-a (pg/mL)
20
15
10
5
0
0
50
100
150
FEV1pp_Avg

Figure 7. IL8 and TNF-α production *in vitro* is associated with lung function. (A) IL8 production at 72 hours post RSV infection in homozygous p.Phe508del CFTR cells negatively correlates with FEV_1 (% pred), R = -0.685, $p = 0.03$. (B) TNF-α production at 72 hours post RSV infection in homozygous p.Phe508del CFTR cells negatively correlates with FEV_1 (% pred), R = -0.818, $p = 0.006$. Spearman Correlation test was used to analyze the data.

Online Data Supplement

Inflammatory Epithelial Cytokines Post *in vitro* Respiratory Syncytial Viral Infection Are Associated with Reduced Lung Function

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Key words: Respiratory Syncytial Virus, Cystic Fibrosis, Pattern Recognition Receptors, cytokines, lung function

Methods

Lung function data:

Forced expired volume in 1 second (FEV1) values were obtained from our local clinical database. All measurements were performed in the clinical laboratory using GLI (global lung initiative) reference equations to determine % predicted values. FEV1 for each CF participant was determined by averaging all values in the year obtained prior to the nasal brushing (subjects are seen every 3 months).

Nasal brushing and Air-Liquid interface (ALI) culture

A 3-mm diameter sterile cytology brush (MP Corporation, Camarillo, CA) was used to brush the inferior turbinate. The human primary nasal epithelial cells (PNECs) were dissociated from the brush and seeded on a collagen-coated flask (P0). Cultures were maintained in basal epithelial growth media (BEGM, Lonza, Walkersville, MD) with antibiotics in an incubator at 37 °C with a humidified 5% CO2 atmosphere. Cells were subsequently expanded into a larger flask (P1) when confluent.

Once P1 cells were 70-80% confluent, they were seeded on collagen coated Transwell inserts (6.5mm diameter, 0.4 µm pore size, Corning, Tewksbury, MA) and cultured in BEGM on both the apical and basolateral sides until confluent. Subsequently, ALI medium (PneumaCult, StemCell Technologies, Vancouver, Canada) was applied on the basolateral side and the cells were maintained for an additional 21 days. The apical side was washed weekly with phosphate-buffered saline (PBS).

Human bronchial epithelial (HBE) cell culture

Cells were expanded to confluence in T-75 flasks in EMEM (Wisent, St-Bruno, Canada) with 10% FBS (Gibco, Gaithersburg, MD), and then seeded on collagen-coated 6.5 mm Transwell inserts. Once confluent on the insert, cells were cultured in ALI media for an additional 21 days.

RSV propagation and purification

HEp-2 cells (ATCC) were seeded in 10% FBS EMEM in tissue culture flasks and grown at 37 ºC in a 5% CO2 incubator overnight. On the next day, the cells were infected with RSV-A2 (ATCC) and incubated at 37 ºC in a 5% CO2 incubator. Virus was then harvested and purified using 30% sucrose via ultracentrifugation. Purified virus was stored at –80 ºC or in liquid nitrogen.

To propagate RSV-GFP, HEp-2 cells were seeded in 10% FBS EMEM and grown at 37 ºC in a 5% CO2 incubator overnight. Cells were infected with RSV-GFP and incubated at 37 ºC for another 3 days. On the day of harvest, cells were scraped off the flasks, transferred to a Falcon 50 mL tube and centrifuged at 820g for 10 min. The supernatant was aliquoted and kept at –80 ºC or in liquid nitrogen.

RSV infection and Transepithelial resistance (TER) measurement

Infected cells were incubated at 37 ºC in a 5% CO2 incubator and rocked manually every 15 min. After 2 hours of incubation, RSV/PBS was gently removed, and inserts were washed with warm PBS 4 times. At the 2 hour time point, 200 µL fresh PBS was added apically and was incubated for 10 min at 37 ºC. PBS washes and basolateral medium were then collected, frozen immediately and stored either in liquid nitrogen or at -80 ºC. Medium change was performed at 24, 48 and 72 hours post infection (hpi). PBS apical washes and basolateral medium were collected as previously described. TER was measured to evaluate epithelial barrier function

before adding RSV and at 24, 48 and 72hrs post RSV infection. At the 72 hour time point, RNA was harvested in Buffer RLT (Qiagen, Hilden, Germany), protein was harvested in RIPA buffer and cells were fixed for immunofluorescence (IF).

