Newborn Cystic Fibrosis Pigs Have a Blunted Early Response to an Inflammatory Stimulus

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Abstract

Rationale: Studies suggest that inappropriate responses to proinflammatory stimuli might contribute to inflammation in cystic fibrosis (CF) lungs. However, technical challenges have made it difficult to distinguish whether altered responses in CF airways are an intrinsic defect or a secondary effect of chronic disease in their tissue of origin. The CF pig model provides an opportunity to study the inflammatory responses of CF airways at birth, before the onset of infection and inflammation.

Objectives: To test the hypothesis that acute inflammatory responses are perturbed in porcine CF airways.

Methods: We investigated the inflammatory responses of newborn CF and non-CF pig airways following a 4-hour exposure to heat-killed *Staphylococcus aureus*, *in vivo* and *in vitro*.

Measurements and Main Results: Following an *in vivo* *S. aureus* challenge, markers of inflammation were similar between CF and littermate control animals through evaluation of bronchoalveolar lavage and tissues. However, transcriptome analysis revealed genotype-dependent differences as CF pigs showed a diminished host defense response compared with their non-CF counterparts. Furthermore, CF pig airways exhibited an increase in apoptotic pathways and a suppression of ciliary and flagellar biosynthetic pathways. Similar differences were observed in cultured airway epithelia from CF and non-CF pigs exposed to the stimulus.

Conclusions: Transcriptome profiling suggests that acute inflammatory responses are dysregulated in the airways of newborn CF pigs.

Keywords: inflammation; airway epithelia; transcriptional profiling; *Staphylococcus aureus*

There is broad agreement that inflammation is an early and important feature of lung disease in people with cystic fibrosis (CF). CF lungs are characterized by chronic neutrophilic airway inflammation, which

contributes to injury and remodeling over time (1, 2). Although it is known that inflammation is present and persistent in CF airways, there is longstanding debate as to why. One possibility is that airway cells

lacking CF transmembrane regulator (CFTR) exhibit abnormally exuberant responses to inflammatory stimuli (e.g., inhaled microbes). Alternatively, the heightened inflammation in CF may

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At a Glance Commentary

Scientific Knowledge on the

Subject: Inflammation is an early feature of lung disease in cystic fibrosis (CF) and contributes to disease onset and progression. However, studies designed to ask whether inflammatory responses are abnormal in CF airways have sometimes produced conflicting results. Furthermore, it is unclear whether altered inflammation in the CF lung is a primary defect resulting from loss of CF transmembrane regulator function or a secondary consequence of chronic inflammation during progression of CF lung disease.

What This Study Adds to the

Field: Here, these issues are addressed by studying the acute pulmonary inflammatory responses of newborn CF pigs at a time point before the onset of infection and chronic inflammation. We report that, whereas CF and non-CF pigs present similar tissue and bronchoalveolar lavage profiles in response to a 4-hour exposure to an inflammatory stimulus, they exhibit differing transcriptional profiles in their airway tissues. Taken together, our studies suggest that acute inflammatory responses of airway epithelia are different in the absence of functional CF transmembrane regulator.

represent a normal response to persistent bacterial infection and colonization. A third possibility is that the acute inflammatory response is reduced in CF airways, leading to enhanced initial infection by bacteria and ultimately increased inflammation.

This debate has been difficult to resolve. Early onset of pulmonary infection and inflammation in CF makes it challenging to address this question in humans. Technical and ethical obstacles limit sampling the lungs of newborn babies; studies at later time points are confounded by inherent variability in the levels of inflammation arising from age, disease course, and other factors. Furthermore, it is difficult to identify an appropriate matched control (non-CF) population for human studies. Experiments using cultured CF and non-CF airway

epithelia have produced widely varying results, possibly because of inherent genetic differences between cell lines and primary cells, changes induced in cells by culture and/or passage, or epigenetic changes resulting from chronic inflammation before culture. Additionally, cultures lack the diversity of epithelia, immune cells, and other cells that act in concert to mediate innate immunity and inflammation *in vivo*.

