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## Mucoid *Pseudomonas aeruginosa* and regional inflammation in the cystic fibrosis lung

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### Abstract

**Background:** *Pseudomonas aeruginosa* is the prominent bacterial pathogen in the cystic fibrosis (CF) lung and contributes to significant morbidity and mortality. Though *P. aeruginosa* strains initially colonizing the CF lung have a nonmucoid colony morphology, they often mutate into mucoid variants that are associated with clinical deterioration. Both nonmucoid and mucoid *P. aeruginosa* variants are often co-isolated on microbiological cultures of sputum collected from CF patients. With regional variation in bronchiectasis, tissue damage, inflammation, and microbial colonization, lobar distribution of nonmucoid and mucoid *P. aeruginosa* variants may impact local microenvironments in the CF lung, but this has not been well-studied.

**Methods:** We prospectively collected lobe-specific bronchoalveolar lavage (BAL) fluid from a CF patient cohort (n=14) using a standardized bronchoscopic protocol where collection was performed in 6 lobare regions. The lobar BAL specimens were plated on *P. aeruginosa*-selective media and proinflammatory cytokines (IL-1, TNF, IL-6 and IL-8) were measured via cytokine array. Correlations between infecting *P. aeruginosa* variants (nonmucoid, mucoid, or mixed-variant populations), the lobar regions in which these variants were found, and regional proinflammatory cytokine concentrations were measured.

**Results:** *P. aeruginosa* mucoid and nonmucoid variants were homogenously distributed throughout the CF lung. However, infection with mucoid variants variants (found within single-or mixed-variant populations) was associated with significantly greater regional inflammation. The upper and lower lobes of the CF lung did not exhibit differences in inflammatory cytokine concentrations.

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**Conclusions:** Mucoïd *P. aeruginosa* infection is a microbial determinant of regional inflammation within the CF lung.

### Keywords

airway; cystic fibrosis; *Pseudomonas aeruginosa*; lung; mucoïd; inflammation; cytokines

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## 1. Introduction

Cystic fibrosis (CF) is a life-shortening genetic disease that affects approximately 30,000 patients in the United States and more than 70,000 worldwide (1). CF patients suffer from devastating, chronic pulmonary infections associated with hyperinflammation and irreversible damage to the lower airways (2). As CF patients age, *Pseudomonas aeruginosa* becomes the predominant pathogen in the respiratory tract during the second decade of life for the majority of the patient population (3, 4). During the initial acquisition period, *P. aeruginosa* strains have a nonmucoïd colony morphology (5). Once chronic infection is established, these bacteria may acquire mutations leading to the emergence of mucoïd *P. aeruginosa* variants, which overproduce the exopolysaccharide, alginate, and exhibit enhanced recalcitrance to antimicrobial therapy (6-9). *P. aeruginosa* mucoïd conversion is associated with a worse prognosis in CF, manifested as a precipitous decline in pulmonary function and heightened mortality (10-16).

Mucoïd and nonmucoïd variants are often co-isolated on microbial culture of CF sputum, suggesting a mixed-variant populations may have selective advantages withstanding endogenous and exogenous factors in the CF lung (i.e. from host immunity and/or antimicrobials, respectively) (17-27). Recent work from our laboratory demonstrates that co-cultures of mucoïd and nonmucoïd *P. aeruginosa* strains are more resistant to innate antimicrobials, such as reactive oxygen species (e.g. H<sub>2</sub>O<sub>2</sub>) and a cationic antimicrobial peptide, LL-37, compared to mono-cultures of either variant (28).

CF lung disease is heterogeneous with a regional distribution of bronchiectasis, tissue damage, and inflammation. Radiographic and histopathology studies show an upper lobe predominance in the CF lung, which are marred by greater architectural changes (e.g. bronchiectasis, air trapping, and emphysema) compared to the lower lobes (29-36). The anatomic, microbial, and intrinsic immune factors contributing to interlobar variations in CF lung disease are poorly understood. Using bronchoalveolar lavage (BAL) fluid, studies have shed some light on the regional distribution of host and microbial factors contributing to the heterogeneity of CF lung disease (37-40). Neutrophils and neutrophil-derived antimicrobials (e.g. elastase) are at higher concentration levels in BAL fluid from upper lobes compared to lower lobes (37). Regional compartmentalization of bacteria has been reported in the CF lower airways with either homogenous or heterogenous distribution of bacterial species across lung lobes (38, 39, 41-44). However, few studies have examined the effect of regional *co-localization* of both bacterial and host products within the CF lung to investigate dynamic microbial-immune interactions and local inflammation. More importantly, the lobar distribution and associated regional inflammatory response to mixed communities of mucoïd and nonmucoïd variants of *P. aeruginosa* has not been studied.

