Data Supplement

The Effect of Polarized Release of CXC-chemokines from Wild-type and Cystic Fibrosis Murine Airway Epithelial Cells

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MATERIALS AND METHODS

Mice. ΔF508 CFTR mice backcrossed into a C57Bl/6 background and wild-type littermates, 6 to 8 weeks of age, were studied [**20**]. All mice were maintained in micro-isolator units, and housed in the full-barrier, pathogen-free animal facility at Washington University School of Medicine and examined twice weekly by veterinary staff for signs of illness. The Animal Studies Committee at Washington University approved all studies.

***In vitro* mouse primary airway epithelial cell preparations.** Primary mTEC preparations were cultured as previously described [**18**]. Briefly, mouse tracheas were resected and incubated in media containing 1.5 mg/ml Pronase (Roche Molecular Biochemicals, Indianapolis, IN) for 18 h at 4°C. The released cells were collected and incubated in tissue culture plates (Primeria, Becton Dickinson Labware, Franklin Lakes, NJ) for 3-4 h in 5% CO_2 at 37˚C to remove fibroblasts by adherence. Non-adherent cells were collected and seeded onto supported polycarbonate and polyester porous (0.4 μm) membranes (Transwell, Corning-Costar, Corning, NY), coated with filter-sterilized 50 mg/ml type I rat tail collagen (Becton Dickinson, Franklin Lakes, NJ). Media to stimulate proliferation was provided in the upper and lower chambers until cells were confluent. Media was then evacuated from the upper chamber to establish an air-liquid interface (ALI), and fresh Delbecco Modified Eagle's Media/Ham's F-12 (1:1 vol:vol) supplemented with 2% NuSerum (Becton Dickinson) antibiotics, and 0.01 mM retinoic acid was added to the lower chamber to induce differentiation. Scanning electron microscopy and immunohistochemical staining of mTEC for β-tubulin-IV, and Clara cell secretory protein (CCSP) were performed as per established protocols [**18**].

Electrophysiology. Electrophysiological measurements of mTEC were performed using Ussing chambers adapted for Transwell filters, modified from previously published protocols [**21**]. mTEC filters were perfused with standard Kreb's bicarbonate Ringer's solution for the measurement of short-circuit current (I_{sc}) under basal conditions and following sequential treatments with 10 μM amiloride (to block apical membrane sodium channels), 10 μM forskolin

(to stimulate intracellular cAMP) and 100 μM uridine triphosphate (UTP, to stimulate intracellular calcium).

***Pseudomonas aeruginosa* culture and cell infection.** *P. aeruginosa* PAO1 was streaked from frozen stock on tryptic soy agar plates and allowed to grow overnight. Individual colonies were transferred to 5 ml LB Broth and grown overnight until OD 600 measured 0.7 to 0.8. The broth was diluted in mTEC media without NuSerum to achieve bacterial concentrations. Epithelial cell preparations were apically treated with 50 μl PAO1 (0, 10^4, 10^6, and 10^8 cfu/ml) for 1 h at 37° C. Cells were then washed three times with PBS, and serum free media containing gentamicin was placed in both chambers of the Transwell for 0-7 d [**18**]. At each time point basolateral conditioned media and apical washings (100 μl) were collected and assayed separately.

RNA extraction and oligonucleotide array analyses. Total cellular RNA was isolated from treated and control cell preparations using Trizol reagent (Invitrogen, Carlsbad, CA) then re-purified (Qiagen, Valencia, CA). To minimize biologic variability pools of RNA samples from multiple mTEC preparation of each genotype and experimental condition were analyzed. Three replicate pools from each condition were hybridized to Illumina BeadChips that target nearly 25,400 well-annotated RefSeq transcripts derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36.2, Release 22). The resulting images were imported into the Illumina BeadStudio application. The application tracked the performance of the controls and normalized the data. Normalization was accomplished in six steps -- self-normalization used information contained within the array itself (normalization ID) followed by additional outlier removal, background estimation, rotational estimation, shear estimation, and scaling estimation (BeadStudio Gene Expression Module v3.4 User Guide). Data was imported in Partek Genomic Suite for ANOVA analysis with step up False Discovery Rate (Benjamini and Hochberg) and multiple testing correction [**22**].

TaqMan RealTime polymerase chain reaction. Total RNA from mTEC cultures was reverse transcribed as per the manufacturer's recommended protocols (Applied Biosystems, Foster

City, CA). Oligonucleotide primers and TaqMan MGB probes for CXC-chemokine genes were obtained from Applied Biosystems. Aliquots of this cDNA (in triplicates) were subjected to real time PCR (qRT-PCR) for relative quantitation using the Applied Biosystems 7500 Real Time PCR System and software, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. Standard qRT-PCR protocols were followed.

Enzyme-linked immunosorbent assay for secreted mouse cytokines. Pro-inflammatory CXC-chemokines (i.e., MIP-2, KC, LIX, and IP-10) were measured using commercially available ELISA kits (R & D Systems, Minneapolis, MN) as per the manufacturer's instructions [**23**]. Apical surface fluid volumes were calculated based on the volume equation for a right circular cylinder: $V = \pi r^2 h$, where V indicates volume, r denotes the cylinder radius, and h is fluid depth, using Transwell surface area (0.33 cm^2) and previously measured fluid depths (WT 7.9 μ; CF 3.9 μ) [**24,25**]. Chemokine concentrations were then determined by dividing the amount of mediator secreted into the specific compartment divided by fluid volume.

***In vitro* neutrophil chemotaxis assays:** Murine bone marrow cell suspensions were isolated from the femurs and tibia of wild-type mice by flushing with PBS (**26**). Erythrocytes were lysed with LCK lysis buffer (Lonza, Williamsport, PA). Cells were resuspended in RPMI 1640 medium supplemented with 10% FBS and gentamicin, and counted. Six hours after apical stimulation of mTEC preparations with *P. aeruginosa*, 1.0 x 10^7 bone marrow cells were added to the basolateral compartment and incubated for 1 hour. Cells were detached from their Transwell membranes using trypsin-EDTA and cell-dissociation solution (Sigma-Aldrich, St. Louis, MO) then collected. The released cells were then washed with FACS buffer (PBS with 2% FBS), blocked with purified rat anti-mouse CD16/CD32 antibody (Mouse BD Fc Block, Becton Dickinson Biosciences, San Jose, CA), stained with Gr1 antibody or Rat IgG2b isotype antibody control (eBioscience, San Diego, CA) for 30 min and washed with FACS buffer. A cell analysis was performed on a FACSCalibur flow cytometer using CELLquest software (Becton Dickinson Biosciences, San Jose, CA). The numbers represent the percent of Gr1-expressing cells within the cell layers on the upper side of the Transwell membranes.

Statistical analysis. Data are expressed as means ± standard error (SEM). Statistical comparisons between CF and non-CF groups were made using unpaired two-tailed Student's *t* tests or single factor analysis of variance (ANOVA).