Electrolyte transport properties in distal small airways from cystic fibrosis pigs with implications for host defense

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Cystic fibrosis (CF) is caused by defects in the membrane-bound channel cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel that is permeable to both Cl− and bicarbonate (HCO3−) (52, 61). CF lung disease is the main cause of morbidity and mortality in people with CF. The main features of CF lung disease are bacterial colonization, chronic infection, and mucus plugging in both large and small conducting airways (1, 14, 43, 61). Genetically modified pigs with either deletion or expression of ΔF508 CFTR mutant have provided significant insight into the mechanism by which loss of CFTR function results in CF lung disease (42, 60). In large proximal airways of CF pigs, lack of CFTR results in host defense defects, including reduced mucociliary transport (MCT) and impaired bacterial killing, due in part to decreased airway surface liquid (ASL) pH (30, 43, 61). However, few studies have explored the role of the small airway in CF etiology, and the effect of loss of CFTR function on ASL pH, mucus viscosity, and antimicrobial activity in the distal small airways is not known.

Histopathology data show small airway abnormalities in early stages of CF lung disease (9, 41, 58). In addition, pulmonary function tests in people with CF demonstrate a progressive decline in 25–75% forced expiratory volume (FEV1), which reflects small airway function, while FEV1, which more reflects central airway function, remains relatively stable in early stages of disease (6). Despite the fact that the distal small airways contribute to over 85–90% of the total surface area of airway epithelia (11, 25, 67) and the clear evidence that people with CF have some disease manifestation in the small airways, the field of small airway functional studies in CF has not significantly progressed due in part to the lack of experimental models.

Compared with extensive studies on CF large airways, previous studies on CF small airways are limited because it is difficult to access the small airways for detailed mechanistic studies (4, 7, 8, 13, 50, 51). We adapted a method to isolate, expand, and culture small airway epithelial cells from newborn CF and non-CF pigs, before the onset of lung inflammation and infection (47). This method allowed us to characterize the bioelectric properties of both large and small airways and to investigate how CFTR deficiency affects ASL pH and host defense properties.

MATERIALS AND METHODS

Isolation, expansion, and culture of airway epithelia. All animal studies were reviewed and approved by the University of Iowa Animal Care and Use Committee. Generation of CFTR−/− pigs has been previously reported (48). Animals were produced by mating CFTR−/− male and female pigs. Newborn CFTR−/− or CFTR−/− (non-CF) piglets were obtained from Exemplar Genetics (Exemplar Genetics, Sioux Center, IA). We studied newborn pigs within 12 h after birth because their lungs have not manifested changes secondary to chronic infection and inflammation (36). Also in contrast to gut-corrected CFTR−/− pigs (62), the CFTR−/− pigs in this study develop severe meconium ileus, and it is difficult to keep them alive beyond 48 h after birth. Newborn piglet lungs were excised, and the whole airway tree was microdissected by carefully combing off the parenchymal tissue. Subsequently, the vascular tissue was excised, and the whole airway tree was microdissected by carefully combing off the parenchymal tissue.
was separated from the airway tree by blunted dissection. Proximal large airways, including trachea and main stem bronchi, and distal small airways (diameter ~200 μm) were dissected out separately from the airway tree. Next, primary porcine airway epithelia were isolated according to an adapted procedure originally developed for tracheal airway cells. Isolated large and small airway cells were expanded using a method developed to conditionally reprogram airway epithelial cells (39, 63). Briefly, large and small airway cells were separately cultured in F media in the presence of 10 μM Y-27632, a Rho kinase (ROCK) inhibitor, and low passages of irradiated fibroblast feeder cells NIH-3T3-J2 (obtained from Dr. H. Green’s laboratory at Harvard University) (46) and maintained at 37°C with 5% CO₂.

For histological analysis of native large and small airways and expanded cells cultured at an air-liquid interface, samples were fixed in 10% neutral buffered formalin for 48–96 h. Tissues were then permeabilized with 0.2% Triton X-100 (Pierce, Rockford, IL). Apical and basolateral chambers of cell cultures grown in Transwell (Corning no. 3470) at a density of 10⁶ cells/cm² were separated from feeder cells and seeded on collagen-coated semipermeable membranes (Corning no. 3470) at a density of 10⁶ cells/cm² and cultured at the air-liquid interface at 37°C in a 5% CO₂ atmosphere as previously described (70) for 2–3 wk in the absence of feeder cells and ROCK inhibitor. In the 1st wk of seeding at the air-liquid interface, cells were maintained in Small Airway Growth Media (Lonza, Basel, Switzerland) supplemented with 10 ng/ml blast feeder cells NIH-3T3-J2 (obtained from Dr. H. Green’s laboratory at Harvard University) (46) and maintained at 37°C with 5% CO₂.