The goals of this study were two-fold. First, identify if *P. aeruginosa* mucoid or nonmucoid variants, either in single- or mixed-variant populations, preferentially localize to certain lobes of the CF lung. Second, determine if mucoid and nonmucoid variants are associated with differential patterns of regional inflammation, as measured by proinflammatory cytokines (i.e. IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8). We collected lobar BAL fluid from CF patients, quantitated microbial density and *P. aeruginosa* colony variants by culture-based methods, and determined proinflammatory cytokine concentrations via multiplex array. We demonstrate that though mucoid and nonmucoid *P. aeruginosa* variants are homogeneously distributed throughout the CF lung, infection with mucoid variants (in single- or mixed-variant populations) correlates with greater lobar inflammation compared to nonmucoid variants. Our findings herein contribute to a growing understanding of host-pathogen interactions that shape regional microenvironments within the CF lung.

## 2. Materials and Methods

### 2.1. Study population and ethics statement.

The study was approved by the Nationwide Children's Hospital (NCH) Institutional Review Board (IRB07-00396 and IRB15-00800) with informed consent and assent obtained from all patient volunteers.

### 2.2. Bronchoalveolar lavage (BAL) protocol.

While undergoing clinically-indicated surgical procedures, BAL fluid was collected from 14 patients with CF via flexible fiberoptic bronchoscopy (FFB). All volunteers had clinically stable CF lung disease, had performed spirometry to assess pulmonary function [e.g. forced expiratory volume in 1 second (FEV1)], and were not acutely ill or requiring antimicrobial treatment for pulmonary symptoms. Each subject had an endotracheal tube placed and received mechanical ventilation prior to their surgical procedure and during the FFB. The BAL fluid samples were collected within 10 minutes of initiation of mechanical ventilation for the entire cohort. For each subject, BAL fluid was obtained from subsegmental airway of the right upper lobe (RUL), right middle lobe (RML), right lower lobe (RLL), left upper lobe (LUL), lingula (LIN), and left lower lobe (LLL). Twenty mL of sterile normal saline was instilled sequentially into each subsegmental bronchus and immediately recovered by manual suction with no dwell time. The BAL fluid was placed on ice and transported to the research laboratory. Demographics of all patients in this study are delineated in Table 1.

### 2.3. Microbiological analyses.

Patient BAL fluid specimens were maintained on ice immediately following FFB and throughout specimen processing. Within 2 hours of specimen collection, each BAL fluid sample was serially-diluted in Phosphate Buffered Saline [(PBS), Gibco™, pH 7.4] and plated for colony forming units (CFUs/mL) on nonselective [Luria Agar (LA), 10g/L tryptone, 5g/L yeast extract, 10g/L NaCl solidified with 1.5% agar] and *P. aeruginosa*-selective [*Pseudomonas* Isolation Agar (PIA)] medium. After 48 hours incubation at 37°C, bacterial colonies were counted on LA and PIA, and total bacterial CFUs/mL and *P. aeruginosa* CFUs/mL were determined from each type of medium, respectively. A minimum of 10 colonies of growth on LA and PIA was used as the limit-of-detection.

*P. aeruginosa* variants (nonmucoid, mucoid, or mixed nonmucoid and mucoid) were identified and counted based on colony morphology on PIA. PIA, which contains Triclosan, facilitates growth and maintenance of mucoid *P. aeruginosa* variants more effectively compared to other culture media (i.e. given the instability of alginate production by clinical isolates grown *in vitro*) (18, 45-47). As such, PIA was specifically chosen here to identify and differentiate mucoid and nonmucoid *P. aeruginosa* variants within patient specimens.

Non-*Pseudomonas* CFUs were calculated by subtracting *P. aeruginosa* colonies on PIA from all colonies counted on LA. Percentage of *P. aeruginosa* colonies (%*P.a.* isolates) was calculated by taking *P. aeruginosa* growth on PIA as a percentage of total microbial growth on LA. Percent mucoid colonies (%Mucoidy) was calculated by taking mucoid colonies identified on PIA as a percentage of total *P. aeruginosa* colonies. Each lobar specimen (regardless of patient-of-origin) was stratified into non-overlapping groups based on microbial populations as follows: No *P. aeruginosa* growth (i.e. only observed microbial growth on LA), and only nonmucoid, only mucoid, or mixed *P. aeruginosa* morphotypes.

#### 2.4. Proinflammatory cytokine arrays.

On the same day of specimen collection and after plating for microbial growth, BAL fluid was centrifuged and filter-sterilized to remove host and bacterial cells. Cell-free fractions of BAL fluid were subsequently stored at  $-80^{\circ}\text{C}$ . Proinflammatory cytokine concentrations within these BAL samples were subsequently quantitated using V-PLEX Proinflammatory Panel 1 Human Cytokine Arrays (Meso Scale Diagnostics, LLC). All cytokine arrays were performed by The Ohio State University Clinical Research Center based on manufacturer's specifications.

#### 2.5. Statistical analyses.

Statistical analyses were performed using GraphPad Prism v.7 (Graphpad Software). Statistical significance was determined using a p-value  $<0.05$ . All assays with patient specimens were performed in duplicate.

