Infection Is Not Required for Mucoinflammatory Lung Disease in CFTR-Knockout Ferrets

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Abstract

Rationale: Classical interpretation of cystic fibrosis (CF) lung disease pathogenesis suggests that infection initiates disease progression, leading to an exuberant inflammatory response, excessive mucus, and ultimately bronchiectasis. Although symptomatic antibiotic treatment controls lung infections early in disease, lifelong bacterial residence typically ensues. Processes that control the establishment of persistent bacteria in the CF lung, and the contribution of noninfectious components to disease pathogenesis, are poorly understood.

Objectives: To evaluate whether continuous antibiotic therapy protects the CF lung from disease using a ferret model that rapidly acquires lethal bacterial lung infections in the absence of antibiotics.

Methods: *CFTR* (cystic fibrosis transmembrane conductance regulator)-knockout ferrets were treated with three antibiotics from birth to several years of age and lung disease was followed by quantitative computed tomography, BAL, and histopathology. Lung disease was compared with CFTR-knockout ferrets treated symptomatically with antibiotics.

Measurements and Main Results: Bronchiectasis was quantified from computed tomography images. BAL was evaluated for cellular differential and features of inflammatory cellular activation, bacteria, fungi, and quantitative proteomics. Semiquantitative histopathology was compared across experimental groups. We demonstrate that lifelong antibiotics can protect the CF ferret lung from infections for several years. Surprisingly, CF animals still developed hallmarks of structural bronchiectasis, neutrophil-mediated inflammation, and mucus accumulation, despite the lack of infection. Quantitative proteomics of BAL from CF and non-CF pairs demonstrated a mucoinflammatory signature in the CF lung dominated by Muc5B and neutrophil chemoattractants and products.

Conclusions: These findings implicate mucoinflammatory processes in the CF lung as pathogenic in the absence of clinically apparent bacterial and fungal infections.

Keywords: cystic fibrosis; mucus; inflammation; airway obstruction; ferret

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At a Glance Commentary

Scientific Knowledge on the

Subject: The pathogenesis of cystic fibrosis (CF) lung disease involves defects in innate immunity, mucociliary clearance, and excessive inflammation, all of which contribute to the emergence of bacterial infections that cannot be eradicated. Bacterial colonization of the CF lung has been thought to be the primary catalyst for excessive mucus production and inflammation. However, the degree to which noninfectious processes contribute to lung disease progression in CF remains unclear.

What This Study Adds to the

Field: We observed that lifelong use of multiple broad-spectrum antibiotics prevented microbial infection in CF ferret lungs for the life of animals (up to 4 yr); however, these animals still developed mucus obstruction, bronchiectasis, and inflammatory lung disease. Proteomics analysis of BAL defined a CF mucoinflammatory signature dominated by neutrophil pathways. Thus, despite prevention of infection, mucus obstruction and inflammation are significant contributors to disease pathogenesis in the CF ferret lung.

Cystic fibrosis (CF) results from mutations in CFTR (cystic fibrosis transmembrane conductance regulator), an anion channel responsible for regulating the composition of fluid on epithelial surfaces (1). Mortality is most often caused by respiratory infections (2). Although defects in lung innate immunity certainly contribute to CF lung disease, decades of research have also implicated an abnormal and heightened inflammatory response in CF lung pathogenesis (3). For example, studies in CF infants have shown that levels of inflammatory cytokines and neutrophils are elevated in BAL, even under culture-negative circumstances (4–6). Other studies, however, have suggested that inflammation occurs only after airway infection, and that it is reversed after treatment (7). Differentiating the pathophysiologic contributions of inflammation and infection in the CF lung

has been difficult, given the intertwined nature of these two processes.

The evolution of bacterial communities in the CF lung also seems to be closely linked to adaptive changes in both the host and microbe (8); this is seen clinically in patients when antibiotic-resistant organisms emerge (9). Recent studies in chronically infected G551D-CFTR subjects treated with a CFTR potentiator (ivacaftor) have shown only transient reductions in sputum bacterial load during the first year, despite a sustained reduction in inflammatory markers (10). These findings suggest that disease-associated alterations to the CF lung may enable bacteria to persist after restoration of CFTR function. They also suggest that even in the presence of persistent infection, restoring CFTR function can have a beneficial effect on inflammation in the CF lung. Understanding the pathophysiologic contributions of infection and inflammation to CF lung disease is critical to developing effective treatments and understanding their potential limitations.