The CF pig model provides a unique opportunity to overcome some of these limitations. Newborn CF pig airways exhibit a host defense defect and within weeks to months, spontaneously develop lung disease with bacterial infection and inflammation similar to that in people with CF (3, 4). Of note, at birth CF pig lungs show no signs of inflammation based on histologic, biochemical, and global gene expression analyses (3, 4). Moreover, transcriptional profiling shows no enrichment of molecular signatures or pathways associated with inflammation in newborn CF pig airways (4). These observations make the newborn CF pig well suited to investigate the responses to a stimulus in the absence of confounding factors, such as preexisting inflammation or infection.

The goal of this study was to test the hypothesis that the inflammatory response is perturbed in the absence of functional CFTR. To address this hypothesis, we investigated the acute responses of newborn CF pigs to an airway challenge with heat-killed *Staphylococcus aureus*. We found that, whereas cell and tissue markers of inflammation were similar between CF and non-CF pigs after *S. aureus* challenge, there were genotype-dependent differences at the level of gene transcription, with possible implications for the processes of inflammation, immunity, apoptosis, and ciliary biogenesis. Some of the results of these studies have been previously reported in the form of abstracts (5, 6).

Methods

Preparation of Bacteria for Inflammatory Challenges

Experiments were performed using *S. aureus* 43, a clinical isolate originally obtained from the lungs of a *CFTR*^{-/-} pig and made chloramphenicol-resistant by introduction of plasmid pCM1 (described in Reference 4). For detailed information

on heat inactivation and preparation of bacteria for *in vivo* and *in vitro* inflammatory challenges, please refer to MATERIALS and METHODS in the online supplement.

In Vivo S. aureus Challenge Experiments

All studies were approved by the University of Iowa Animal Care and Use Committee. Piglets were delivered at term by caesarean section, and experiments were performed 4–6 hours after birth to minimize environmental influences on tissues and to synchronize the timing of the experimental interventions. Littermates from *CFTR*^{+/-} matings were obtained from Exemplar Genetics (Sioux Center, IA). Heat-inactivated *S. aureus* (Strain SA43) was administered via aerosol to the intrapulmonary airways of newborn CF and non-CF piglets using a microsyringe with high-pressure syringe (Penn-Century, Inc., Wyndmoor, PA). More details regarding *S. aureus* delivery, tissue collection, and analysis of inflammatory markers in *S. aureus*-challenged animals can be found in the MATERIALS and METHODS in the online supplement.

Stimulation of Porcine Primary Airway Epithelial Cultures with *S. aureus*

Porcine airway epithelia were cultured at the air-liquid interface (7) and stimulated with heat-killed *S. aureus* as described in the online supplement.

Expression Profiling

Expression profiling using RNAseq (*in vivo* experiments) or microarray (*in vitro* studies) was performed by the University of Iowa DNA Facility. For both datasets, global gene expression profiles between treatment groups were compared using the hierarchical clustering tool in Gene Pattern (8, 9). Differentially expressed genes were analyzed by pathway analysis using the online tools Database for Annotation, Visualization and Integrated Discovery version 6.7 (DAVID) (8, 10, 11) and Gene Ontology Enrichment Analysis and Visualization Tool (GORilla) (8, 12, 13). Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was used to validate the results of the bioinformatic analyses for select genes of interest. Detailed methods can be found in the MATERIALS and METHODS in the online supplement.

Results

We previously reported that CF pig lungs lack inflammation at birth (3, 4). Consistent with this observation, we found no significant differences in the resident immune/inflammatory cell types in late gestation fetal CF and non-CF pigs (see Figure E1 in the online supplement). We also found no evidence of systemic inflammation in the newborn CF pigs, based on measurements of proinflammatory cytokines in newborn CF and non-CF serum (see Figure E2). To investigate the responses of CF and non-CF lungs at an early time point, we performed an inflammatory challenge in newborns. Animals were delivered by caesarean section to facilitate precise timing of the exposure and ensure that the animals' lungs would be free of bacteria that are normally acquired in the birth canal. We hypothesized that in the absence of preexisting inflammation, the response of CF and non-CF lungs to microbial stimuli would be similar.