Cells were separated from feeder cells and seeded on collagen-coated semipermeable membranes (Corning no. 3470) at a density of 10⁶ cells/cm² and cultured at the air-liquid interface at 37°C in a 5% CO₂ atmosphere as previously described (70) for 2–3 wk in the absence of feeder cells and ROCK inhibitor. In the 1st wk of seeding at the air-liquid interface, cells were maintained in Small Airway Growth Media (Lonza, Basel, Switzerland) supplemented with 10 ng/ml blast feeder cells NIH-3T3-J2 (obtained from Dr. H. Green’s laboratory at Harvard University) (46) and maintained at 37°C with 5% CO₂.

**Bioelectrical properties.** Ussing chamber studies were performed as previously described (19, 54, 55). Apical and basolateral chambers contained the same bathing solution with symmetrical Cl⁻ concentrations. CFTR-mediated Cl⁻ current and amiloride-sensitive Na⁺ current were measured using a previously described protocol (19).

First, Na⁺ current was inhibited with apical amiloride (100 μM), an inhibitor of the epithelial sodium channel (ENaC), which hyperpolarizes the apical membrane and increases the driving force for Cl⁻ secretion through CFTR. Second, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS, 100 μM) was added apically to block the calcium-activated Cl⁻ channels to specifically examine the CFTR-mediated Cl⁻ current. Next, CFTR activity was increased by raising cellular levels of cAMP with forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM). GlyH-101 (GlyH, 100 μM), a CFTR inhibitor, was added apically to block the CFTR-mediated Cl⁻ current (19). Finally, the transepithelial Cl⁻ current was reduced by inhibiting the Na⁺/K⁺ -Cl⁻ cotransporter with basolateral bumetanide (100 μM). The following parameters were calculated as previously described (38): decrease in current after apical addition of amiloride, decrease in current after apical addition of DIDS, cAMP-stimulated current after apical addition of forskolin and IBMX to epithelia already in the presence of apical amiloride and DIDS, and decrease in current after addition of GlyH-101 to the previous solutions. To study HCO₃⁻ transport, we used Cl⁻–free Krebs solution containing (in mM): 118.9 sodium gluconate, 25 NaHCO₃, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 5 calcium gluconate, 1 magnesium gluconate, and 5 dextrose and gassed with 5% CO₂ as previously described. The sequence of drug administration was the same as above.

**Gene expression profiles.** Total RNA was isolated from non-CF expanded large and small airway epithelial cells at passage 4 with TRIZol Reagent (Invitrogen). After confirming RNA integrity, 5 μg of total RNA was used to generate biotinylated cRNA using the Affymetrix Gene Chip one-cycle target labeling kit (Affymetrix, Santa Clara, CA) and then hybridized to the Affymetrix Porcine GeneChip (23,937 probe sets that interrogate ~23,256 transcripts from 20,201 Sus scrofa genes). All samples were hybridized on the same day. The arrays were scanned using the Affymetrix Model 450 Fluidics Station and Affymetrix Model 3000 scanner, and data were collected using the GeneChip Operating Software, version 1.4, and analyzed with Partek Genomics Suite Software (Partek, St. Louis, MO).

**Quantitative real-time PCR.** Quantitative RT-PCR with SYBR Green chemistry and an ABI 7500 Fast Real-time PCR System were used to measure SP-D mRNA levels in tissue excised from native large and small airways. Briefly, tissues were collected in RINalater (Ambion), and total RNA was isolated using the Purelink RNA kit (Invitrogen) and RNAeasy Mini Kit (Qiagen). First-strand cDNA was synthesized with random hexamers (SuperScript III; Invitrogen). Sequence-specific primers and probes were from IDT (Coralville, IA). SP-D mRNA levels were normalized to ZO-1 mRNA. The primer sequences of SP-D and ZO-1 genes were as follows: pig SP-D forward, 5'-AGC GGA GCA GAG AAC TGT GTG-3'; pig SP-D reverse, 5'-CTC AGA ACT CGC AGA TCA CG-3' (53); ZO-1 forward, 5'-AGA CCC GCA CAA GTG TGA TAG GA-3'; and pig ZO-1 reverse, 5'-ACG GGC GTG GGC TCT CAT AG-3'. SP-D mRNA was measured using real-time fluorescent pH indicator SNARF conjugated to dextran (Molecular Probes, Eugene, OR) as previously reported (43). Briefly, the pH indicator was applied to the apical surface, and forskolin (10 μM) and IBMX (100 μM) were applied to basolateral medium. Two hours later epithelia were placed in a humidified chamber with 5% CO₂ atmosphere at 37°C and examined by confocal microscopy (Zeiss 510 Meta NLO). pH was calculated as previously described (43).