The development of CF pig and ferret models has provided new opportunities to interrogate the links between infection and inflammation in the CF lung (11–13). In both of these CF models, the lungs are rapidly colonized by bacteria within weeks to months (14, 15). End-stage lung disease in CF ferrets is associated with significant IL-8-centric inflammation (16). We hypothesized that preventing infection in the CF ferret lung would also prevent inflammatory lung disease. To test this, we raised CF ferrets and age-matched non-CF control animals on combination broad-spectrum antibiotics from birth to up to 4 years of age with the goal of preventing the emergence of lung disease. We followed the natural history of structural lung disease by quantitative computed tomography (QCT) imaging and lung inflammation by quantitative proteomics analysis of BAL. Surprisingly, CF ferrets treated with combination broad-spectrum antibiotics still developed neutrophil-dominated inflammatory and structural lung disease with mucus obstruction, despite the lack of detectable bacteria or fungi above that of active controls. CF ferrets symptomatically treated with antibiotics developed more severe inflammation and mucus obstruction early in life than those treated by continuous antibiotics, and had a significantly shorter life span.

Thus, although antibiotics prolong the life of CF ferrets by preventing infection, they did not halt disease progression. These findings suggest that mucoinflammatory processes in the CF lung independently contribute to disease pathogenesis in the absence of infection. Some of the results of these studies have been previously reported in the form of an abstract (17, 18).

Methods

Rearing of Ferrets with CF

This study was performed according to protocols approved by the IACUC of the University of Iowa. Ferrets with a *CFTR* exon-10 disruption (12) and non-CF littermates (see Tables E1–E3 in the online supplement) were reared as previously described (19), with changes per the online supplement.

Collection and Processing of Bronchoscopy Samples

CF and non-CF control ferrets underwent serial bronchoscopy to collect BAL using a weight-based volume of sterile saline into the left upper lobe. For details, including processing and microbiology, see the online supplement.

Proteomics Analysis of BAL

Quantitative proteomics was conducted as previously described (20) on BAL obtained from adult non-CF and CF ferrets after removal of cells and nonsoluble mucins. Quantitation was performed between age-matched pairs of non-CF and CF animals using peptide labeling with light formaldehyde (CH_2O) and heavy formaldehyde ($^{13}\text{CCD}_2\text{O}$). For details, see the online supplement.

Computed Tomography Acquisition and Analysis

Lung computed tomography images were obtained via either a SOMATOM Definition Flash or SOMATOM Force dual-source scanner (Siemens). Quantification was performed by an investigator blinded to the genotype according to prospectively determined measures. Measurements for bronchial wall thickness, ratio of airway to vessel diameter, and the distance of distal cartilaginous airways from the pleural surface were obtained from the left cranial and right caudal lobes. For details, see the online supplement.

Histopathology

Formalin-fixed, paraffin-embedded lungs were sectioned (5 μ m) and processed according to routine protocols, stained with hematoxylin and eosin or diastase-resistant periodic acid-Schiff. Bacterial histochemical stains used a standard Gram stain. Histochemical stains were conducted as reported in previous studies (19) and quantified in a blinded fashion (21). For details, see the online supplement.

Statistical Analysis

Results are reported as mean $\pm$ SEM. Statistical analysis was conducted using Prism version 7.0c (GraphPad Software) and R (version 3.3.3, <http://www.R-project.org>; see online supplement for packages used). Comparisons used the nonparametric two-sample or paired Wilcoxon, as indicated in the figure legend. Repeated-measures analysis was used for data that included more than one measure per animal, where indicated in the figure legend. Data were considered significant at P less than 0.05. For additional details, see the online supplement.

Results

CF Ferrets Maintained on Lifelong Antibiotics Still Develop Bronchiectasis

Seven pairs of age-matched CF and non-CF ferrets were reared to ages 1–4 years (see Table E1), with animals receiving three antibiotics twice daily (see online supplement). Ferrets underwent serial QCT imaging (Figure 1), and lung disease was characterized in the final imaging examination to test the hypothesis that lifelong broad-spectrum antibiotics from birth would protect the CF lung from disease progression. Contrary to expectations, CF ferrets developed features of bronchiectasis (Figures 1A–1H), differing significantly in this respect from their non-CF control animals. Quantitative assessment of bronchiectasis parameters demonstrated significant differences between genotypes including a CF-associated increase in airway wall thickness (Figure 1I), increase in ratio of airway to vessel diameter (Figure 1J), and a reduction in the distance of small airways to pleural surface (Figure 1K). As an additional control, quantitation of these imaging parameters in non-CF ferrets that did not receive antibiotic treatment