We selected *S. aureus* as a proinflammatory stimulus because it is a frequent pathogen in people with CF, particularly during the first decade of life (14). In addition, *S. aureus* frequently infects the airways of CF pigs (4). The strain selected was isolated from a CF pig with lung disease (4) and used previously to investigate the host defense defect in CF pigs (4, 15). To investigate the CF and non-CF responses to a proinflammatory stimulus, we heat-inactivated the bacteria, thereby minimizing confounding factors arising from preferential infection and growth in CF lungs (4).

S. aureus Elicits Similar Histologic and Bronchoalveolar Lavage Profile Responses in CF and Non-CF Pigs

We administered a challenge dose of heat-killed *S. aureus* (10^9 CFU equivalents/animal) to newborn CF piglets and non-CF littermate control animals. This dose was chosen based on previous dose response experiments performed in newborn wild-type piglets (see Figure E3). At 4 hours post-stimulation, inflammatory markers were elevated in bronchoalveolar lavage (BAL) fluid from both CF and non-CF piglets, with both groups displaying significant polymorphonuclear leukocyte (PMN) recruitment (Figures 1A and 1B)

Figure 1. Inflammatory markers in bronchoalveolar lavage (BAL) from cystic fibrosis (CF) and non-CF piglets exposed to an inflammatory challenge. Newborn CF and non-CF littermates were exposed to aerosolized heat-killed *Staphylococcus aureus* at a dose of 10^9 CFU equivalents/animal via intratracheal instillation. At 4 hours postinstillation, animals were killed and BAL assayed for markers of inflammation, including recruitment of inflammatory cells (A and B) and IL-8 production (C). (A–C) Error bars represent means $\pm$ SEM. Statistical significance was determined by one-way analysis of variance coupled with Tukey *post hoc* test (* $P < 0.05$; *** $P < 0.001$). Non-CF vehicle control group, $n = 7$ pigs; non-CF *S. aureus* group, $n = 8$ pigs; CF vehicle control group, $n = 6$ pigs; CF *S. aureus* group, $n = 6$ pigs. (D) Representative images of hematoxylin and eosin–stained (original magnification $\times 600$) tracheal tissue from CF and non-CF pigs challenged with heat-killed *S. aureus*. In both the CF and the non-CF piglets, polymorphonuclear leukocytes (arrows) were readily observed in the epithelial layer and the lamina propria of the trachea at 4 hours postinstillation. (E) Summary of histopathologic scoring for the abundance of polymorphonuclear leukocytes in both the intraepithelial and submucosal spaces of the tracheas of challenged piglets. Error bars indicate mean $\pm$ SEM. Statistical significance was determined by Kruskal–Wallis one-way analysis of variance with Dunn *post-tests* ($P = 0.0096$). Non-CF vehicle control group, $n = 5$; non-CF *S. aureus* group, $n = 8$; CF vehicle control group, $n = 6$; CF *S. aureus* group, $n = 6$. ns = not significant; S.a. = *Staphylococcus aureus*.

and IL-8 production (Figure 1C). The only statistically significant difference between the challenged CF and non-CF animals was the slightly greater IL-8 response in the *S. aureus*–challenged CF pigs (Figure 1C). In agreement with the BAL data, histopathologic analysis of the airways from

challenged animals suggested that the *S. aureus* exposure elicited an inflammatory response in both the CF and non-CF piglets. We observed PMNs infiltrating the epithelial layer and the submucosal spaces of challenged animals (Figure 1D), but the PMN abundance in the tracheal wall was

increased similarly in both CF and non-CF animals (Figure 1E).

The Early Transcriptional Response to *S. aureus* Challenge in CF Pigs Differs from Non-CF Pigs

To learn whether loss of CFTR function altered the transcriptional responses, we harvested distal trachea from both challenged and untreated control pigs. Total RNA was isolated from this tissue and gene expression profiling performed using RNA-seq. We performed unsupervised hierarchical clustering (Gene pattern) to assess similarities in global gene expression between the samples. Clustering of control samples revealed that unstimulated CF and non-CF tracheas were indistinguishable in their global gene expression profiles (Figure 2A), as previously reported (4). However, clustering of *S. aureus*-challenged samples showed clear genotype-based segregation (Figure 2B), indicating genotype-dependent differences in responses.