### 3. Results

#### 3.1. Single- and mixed-variant populations of mucoid and nonmucoid *P. aeruginosa* were isolated from CF patient specimens.

Previous studies had shown that both mucoid and nonmucoid variants of *P. aeruginosa* are often isolated in mixed communities from chronically-infected CF patients (17-27); however, the anatomic distribution of these variants within the CF lung has not been well-studied.

From multiple patient specimens, we were able to isolate both mucoid and nonmucoid variants of *P. aeruginosa*, both in single- and mixed-variant populations (Fig. S1A, B, and C). The mixed-variant colony morphologies of one CF patient's *P. aeruginosa* infection are shown, visualized directly upon medium surface by handheld camera and with standard light microscopy (10X magnification) (Fig. S1A). Conversely, a proportion of patients were not infected with *P. aeruginosa* (indicated as "No *P.a.*") at the time of specimen collection (Fig.

S1B); however, in all instances, specimens from these CF patients did produce microbial growth on LA, suggesting the presence of non-*P. aeruginosa* species, which were not specifically identified for the purposes of this study.

Of all patients infected with mucoid variants (“mucoid *P.a.* + patients”), a small majority (56%) showed mixed mucoid/nonmucoid *P. aeruginosa* populations, whereas others were infected only with mucoid variants (Fig. S1C). In total, these data indicated that mixed-variant *P. aeruginosa* communities were well-represented within our patient cohort, thereby enabling us to study regional and inflammatory correlates with infection.

### 3.2. Both mucoid and nonmucoid *P. aeruginosa* morphotypes were distributed throughout the CF lung.

To determine the lobar distribution of mucoid, nonmucoid, and mixed-variant populations of *P. aeruginosa*, BAL fluid from 6 lobar regions (RUL, RML, RLL, LUL, LIN, and LLL), regardless of patient-of-origin, were independently examined for total bacterial, total *P. aeruginosa*, and total mucoid/nonmucoid variant CFUs. This approach was justified because some patients had distinct microbial populations in different lobar specimens (Table S1). For instance, some patients were infected with only mucoid *P. aeruginosa* in certain lobes but had mixed variant populations in other regions. There were also patients who had no *P. aeruginosa* isolates in some lobar specimens but nonmucoid isolates in others. As such, each lobar specimen was treated independent of patient-of-origin to determine density and localization of the microbial populations.

However, there were no statistically significant trends for bacterial density across the different lung lobes of the entire cohort (Fig. 1A, B, and C). Similarly, we examined two other calculated measures of microbial populations: %*P. aeruginosa* isolates (calculated as a percentage of *P. aeruginosa* colonies of total microbial growth) and %mucoidy (calculated as a percentage of mucoid *P. aeruginosa* isolates out of total *P. aeruginosa* growth). Again, there were no differences for either the relative presence of *P. aeruginosa* (%*P.a.*) or mucoid variants (%mucoidy) throughout different regions of the CF lung (Fig. 1D and E). Furthermore, by examining all strata used to categorize each lobar specimen based on microbes isolated- no *P. aeruginosa* infection (i.e. infection with non-*P. aeruginosa* species), and infection with nonmucoid, mucoid, and mixed-*P. aeruginosa* variants, we observed no trends for lobe-dependent frequency of any of these microbial communities (Fig. 1F). These results suggested that within our patient cohort, there was no detectable propensity of *P. aeruginosa* variants to localize within certain anatomic lobes of the CF lung.

### 3.3. Independent of microbial infection, regional differences in proinflammatory cytokine concentrations were not observed.

Given that we did not observe significant regional differences in bacterial burden and *P. aeruginosa* variants in our CF patient cohort, we next queried the regional inflammatory status of these patients’ lungs by measuring proinflammatory cytokines within BAL fluid specimens. Based on aforementioned studies, we hypothesized that there would be intrinsic differences between the upper and lower lobes of CF lungs in concentrations of inflammatory indices used here: IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-8. All four cytokines have been

quantitated previously within the CF airway, shown to be relevant to the CF disease process (at the host-microbial interface), and found to be reliable indicators of inflammation in other diseases as well (37, 48-52).

Within our patient cohort, we did not observe any statistically-significant, interlobar differences in proinflammatory cytokine concentrations (Fig. 2A, B, C, and D). There were subtle, but noticeable differences, between the upper and lower lobes, wherein IL-1 $\beta$  concentrations were generally higher in the LUL compared to LLL and TNF- $\alpha$  concentrations were higher in the RUL and LUL compared to RLL and LLL respectively; however, these differences fell short of significance ( $p>0.05$ ) (Fig. 2A and B). Concentrations of IL-8, a potent neutrophil chemokine, were high across all patient specimens and all lung lobes (Fig. 2D); this observation aligns with previous work demonstrating IL-8 as a hallmark of neutrophil-dominated, CF lung immunopathology (53). These data suggested that independent of microbial infection, there were no regional differences in proinflammatory cytokines within CF patient lungs.