Figure 1. Cystic fibrosis (CF) ferrets develop bronchiectasis despite lifelong maintenance of antibiotics. (A–H) Representative computed tomography images acquired from non-CF (A–D) and CF (E–H) ferrets maintained on three antibiotics for life (age range of images shown: 515–1,025 d). In each case, inset panels (B–D and F–H) show representative details of three core findings that are diagnostic of bronchiectasis: increased airway wall thickness (B and F) (marked by solid lines), increased ratio of airway to vessel diameter (C and G) (marked by solid lines), and distance of the cartilaginous airways to the pleural surface (D and H) with the most distal airway marked by arrows. All images quantified are from age-matched ferrets and were acquired under identical conditions. (I–K) Blinded quantification of the previously described parameters from computed tomography examinations performed while on antibiotics. Each data point represents one ferret ($n = 7$; P value by paired two-sided Wilcoxon rank sum test). (I) Wall thickness in CF versus non-CF control animals, $P = 0.016$. (J) Ratio of airway diameter to that of adjacent vessel, $P = 0.031$. (K) Distance from cartilaginous airways to pleural surface, $P = 0.047$. Graphs show mean $\pm$ SEM. * $P < 0.05$.

demonstrated no differences in any of the endpoints (see Figure E1).

Bronchiectasis Develops in the CF Ferret Lung despite Antibiotic Protection from Microbial Colonization

Hypothesizing that bacterial infection and antibiotic-resistance were the underlying cause of bronchiectasis, we evaluated the bacterial load in the BAL using culture methods and bacterial genomic DNA quantitation (16S rDNA). Bacterial colony-forming units (CFU) in the BAL did not significantly differ between the CF and non-CF control animals (Figure 2A). Only one of the seven CF animals exhibited transient

colonization on two sequential BALs ($2\text{--}3 \times 10^4$ CFU/ml), but not the third (Figure 2A; see Table E1). Additionally, bacterial CFU counts in the BAL from naive non-CF animals lacking exposure to antibiotics (CFU/ml 133 ± 92 ; $n = 3$) was not significantly different than non-CF control ferrets on antibiotics. Bacterial load in the BAL and BAL pellets, as determined using 16S qPCR that detects all bacteria and mycobacteria, was also not significantly different between genotypes (Figures 2B and 2C). Reasoning that continuous therapy with broad-spectrum antibiotics could confer a selective advantage to fungal infection, we used a qPCR assay, measuring fungal copies of internal transcribed spacer 1 in BAL (22).

Figure 2. Lifelong maintenance of antibiotics prevents microbial colonization of the cystic fibrosis (CF) ferret lung and prolongs survival. (A) Colony-forming units (CFU) per milliliter in BAL in non-CF and CF ferrets maintained on antibiotics ($n = 17$ for seven paired animals; mixed effects negative binomial modeling using R package glmmTMB; the ratio of non-CF to CF is 0.76) (95% confidence interval [CI], 0.19–3.04; $P = 0.69$). (B and C) Number of copies of bacterial genome, as assessed by analysis of 16S ribosomal DNA, in BAL supernatant (B) and pellet (C) ($n = 17$ for seven paired animals for both). Based on mixed effects negative binomial modeling, for BAL supernatant (B), the ratio of non-CF to CF is 0.7 (95% CI, 0.29–1.87; $P = 0.48$). In the BAL pellet (C), the ratio is 0.79 (95% CI, 0.35–1.78; $P = 0.57$). (D) Number of copies of fungal internal transcribed spacer-1 per milliliter BAL, as assessed by quantitative PCR ($n = 6$ –7 animals per genotype; P value by two-sample Wilcoxon rank sum test, $P = 0.5$). (E) Bacterial CFU/mg protein of lung lysates from non-CF and CF ferrets treated with two different antibiotic regimens: group 1 ($n = 9$ –10; P value by two-sample Wilcoxon rank sum test, $P < 0.0001$) was treated symptomatically based on weight changes, whereas group 2 ($n = 6$; P value by paired Wilcoxon rank sum test, $P > 0.9999$) was reared on continuous antibiotics from birth. (F) Kaplan-Meier survival curves of CF ferrets reared on the different antibiotic regimens as described in E, P value by log-rank test. Graphs in A–E show mean $\pm$ SEM. ITS1 = internal transcribed spacer-1.

We found no more fungal DNA in CF animals than in control animals (Figure 2D).