To characterize these genotype-based differences, we first generated two gene lists: all genes that were differentially expressed between unstimulated and *S. aureus*-challenged non-CF samples; and all genes that were differentially expressed between unstimulated and *S. aureus*-challenged CF samples ($P < 0.05$; fold change > 1.2 ; Partek Genomic Suite) (Figure 2C). Each of these gene lists was further subdivided into two datasets based on directionality of change. This yielded a total of four datasets for analysis: (1) all genes showing significantly increased expression in response to *S. aureus* in non-CF pigs, (2) all genes showing significantly increased expression in response to *S. aureus* in CF pigs, (3) all genes showing significantly decreased expression in response to *S. aureus* in non-CF pigs, and (4) all genes showing significantly decreased expression in response to *S. aureus* in CF pigs. Each resulting list was then used as an independent query for the online tools DAVID and GOrilla (Figure 2C).

CF Pig Airways Exhibit Diminished Host Defense and Increased Apoptotic Responses to the *S. aureus* Challenge

To investigate differences in the innate immune and host defense responses of CF and non-CF tissues in this experiment, we

Figure 2. Loss of cystic fibrosis (CF) transmembrane regulator alters the transcriptomic response to a proinflammatory stimulus in newborn CF pig airways. RNA harvested from newborn CF and non-CF pig trachea was used to profile for transcriptome changes induced by a 4-hour heat-killed *Staphylococcus aureus* challenge (non-CF group, $n = 5$; CF group, $n = 6$; non-CF *S. aureus* group, $n = 5$; CF *S. aureus* group, $n = 6$). Fragments per kilobase of exon per million fragments mapped gene counts from RNA sequencing were used for unsupervised hierarchical clustering to segregate (A) all CF and non-CF control samples, and (B) all *S. aureus*-challenged CF and non-CF samples. (C) Schematic describing the workflow used in the analysis of the gene lists generated from the transcriptome profiling experiments. Gene lists represent gene products significantly up-regulated or down-regulated ($P < 0.05$; fold change > 1.2) in response to the heat-killed *S. aureus* challenge in CF and non-CF samples. Lists of differentially expressed genes were analyzed using the pathway analysis tools DAVID and GOrilla, as described in the METHODS section. *S.a.* = *Staphylococcus aureus*.

focused on pathways (enriched with differentially expressed genes) rather than individual genes. Analysis with both DAVID (see Table E1) and GOrilla (data not shown) indicated striking differences in pathways that were increased in CF airways in response to the *S. aureus* challenge. Notably, non-CF pigs exhibited significant increases in expression of genes mapping to host defense response pathways (see Table E1A, highlighted in blue). In contrast, CF animals showed a blunted induction of these same pathways (see Table E1B, highlighted in blue). To represent these results visually, we generated a heat map by plotting the fragments per kilobase of exon

per million fragments mapped gene counts (16) for transcripts constituting all host defense pathways increased in both CF and non-CF samples. As shown in Figure 3A, we observed a significant increase in host defense genes in the *S. aureus*-challenged non-CF samples relative to the other three groups (red). Additionally, our pathway analysis revealed a significant enrichment of apoptotic pathways in the *S. aureus*-challenged CF animals (see Table E1B, highlighted in yellow). Although there was also a trend for some genes in this pathway to show increased expression in *S. aureus*-challenged non-CF animals, this phenomenon was significantly more

Figure 3. Cystic fibrosis (CF) airways exhibit altered host defense responses to an inflammatory stimulus. (A) RNA harvested from newborn CF and non-CF pig trachea was used to profile for transcriptome changes induced by a 4-hour heat-killed *Staphylococcus aureus* challenge. A heat map was generated by plotting the fragments per kilobase of exon per million fragments mapped

pronounced in the CF pig airways (Figure 4A).