#### **3.4. Mucoïd and mixed-variant *P. aeruginosa* infections were associated with elevated proinflammatory cytokine concentrations within regional BAL fluid.**

Multiple laboratory and patient studies have demonstrated that bacterial infection is a critical driver of the inflammatory process in CF (54-56). Though, we did not find significant interlobar variations in inflammation or in the density and distribution of microbial populations independently, we postulated that regional inflammation and microbes may still be interrelated in our cohort. Specifically, we hypothesized that *P. aeruginosa* colony variants may be driving inflammation differentially, independent of their location within the CF lung.

As described earlier, each lobar BAL specimen was grouped only based on microbes isolated therein (i.e. infected with no *P. aeruginosa* or with nonmucoïd, mucoïd, or mixed-variants of *P. aeruginosa*). Subsequently, proinflammatory cytokine concentrations in each of these groups were analyzed. Indeed, regional BAL specimens from which we isolated *P. aeruginosa* mucoïd or mixed variants contained higher concentrations of IL-1 $\beta$ , TNF- $\alpha$ , and IL-8 compared to specimens that were culture-negative for *P. aeruginosa* or contained nonmucoïd *P. aeruginosa* variants only (Fig. 3A, B, and D). We did not observe any statistically-significant differences among these groups for IL-6 concentration (Fig. 3C).

BAL fluid samples containing mixed-variant *P. aeruginosa* isolates differed in the relative density of mucoïd and nonmucoïd constituents (i.e. CFUs of mucoïd and nonmucoïd variants or “% mucoïdy” for these specimens varied significantly); some specimens within this group showed predominantly mucoïd isolates, whereas others contained mostly nonmucoïd *P. aeruginosa*. As such, we performed linear regression analyses to determine associations between total CFUs of mucoïd/nonmucoïd isolates and proinflammatory indices across all BAL specimens. We found statistically-significant, positive correlations between mucoïd *P. aeruginosa* CFUs and IL-1, TNF $\alpha$ , and IL-8 (Fig. 4A, B, C, and D); in contrast, density of nonmucoïd *P. aeruginosa* variants was not positively correlated with any proinflammatory cytokine (Fig. 4E, F, G, and H). However, there was a negative correlation between nonmucoïd *P. aeruginosa* CFUs and IL-6 concentration (Fig. 4G).



Additional associations between microbial variables (e.g. total bacterial CFUs, non-*P. aeruginosa* CFUs, % mucoidy, etc.) and proinflammatory cytokines were documented as well (Table S2). There were positive correlations between total bacterial burden as well as non-*P. aeruginosa* CFUs with cytokine concentrations, suggesting that future studies should examine the regional impact of other bacterial and fungal species upon the CF lung as well. Importantly, however, only % mucoidy was positively correlated with all four proinflammatory cytokines examined (Table S2). In total, these data suggested that *P. aeruginosa* variants differentially impact the inflammatory microenvironment of the CF lung, and specifically, mucoid variants are associated with greater regional inflammation than nonmucoid variants.

#### 4. Discussion

The regional heterogeneity of the CF lung with respect to bronchiectasis, tissue damage, and microbial colonization has been well described with the pathophysiologic mechanisms being poorly understood (29-36). The etiology of interlobar variations in the CF lung often manifested with upper lobes predominance may be attributable to host-pathogen interactions. However, few studies have addressed co-localization of bacterial pathogens and host immune factors as an influencing factor in this clinical scenario of the CF airway. Given the importance of *P. aeruginosa* in the CF and the important clinical implications of mucoid conversion, we successfully examined the regional distribution of mucoid and nonmucoid variants of *P. aeruginosa* to improve our understanding how these bacterial morphotypes may differentially impact the inflammatory microenvironment of the CF lung.

This study made three important observations: 1) Mucoid and nonmucoid variants of *P. aeruginosa* (including mixed populations) were distributed throughout the CF lung, without preferential localization to an anatomic lobe/region; 2) Independent of bacterial infection, the upper lobes of the CF lung showed marginally greater proinflammatory cytokine concentrations compared to the lower lobes, though these differences did not meet statistical significance; 3) Mucoid variants of *P. aeruginosa* (and to a lesser extent, mixed-variant populations) were associated with greater regional inflammation compared to nonmucoid variants.

As indicated by published studies, more airway destruction is seen in the CF lung where greater concentrations of neutrophils and neutrophil-derived products exist (37). Similarly, mixed variant populations of mucoid and nonmucoid *P. aeruginosa* exhibit enhanced resistance to neutrophil antimicrobials (i.e. LL-37 and H<sub>2</sub>O<sub>2</sub>) *in vitro* (28). Although the isolation frequency of mucoid, nonmucoid, and mixed *P. aeruginosa* variants among different lobes did not achieve statistical significance in the current study, we believe further analysis is needed in larger CF cohorts as mixed communities of both *P. aeruginosa* variants may be an important factor why an upper lobe predominance occurs in CF.