Continuous Antibiotic Treatment Dramatically Improves Survival of CF Ferrets as Compared with Symptomatic Treatment with Antibiotics

CF kits reared without antibiotics rapidly succumb to lung infections within weeks (14). By contrast, symptomatic use of antibiotics can significantly prolong the lifespan of CF ferrets (19). However, all CF animals treated symptomatically with antibiotics, or removed from antibiotics at 6 months of age, developed lethal bacterial lung infections with culturable bacteria in lung lysates significantly ($P < 0.0001$) and substantially ($> 6 \times 10^6$ -fold) above that of non-CF control animals treated with the same antibiotic regimen (group 1,

Figure 2E) (19). It is important to note that only 2 of 10 CF animals in this cohort survived to 6 months of age, at which time antibiotics were removed; these animals developed terminal lung infections 73 and 77 days after removal of antibiotics. The remaining eight of the CF animals succumbed to lung infection despite antibiotics. In terminally ill CF animals reared continuously on three antibiotics from birth, the levels of culturable bacteria in lung lysates were at the background levels seen in lung lysates of non-CF control animals (group 2, Figure 2E; see Table E1). These findings are consistent with the lack of significant bacteria in the BAL of CF animals continuously treated with combinational antibiotics and suggest that bacterial resistance did not occur (23, 24). Notably, continuous treatment with combinational antibiotics in our current

study led to an average survival of more than $1,143 \pm 77$ days ($n = 7$; $\pm$ SEM), whereas CF ferrets treated symptomatically with antibiotics (19) survived 105 ± 27 days ($n = 10$; $\pm$ SEM). Thus, continuous combinational antibiotics led to an approximately 10-fold increased life span (Figure 2F).

Excessive Mucus and Neutrophil-dominated Inflammation in the CF Ferret BAL despite Continuous Bacterial Eradication

The apparent disconnect between our BAL and lung microbiologic data and QCT findings prompted us to investigate whether CF ferrets reared on continuous antibiotics experience neutrophil-predominant inflammation in the lungs. To this end, we performed further analyses on the BAL collected from CF and non-CF animals. BAL samples retrieved from CF ferrets were

starkly different from their active control animals, with large amounts of nonsoluble mucocellular material (Figure 3A). Analysis of BAL cellular material (Figures 3B–3I) revealed that the number of neutrophils was significantly higher in CF versus non-CF BAL (Figure 3B) ($P = 0.016$).

We next evaluated the abundance of the active form of neutrophil elastase (NE) in both phases of the BAL (pellet and supernatant) using an activity-based immunoassay (25) verified to be reactive to ferret NE (*see* Figure E3). Unexpectedly, we found no significant differences in active NE between non-CF and CF (Figures 3C and 3D). Although the number of alveolar

macrophages was non-significantly lower in CF versus non-CF BAL (Figure 3E) ($P = 0.061$), there were significantly more activated macrophages in CF samples (Figures 3F and 3G) ($P = 0.0085$). Lymphocytes and the total number of cells overall did not differ significantly between CF and non-CF ferrets (Figures 3H and 3I). Despite the removal of nonsoluble mucinous material in the BAL pellet, CF BAL still retained far more soluble Muc5B ($\text{Log}_2\text{FC} = 14.6$; $P < 0.0001$) as determined by quantitative liquid chromatography tandem-mass spectrometry (20, 26), as compared with non-CF. Consistent with elevated neutrophil counts in CF ferret

lungs, the BAL secretory proteome (Figure 4A; *see* Table E4A) was characterized by significant increases in the expression of neutrophil chemotactic proteins (S100 proteins), matrix metalloprotease 8 and 9, and other indicators of neutrophil-mediated inflammation (denoted by asterisk: CD177, MPO, LTF, CAMP, CTSG) (Figures 4B and 4C). NE inhibitors (27) were also elevated in CF samples, primarily OLFM4 (28) ($>100,000$ -fold increase in CF/non-CF) and mucin (29), consistent with the lack of increased active form of NE in CF BAL (Figures 3C and 3D) and the presence of NE inhibitory activity in the BAL (*see* Figure E3). This proteomic signature is strikingly