**ASL viscosity measurement.** ASL viscosity was assayed using fluorescence recovery after photobleaching as previously described (18) and our recent study (64).

**Antimicrobial activity measurement.** Antimicrobial activity was examined using two separate assays as follows: 1) a luminescence-based antibacterial assay (2) modified for use on cells and 2) a grid-based assay (43). For the luminescence antibacterial assay, *Staphylococcus aureus* Xen-29 (Caliper LifeSciences Bioware), which contain a stable copy of the modified Photorhabdus luminescens luxABCDE operon, were grown to the exponential phase. Bacteria were harvested by centrifugation and suspended in a 1% tryptic soy broth buffer (2). Epithelial cells grown in a 24 Transwell plate (insert area 0.33 cm²) were exposed to bacteria (5 × 10⁸ colony-forming units in 10 μl) for 2 min, followed by measurement of luminescence with a luminometer (Spectra Max L; Molecular Devices, Sunnyvale, CA). Data are reported as relative light units as a percentage of control (dead epithelial cells with no bacterial killing ability). For the grid assay, bacteria-coated grids were prepared as previously described (43) using *S. aureus* isolate 43SA (isolated from a CF pig with pulmonary infection) (60). Grids were place on the
apical surface of epithelia in a humidified chamber with 5% CO₂ at 37°C for 2 min, followed by assessment of bacterial killing using the Live/Dead BacLight Bacterial Viability kit (Invitrogen) per the manufacturer’s instructions. All live and dead bacteria were counted in four to five individual fields (60×H11003), and the percentage of live bacteria in each field was averaged.

Statistics. Data are expressed as means ± SE. For microarray studies, a one-way ANOVA was used. P values are presented in legends for Figs. 1–6. For analyses that compared large and small airways from the same animal, we used a nonparametric Wilcoxon signed-rank test. For comparisons of different genotypes, we used the Wilcoxon rank-sum test. All analyses were done using Prism software version 6.0.

RESULTS

Isolation, expansion, and culture of distal small airway cells. In human adults, “small airways” are defined as airways with an internal diameter of <2 mm (31), usually distal to the sixth generation of the bronchial tree. Because the size of the small airways is species and age specific, we defined small airways in newborn pigs based on histology. Previous work defined the airway tree nomenclature in newborn pigs (3). In newborn pigs, we define the small airways as distal airways, bronchioles without submucosal glands and complete cartilage ring support, with an internal diameter of <200 μm (Fig. 1). Newborn pigs were chosen because CF newborn pigs show no infection or inflammation. Figure 1A is an image of the intact airway tree dissected from the left lung of a non-CF newborn pig. The red square denotes the region where large airways were taken from while the red circle denotes the region where small airways were taken from for epithelial cell expansion. Large airways revealed a cartilaginous wall lined by pseudostratified epithelium with submucosal glands and smooth muscle (Fig. 1, B and E). In contrast, the small airways lacked submucosal glands and complete cartilage ring support and displayed thin bands of smooth muscle and low columnar to cuboidal lining epithelium (Fig. 1C). Figure 1D shows staining of an airway tree dissected from the right lung from a newborn pig; it was stained with Alcian blue, which mainly stains cartilages. The small airways contained only isolated cartilage plates and were devoid of complete cartilage ring support (Fig. 1F).

Whereas sufficient numbers of large airway cells can be obtained from newborn pig trachea for direct in vitro studies, substantially fewer cells can be recovered from the small airways. To perform a comparative analysis of large and small airway epithelia, we applied a recently developed method (39, 63) to expand and culture both large and small airway cells at the air-liquid interface. This method includes coculture with low-passage irradiated NIH 3T3-J2 fibroblast “feeder cells” and treatment with a ROCK inhibitor (39, 46).