To validate these observations, we selected 11 genes each from the host defense response and apoptotic pathways for confirmation by RT-qPCR. The genes selected are well-studied from the perspective of host defense and apoptosis, and corroborate significant genotype-dependent differences in their expression in response to the *S. aureus* stimulus. Consistent with our expression profiling results, the airways of the non-CF animals showed more significant induction of IL8, IL6, IL1A, IL1B, HIF1A, ICAM1, COX2, and NFKBIA mRNAs on receiving *S. aureus* than did their CF counterparts (Figure 3B). The converse was observed for apoptotic genes, wherein induction of CASP3, CASP6, CASP7, BCL10, BCLAF1, MSX1, ROCK1, TGFBI, FASLG, and FASR mRNA expression was greater in CF animals than in non-CF following *S. aureus* challenge (Figure 4B). These results suggest that loss of CFTR reduced the early transcriptional host defense/inflammatory/nuclear factor- κ B (NF- κ B)-inducible responses in CF airways, and altered expression of apoptotic factors.

CF Pig Airways Exhibit Decreased Expression of Ciliary and Flagellar Biogenesis Genes in Response to the *S. aureus* Challenge

We investigated pathways showing reduced expression in CF and non-CF animals in response to the *S. aureus* challenge. Of note, the pathways decreased in challenged non-CF animals failed to show significant enrichment in “GO biologic process” terms. However, we unexpectedly observed in CF animals receiving the *S. aureus* challenge that there were significant reductions in pathways involved in ciliary and flagellar biogenesis/function, as indicated by both DAVID (see Table E2B, highlighted in green) and GOrilla (see Figure E4). A heat map of genes comprising all the ciliary and flagellar pathways with reduced expression revealed that expression of these genes was significantly reduced in *S. aureus*-challenged CF animals (Figure 5A). To confirm these findings, we selected 11 genes with well-studied roles in ciliary biogenesis and measured their expression by RT-qPCR. All 11 genes showed significantly reduced expression in CF animals that received the *S. aureus* stimulus (Figure 5B).

Cultured CF and Non-CF Airway Epithelia Also Exhibit Different Transcriptional Responses to *S. aureus*

We also tested the responses to the *S. aureus* stimulus of well-differentiated air-liquid interface primary cultures of tracheal epithelia. Here, cells form a pseudostratified airway epithelium containing ciliated and nonciliated columnar cells, goblet cells, and basal cells; however, submucosal glands and phagocytes, such as neutrophils and macrophages, are absent (7). We challenged cultured epithelia with heat-killed *S. aureus* and at 4 hours post-stimulation, RNA was harvested and the transcriptome profiled using the Affymetrix Porcine Array (Affymetrix, Santa Clara, CA). Using an analysis strategy similar to that described in Figure 2C, we observed considerable enrichment in pathways related to host defense responses in non-CF epithelia. In contrast, CF epithelia showed a significantly diminished host defense response to the *S. aureus* challenge (see Figure E5). Also corroborating the *in vivo* findings, we observed a significant increase in apoptotic genes in the *S. aureus*-challenged CF samples relative to the other three groups (see Figure E6). Down-regulation of ciliary/flagellar biosynthesis genes in the *S. aureus*-challenged CF epithelia was not apparent in the microarray profiling results, probably because many of these genes were not on the porcine Affymetrix array. However, we observed decreased expression of select target genes mapping to these pathways in the CF epithelia when we measured their expression directly by RT-qPCR (see Figure E7). Thus, cultured CF and non-CF tracheal epithelia recapitulated the genotype-dependent transcriptional differences observed in the *in vivo* pig airways after exposure to heat-killed *S. aureus*.

Discussion

Here, we report differing transcriptional responses to an acute inflammatory

stimulus (heat-killed *S. aureus*) in the airways of newborn CF and non-CF pigs. Although the basal gene expression profiles in both genotypes were similar, the induction of numerous inflammatory/host defense/NF- κ B pathway genes was blunted in CF animals relative to non-CF littermates, whereas expression of apoptosis pathway genes was increased. Additionally, the inflammatory challenge resulted in decreased expression of genes involved in ciliogenesis in CF animals, but not in non-CF control animals. Importantly, an experimental stimulus was required to uncover these genotype-dependent differences.

Interestingly, these trends were mirrored in cultured CF and non-CF airway epithelia exposed to *S. aureus*. These transcriptional differences were manifest in the absence of phagocytes or other immune cells, suggesting that airway epithelia drove the transcriptional responses observed *in vivo* following the inflammatory stimulus. This is consistent with literature implicating CF airway epithelia in the dysregulated inflammatory and host defense responses that characterize the CF lung (17–29).

