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Innate immune response in CF airway epithelia: hyperinflammatory?

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Machen, Terry E. Innate immune response in CF airway epithelia: hyperinflammatory? *Am J Physiol Cell Physiol* 291: C218–C230, 2006; doi:10.1152/ajpcell.00605.2005.—The lack of functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR) in the apical membranes of CF airway epithelial cells abolishes cAMP-stimulated anion transport, and bacteria, eventually including *Pseudomonas aeruginosa*, bind to and accumulate in the mucus. Flagellin released from *P. aeruginosa* triggers airway epithelial Toll-like receptor 5 and subsequent NF- κ B signaling and production and release of proinflammatory cytokines that recruit neutrophils to the infected region. This response has been termed hyperinflammatory because so many neutrophils accumulate; a response that damages CF lung tissue. We first review the contradictory data both for and against the idea that epithelial cells exhibit larger-than-normal proinflammatory signaling in CF compared with non-CF cells and then review proposals that might explain how reduced CFTR function could activate such proinflammatory signaling. It is concluded that apparent exaggerated innate immune response of CF airway epithelial cells may have resulted not from direct effects of CFTR on cellular signaling or inflammatory mediator production but from indirect effects resulting from the absence of CFTRs apical membrane channel function. Thus, loss of Cl^- , HCO_3^- , and glutathione secretion may lead to reduced volume and increased acidification and oxidation of the airway surface liquid. These changes concentrate proinflammatory mediators, reduce mucociliary clearance of bacteria and subsequently activate cellular signaling. Loss of apical CFTR will also hyperpolarize basolateral membrane potentials, potentially leading to increases in cytosolic $[\text{Ca}^{2+}]$, intracellular Ca^{2+} , and NF- κ B signaling. This hyperinflammatory effect of CF on intracellular Ca^{2+} and NF- κ B signaling would be most prominently expressed during exposure to both *P. aeruginosa* and also endocrine, paracrine, or nervous agonists that activate Ca^{2+} signaling in the airway epithelia.

Pseudomonas aeruginosa; Toll-like receptor; NF- κ B; oxidative stress; acidic airway surface liquid; calcium

ARE INNATE HOST RESPONSES IN CF “HYPERINFLAMMATORY”?

UNDER NORMAL CONDITIONS, the airways remain relatively sterile due to the efficient action of the mucociliary escalator. The lack of functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR) in the apical membranes of CF airway epithelial cells abolishes cAMP-stimulated anion transport, and bacteria, eventually including *Pseudomonas aeruginosa*, accumulate in the mucus (180) and trigger a dramatic inflammatory response to the infection. The innate immune response of the epithelial cells to these bacteria in the airway surface liquid (ASL) involves the activation of receptors and signaling pathways, production, and release of proinflammatory cytokines and the recruitment of macrophages and neutrophils to the infected region. The most important *P. aeruginosa* product triggering the early inflammatory responses is flagellin, the monomer that comprises the structural shaft of the flagellum (134). These flagellin subunits activate Toll-like

receptor (TLR)-5 (58) in the apical membranes of airway epithelial cells (179, 199). Signaling through MyD88-IRAK-TRAF and p38 MAP kinases (199), and perhaps Ca^{2+} (1, 137), activates NF- κ B and AP-1 transcription factors that regulate proinflammatory genes. Although TLRs 2, 6, 9, and 10 and perhaps others are also expressed (5, 52, 61, 199), they appear to be much less important in early responses to the luminal bacteria (59, 179, 199). In particular, LPS and other released bacterial products (10, 52, 112) appear only to activate weak inflammatory responses in airway epithelia (179), and then only at very high concentrations (59, 71). It should be noted that although the earliest inflammatory signaling in response to *P. aeruginosa* seems to be controlled by flagellin-TLR5-NF- κ B, other bacterial products and epithelial signaling pathways may be important during infections with other common CF pathogens, including *Staphylococcus aureus* (50, 137), *Haemophilus influenzae* (19, 138), or *Burkholderia cepacia* (181). This may also pertain to the situation occurring during persistent, extended infections with *P. aeruginosa*, because under these conditions the bacteria become immotile, presumably resulting from the loss of flagella (see 152). In this circumstance, inflammation may be maintained by *P. aeruginosa* secreting quorum-sensing homoserine lactones (162),

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alginate (30), pyocyanin (34, 136), and/or other secreted factors like proteases and exotoxin A: any or all of these may trigger proinflammatory signaling in airway epithelial cells. *P. aeruginosa* also secrete other virulence factors that may contribute to the proinflammatory state (see Ref. 152 for review).

