# The effects of IFN-λ on epithelial barrier function contribute to *K. pneumoniae* ST258 pneumonia

Danielle Ahn, Matthew Wickersham, Sebastian Riquelme, and Alice Prince

ONLINE DATA SUPPLEMENT

## Supplemental Methods

### Cell lines

16HBE cells, a human bronchial epithelial cell line (D. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA), were grown at 37°C and 5% $CO_2$ in BronchiaLife B/T medium with 1% fetal bovine serum, and 1% streptomycin/penicillin. Where indicated, the 16HBE cells were grown on 3-μm pore size Transwell-Clear filters with an air-liquid interface to form polarized layers. Cells were stimulated with purified IL-22 (20 ng/mL) or IFN-λ (20 ng/mL) protein 24 hours prior and at the time of infection.

### Mouse Studies

Mice were anaesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine, infected intranasally with *K. pneumoniae* ($10^8$ CFU in 50 μL of PBS) and sacrificed between 4 hours and 96 hours after infection as previously described (3). After euthanasia and cannulation of the murine trachea, sterile $PBS^{++}$ was instilled in the airway in 1 mL aliquot x 3. After an aliquot was used to enumerate CFU in total BALF, the samples was then spun at 1000 rpm (218 rcf) for 10 minutes. The supernatant was then isolated and frozen for use in cytokine assays or blast proteomics. The cellular pellet was then set aside for characterization by flow cytometry.

**Lung Immunofluorescence**

For lung immunofluorescence, mouse lungs were removed from the thoracic cavity en bloc and fixed in non-paraffin fixative for 1 hour and then transferred to 70% ethanol. Tissue was then placed in paraffin blocks in the usual fashion. Five mm sections were then mounted on glass slides and deparaffinization and rehydration was performed in the usual fashion with citrate butter and antigen retrieval using a microwave. ZO-1 Antibody (Abcam, anti-Rabbit) was incubated 1:400 over night at 4$^{o}$C, then secondary Donkey anti-Rabbit AF567. DAPI counterstain (1:1000) was applied for 10 min. After mounting, sections were imaged on Leica DMi8 S Platform.

**Analysis of immune cell populations**

To further delineate immune cell populations, analysis of cell populations in BALF or single cell suspension of lung homogenate was conducted using multi-color FC on a BD LSR II. Cells were labeled with a combination of flourophores detailed in **Supplemental Table 1**. LIVE/DEAD Fixable Dead Cell Stain Kits, blue fluorescent dye (Molecular Probes) and Fc block (CD16/32) were added to each sample. Cellular populations are defined as Alveolar macrophages (**Alv Macs**) ($CD45^{+}SiglecF^{+}CDII11b^{lo\text{-}mid}$), granulocytic-myeloid derived suppressor cells/neutrophils (**G-MDSCs/NEUTs)** ($CD45^{+}CD11b^{+}MHCII^{lo}Ly6C^{hi}Ly6G^{hi}$) and monocytic-myeloid derived suppressor cells (**M-MDSCs**) ($CD45^{+}CD11b^{+}MHCII^{lo}Ly6C^{hi}Ly6G^{lo}$) as previously shown (1). Uniform dyed microspheres (Bangs Laboratories) were added to calculate the concentration of

cellular components. All flow data was analyzed on FlowJo (ver 10.0.8).

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**Bone marrow MDSC (BM/MDSC) differentiation and infection**

BM/MDSCs were differentiated as previously described(1) from WT and *Il22*$^{-/-}$ mice. Briefly, 5 x $10^6$ bone marrow cells were seeded into 100 mm dishes in 10 mL of RPMI 1640 2 mM L-glutamine, 10 mM HEPES, 20 µM 2-ME, 1% streptomycin/penicillin, and 10% heat-inactivated FBS supplemented with GM-CSF (40 ng/mL) and G-CSF (40 ng/mL) (Peprotech). Cells were maintained at 37ºC and 5% $CO_2$ for 5 days. On day 5, cells were washed twice and resuspended in RPMI 1640 + FBS 10%. Five x $10^5$ cells/well were then seeded into 24-wells plate and stimulated with PBS or KP35 (MOI 10) for 4 hours at 37ºC and 5% $CO_2$. Filtered and supernatants treated with gentamicin (500 µg/mL) after 4 hours were used for dextran transmigration assays.

**qRT-PCR**

Total RNA was isolated from cultured cells and lung homogenates using the eZNA Total RNA kit (Omega bio-tek) followed by DNase treatment with DNAfree (Life Technologies). All cDNA was produced with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Power SYBR Green PCR

Master Mix (Applied Biosystems) in a StepOne Plus thermal cycler (Applied Biosystems) was used to perform qRT-PCR. Sequences were designed using Primer3-BLAST (NCBI/NIH). Samples were normalized to β-actin (**Supplemental Table 2**).

**Immunoblots**

Cell lysates (cell culture or lung homogenate) were studied by Western blot in the usual fashion. The antibodies used were IL-22 (Abcam; 1:1000), Ezrin, IL-10Rβ and occludin (Santa Cruz Biotechnology; 1:200). Actin or GAPDH antibodies (Santa Cruz Biotechnology; 1:1000) were used as loading controls. Blots were exposed using the ProteinSimpleM apparatus. Densitometry was measured using ImageJ64 software (1.48v) and presented as image intensity relative to actin control from the same immunoblot.