We first determined that expanded large and small airway cells cultured at the air-liquid interface formed tight junctions and had the capacity to differentiate into ciliated (acetylated α-tubulin positive) (Fig. 2, A and B) and goblet (Muc5AC-positive) (Fig. 2, C) epithelia.
positive) (Fig. 2, C and D) cells. This is consistent with the cellular composition of the native large and small airways, which were mostly covered by ciliated cells, with fewer goblet cells in the small airway compared with large airway (Fig. 2, E–H).

To further characterize the expanded small airway cells, we compared the gene expression profiles of non-CF small and large airways using a porcine gene microarray. We found that the expression of several genes was significantly different between expanded large and small airway epithelial cells (Fig. 3A). For example, SP-D was robustly expressed (17.4-fold higher) in expanded small airway epithelial cells (Fig. 3A), which is consistent with previous studies of SP-D distribution in pig, rat, and human small airways (21, 29, 40). Using real-time PCR and immunocytochemistry, we validated that SP-D expression was higher in excised native small airway tissue, with no SP-D-positive staining detected in large airway surface epithelia (Fig. 3, B–D). Other genes, such as complement component 9 and integrin-α9, had at least 10 times higher expression in the expanded small airway epithelia relative to the large airway cells (Fig. 3A). Conversely, there are some genes, such as glutathione-S-transferase α1 (GSTA1) (Fig. 3A), that have lower gene expression levels in small airways. The complete list of gene array data is included in the Supplemental Table S1 (Supplemental Material for this article is available online at the Journal website.). These data demonstrate that expanded epithelial cells retain some characteristics of the native tissue and small airway epithelia have a distinct gene expression profile compared with large airway epithelia.

**Differential electrolyte transport properties in expanded large and small airway epithelia.** The physical and chemical properties of ASL, which play important roles in host defense and MCT, are dependent on transepithelial ion transport. Thus, we investigated the electrolyte transport properties in expanded large and small airway epithelia from both non-CF and CF pigs under short-circuit conditions. Expanded large and small airway epithelia from CF and non-CF pigs were used for Ussing chamber studies as previously described (54). The baseline transepithelial resistances were not different among groups of expanded cells studied: non-CF large airway epithelia (792.8 ± 146.7 Ω·cm²); non-CF small airway epithelia (907.8 ± 204.3 Ω·cm²); CF large airway epithelia (726.0 ± 128.0 Ω·cm²); and CF small airway epithelia (1014 ± 125.6 Ω·cm²). Summarized short-circuit current (Isc) data in non-CF and CF large and small airway epithelia are presented in Fig. 4, A and B. Data for large and small airways from the same animal were paired for each pig, allowing direct comparison within each genotype. Amiloride-sensitive ENaC current was similar in expanded large and small airway epithelia from CF and non-CF pigs (Fig. 4C). We found no difference in the DIDS-sensitive Ca²⁺-activated Cl⁻ channel activity in large and small CF and non-CF airway epithelia (Fig. 4D). However, we detected greater cAMP-stimulated Cl⁻ current in non-CF small airway epithelia compared with large airway (Fig. 4E). Minimal cAMP-stimulated current was observed in either large or small CF airway epithelia (Fig. 4E). In line with these findings, the CFTR blocker GlyH produced a greater decrease in current in non-CF small airway cells relative to large airway cells, and application of GlyH had no effect in CF epithelia (Fig. 4F).

To determine whether small airway epithelia have differences in HCO₃⁻ transport, we measured electrogenic HCO₃⁻ transport by Ussing chamber using Cl⁻-free Krebs solution gassed with 5% CO₂. Representative Isc traces in non-CF and CF large and small airway epithelia are presented in Fig. 5, A and B. Compared with expanded non-CF large airway epithelia, addition of 10 μM forskolin and 100 μM IBMX
and the addition of 100 μM GlyH to non-CF small airway epithelia resulted in a greater change in current (Fig. 5, A, C, and D). These data suggest that the greater CFTR activity in small airway epithelia is associated with greater HCO₃⁻ transport. The minimal response to treatments (forskolin and IBMX; GlyH) in CF large and small airway epithelia confirms the role of CFTR in HCO₃⁻ transport (Fig. 5, B, C, and D).