Figure 3. BAL from cystic fibrosis (CF) ferrets maintained on antibiotics exhibits a neutrophil-predominant mucoinflammatory signature despite normal levels of microbes in the lung. (A) Photographs of nonsoluble material in CF and non-CF BAL samples, as indicated. Arrows in main panels and arrowheads in insets indicate the top of the nonsoluble material pellet. Inset, when present, provides a contrasted image to emphasize the solid pellet. (B, E, H, and I) Differential of cell types in BAL from non-CF and CF ferrets ($n = 13$ –14 for seven paired animals; P value by mixed effect β modeling [B, E, and I] or linear mixed effects model [H], $*P < 0.05$ for neutrophils). (B) Percentage of neutrophils in BAL had an estimated difference in proportion for non-CF versus CF of -0.84 (95% confidence interval [CI], -1.51 to -0.16). Active neutrophil elastase in the BAL supernatant (C) and cell pellet (D) ($n = 7$ animals for each genotype; P value by linear mixed effects model, $P = 0.48$ and 0.15 , respectively). (E) Macrophage percent in BAL had an estimated difference in proportion for non-CF versus CF of 0.1 (95% CI, 0.00 to 0.21 ; $P = 0.061$). (F) Macrophage morphology seen in BAL cell pellets (by Diff-Quick stain). Scale bar = $25 \mu\text{m}$ for both micrographs. (G) Percent of macrophages in BAL exhibiting activated phenotype in non-CF and CF samples ($n = 7$ animals for each genotype; P value by linear mixed effects model, $**P = 0.0085$). Estimated change from CF to non-CF was -33.1 (95% CI, -53.82 to -12.46). (H) Lymphocyte percent in BAL had an estimated difference in proportion for non-CF versus CF of 0.005 (95% CI, -0.004 to 0.015 ; $P = 0.28$). (I) Total cell counts for BAL in non-CF and CF BAL with an estimated ratio for non-CF versus CF of 0.78 (95% CI, 0.42 to 1.46 ; $P = 0.43$). Graphs show mean $\pm$ SEM.

Figure 4. BAL from cystic fibrosis (CF) ferrets maintained on antibiotics exhibits a mucoinflammatory proteomic signature despite normal levels of microbes in the lung. (A) Volcano plot of more than 275 proteins whose levels were evaluated by quantitative proteomics of BAL from non-CF and CF ferrets (open circles, not statistically significant; solid circles, statistically significant; $n = 13$ samples from seven animals of each genotype; P value by Scaffold software t test). (B and C) Proteins from A with statistically significant P value ($P < 0.05$) and $\log_2$ (fold change) CF/non-CF of ≥ 2 (B) or ≤ -2 (C). Asterisk identifies neutrophil-associated proteins in BAL proteome. Heatmap depicts the $\log_2$ (fold-change) in protein content of the BAL with red increased and blue decreased in CF. See Table E4A for all proteins identified, $\log_2$ fold-change, and P value. Ingenuity pathway analysis was conducted on the proteome identified in A and arranged by disease (D) and canonical (E) pathways. Shown are selected top pathways ranked by increasing statistical significance. See Tables E4B and E4C for all pathways identified by ingenuity pathway analysis. COPD = chronic obstructive pulmonary disease; FC = fold change; ROS = reactive oxygen species.

similar to that observed in BAL from colonized human CF subjects showing elevations in Muc5B, S100 proteins, matrix metalloprotease 9, LTF, ANXA, and declines in TTR, VTN, KNG1, and SFTPA1 (see Table E4A) (30).

Ingenuity pathway analysis confirmed significant upregulation of inflammatory response pathways that promote the chemotaxis, adhesion, and activation of leukocytes in CF ferrets (Figure 4D and 4E; see Tables E4B and E4C). Among the top diseases or functions pathways were

leukocyte migration ($P = 1 \times 10^{-17}$), inflammatory response ($P = 2.7 \times 10^{-17}$), cellular infiltration ($P = 2.2 \times 10^{-13}$), adhesion of neutrophils ($P = 7.7 \times 10^{-13}$), and severe acute respiratory syndrome ($P = 1.1 \times 10^{-12}$). Top canonical pathways included acute phase response signaling ($P = 1.6 \times 10^{-21}$), LXR/RXR activation ($P = 5 \times 10^{-21}$), and complement system ($P = 2 \times 10^{-17}$); also, IL-8 signaling reached significance ($P = 8.3 \times 10^{-3}$). These findings demonstrate that there is significant neutrophil-dominated

inflammation in the CF ferret lung despite continuous bacterial eradication.