**Dextran Permeability Assays and Transepithelial Electrical Resistance (TEER)**

16HBE cells were grown on a 3-μm pore size Transwell-Clear filters with an air-liquid interface. After polarized monolayers formed, the cells were basolaterally stimulated with purified IL-22 (20 ng/mL), IFN-λ (20 ng/mL) or supernatants from MDSCs for 24 hours. Cells were then treated with PBS or KP35 (MOI 10) for 4 hours before adding dextran-fluorescein (MW 3,000) apically for 1 hour. Fluorescence (ex 485/em 535) in the basal compartment was measured on a SpectraFluor Plus fluorometer (Tecan). For TEER, 200 μL of media was added to the apical compartment prior to TEER measurements with EVOM$^2$ epithelial

voltometer (World Precision Instruments). Three values were obtained and averaged per well before and after stimulation with cytokine or control condition. EDTA served as a positive control.

**Shotgun Proteomics**

**Mice**

C57BL/6J mice were given KPPR1 ($10^5$ cfu), KP35 ($10^8$ cfu) or PBS and BALF harvested at 48 hours post inoculation. Equivalent inocula were recovered ($10^6$ CFU) at this time point despite disparate initial inocula. Proteomic analysis was performed on BALF pooled from 3 mice per experimental condition.

**Material**

HPLC grade LC buffers, dithiothreitol (DTT), acetonitrile (ACN), ammonium bicarbonate, trifluoretic acids (TFA), and iodoacetamide (IAA) were purchased from Thermo Fisher Scientific (Waltham, MA). Trypsin Gold, mass spectrometry grade, was purchased from Promega (Madison, WI). Nanopure water was prepared with use of Milli-Q water purification system (Millipore, Billerica, MA).

**In Solution Protein Digestion**

Proteins were precipitated from 500 µl of BALF using methanol/chloroform and protein concentrations were determined by the Qubit assay according to the manufacture's instruction (Life Technologies). 50 µg of proteins were reduced, alkylated, and digested with 500 ng Trypsin Gold in 200 µl of 50 mM ammonium bicarbonate at 37°C for 16 hours. After digestion, the peptide mix was centrifuged subsequently for 30 minutes at 14,000 rpm (20,817 rcf), and the cleared supernatants were transferred to fresh tubes to be dried and

resuspended in 0.1% TFA for subsequent peptide fractionation using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher). 7 peptide fractionations were collected and lyophilized for LC-MS/MS analysis.

**Sample Preparation**

Proteins from pooled samples of mouse BALF (total volume 500 μl) were precipitated by methanol/chloroform and resuspended in 20 μl of 4 M urea in 50 mM ammonium bicarbonate. Protein concentration in the mouse lavage was determined by the EZQ Protein Quantification Assay (Life Technology Corp. CT). 2 μg of protein from mouse lavage were digested with 150 ng of trypsin (1:40) along with 2 mM $CaCl_2$ and incubated at 37$^{o}$C for 16 hours. Samples were centrifuged for 30 minutes at 14,000 rpm (20,817 rcf), and the cleared supernatants were transferred to fresh tubes to be acidified with 90% formic acid (2% final) to stop proteolysis. The soluble peptide mixtures were collected for LC-MS/MS analysis.

**LC-MS/MS Analysis**

The concentrated peptide mix was reconstituted in a solution of 2 % acetonitrile (ACN), 2 % formic acid (FA) for MS analysis. Peptides were loaded with the autosampler directly onto a 2 cm C18 PepMap pre-column and were eluted from the 50 cm x 75 μm ID PepMap RSLC C18, 2 μm column using a Thermo Dionex 3000 with a 98 min gradient from 2% buffer B to 30 % buffer B (100 % acetonitrile, 0.1 % formic acid). The gradient was switched from 30 % to 85 % buffer B over 5 min and held constant for 1 minute. Finally, the gradient was changed from 85 % buffer B to 98 % buffer A (100% water, 0.1% formic acid)

over 2 minutes, and then held constant at 98 % buffer A for 25 more minutes. The application of a 2.0 kV distal voltage electrosprayed the eluting peptides directly into the mass spectrometer equipped with an Easy-spray source (Thermo Finnigan, San Jose, CA). Full mass spectra (MS) was recorded on the peptides over a 400 to 1500 m/z range at 120,000 resolution, followed by tandem mass (MS/MS) CID (collision induced dissociation) events for a total of a 3 sec cycle. Charge state dependent screening was turned off, and peptides with a charge state of 2-6 were analyzed. Mass spectrometer-scanning functions and HPLC gradients were controlled by the Xcalibur data system (Thermo Finnigan, San Jose, CA). Three technical replicates were run for each sample, and MS/MS data from technical replicates were merged for subsequent database search.