Fig. 3. Gene expression profile between expanded large and small airway epithelia is different. A: top five significantly upregulated and downregulated genes in expanded small vs. large airway epithelia from non-CF pigs, N = 3 non-CF piglets. Three Transwell inserts of expanded epithelia from each pig were pooled to isolate RNA. ANOVA was used, and the P value was listed. B: validation of surfactant protein D (SFTPD) mRNA changes in native large and small airway tissue by qRT-PCR. Data were normalized to ZO-1. N = 6 non-CF piglets. *P = 0.0498; Ratio-paired t-test. C and D: immunostaining of SP-D in native large and small airway tissue. The white arrowhead in D indicates the SP-D-positive surface epithelia in small airway but not in large airways. Scale bar = 60 μm in C and 40 μm in D.

Fig. 4. Non-CF small airway cells have increased cystic fibrosis transmembrane conductance regulator (CFTR)-mediated cAMP-stimulated Cl⁻ current. A and B: summarized short-circuit current (Isc) data of expanded large and small airway epithelial cells from non-CF (A) and CF (B) pigs. C: summary data of amiloride-sensitive ΔIsc (ΔIscAmil) in expanded large and small airway epithelia from non-CF and CF pigs. D: summary data of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)-sensitive ΔIsc (ΔIscDIDS) in expanded large and small airway epithelia from non-CF and CF pigs. E: summary data of forskolin (F) + 3-isobutyl-1-methylxanthine (I)-stimulated ΔIsc (ΔIscF&I) in expanded large and small airway epithelia from non-CF and CF pigs. F: summary data of GlyH-101 (a CFTR inhibitor)-sensitive ΔIsc (ΔIscGlyH) in expanded large and small airway epithelia from non-CF and CF pigs. N = 11 non-CF piglets and 11 CF piglets. One Transwell insert of expanded epithelia from each pig was used. *P = 0.002 vs. non-CF large airway epithelia, Wilcoxon signed-rank test. F: summary data of GlyH-101 (a CFTR inhibitor)-sensitive ΔIsc (ΔIscGlyH) in expanded large and small airway epithelia from non-CF and CF pigs. N = 11 non-CF piglets and 11 CF piglets. One Transwell insert of expanded epithelia from each pig was used. *P = 0.0029 vs. non-CF large airway epithelia, Wilcoxon signed-rank test.
ASL pH is higher in non-CF small airway epithelia than in expanded large airway epithelia. Changes in HCO$_3^-$ transport will alter ASL pH given a constant P$_{CO_2}$ (43). Thus, we examined if the differences in HCO$_3^-$ transport in large and small airway epithelia result in a change in ASL pH. We used the dextran-conjugated fluorescent ratiometric pH indicator SNARF to measure ASL pH, and the calibration curve is shown in Fig. 5E. We found that non-CF small airway epithelia had a higher ASL pH compared with non-CF large airway epithelia under 5% CO$_2$ conditions (Fig. 5F). These data are consistent with the differences in CFTR activity (Fig. 4, E and F) and HCO$_3^-$ transport (Fig. 5, C and D) in large vs. small airway epithelia from non-CF pigs.

CF small airway epithelia have lower ASL pH, higher viscosity, and impaired bacterial killing. Previous studies in large airway epithelia of pig and human origin have established that loss of CFTR results in decreased HCO$_3^-$ secretion and reduced ASL pH (19, 43, 56, 59). We therefore compared ASL pH in expanded CF and non-CF pig small airway epithelia and found that ASL pH was lower with CFTR deficiency (Fig. 5F), consistent with that CF ASL pH is more acidic (43).

Studies in newborn CF pigs identified at least two respiratory host defense defects in the large airway that were present at birth: 1) impaired MCT (30) and 2) impaired endogenous bacterial killing in ASL lining fluid due to lower pH (43, 61). Because we observed lower pH in CF vs. non-CF small airway epithelia, we next examined ASL viscosity and bacterial killing to assess if these same defects are present in the CF small airways. We found that ASL viscosity was higher in CF small airway epithelia compared with non-CF cells (Fig. 6A), consistent with a role for CFTR in MCT defects. In addition, we used two independent methods to evaluate bacterial killing: luminescence-based assessment of bacterial viability (2) and bacteria-coated grids placed on the apical surface of expanded airway epithelia (43). Compared with expanded non-CF small airway epithelia, both methods demonstrated a significant impairment of bacterial killing in CF small airway cells (Fig. 6, B and C). These data highlight the important implications for host defense of CFTR deficiency in small airway epithelia.