HBE cells were treated with RSV-GFP in the same way as nasal cells. At 24, 48 and 72 hours post RSV-GFP infection, cells were imaged with an inverted epifluorescence microscope (Nikon TE-2000) and analyzed using ImageJ (NIH). The percentage of GFP intensity (GFP/total nuclei) was determined from 4 random fields per well (4x magnification). The medium was harvested at 72 hpi for ELISA.

Real-Time PCR (qRT-PCR)

mRNA was reverse transcribed (RT) to cDNA using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Cedar Creek, Texas) with program settings of 25°C for 5 minutes, 42°C for 15 minutes and 95°C for 5 minutes. The primers were listed in Table S1. qPCR reactions were run on Stratagene Mx3000P and the relative mRNA expression levels were analyzed using MxPro-Mx3000P software (Agilent Technologies, Cedar Creek, Texas).

Plaque assay

PBS washes on the apical side 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 ºC. Formalin was added to each well for 30 min and then the agarose was flicked off. Subsequently, neutral red was added to each well and incubated for 1 hr. The plaques were quantified using a dissecting scope.

Immunofluorescence (IF)

Primary nasal epithelial cells were fixed with 2% paraformaldehyde and 0.01% glutaraldehyde. Cells were then gently scraped from the filter membrane and neutralized in 0.1 M phosphate buffer with 0.15 M glycine and 80 mM NH_4Cl. Subsequently, cells were permeabilized in 0.1 M phosphate buffer with 0.2% Triton X100 and washed with 0.15 M glycine 3 times. Then, the samples were blocked with 4% BSA and incubated with primary antibodies, zonula occludens-1 (ZO-1, Thermo Fisher Scientific, Waltham, MA), E-cadherin (Cell Signaling Technology, Danvers, MA), β-Catenin (Abcam, Cambridge, MA) and β-Tubulin (Sigma-Aldrich, St. Louis, MO). DAPI was applied as a nuclear stain. Confocal images were captured using the Olympus IX81 inverted fluorescence microscope (60x magnification). Images were analyzed with the Volocity software suite (Perkin Elmer).

Western blots

After electrophoresis on SDS-PAGE gels, protein samples were transferred onto nitrocellulose membranes. Blocking was performed using 5% milk. Primary antibody incubations (antibodies to ZO-1 (1:1000), E-cadherin (1:1000), β-Catenin (1:1000), β-Tubulin (1:2000) and Calnexin (CNX, 1:5000)) were performed overnight in blocking buffer. Membranes were then incubated with the appropriate secondary antibodies in blocking buffer at room temperature for 1 hour. Amersham ECL Prime Western Blotting Detection Reagents (Thermo Fisher Scientific, Waltham, MA) were used for band detection. Images were captured and analyzed using Image Lab 6.0 (Bio-Rad Laboratories, Hercules, California).

Cytokine measurements

Basolateral medium was collected at 72 hpi and cytokine expression was measured using the Bio-Plex Pro™ Human Cytokine 27-plex assay and Bio-Plex Pro Treg Cytokine 12-Plex assay, according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, California). Data was read with the Bio-Plex Systems 100 (Bio-Rad, CA, USA).

Fig S1.