CF Pig Airways Display Diminished Induction of Inflammatory and Host Defense Genes in Response to Inflammatory Challenge

Overall, our transcriptional profiling results do not support the idea that inflammatory responses are exaggerated in CF airways at the early time point used in this study; rather, they point to a tendency for CF airways to be hyporesponsive to the *S. aureus* stimulus with respect to induction of host defense/inflammatory/NF- κ B-inducible gene expression. Our results differ somewhat from many earlier studies suggesting that CF airway epithelia are intrinsically proinflammatory or hyperinflammatory. Reports describe overproduction of proinflammatory mediators, particularly IL-8, in CF cell lines,

primary CF airway cells, and subepithelial spaces of human fetal CF airway grafts (17, 19, 22, 24, 27–30). Studies using CF mice report altered regulation of several inflammatory mediators (23, 31, 32), and enhanced proinflammatory cytokine production in response to pulmonary *Pseudomonas aeruginosa* exposure compared with wild-type counterparts (33). Overactivity of NF- κ B, or diminished levels of the NF- κ B inhibitor I κ B α , have also been reported in CF cells and tissues (17, 18, 20, 28, 29, 34–36). Although it is unclear how loss of CFTR might perturb inflammatory signaling, it has been posited that accumulation of misfolded CFTR in the endoplasmic reticulum (ER) leads to a cell stress response that includes activation of NF- κ B (37), or that CFTR is a negative regulator of the NF- κ B pathway (38).

Although many studies suggest that CF airway epithelia exhibit exaggerated inflammatory responses, others have reached different conclusions. Massengale and colleagues (25) observed that cells expressing mutant CFTR secreted less IL-8 than those expressing wild-type CFTR, both under baseline conditions and after IL-1 β treatment; in a microarray study examining the responses of CF and non-CF cell lines to *P. aeruginosa* stimulation, expression of several NF- κ B-inducible gene products was reduced in the absence of CFTR (26). Other investigators have detected no significant differences in cytokine production between CF and non-CF cells, before or after treatment with proinflammatory stimuli (39–41). Overall, differences in cell culture methods and experimental models have made it difficult to draw firm conclusions about the presence of an inherent defect in inflammatory signaling in CF airway epithelia. There is evidence that some of the differences documented in earlier studies may arise from genetic heterogeneity in donor cells (42, 43). Another possible confounding variable is that cells derived

Figure 3. (Continued). gene counts for genes constituting all the host defense pathways that were up-regulated in CF and non-CF samples in response to the heat-killed *S. aureus* challenge. In constructing heat maps, samples were not clustered by sample ID. Instead, genes were clustered using pairwise complete-linkage and distance measured by Pearson correlation. Rows were normalized by subtracting the mean of each row from each value in that row. Red denotes increased expression relative to the mean of each row; blue denotes decreased expression. (B) We measured mRNA expression levels for selected target genes from host defense response pathways by reverse-transcriptase quantitative polymerase chain reaction, using RNA harvested from *S. aureus*-challenged and control newborn CF and non-CF pig trachea. Error bars indicate mean $\pm$ SEM. Statistical significance was determined by Kruskal–Wallis one-way analysis of variance coupled with Dunn *post hoc* test (* P < 0.05; ** P < 0.01; *** P < 0.001). Non-CF group, n = 5; CF group, n = 6; non-CF *S. aureus* group, n = 5; CF *S. aureus* group, n = 6. Influence of *S. aureus* on gene expression between non-CF and CF groups was determined by two-way analysis of variance (* P < 0.05; ** P < 0.01; *** P < 0.001). ns = not statistically significant; NT = no treatment; S.a. = *Staphylococcus aureus*.