Mucus Accumulation and Inflammation in the CF Ferret Airway Occurs despite Continuous Bacterial Eradication

To gain a better understanding of the structural nature of lung disease in CF ferrets maintained on combinational antibiotic for life, we examined lung histopathology in those animals that became terminally ill during this study (six pairs)

together with animals treated symptomatically with antibiotics (see Tables E1 and E3). One CF animal was not terminally ill but failed to adequately recover following a procedure (771 d); the other CF animals became terminally ill because of severe nonpulmonary diseases and failure to thrive (see Figures E3–E5 and Table E1). Whereas the lungs of non-CF ferrets did not exhibit lung pathology at necropsy (Figures 5A and 5B), those of CF ferrets maintained on antibiotics from birth were characterized by airway mucus obstruction (Figures 5C–5E), and neutrophil-predominant inflammation in the airways and alveolar spaces (Figures 5F–5I). Although bacteria were visible in airway mucus of CF animals symptomatically treated with antibiotics (see group 1, Figures E2A–E2C), the airways of CF animals reared on combination antibiotics from birth did not have visible bacteria despite accumulated mucus (see group 2, Figures E2D–E2I).

To evaluate the progression of lung disease between CF animals symptomatically

treated with antibiotics (group 1) and those reared on combination antibiotics (group 2), we used blinded semiquantitative histopathology to score lung sections for inflammation and mucus obstruction (Figure 6A) across ages (15–1,417 d) and treatment groups (non-CF, group 1 CF, and group 2 CF). These studies confirmed that continuous antibiotics failed to prevent mucoinflammatory lung disease in the CF ferret (Figure 6B). When younger age-matched CF ferrets treated with symptomatic and continuous antibiotic therapy were compared with non-CF control animals, both CF treatment groups demonstrated an enhanced inflammatory score (Figure 6C). However, at this younger age only symptomatically treated CF ferrets retained significantly greater mucus in the airways as compared with control animals (Figure 6C). Age-dependent analysis of histologic measures and groups evaluated revealed a more rapid trajectory in symptomatically treated CF ferrets

(group 1) for all indexes (Figure 6D), suggesting that antibiotics indeed slow disease progression. Interestingly, inflammation appeared early in the continuous antibiotic therapy treated group (group 2) and was relatively constant with age, whereas mucus accumulation demonstrated an age-dependent trend (Figure 6D). Although these trends remain speculative and the studies are underpowered to draw statistical age-dependent conclusions, they provide a framework for future studies dissecting the link between inflammation and mucus accumulation in disease progression and how infection impacts these processes. Together, these histologic findings support the earlier observations by QCT and BAL analysis in these animals and demonstrate significant inflammation and mucus obstruction despite continuous bacterial eradication.

Discussion

These findings in the CF ferret model add to the understanding of CF lung pathogenesis in several ways. Before this study, it had been generally accepted that continual bacterial eradication in the CF lung would stop disease progression. Certainly, this statement is partially true, because CF ferrets on lifelong antibiotics lived on average more than 10-fold longer than those without antibiotics. However, lifelong bacterial eradication did not halt disease progress, and in general, lung pathologies were similar to CF animals symptomatically treated with antibiotics (i.e., mucus accumulation, airway obstruction, and neutrophil-mediated inflammation), although less severe in nature and with a delayed onset.

Second, continuous antibacterial selective pressure was key to preventing bacterial adaptations that allow them to flourish in the CF ferret lung, whereas symptomatic use of layered antibiotics failed to prevent the eventual overgrowth of bacteria (19), even when animals were on the same three antibiotics used here and a fourth antibiotic (cefazolin) at end-stage disease. These findings suggest that prevention of a bacterial foothold in the CF lung is key to sustained bacterial eradication. Studies that demonstrate the eventual reoccurrence of bacterial colonization of ivacaftor-treated G551D-CFTR

Figure 5. Cystic fibrosis (CF) ferrets maintained on antibiotics still develop mucus obstruction and inflammation in the lung despite lack of bacterial colonization. (A and B) Sections of large airways and adjacent alveoli from non-CF ferrets maintained on antibiotic therapy, stained with hematoxylin and eosin. (C–E) Sections from large airways of CF ferret stained with hematoxylin and eosin (C) or diastase-resistant periodic acid–Schiff (D and E). Mucus is marked by asterisk. (F and G) Sections of large (F) and small (G) airways from CF ferrets stained with hematoxylin and eosin, revealing inflammatory cells (arrows) and mucus (asterisk) within the lumen. Boxed regions in F and G are enlarged in panels H and I, respectively. Scale bars: A–F, 100 μ m; G–I, 50 μ m.