Recent work investigated interlobar variations in microbial communities in the CF lung that examined regionality of genotypic and phenotypic variants of bacterial pathogens. While some studies showed regional compartmentalization of clonally-related variants of *P. aeruginosa* (and of other bacterial species), others found a more homogenous distribution of

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microbes (38, 39, 41-44). To our knowledge, the lobar distribution of mucoid and nonmucoid variants of *P. aeruginosa* has not been previously explored. Bjarnsholt et al. showed differences in the presence of mucoid and mixed mucoid/nonmucoid-variant populations within the conductive and respiratory zones of explanted CF lungs (21); however, both zones are represented in each lung lobe [i.e. the conductive zones within proximal, larger airways (e.g. segmental bronchi), and the respiratory zones within the terminal bronchioles/alveoli]. The current study examined *P. aeruginosa* morphotypes isolated from all lobes of the lung including the lingula to better understand aforementioned evidence of local interlobar differences in the CF lung and found mucoid and nonmucoid variants were not geographically restricted by the lobe.

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No significant differences in concentrations of proinflammatory cytokines, IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-8, across different lung lobes were found in our analysis (Fig. 2A, B, C, and D). However, there were slight trends towards higher concentrations of some of these inflammatory indices within the upper lobes. Independent of lung lobe, *P. aeruginosa* variants may differentially influence local inflammation in the CF lung. Regional BAL fluid specimens containing mucoid (and also mixed-mucoid/nonmucoid) isolates had higher concentrations of proinflammatory cytokines compared to specimens that were culture-negative for *P. aeruginosa* or contained nonmucoid variants only (Fig. 3A, B, and D). Linear regression analyses demonstrated a direct relationship between the burden of mucoid variants and cytokine concentrations, indicating that the mucoid phenotype was the likely driver of inflammation in both single- and mixed-variant *P. aeruginosa* infections. These findings add further insight to a large body of clinical literature demonstrating deleterious impacts of mucoid conversion upon CF patients, thereby necessitating the development of therapeutics specifically targeting mucoid *P. aeruginosa* strains.

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Surprisingly, we found that nonmucoid variants of *P. aeruginosa* were inversely correlated with IL-6 concentration in the BAL fluid analyzed (Fig. 4G). Bonfield et al. also reported that CF patients colonized with *P. aeruginosa* had lower concentrations of IL-6 in BAL fluid samples compared to patients not colonized (48). Based on these observations, we postulate that nonmucoid *P. aeruginosa* suppresses synthesis or abrogates stability of IL-6. To that end, a recent study reported that LasB protease of *P. aeruginosa* specifically degrades IL-6 *in vitro* as well as within a murine model of disease, thereby compromising IL-6-mediated tissue-protective and antimicrobial responses; in contrast, overexpression of IL-6 protects animals from lethal *P. aeruginosa* lung infection (57). Our findings and the aforementioned mechanistic study provide a rationale to further examine *P. aeruginosa*/IL-6 interactions, as these may shed light on *P. aeruginosa* evasion of innate immunity and illuminate avenues for novel therapeutic modalities.

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Our study was limited by the use of a small, single-center cohort. There were also limitations regarding the majority of male patients undergoing surgery during the study period. Therefore, further studies with larger patient cohorts are needed to confirm associations between *P. aeruginosa* colony variants and inflammatory indices. Though not within the immediate scope of this work, correlations between microbial populations/variants, inflammatory indices, and patient demographics (e.g. age, sex, race, genotype, etc.) could also be investigated. Here, we briefly considered an association between patient age,



mucoidity, and inflammation, finding that advanced patient age correlated with higher overall bacterial density (CFUs), greater relative presence of mucoid variants (% mucoidity), as well as higher concentrations of IL-1 $\beta$  and TNF- $\alpha$  in BAL specimens (Table S3). These data validate previous studies, demonstrating that mucoid *P. aeruginosa* becomes more prevalent with age and that CF lung inflammation is likely progressive throughout the course of a patient's lifetime (11, 27, 55, 58-60).

The lung microbiome of the CF lung is very dynamic, so we cannot report on any effect of anesthesia, endotracheal tube placement, and mechanical ventilation on our findings. Given that all of these factors were limited to a short duration (less than 10 minutes for the entire cohort), we believe the effect is not significant. We also showed that total bacterial burden (as well as CFUs of non *P. aeruginosa* species, which were enumerated on non-selective medium but not specifically identified) also correlated with inflammatory cytokine concentrations.