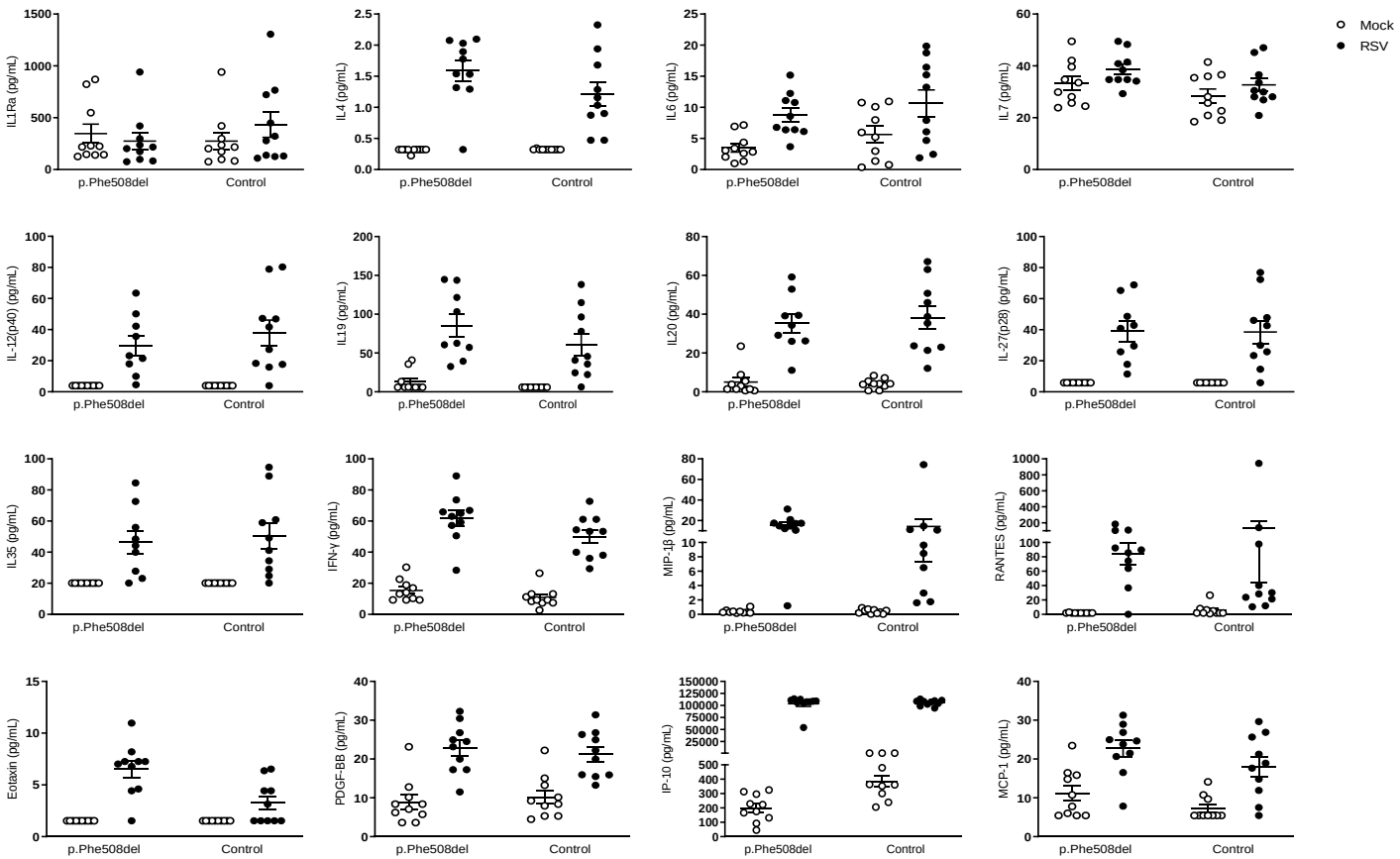
Mock
RSV
IL1Ra (pg/mL)
IL4 (pg/mL)
IL6 (pg/mL)
IL7 (pg/mL)
IL-12(p40) (pg/mL)
IL19 (pg/mL)
IL20 (pg/mL)
IL-27(p28) (pg/mL)
IL35 (pg/mL)
IFN-γ (pg/mL)
MIP-1β (pg/mL)
RANTES (pg/mL)
Eotaxin (pg/mL)
PDGF-BB (pg/mL)
IP-10 (pg/mL)
MCP-1 (pg/mL)
p.Phe508del
Control

Figure S1. The production of other cytokines. The productions of IL1Rα, IL4, IL6, IL7, IL12(p40), IL19, IL20, IL27(p28), IL35, IFN-γ, MIP-1β, RANTES, Eotaxin, PDGF-BB, IP-10 and MCP-1 are not significantly different between homozygous p.Phe508del CFTR cells and control cells post RSV infection. Non-paired data were analyzed by Mann-Whitney test and paired data were analyzed by matched Wilcoxon test (n = 9 – 10).

Fig S2.