Figure 6. Semiquantitative analysis of histopathology demonstrates that muco-inflammatory cystic fibrosis (CF) lung disease develops without bacterial infection. (A) Histopathologic sections that represent severity scores (indicated in left lower corner) assigned to features of inflammation and mucus accumulation (specific components are listed to the left of the panel). Scale bars in scores 0 and 1 are 500 μ m, while scale bars in scores 2 and 3 are 200 μ m. Inset scale bars are 20 μ m in all panels. (B) Inflammation and mucus scores for primary cohort of six older CF ferrets and active non-CF control animals maintained on lifelong bacterial eradication therapy (CF continuous). One CF ferret had lungs that could not be scored for inflammation because of comorbid tumor ($n = 5$ –6 ferrets per group; P value by two-sample Wilcoxon rank sum test, $*P < 0.05$). (C) Scores for comparison of younger animals that either received symptomatic antibiotic therapy (group 1) or continuous antibiotic regimens (group 2); non-CF ferrets are age-matched as best possible to provide comparison (sample size as indicated; P value by two-sample Wilcoxon rank sum test for the indicated groups, $*P < 0.05$ and $**P < 0.01$). (D) Scores from all CF and non-CF ferrets in B and C plotted against age in days. Linear regression was calculated for each group and displayed on the graphs. Note that points were slightly offset only to better display overlapping data. Graphs show mean $\pm$ SEM.

patients supports this notion of bacterial adaptation within the host despite CFTR functional restoration (10). Third, our findings support the notion that mucus accumulation, even in the absence of bacterial infection, is proinflammatory and that these processes can lead to significant structural remodeling in the CF lung

(31, 32). Notably, the proteomics inflammatory signature in BAL from CF ferrets with continuous bacterial eradication was strikingly similar to BAL from colonized human CF subjects (30). Thus, these studies lay the groundwork for understanding processes in both the host and microbe that control bacterial adaptation in the CF lung.

Study Limitations and Strengths

This study has several limitations. First, although our microbial assays can detect bacteria, mycobacteria, and fungi in the lung and these infectious pathogens were not greater in CF as compared with non-CF ferrets, we have not ruled out viral pathogens. However, we believe viral

infections are an unlikely cause of the CF lung disease because our experience with viral outbreaks in CF ferrets demonstrates they are uniformly lethal to CF ferrets even when reared on combinational antibiotics (data not shown). Second, our longitudinal analysis of microorganisms in the BAL only samples a fraction of the lung, thus it is possible that CF animals may have been transiently colonized more often than observed in one CF animal. However, a larger sampling of multiple regions of the CF lung at the time of sacrifice demonstrated the level of culturable aerobic or anaerobic bacteria on multiple medias (33) was no different than that of non-CF control animals. Thus, we believe this limitation is also an unlikely cause of the global inflammation and mucus production we observed over the lifetime of the animals. Furthermore, the same region of the lung was longitudinally sampled for inflammatory factors and microorganisms in the BAL, thus enabling a direct comparison of microbial load and inflammation. Lastly, our proteomics analysis only took into account proteins and mucins within the soluble BAL fraction. Thus, our studies likely grossly underestimate the quantity and composition of mucin in the CF ferret lung. In this regard, our findings that Muc5B was significantly elevated in the BAL of CF animals, whereas Muc5AC was not significantly different between genotypes, may reflect differing solubility of these two mucins and/or protease sensitivities to degradation (34, 35).

Our study also has strengths. This is the first analysis of chronic CF lung disease in an animal model that spans up to 4 years. Second, our analyses used age-matched control animals to quantitatively assess multiple endpoints including structural changes consistent with bronchiectasis, microbe presence, and proinflammatory changes in the BAL proteome. Such an approach is ideal for patient-centered trials in the CF population, but would not be feasible from the standpoint of obtaining non-CF controls for such a broad number of procedures, particularly because non-CF ferret control animals received continuous antibiotics. Third, the proteomic studies in BAL used isobaric tags capable of directly referencing the fold-change in a given protein between a CF animal and its matched control. Lastly, unlike most longitudinal studies in CF subjects, we were

able to study lung histopathology in samples obtained from study animals at the conclusion of the study.

Infection and Inflammation: Two Related but Distinct Pathophysiologic Processes?

Our multiyear experiments challenge the concept that infection is required for excessive inflammation in the CF lung. Although sustained antibiotic therapy in CF ferrets kept the bacterial burden at control levels in nearly all animals and prevented permanent colonization, we cannot exclude the contribution of normal levels of bacterial exposure as the inciting event that catalyzes mucoinflammatory disease progression. Distinguishing these two processes completely would require the generation of gnotobiotic ferrets, which is currently not feasible. Thus, two potential mechanisms can explain our results. First, mucus obstruction and airway dehydration in CF occurs in the absence of infection and is itself proinflammatory. Alternatively, normal bacterial exposure may initiate mucus secretion and in the context of CF these secretions are abnormal and could begin a proinflammatory cascade that further enhances mucus secretion and obstruction. Nonetheless, our results demonstrate that preventing microbial infection is not sufficient to prevent CF lung disease.