In conclusion, mucoid and nonmucoid variants of *P. aeruginosa* are regionally distributed throughout the CF lung, and lobar infection with mucoid variants is correlated with greater inflammation than is infection with nonmucoid variants or non-*Pseudomonas* species. In addition to expanding the cohort size to optimize statistical power, future work in this area will include longitudinal studies to define dynamic host-pathogen interactions and account for non-*Pseudomonad* species, including *Staphylococcus aureus* and *Burkholderia cenocepacia*, which are also important bacterial pathogens in the CF population (61).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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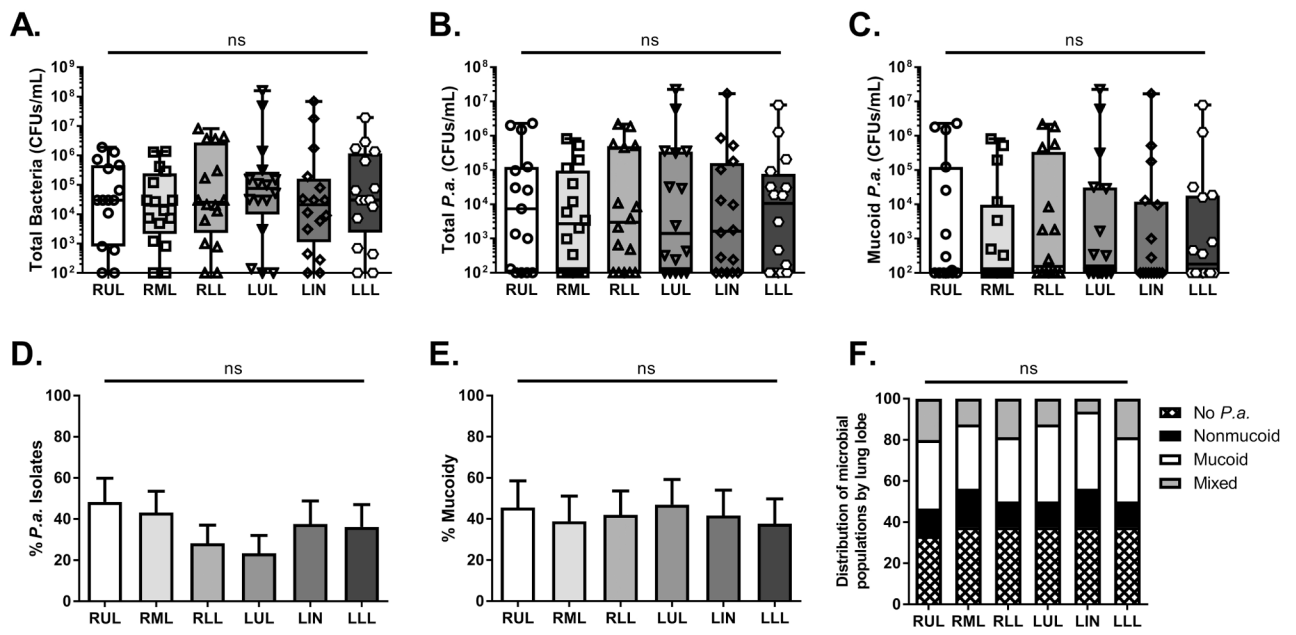
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**Highlights:**

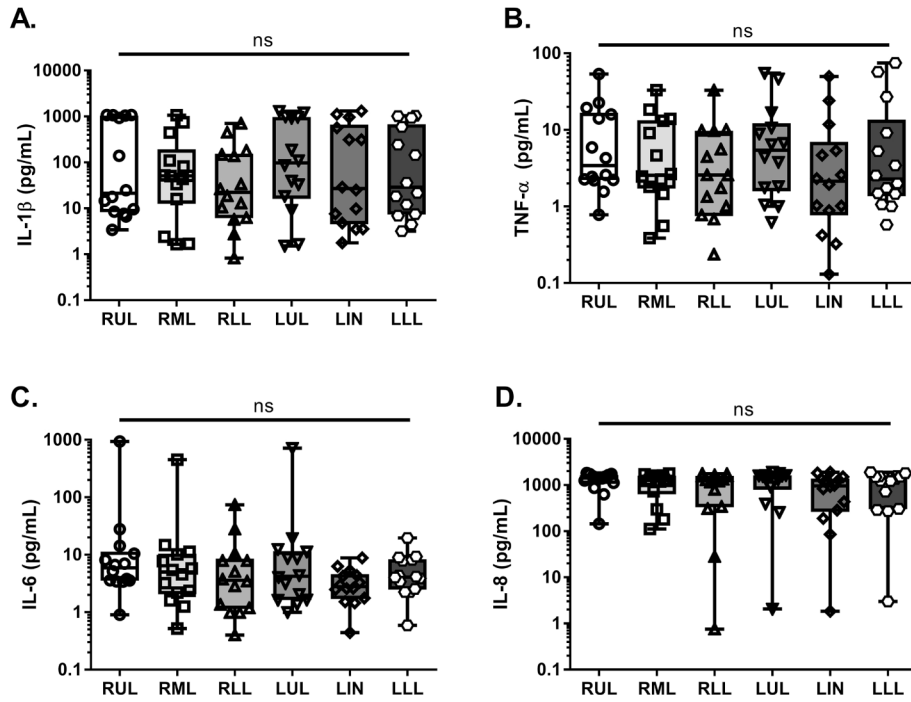
- *P. aeruginosa* mucoid/nonmucoid variants are distributed throughout the CF lung
- CF lung upper lobes are slightly more inflamed compared to the lower lobes
- *P. aeruginosa* mucoid variants are associated with greater regional inflammation