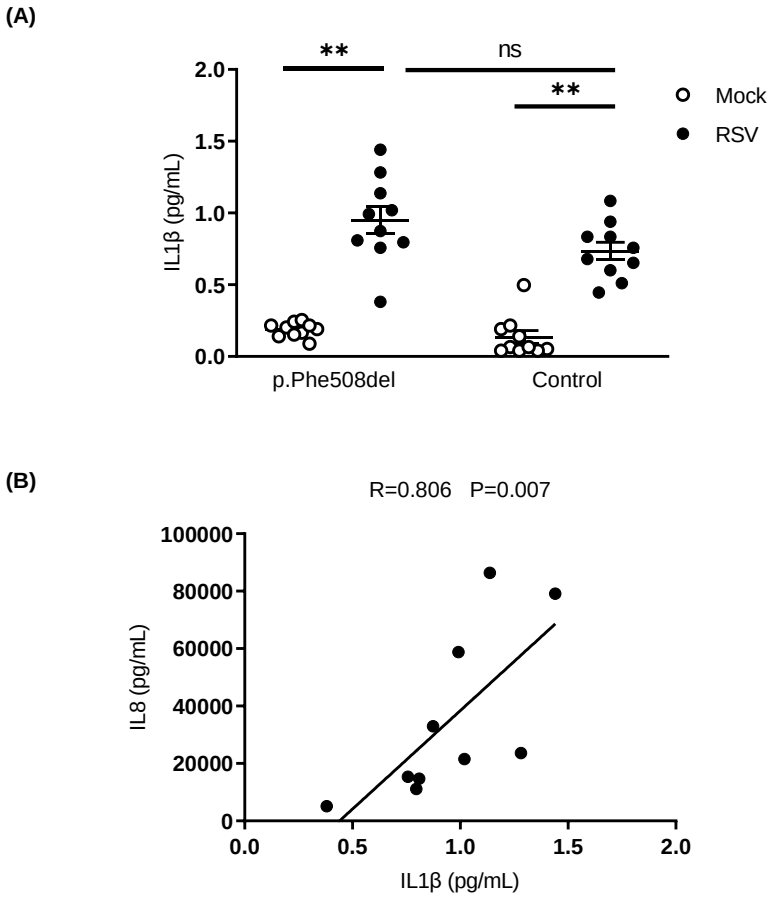
(A)
**
ns
**
Mock
RSV
IL1β (pg/mL)
2.0
1.5
1.0
0.5
0.0
p.Phe508del
Control
(B)
R=0.806 P=0.007
IL8 (pg/mL)
100000
80000
60000
40000
20000
0
0.0
0.5
1.0
1.5
2.0
IL1β (pg/mL)

Figure S2. IL1β and IL8 production. (A) RSV promotes IL1β production at 72hpi in both p.Phe508del CFTR and control cells, however, no significant difference is seen between RSV infected p.Phe508del CFTR and control cells (n = 10). The analysis was performed using Mann-Whitney test. ** $p < 0.01$; ns=not significant (B) Positive correlation is seen between the production of IL1β and the production of IL8, R = 0.806, $p = 0.007$. Spearman Correlation test was used to analyze the data.

Table S1. Primers for Real-Time PCR

RSV-NS1	Forward 5'-AGAGATGGGCAGCAATTCAT-3'
	Reverse 5'-ACTGGCATTGTTGTGAAATTGG-3'
RIG-I	Forward 5'-CAGAGCACTTGTGGACGCTT-3'
	Reverse 5'-AGCAACTGAGGTGGCAATCA-3'
MDA-5	Forward 5'-GAGCAACTTCTTTCAACCACAG-3'
	Reverse 5'-CACTTCCTTCTGCCAAACTTG-3'
ISG56	Forward 5'-CAGCAACCATGAGTACAAAT-3'
	Reverse 5'-AAGTGACATCTCAATTGCTC-3'
TLR2	Forward 5'-GTGTTTGGTGTTGCAAGCAGGATC-3'
	Reverse 5'-CACCATCCACAAAGTATGTGGCATTG-3'
TLR3	Forward 5'-CTGAACTCCATCTCATGTCCAACTCAATC-3'
	Reverse 5'-GAAGCTCTTGGAGATTTTCCAGCTGAAC-3'
TLR4	Forward 5'-CCAAGAACCTGGACCTGAGCTTTAATC-3'
	Reverse 5'-GAGAGGTGGCTTAGGCTCTGATATG-3'
TLR7	Forward 5'-CAACCAGACCTCTACATTCCATTTTGGAA-3'
	Reverse 5'-TCTTCAGTGTCCACATTGGAAAC-3'
TLR9	Forward 5'-CCCTGTAGCTGCTGTCCAGTCTG-3'
	Reverse 5'-CAGGAACAGCCAGTTGCAGTTCAC-3'
GAPDH	Forward 5'-GAAGGTGAAGGTCGGAGTCA-3'
	Reverse 5'-TTGAGGTCAATGAAGGGGTC-3'
TBP	Forward 5'-CAAACCCAGAATTGTTCTCCTT-3'
	Reverse 5'-ACGTCGTCTTCCTGAATCCCT-3'