Our findings complement recent studies in the β -ENaC-overexpressing (36–38) and SLC26A9-knockout (39) mouse models, where mucus plugging and inflammation develop in the absence of detectable infection (40). However, our data diverge from these studies in that elevated active NE was not observed in the CF as compared with non-CF BAL (38). This finding may be due to the fact that submucosal glands in the ferret are found throughout the cartilaginous airways, in contrast to mice, and could provide larger amounts of Muc5B and other inhibitors that prevent NE activation (27, 29). On the whole, however, these studies also implicate mucus accumulation in the lung as proinflammatory (31, 32). Furthermore, a recent study in chronically infected adult G551D-CFTR CF patients treated with ivacaftor (Kalydeco, VX770) demonstrated only transient (<1 yr) reductions in sputum bacterial loads, but sustained reductions in inflammatory factors (10). This interesting finding also

suggests an apparent disconnect between infection and inflammation after the restoration of CFTR function, similar to our observations in CF ferrets reared under conditions of continuous bacterial eradication. One potential interpretation of this study (10), in light of our current findings, is that restoration of CFTR function assists in mediating improved mucus clearance and thus reduces inflammation, whereas the inability to eradicate bacteria is the result of evolutionary adaptation that allows bacteria to persist in remodeled CF lung even in the presence of CFTR restoration. Our findings present the opposite scenario, where bacteria were never allowed the opportunity to evolve because of antibiotic suppression yet mucus abnormalities persist causing inflammation.

Excessive Mucus Production and Inflammation in the Absence of Infection

A major remaining question of this study is the causative link between excessive mucus production and inflammation. Without question, there was significant accumulation of mucus and airway obstruction histologically in the CF ferret lung lacking culturable bacteria. However, it remains unclear if mucus accumulation itself is proinflammatory, potentially through altered physical properties at the airway surface, or if CFTR dysfunction itself is proinflammatory and leads to excessive mucus production in the CF ferret lung.

The two major secreted mucins in the lung are Muc5AC and Muc5B (32, 41, 42). Muc5AC is predominantly produced by goblet cells in the surface airway epithelium, whereas Muc5B is predominantly produced by the submucosal glands (43, 44). Submucosal glands express abundant CFTR (45) and fail to secrete properly in CF humans (46) and ferrets (14). Our BAL proteomics studies demonstrated elevation of Muc5B in the CF lung, as compared with age-matched non-CF control animals, whereas there is no significant difference in the quantity of Muc5AC. Although this is somewhat contrary to the known gland secretion defects in CF, others have shown that Muc5B is expressed by the surface epithelial goblet cells in the context of disease (47, 48) and specifically in later stages of CF lung disease (48–50). We also observed both Muc5B and Muc5AC

expression in the surface airway cells of CF ferrets in this study (data not shown). Thus, the lack of a significant change in Muc5AC content in BAL between CF and non-CF animals is intriguing.

It is possible that Muc5B resides primarily in the soluble fraction of retrieved BAL, whereas Muc5AC remains more insoluble and thus not adequately evaluated by our proteomics studies. Whether a difference in solubility of these mucins exists and contributes to inflammation in the CF lung remains an open question. Recent studies in Muc5B-deficient mice demonstrate that this mucin subtype is required for airway defense of the murine lung (51). Furthermore, Muc5AC is the secreted mucin that predominantly responds to a T-helper cell type 2-centered response seen in asthma (52, 53), where airway obstruction is seen in severe and fatal cases (54). Further

studies in Muc5B- and Muc5AC-knockout ferrets may lend mechanistic insights into how each of these mucin subtypes contributes to airway obstruction and/or inflammation in the CF ferret lung when the confounding contributions of bacterial infection are removed from the equation.

Our data clearly demonstrate that a pathogenic process occurs in the CF ferret lung even in the absence of clinically apparent infections. However, it remains unclear in the uninfected CF ferret lung whether reduced mucus clearance is the cause of enhanced inflammation or if enhanced proinflammatory responses to normal levels of bacteria leads to excessive mucus secretion. Nonetheless, these findings emphasize the importance of airway clearance as a means of improving health of the CF lung. We propose that the pathogenesis of CF lung

disease shares mucoinflammatory components (55) with other forms of COPD, such as chronic bronchitis (31, 32, 56, 57) and severe asthma (52, 58) and thus strengthens the case for developing new therapeutics targeting these aberrant responses. ■

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