**Database Search and Interpretation of MS/MS Data**

Tandem mass spectra from raw files were searched against a human protein database using the Proteome Discoverer 1.4 (Thermo Finnigan, San Jose, CA). The Proteome Discoverer application extracts relevant MS/MS spectra from the .raw file and determines the precursor charge state and the quality of the fragmentation spectrum. The Proteome Discoverer probability-based scoring system rates the relevance of the best matches found by the SEQUEST algorithm (2). The mouse database was downloaded as FASTA-formatted sequences from Uniprot protein database (database released on December, 2014). The peptide mass search tolerance was set to 10 ppm. A minimum sequence length of 7 amino acids residues was required. Only fully tryptic peptides were considered. To calculate confidence levels and false positive rates

(FDR), Proteome Discoverer generates a decoy database containing reverse sequences of the non-decoy protein database and performs the search against this concatenated database (non-decoy + decoy) (3). The discriminat score was set at 1% FDR determined based on the number of accepted decoy database peptides to generate protein lists for this study. Spectral counts used to identify each protein were used for expression profiling analysis. Qlucore Omics Explorer (Qlucore AB, Sweden) was used to perform statistical analysis of quantifiable proteins among biological replicates (t-test, $p<0.05$). Differentially expressed proteins were analyzed using DAVID (4).

**Neutrophil Migration Assay**

Neutrophil migration assays were performed as previously described (5) with some modifications. 16HBE cells were plated on the underside of 3 μm Transwells. When confluent, cells were polarized across an air liquid interface for 5 days by removing the 500 μl of media in the lower chamber. Cells were pretreated basolaterally with purified IL-22 (20 ng/mL) or IFN-λ (20 ng/mL) 24 hours prior to stimulating cells with KP35 ($2 \times 10^6$ CFU) on the apical surface. After 2 hours of infection, $10^6$ neutrophils were added to the basolateral side. These neutrophils were isolated from the blood of healthy human volunteers (IRB Protocol AAAR1395) using dextran sedimentation and Hypaque-Ficoll (Sigma) density-gradient separation and hypotonic lysis of erythrocytes. After 2 hours, neutrophils on the apical side were collected and stained using TrypanBlue and counted using a hemocytometer.

## References


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## Supporting Information Legends

**Table 1. Primers used for qRT-PCR.**

**Table 2. Fluorescently tagged antibodies used for flow cytometry.** The multicolor flow panel used for immune cell enumeration in Figureure 5B, F.

## Supplemental Tables

**Table 1**

| Gene | Species | Forward Primer | Reverse Primer |
|---|---|---|---|
| *ACTB* | Human | 5'-GTG GGG CGC CCC AGG CAC CA-3 | 5'-CGG TTG GCC TTG GGG TTC AGG GGG G-3' |
| *EZR* | Human | 5'-TTC TGC TCT GAC TCC AGG TTG-3' | 5'-CAG TTG TAT TTG GCT GGA TTG C-3' |
| *IL22* | Human | 5'-CAG CAA CAG GCT AAG CAC AT-3' | 5'-TTC AGC TTT GCT CTG GTC AA-3' |
| *IFNL2/3* | Human | 5'-AGC TGC AGG TCC AAG AGC G-3' | 5'-GGT GGT CAG GGC TGA GTC ATT-3' |
| *OCLN* | Human | 5'-CGG TTG GCC TTG GGG TTC AGG GGG G-3' | 5'-ATG AAG CCC TTT GCT GCT CT-3' |
| *Actb* | Mouse | 5'-CCT TTG AAA AGA AAT TTG TCC-3' | 5'-AGA AAC CAG AAC TGA AAC TGG-3' |
| *Il22* | Mouse | 5'-AGA CAG GTT CCA GCC CTA CA-3 | 5'-CAG GTC CAG TTC CCC AAT CG-3' |
| *Ifnl2/3* | Mouse | 5'-AGC TGC AGG CTT TCA AAA AG-3' | 5'-TGG GAG TGA ATG TGG CTC AG-3' |
| *Il10rb* | Mouse | 5'-ACC TGC TTT CCC CAA AAC GAA-3' | 5'-TGA GAG AAG TCG CAC TGA GTC-3' |

**Table 2**

| Label | Marker | Clone;Company |
|---|---|---|
| PerCP-Cy5.5 | α-CD11c | N418; Biolegend |
| fluorescein (FITC) | α-MARCO | MCA1849FT; BioRad |
| phycoerythrin (PE)-CF594 | α-Ly6C | AL-21; BD Biosciences |
| PE-Cy7 | α-F4/80 | BM8; Biolegend |
| PE-labeled | α-CD200R | PK136; eBioscience |
| BV421 | α-CD86 | GL-1; Biolegend |
| BV510 | α-CD11b | M1/70; Biolegend |
| BV605 | α-Ly6G | 1A8; BD Biosciences |
| BV650 | α-CD206/MMR | C068C2; Biolegend |
| APC-Cy7 | α-MHCII | M5/114.15.2, Biolegend |
| AF700 | α-CD45 | 30-F11; Biolegend |
| AF647 | α-Siglec F | E50-2440; BD Biosciences |
| Fc Block | α-mouse CD16/32 | 93; Biolegend |