Figure 4. Cystic fibrosis (CF) airways exhibit altered apoptotic responses to an inflammatory stimulus. (A) RNA harvested from newborn CF and non-CF pig trachea was used to profile for transcriptome changes induced by a 4-hour heat-killed *Staphylococcus aureus* challenge. A heat map was generated as described in Figure 3, to visualize all the apoptosis/apoptosis regulation pathways that were up-regulated in CF and non-CF samples in response to the heat-killed *S. aureus* challenge. Red color denotes increased expression relative to the mean of each row; blue denotes decreased expression. (B) We measured mRNA expression levels for selected target genes from apoptosis pathways by reverse-transcriptase quantitative polymerase chain reaction, using RNA harvested from *S. aureus*-challenged and control newborn CF and non-CF pig trachea. Error bars indicate mean $\pm$ SEM. Statistical significance was determined by Kruskal-Wallis one-way analysis of variance coupled with Dunn *post hoc* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Non-CF group, $n = 5$; CF group, $n = 6$; non-CF *S. aureus* group, $n = 5$; CF *S. aureus* group, $n = 6$. Influence of *S. aureus* on gene expression between non-CF and CF groups was determined by two-way analysis of variance (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$). ns = not statistically significant; NT = no treatment; S.a. = *Staphylococcus aureus*.

from people with CF may retain inflammatory signatures, such as increases in proinflammatory signaling molecules, as a result of chronic exposure to inflammation *in vivo*.

We note that our findings differ from those recently reported by Keiser and colleagues (44) in the CF ferret model. They observed differences in the BAL proteomes of CF and non-CF ferrets suggesting that already before birth, CF ferret lungs contain altered levels of cytokines, inflammatory mediators, and other immune factors; furthermore, spontaneous exposure to bacteria (during birth) seemed to induce an inflammatory state in newborn CF ferret lungs that was evident in the first 24 hours of life (44). It is possible that these differences between the pig and ferret models reflect species-dependent differences in the development and regulation of airway innate immunity. Note that in contrast to humans and pigs, ferrets have few ciliated airway epithelia and no submucosal glands at birth (45). Alternatively, methodologic differences in how and when the tissues were sampled and/or analyzed may have contributed to the differing results.

CF Pig Airways Increase the Expression of Genes Involved in Apoptosis Regulation in Response to *S. aureus*

Numerous proapoptotic genes were induced in CF pig airways in response to the *S. aureus* challenge, suggesting that expression of apoptotic factors is dysregulated in CF airway epithelia. Consistent with this, work with CF cell lines and cells cultured from the airways of CF mice indicate that CF airway cells are comparatively more sensitive to apoptotic induction by drugs (46, 47), fungal spores (48), and cigarette smoke (49). Conversely, Gottlieb and colleagues (50) reported that mutant CFTR confers resistance to a proapoptotic drug. In a microarray study, CF cells displayed a less robust induction of proapoptotic genes in response to influenza A infection than control cells (51), and no difference in apoptotic responses was observed between CF and matched non-CF cell lines exposed to *P. aeruginosa* strain PA01 (52). Thus, the relationship between apoptotic signaling and loss of CFTR is unclear, and the response of CF epithelia to proapoptotic stimuli may depend on the nature of the challenge. We speculate that

Figure 5. Cystic fibrosis (CF) pig airway epithelia suppress ciliary and flagellar biosynthesis in response to a proinflammatory stimulus. (A) Heat map generated by plotting the fragments per kilobase of exon per million fragments mapped gene counts for genes constituting ciliary and flagellar biosynthesis pathways that were down-regulated in response to heat-killed *Staphylococcus aureus* in CF and non-CF pigs. The heat map was generated as described in Figure 3. Red denotes increased expression relative to the mean of each row; blue denotes decreased expression. (B) To confirm these results we measured mRNA expression levels for selected target genes by reverse-transcriptase quantitative polymerase chain reaction, using RNA harvested from *S. aureus*-challenged and control newborn CF and non-CF pig trachea. Error bars indicate mean $\pm$ SEM. Statistical significance was determined by Kruskal–Wallis one-way analysis of variance coupled with Dunn *post hoc* test (** $P < 0.01$; *** $P < 0.001$). Non-CF group, $n = 5$; CF group, $n = 6$; non-CF *S. aureus* group, $n = 5$; CF *S. aureus* group, $n = 6$. Influence of *S. aureus* on gene expression between non-CF and CF groups was determined by two-way analysis of variance (## $P < 0.01$; ### $P < 0.001$). ns = not statistically significant; NT = no treatment; S.a. = *Staphylococcus aureus*.