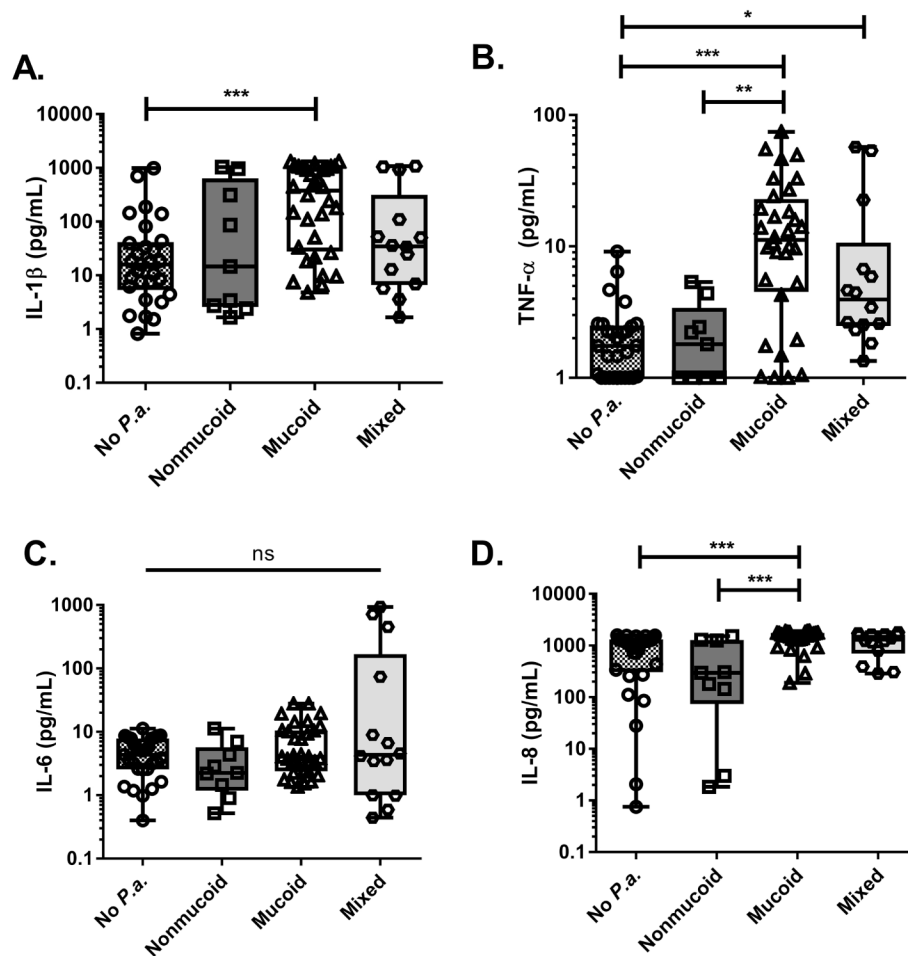
**Figure 1. Mucoid, nonmucoid, and mixed-variant *P. aeruginosa* populations are found throughout the CF lung.**

Regional BAL specimens from CF patients (n=14 patients, 84 lobar BAL fluid samples) were plated on nonselective or selective media to quantitate microbial populations. **A.** Total bacterial colony forming units (CFUs/mL) enumerated on LA (non-selective medium) **B.** Total *P. aeruginosa* CFUs/mL enumerated on PIA **C.** Mucoid variants of *P. aeruginosa* enumerated on PIA **D.** Percentage of *P. aeruginosa* isolates of total microbial growth **E.** Percentage of mucoid variants of total *P. aeruginosa* isolates **F.** Relative frequencies of various microbial populations within regional BAL samples of the CF cohort. Statistical significance was determined via non-parametric, Kruskal-Wallis one-way analysis of variance (ANOVA). No statistically-significant differences were observed (ns). LA- Luria agar, PIA- *Pseudomonas* Isolation Agar, RUL- right upper lobe, RML- right middle lobe, RLL- right lower lobe, LUL- left upper lobe, LIN- lingula, LLL- left lower lobe.



