# Response to acute lung infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis mice

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## ONLINE DATA SUPPLEMENTAL

## METHODS: SUPPLEMENTAL

**Mice**. Breeding pairs of congenic B6.129P2-*Cftr*$^{tm1Unc}$ heterozygotes (stock #2196; *Cftr*$^{+/-}$ mice) and STOCK *Cftr*$^{tm1Unc}$-TgN(FABPCFTR)#Jaw mice (stock #2364) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). *Cftr*$^{+/-}$ siblings were bred, and although pups were sometimes found dead following birth, there was no indication that the deaths favored a particular genotype (data not shown). Pups were genotyped at 7-10 days of life using toe clips as the DNA source, as published elsewhere[E1]. STOCK *Cftr*$^{tm1Unc}$-TgN(FABPCFTR)#Jaw mice were bred as homozygotes for both the FABPCFTR transgene and the *Cftr* mutation, and have a CF lung phenotype comparable to *Cftr*$^{-/-}$ mice [E2]. Therefore, STOCK *Cftr*$^{tm1Unc}$-TgN(FABPCFTR)#Jaw (CF) mice were used, and results were compared to *Cftr*$^{+/-}$ (wild type) mice (littermates to *Cftr*$^{-/-}$ mice). Only female mice were used in these studies.

**Animal husbandry.** Mice were housed in autoclaved Micro-Isolator units (Lab Products, Inc., Seaford, DE), bedded on autoclaved combination size corncob bedding (The Andersons, Maumee, OH), and autoclaved city water was available ad libitum. Breeding females were fed autoclaved Harlan Teklad 9F Sterilizable Rodent Diet (W) 7960 (Harlan Teklad, Madison, WI) while males and experimental mice were fed irradiated Prolab® RMH 3000 (Purina Mills, Inc., St. Louis, MO). Fluorescent lights were cycled 12 hours on, 12 hours off, and ambient temperature (22 ± 1ºC) and relative humidity (40-60%) was regulated. Case Western Reserve University's Health Sciences Animal Resource Center is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility, and mice were maintained in

specific pathogen-free (SPF) conditions, as described previously [E3]. All procedures were approved by Case Western Reserve University's Animal Care and Use Committee.

***Pseudomonas aeruginosa* strains**. Dr. Michael Tosi originally provided mucoid clinical strain PA M57-15. Bacteria were maintained in glycerol stocks and stored at –70°C.

***P. aeruginosa* infection of mice**. Materials and supplies were purchased from Fisher Scientific (Hanover Park, IL). A 10-µl aliquot of bacteria from glycerol stocks was streaked for isolation on tryptic soy agar (TSA) and incubated at 37°C overnight. A 10-µl aliquot from a glycerol stock was used to inoculate 50 ml of tryptic soy broth (TSB) in a 125 ml Erlenmeyer flask, which was placed in a shaking incubator at 37°C overnight. A 0.5 ml aliquot of the bacterial suspension was used to inoculate a second flask of 50 ml of TSB. Bacteria were grown to late log phase (18-21h), in a shaking incubator at 37°C. Bacteria were washed three times with 10 ml of sterile PBS, pH 7.4 prior to inoculation into mice. Optical density ($OD_{600}$) and/or quantitative bacteriology were determined on an aliquot of the bacterial broth. Some aliquots were lightly fixed with 0.5% gluteraldehyde in HBSS for 30 min. to kill the bacteria yet leave the flagella and pili largely intact and others were heat killed at 60ºC for 1h.

Mice were 7.1 ± 1.1 weeks old and weighed 18.0 ± 1.9 g at the time of inoculation. Mice were anesthetized with isoflurane using a vaporizer (VetEquip, Inc., Pleasanton, CA). Mice were inoculated with 0.02 of bacterial broth ($10^5$ to $10^9$ CFU/mouse) by insufflation using a sterile pipet placed just above the nostril. All mice tolerated the procedure well, and none experienced mortality during or immediately after the procedure.

**Evaluation of mice following infection**. Mice were observed daily for clinical signs, such as coat quality, posture, ambulation, hydration status, and body weight. Mice that could not right themselves after being placed in lateral recumbency were considered moribund and were sacrificed before termination of the experiment. Mice were sacrificed using >70% carbon dioxide in air followed by exsanguination by direct cardiac puncture, in accordance with 2000 AVMA Panel on Euthanasia guidelines[E4]. Gross lung pathology was noted.