DISCUSSION

Recent work in the large airway has provided compelling data that ASL pH and MCT are altered by CFTR deficiency, resulting in impaired host defense against bacterial challenge (30, 43, 61). While clinical and pathology data suggest that the small airways are involved in the early development of CF lung disease (65), progress toward understanding the precise role of small airway epithelia has been significantly hampered by the lack of experimental models. Using a novel approach to isolate,
Fig. 6. CF small airway epithelia have higher ASL viscosity and lower bacterial killing ability compared with non-CF small airway epithelia. A: ASL viscosity as determined by fluorescence recovery after photobleaching of FITC-dextran in expanded small airway epithelia from non-CF and CF pigs. $N = 6$ non-CF piglets and 6 CF piglets. One Transwell insert of expanded epithelia from each pig was used. $*P = 0.0043$ vs. non-CF large airway epithelia, Wilcoxon rank-sum test. B: antimicrobial activity of expanded small airway epithelia from non-CF and CF pigs. Data are presented as the relative luminescence (RLU) of Staphylococcus aureus (Xen-29) as a percentage of control. $N = 6$ non-CF piglets and 6 CF piglets. One Transwell insert of expanded epithelia from each pig was used. $*P = 0.0043$ vs. non-CF large airway epithelia, Wilcoxon rank-sum test. C: antimicrobial activity of expanded small airway epithelia from non-CF and CF pigs as evaluated by S. aureus-coated grid assay. Data are presented as the percentage of live bacteria. $N = 5$ non-CF piglets and 5 CF piglets. One Transwell insert of expanded epithelia from each pig was used. $*P = 0.0159$ vs. non-CF small airway epithelia, Wilcoxon rank-sum test.

We found that small airway cells from non-CF pigs have greater CFTR activity, $\text{HCO}_3^-$ transport, and ASL pH relative to large airway cells. Lack of CFTR function in small airway epithelia blunted $\text{HCO}_3^-$ transport, decreased ASL pH, and significantly impaired ASL viscosity and bacterial killing. In summary, this method for expanding and culturing small airway epithelia allowed us to uncover differences in ASL pH, viscosity, and antimicrobial properties between CF and non-CF small airway epithelia.

The majority of published studies of the small airways rely on large animals, namely pigs and sheep. Murine models are not suitable because the size of the small airways precludes the experiment. Using a recently reported approach for culturing primary cells in the presence of a ROCK inhibitor (39, 63), we isolated large and small airway cells from newborn pig lungs and expanded and cultured the expanded cells at the air-liquid interface. We confirmed that our model retains many features of the native tissue, including gene expression profile and distribution of SP-D in the small but not large airway epithelia (21, 29). We also observed that several other genes as listed in Fig. 3A are differentially expressed in large vs. small airway cells, although they do not have obvious roles in ion transport properties and host defense. They likely present an interesting area for further investigation. Moreover, we demonstrate that this approach can be applied to study small airway cells from CF pigs, which facilitates comparative studies of small airway function in CF and non-CF pigs.

A limitation of this study is that expanded airway epithelia may have altered properties compared with native tissue or primary cells. For example, published data from expanded human alveolar cells demonstrate loss of ion channel transport properties and expression of surfactant proteins at higher passage numbers (16). We observed a similar trend in our expanded airway cells. For this reason, we only used expanded cells with passage numbers under six. We also observed a non-negligible batch effect. To overcome this limitation, experiments were performed using matched cells derived from the large and small airways of the same pig, cultured under the same conditions, and at the same passage number.