CF airway epithelia may be more likely than their wild-type counterparts to apoptose in response to bacterial products. Alternatively, exposure to such a stimulus may cause gene expression changes that “prime” the CF airway epithelia for apoptosis, enhancing susceptibility to subsequent proapoptotic stimuli.

CF Pig Airways Down-Regulate Expression of Ciliogenesis Genes in Response to Inflammatory Challenge

The other major finding in this study, that the *S. aureus* challenge lead to suppression of genes involved with ciliary/flagellar biogenesis pathways in CF airways was unexpected. We observed decreases in three

major gene classes in *S. aureus*-treated CF animals: (1) Bardet-Biedl syndrome proteins (BBS1, BBS4, BBS9), (2) microtubule-associated dynein motor proteins (DNAH2, 7, 9, 10, DNAI2), and (3) the cilia associated kinesin family members (KIF6, 7, 27). BBS proteins associate with primary cilia (53) and motile cilia on the airway epithelium (54). Loss of BBSome proteins causes a misshaped appearance of motile cilia, reduced ciliary beat frequency (54), and ciliary bulges in the airway epithelium (53, 54).

Interestingly, patients with BBS may have an increased incidence of asthma (55), and *Bbs2*^{−/−} and *Bbs4*^{−/−} mice are partially protected from airway hyperresponsiveness (54). Therefore, we speculate that decreased expression of BBSome proteins in CF airways may reduce responsiveness to subsequent inflammatory stimuli. Mutations in DNAH2, DNAH7, DNAH9, and DNAI2 have been linked to primary ciliary dyskinesia (56), which is associated with altered mucociliary clearance, infection, and inflammation of the respiratory tract (57). KIF7 and KIF27 are involved in hedgehog signaling and primary cilia formation (58). Together, these results imply that exposure to an inflammatory stimulus induces CF airway epithelia to enact a gene expression program resulting in altered cilia structure and function.

Advantages and Limitations of this Study

The CF pig allowed us to address some limitations of earlier human studies and studies done using cells and tissues derived from humans with CF. First, using CF and non-CF littermates with the same genetic background ensured that the main experimental variable was the presence or absence of CFTR. Second, we provided the inflammatory stimulus within the first few hours after birth by caesarean section to avoid confounding variables arising from bacterial infection and accompanying inflammation. Importantly, we used CFTR null animals for this study; therefore, none of the observed gene expression differences can be attributed to an ER stress response induced by retention of $\Delta F508$ CFTR in the ER.

This study also has limitations. Responses to proinflammatory challenges follow a course of onset and resolution that takes many hours; we focused on a single, early time point, 4 hours post-instillation. This time interval provides valuable insights

regarding the initiation of the inflammatory response, but does not address the resolution phase. A goal of future studies will be to investigate later time points to learn how loss of CFTR affects the peak and resolution phases of inflammation. Another limitation of this study is the use of a single inflammatory stimulus (heat-killed *S. aureus*); it is of interest to learn whether similar differences are observed in response to other types of stimuli (e.g., lipopolysaccharide). Furthermore, although our results point to a primary impairment in the induction of several classes of genes in response to an inflammatory stimulus, they do not preclude the possibility that inflammatory responses are further altered by the onset and progression of CF lung disease. For instance, diminished mucociliary transport likely contributes to the retention of immunostimulatory

material, further amplifying the effects of this defect (4, 15). Similarly, over time the reduced antimicrobial activity of CF airway surface liquid (4, 15) may increase the bacterial burden, presenting more inflammatory stimuli to mucosal surfaces and immune effector cells. Defects in the responses of pulmonary macrophages and PMNs caused by loss of CFTR may also contribute to the *in vivo* findings (59–62).

In summary, our results suggest that following an inflammatory stimulus, multiple gene expression pathways are dysregulated in CF airway epithelia in the early phase of the response. The biologic impact of these early transcriptional differences may not manifest until a later stage of the inflammatory response. These differences arise independently of preexisting inflammation, suggesting they represent an intrinsic property of the

airways and perhaps other cell types lacking functional CFTR. An important avenue for future research is to investigate how loss of CFTR function may lead to these phenomena, in particular the possibility that reduced ASL pH (15) or changes in the properties of secreted mucins (63) may alter the responses of CF airways to inflammatory stimuli. ■

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