**Bronchoalveolar lavage**. Following sacrifice, BAL was performed, and cell counts were performed on resuspended cell pellets and the sterile filtered supernatant was processed for evaluation of mediators, as described elsewhere [E1]. Murine cytokines measured were TNF-α, IL-1β, IL-6, IL-4, IL-5, IL-10, IL-12, and IFN-γ read by a Luminex[100] using murine Linco*plex*[TM] Multiplex immunoassay kits (Linco Research, Inc., St. Charles, MO) and reagents from R&D Systems (Minneapolis, MN) to generate standard curves. The murine neutrophil chemokines, MIP-2 and KC were measured by ELISA, according to the manufacturer's recommendations (R&D Systems) or as with the aforementioned mediators. Values that fell below the limits of detection for the assay were assigned a value equal to the lowest limit of detection for each assay. Values were corrected for urea dilution [E5] and expressed as ng/ml ELF.

**Lung histopathology**. Following BAL, lungs were inflation-fixed with 1 ml of 2% paraformaldehyde in PBS for at least 48 hours, cut midsagitally, and embedded in paraffin. Sections were cut at regular 50μ intervals, and 5μ sections were stained with H&E using standard methods. Adjacent sections were stained with alcian blue and PAS. The area of the lung that was inflamed was assessed from H&E stained sections by

quantitative morphometric techniques, as described elsewhere [E6]. Qualitative production of mucin was assessed in airways and airway epithelial cells from alcian blue/PAS stained slides.

**Bacterial clearance**. The right and left lung were placed in 9 ml of cold, sterile PBS and homogenized. Ten-fold serial dilutions of lung homogenates were plated on tryptic soy agar, in duplicate, and the number of colonies counted 20-24 hours after incubation at 37ºC.

**Statistics**. Statistical software packages SigmaPlot V2.03 (SPSS) or SAS (Cary, NC) were used. Two-group comparisons of means were made using an unpaired Student's t-test or the Mann-Whitney U test. The Bonferroni correction was applied when a single outcome was compared between groups at multiple time points. Comparisons of more than two groups was made using one-way analysis of variance or the nonparametric Kruskal-Wallis test, with Tukey or Bonferroni post-hoc adjustment for multiple pairwise comparisons. When pooling data from more than one experiment, effects of experiment were controlled for using two-way analysis of variance with experiment as one factor, or using the nonparametric van Elteren test [E7], using experiment as a stratifying factor. Data are represented as the means ± standard error of the mean of the raw data. Data from untreated control animals are shown at time point zero.

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**Figure Legends**

**Figure E1.** Leukocytes in BAL fluid (BALF) after a single inoculation with *P. aeruginosa*. Wild type (closed circle) and CF mice (open triangle) were inoculated with a single dose of *P. aeruginosa*. Mice were sacrificed 1h, 2h or 3h later in a single experiment (n = 6/group). BAL was performed and relative (A, C and E) and absolute numbers (B, D and F) of alveolar macrophages (A and B), neutrophils (C and D) and lymphocytes (E and F) were enumerated, as described. Arrows indicate the timing of infection. Data points at the t=0 represent cell counts from BAL fluid collected from untreated mice sacrificed at three different time periods during the course of these studies (n = 12/group). *Significantly different ($P < 0.001$; unpaired Student's t-test) from wild type controls at the same time point. †Significantly different ($P < 0.001$; Kruskal-Wallis test and $P < 0.05$ using Dunn's method to make pairwise comparisons) from wild type controls at the same time point. The Bonferroni method was used to adjust for multiple testing.

**Figure E2:** Area of the lung that was inflamed after a single inoculation with *P. aeruginosa*. CF (open triangles) and wild type mice (closed circles) were inoculated with *P. aeruginosa* by insufflation, and sacrificed 3h, 1d or 2d after infection from a single experiment. The A) left and B) right lungs were evaluated for the area of the lung that was inflamed, as described. C) The data were pooled for the entire lung. There were no significant differences between the CF and wild type mice at any time point evaluated ($P > 0.05$; unpaired Student's t-test).