Our data provide clear evidence that CFTR activity is greater in small airway epithelia compared with large airway epithelia. A previous in situ hybridization study in human lung tissue demonstrated that CFTR expression is higher in small vs. large airways (23). As expected, deficiency of CFTR abrogated Cl$^-$ transport in expanded small airway epithelia. Consistent with the greater CFTR activity in small airways, $\text{HCO}_3^-$ transport was greater in the small compared with the large airway epithelia. Others have measured $\text{HCO}_3^-$ secretion in native small airways using micro-Ussing chamber assays (50), but ours is the first study to directly compare $\text{HCO}_3^-$ transport in large and small airway epithelia. We also confirmed that $\text{HCO}_3^-$ transport in the small airways is CFTR dependent, which has been previously reported in large airway (20, 32, 33, 45, 57). While our data provide evidence that $\text{HCO}_3^-$ transport is mainly mediated by CFTR in the small airways, it remains possible that the channel TMEM16A (17, 22, 49, 69) and the transporter SLC26A9 (10) may contribute to $\text{HCO}_3^-$ transport, perhaps by working in concert with the Cl$^-$/$\text{HCO}_3^-$ exchanger SLC26A4 (26). In addition, ASL pH may be regulated by other mechanisms besides $\text{HCO}_3^-$ secretion. It has been reported that acid secretion can also regulate ASL pH in airway epithelia (24). Our data suggest that higher $\text{HCO}_3^-$ secretion through CFTR can at least partially explain why ASL pH is higher in non-CF small airways. Further studies are needed to elucidate the detailed ASL pH regulation mechanism, such as measurement of anion gaps and proton secretion in non-CF large and small airways (24, 35).

The lower ASL pH in the small airways of CF pigs was associated with increased viscosity and decreased bacterial killing compared with non-CF pigs. These data indicate that the small airways are susceptible to bacterial colonization, which may be caused by the same antibacterial host defense defects present in the large airways. In large CF airways, the submucosal glands secrete mucus strands that remain tethered to gland ducts (30), resulting in impaired MCT. Because the small airways lack submucosal glands, we speculate that acidic ASL pH in CF small airways alters goblet cell-derived mucus properties, providing an alternate mechanism for the MCT defect. The impaired bacterial killing in CF small airways may be explained by the impact of ASL pH on antimicrobial
proteins, such as SP-D, which is exclusively expressed in the small airways. We have reported that acidic pH impairs the ability of two key airway antimicrobial peptides, β-defensin-3 and LL-37, to kill bacteria (2). When these peptides are combined, they exhibit synergistic bacterial killing, but an acidic pH reduced their synergistic effect. Future studies are warranted to understand how changing ASL pH affects SP-D antimicrobial activity and its synergy with other antimicrobial proteins/peptides. Even though we suggest that lower ASL pH in CF small airways could contribute to CF pathogenesis, there could be other factors. For example, several studies of cultured large airway epithelia reported that loss of the CFTR led to ASL height depression (15). Considering that there could be some difference in water permeability between large and small airways (37), ASL height will be important to determine in expanded large and small airways in future studies. However, we did not detect any difference in aquaporin gene expression in our expanded large and small airway epithelia as described in a previous study (37). In addition, it is not clear what the physiological and pathophysiological significance of several other differentially expressed genes in large and small airways is. Interestingly, it has been reported that GSTA1 polymorphism is associated with asthma, which is another small airway disease (44). Similar investigations are needed to determine if these differences contribute to CF lung disease pathogenesis.

Our data leave the interesting question unanswered: why would the small airways have higher CFTR activity and a more alkaline ASL pH than the large airway? This observation is consistent with previous studies (13, 23, 66). We speculate several possibilities. First, because the small airways lack submucosal glands, and the submucosal glands in large airways secrete fluid and HCO$_3^-$, the epithelia of the small airways have to compensate by secreting more liquid and HCO$_3^-$ through elevated CFTR activity (13, 68). Because our culture model lacks submucosal glands, this results in higher ASL pH in the small airways. Second, the small airways are exposed to a constant ~5% CO$_2$ concentration during the respiratory cycle. In contrast, the large airways are exposed to oscillations in CO$_2$, with lower CO$_2$ concentrations during inspiration (27). Thus the average CO$_2$ concentration in small airways is higher than in the large airways during the respiratory cycle. A higher HCO$_3^-$ concentration in the small airway would partially counteract the differences in CO$_2$ concentration. Third, the optimal pH needed for host defense mechanisms, including MCT and antimicrobial capacity, may be different between large and small airways. Finally, because distal airways may have greater susceptibility to particles/bacterial accumulation due to slower MCT in the distal lung region (5), enhanced pH-mediated antimicrobial activity and MCT may be required to maintain sterile epithelia.

Detailed mechanistic studies are needed to test these possibilities and understand why ASL pH is higher in the small airways under normal physiological conditions. Moreover, these questions should be taken into account when developing therapeutic approaches to overcome the effect of CFTR deficiency that leads to CF lung disease.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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