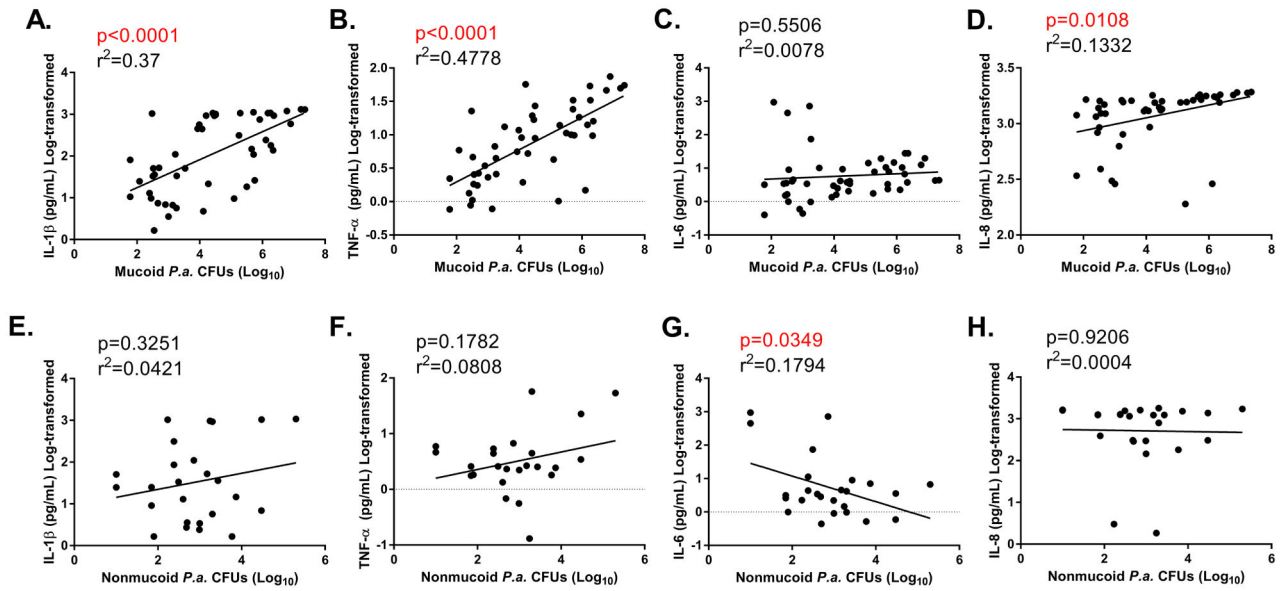


**Fig. 2. Independent of infection, significant interlobar differences in proinflammatory cytokines were not observed.** Proinflammatory cytokine concentrations were measured in regional BAL specimens from all CF patients (n=14 patients= 84 lobar BALs) via V-PLEX array. Data from each specimen were stratified based upon lung lobe of origin. **A.** IL-1β (pg/mL) **B.** TNF-α (pg/mL) **C.** IL-6 (pg/mL) **D.** IL-8 (pg/mL). Statistical significance was determined via non-parametric, Kruskal–Wallis one-way analysis of variance (ANOVA). No statistically-significant differences were observed (ns). RUL- right upper lobe, RML- right middle lobe, RLL- right lower lobe, LUL- left upper lobe, LIN- lingula, LLL- left lower lobe.



**Fig. 3. Mucooid or mixed-variant *P. aeruginosa* infections were associated with higher proinflammatory cytokines within regional BAL fluid.**

Proinflammatory cytokine concentrations were measured in regional BAL specimens from all CF patients (n=14 patients= 84 lobar BALs) via V-PLEX array. Data from each specimen were stratified based upon type of microbial infection [No *P.a.* (n=29), Nonmucooid (n=9), Mucooid (n=32), Mixed (n=14)]. **A.** IL-1 $\beta$  (pg/mL) **B.** TNF- $\alpha$  (pg/mL) **C.** IL-6 (pg/mL) **D.** IL-8 (pg/mL). Statistical significance was determined via non-parametric, Kruskal–Wallis one-way analysis of variance (ANOVA). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, ns- not statistically significant.



**Fig. 4. Infection with mucoid *P. aeruginosa* directly correlates with higher proinflammatory cytokine concentrations within regional BAL specimens.**

Proinflammatory cytokine concentrations were measured in regional BAL specimens from all CF patients (n=14 patients= 84 lobar BALs) via V-PLEX array. Cytokine concentrations are plotted versus mucoid *P. aeruginosa* CFUs/mL (A.-D.) or nonmucoid *P. aeruginosa* CFUs/mL (E.-H.) for each patient specimen. Correlation plots for A./E. IL-1β (pg/mL), B./F. TNF-α (pg/mL), C./G. IL-6 (pg/mL), D./H. IL-8 (pg/mL). R<sup>2</sup> and slope p-values are shown. Statistically significant correlations (p<0.05) are highlighted in red text.

**Table 1.****Cohort demographics.**

BMI- body mass index. FEV<sub>1</sub>- forced expiratory volume in 1 second.

	CF patients
n=	14
Females (%)	30%
Age (years)	
Median	23
Range	14-40
Pancreatic insufficiency (%)	100
Genotype (%)	
F508del homozygous	57%
F508del heterozygous	29%
FEV <sub>1</sub> (L)	
Median	2.36
Range	0.95-3.87
FEV <sub>1</sub> (%Predicted)	
Median	71
Range	22-103
BMI	
Median	19.9
Range	15.4-30.4

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