**Table E1.** Lung inflammation in response to a 5 min exposure to isoflurane in of CF and wild type mice (n = 3/group).

| | Mouse | Untreated | Time post anesthesia exposure | | | | |
|---|---|---|---|---|---|---|---|
| Parameter | Phenotype | Control | Immediate | 1h | 3h | 6h | 24h |
| Leukocytes ($10^3$ cells/ml BALF) | WT | 63.3 ± 13.8 | 85.0 ± 39.7 | 60.0 ± 17.5 | 95.8 ± 16.6 | 56.5 ± 15.3 | 83.3 ± 13.8 |
| | CF | 53.3 ± 21.3 | 66.7 ± 11.8 | 10.8 ± 52.9 | 70.0 ± 31.3 | 73.3 ± 30.6 | 54.2 ± 12.3 |
| %AM | WT | 99.2 ± 1.1 | 99.0 ± 1.0 | 99.1 ± 1.0 | 99.2 ± 0.5 | 99.8 ± 0.2 | 96.0 ± 6.9 |
| | CF | 99.2 ± 0.4 | 99.3 ± 1.2 | 99.8 ± 0.4 | 99.4 ± 0.5 | 99.4 ± 0.5 | 99.9 ± 0.2 |
| %PMN | WT | 0.2 ± 0.2 | 0.2 ± 0.2 | 0.2 ± 0.4 | 0.1 ± 0.2 | 0.1 ± 0.2 | 2.3 ± 4.0 |
| | CF | 0.3 ± 0.6 | 0.3 ± 0.6 | 0.1 ± 0.2 | 0.0 ± 0.0 | 0.1 ± 0.2 | 0.1 ± 0.2 |
| %Lymphocytes | WT | 0.6 ± 1.0 | 0.8 ± 0.8 | 0.7 ± 0.7 | 0.7 ± 0.3 | 0.1 ± 0.2 | 1.7 ± 2.9 |
| | CF | 0.4 ± 0.5 | 0.3 ± 0.6 | 0.1 ± 0.1 | 0.6 ± 0.5 | 0.4 ± 0.4 | 0.0 ± 0.0 |
| IL-6 (ng/ml ELF) | WT | 1.70 (n = 2) | 1.18 ± 0.24 | 1.15 ± 0.26 | 1.59 ± 0.62 | 1.08 ± 0.25 | 1.46 ± 0.26 |
| | CF | 1.42 ± 0.53 | 0.53 ± 0.03 | 0.61 ± 0.07 | 0.96 ± 0.06 | 0.82 ± 0.07 | 1.26 ± 0.26 |

Data are expressed as means ± standard deviation.

