1 **Online Data Supplement**

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

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20 cytokines, lung function

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22 **Methods**

23 ***Lung function data:***

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

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30 ***Nasal brushing and Air-Liquid interface (ALI) culture***

31 A 3-mm diameter sterile cytology brush (MP Corporation, Camarillo, CA) was used to brush the
32 inferior turbinate. The human primary nasal epithelial cells (PNECs) were dissociated from the
33 brush and seeded on a collagen-coated flask (P0). Cultures were maintained in basal epithelial
34 growth media (BEGM, Lonza, Walkersville, MD) with antibiotics in an incubator at 37 °C with
35 a humidified 5% CO_2 atmosphere. Cells were subsequently expanded into a larger flask (P1)
36 when confluent.

37 Once P1 cells were 70-80% confluent, they were seeded on collagen coated Transwell inserts
38 (6.5mm diameter, 0.4 μm pore size, Corning, Tewksbury, MA) and cultured in BEGM on both
39 the apical and basolateral sides until confluent. Subsequently, ALI medium (PneumaCult,
40 StemCell Technologies, Vancouver, Canada) was applied on the basolateral side and the cells
41 were maintained for an additional 21 days. The apical side was washed weekly with phosphate-
42 buffered saline (PBS).

43

44 ***Human bronchial epithelial (HBE) cell culture***

45 Cells were expanded to confluence in T-75 flasks in EMEM (Wisent, St-Bruno, Canada) with
46 10% FBS (Gibco, Gaithersburg, MD), and then seeded on collagen-coated 6.5 mm Transwell
47 inserts. Once confluent on the insert, cells were cultured in ALI media for an additional 21 days.
48
49 ***RSV propagation and purification***
50 HEp-2 cells (ATCC) were seeded in 10% FBS EMEM in tissue culture flasks and grown at 37 ºC
51 in a 5% CO2 incubator overnight. On the next day, the cells were infected with RSV-A2 (ATCC)
52 and incubated at 37 ºC in a 5% CO2 incubator. Virus was then harvested and purified using 30%
53 sucrose via ultracentrifugation. Purified virus was stored at –80 ºC or in liquid nitrogen.
54 To propagate RSV-GFP, HEp-2 cells were seeded in 10% FBS EMEM and grown at 37 ºC in a
55 5% CO2 incubator overnight. Cells were infected with RSV-GFP and incubated at 37 ºC for
56 another 3 days. On the day of harvest, cells were scraped off the flasks, transferred to a Falcon
57 50 mL tube and centrifuged at 820g for 10 min. The supernatant was aliquoted and kept at –80
58 ºC or in liquid nitrogen.
59
60 ***RSV infection and Transepithelial resistance (TER) measurement***
61 Infected cells were incubated at 37 ºC in a 5% CO2 incubator and rocked manually every 15
62 min. After 2 hours of incubation, RSV/PBS was gently removed, and inserts were washed with
63 warm PBS 4 times. At the 2 hour time point, 200 µL fresh PBS was added apically and was
64 incubated for 10 min at 37 ºC. PBS washes and basolateral medium were then collected, frozen
65 immediately and stored either in liquid nitrogen or at -80 ºC. Medium change was performed at
66 24, 48 and 72 hours post infection (hpi). PBS apical washes and basolateral medium were
67 collected as previously described. TER was measured to evaluate epithelial barrier function

68 before adding RSV and at 24, 48 and 72hrs post RSV infection. At the 72 hour time point, RNA
69 was harvested in Buffer RLT (Qiagen, Hilden, Germany), protein was harvested in RIPA buffer
70 and cells were fixed for immunofluorescence (IF).
71 HBE cells were treated with RSV-GFP in the same way as nasal cells. At 24, 48 and 72 hours
72 post RSV-GFP infection, cells were imaged with an inverted epifluorescence microscope (Nikon
73 TE-2000) and analyzed using ImageJ (NIH). The percentage of GFP intensity (GFP/total nuclei)
74 was determined from 4 random fields per well (4x magnification). The medium was harvested at
75 72 hpi for ELISA.
76
77 ***Real-Time PCR (qRT-PCR)***
78 mRNA was reverse transcribed (RT) to cDNA using AffinityScript QPCR cDNA Synthesis Kit
79 (Agilent Technologies, Cedar Creek, Texas) with program settings of 25°C for 5 minutes, 42°C
80 for 15 minutes and 95°C for 5 minutes. The primers were listed in Table S1. qPCR reactions
81 were run on Stratagene Mx3000P and the relative mRNA expression levels were analyzed using
82 MxPro-Mx3000P software (Agilent Technologies, Cedar Creek, Texas).
83
84 ***Plaque assay***
85 PBS washes on the apical side were diluted and added onto HEp-2 cells in 6-well plates.
86 DMEM-F12/agarose was overlaid onto the cells and incubated for 6 days at 37 ºC. Formalin was
87 added to each well for 30 min and then the agarose was flicked off. Subsequently, neutral red
88 was added to each well and incubated for 1 hr. The plaques were quantified using a dissecting
89 scope.
90

91 ***Immunofluorescence (IF)***

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

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103 ***Western blots***

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

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113 ***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).

137 **Figure Legends**

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

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

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Table S1. Primers for Real-Time PCR

Gene	Primer
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'

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