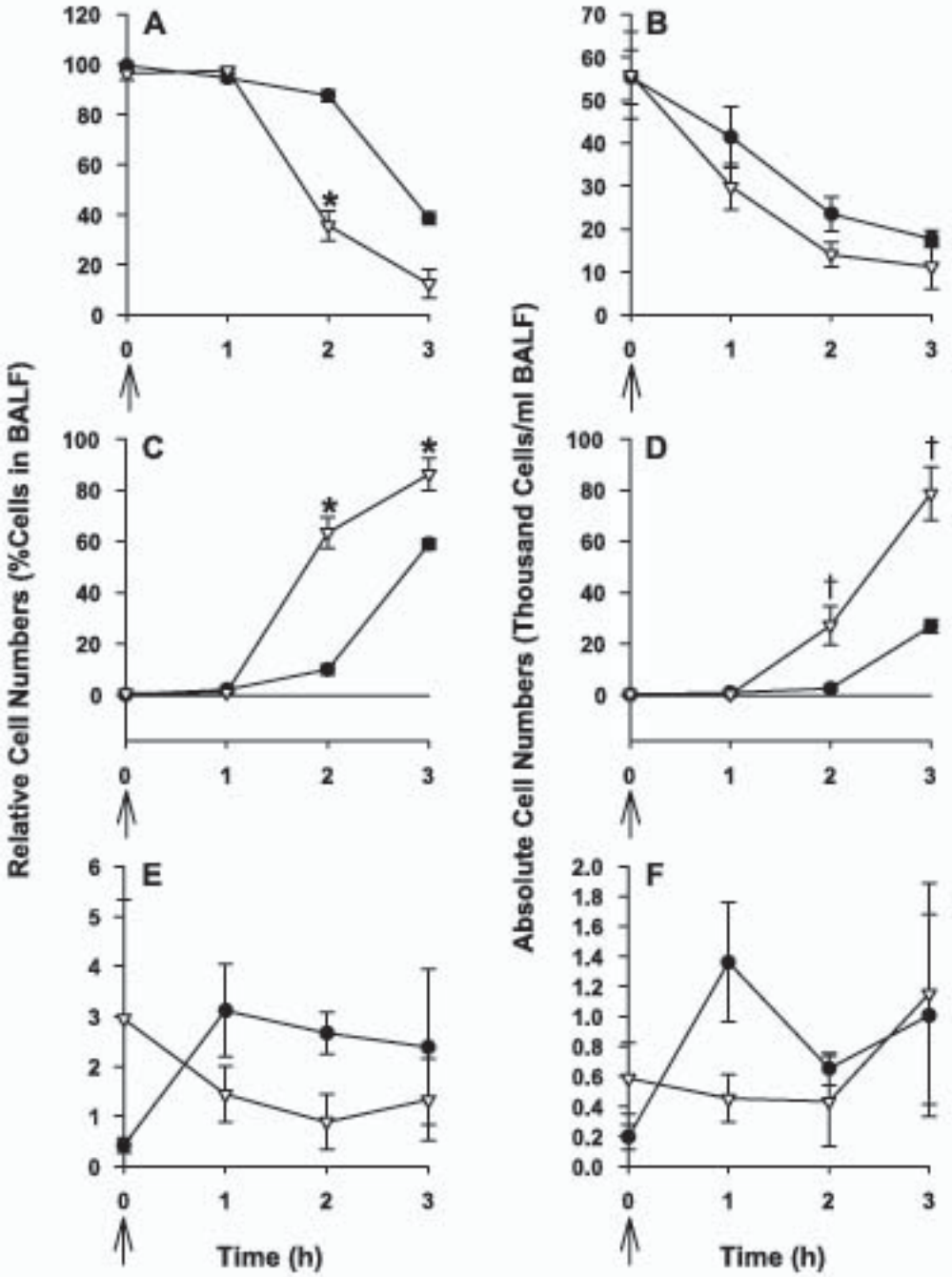


Figure E1: van Heeckeren et al.

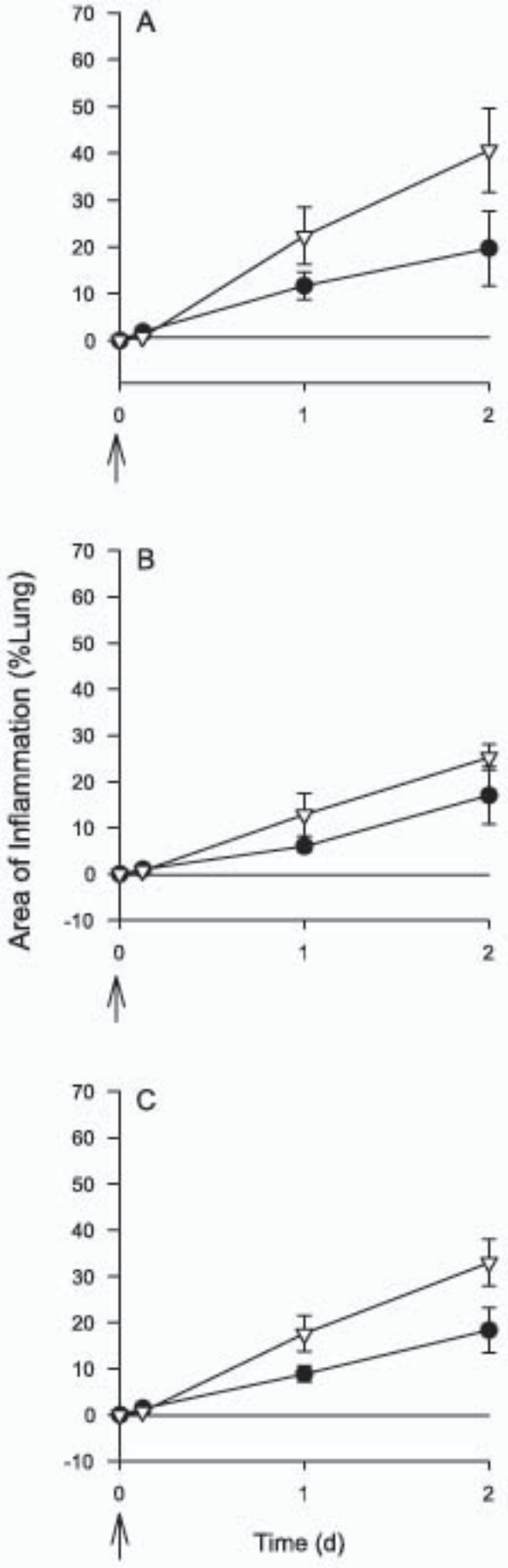


Figure E2: van Heeckeren et al.