Although the inflammatory responses to the bacteria occur on a grand scale in CF airways, there is great uncertainty whether the CF airway epithelia exhibit such a large inflammatory response because so many bacteria have accumulated, or, alternatively, because the epithelia have an inherent defect leading to a hyperinflammatory state, in which there is constitutive production and secretion of inflammatory cytokines and increased responses to the presence of bacteria.

Some studies have found increased numbers of inflammatory cells and IL-8 in bronchoalveolar lavage (BAL) from CF patients with either mild disease symptoms or in the absence of demonstrable microorganisms (16, 17, 80, 111, 115, 150, 176). There is also *in vivo* evidence of reduced production of anti-inflammatory products like IL-10 (16, 17) and lipoxins (76). Many studies have found that CF airway epithelial cells in culture have constitutively active NF- κ B and upregulated expression and secretion of IL-8 and other inflammatory mediators (12, 23, 33a, 35, 36, 41, 54, 90, 167, 171, 174, 183, 186, 190). In some cases, these phenotypes were largely reversed in CFTR-corrected cells or in cells incubated at 25°C, which increased mutant CFTR movement from the ER to the plasma membrane (e.g., 41, 159). This apparent inherent hyperinflammatory state may be further stimulated by the presence of *P. aeruginosa* (39, 176), though not always (23).

Microarray methods have recently been used to test for differences in gene expression in CF vs. non-CF or CFTR-corrected cells. Some experiments have shown that *patterns* of gene expression (i.e., specific genes) were different in CF vs. CFTR-corrected cell (41, 141, 167). Whitsett and colleagues (197, 198) have described similar differences in expression of specific genes in the lungs of wild-type, CF knockout, and Δ F508CFTR mice at different stages of development. The most prominent effects of the lack of wild-type CFTR expression were on genes related to redox balance and regulation [particularly in genes related to glutathione (GSH) homeostasis], heat shock or stress, ion transport, and CFTR-interacting proteins (198). Although there were increases in expression of some pro-inflammatory genes (e.g., IL-1 β , TNF- α -induced protein 3, colony stimulating factor-3 receptor), most of these changes were relatively small (<2-fold) and there were no increases in the well-known proinflammatory mediators like IL-8 and TNF- α (197). Overall, these microarray studies of the lungs of mice were consistent with the studies of cells in culture in showing that the absence of CFTR had selective effects to regulate specific genes, but, unlike the *in vitro* studies, the *in vivo* results indicated that genes involved in regulating inflammatory processes were not prominently affected.

In contrast, Perez and Davis (124) found similar gene expression patterns but different *magnitudes* of responses to *P. aeruginosa* strain PAO1 in CF vs. non-CF cells. Kelley and colleagues (78, 79, 88, 168) found that CF cells have inefficient Jak-stat1 signaling, reducing nitric oxide synthase 2 expression. Exuberant NF- κ B signaling (174) or reduced nitric oxide

production leading to destabilized I κ B and increased NF- κ B activity (84) in CF have been proposed to explain these hyperinflammatory phenotypes.

In contrast to these studies showing apparent intrinsic hyperinflammation in CF cells, Dakin et al. (32) showed that early infection in CF was the likely explanation for the enhanced inflammatory responses in CF lungs. This result was consistent with other *in vivo* measurements of inflammatory mediators in BAL fluids showing that increased inflammation in CF followed bacterial infections (5). Recent studies using terminal restriction fragment length polymorphism profiling of sputum from both adult (149) and pediatric (148) CF patients have shown many (>40) bacterial species that have not been previously identified in CF. Most of these bacteria were metabolically active, indicating that they could potentially play a role in pathogenesis. It therefore seems possible that previous *in vivo* studies that observed inflammation in the apparent absence of infection may have suffered from undetected bacteria.

Technical differences may also explain the apparent hyperinflammatory phenotype observed *in vitro*. Thus a comprehensive study by Aldallal et al. (3) compared the cell lines used by many research groups, and also used adenovirus to express CFTR in CF cells to ensure isogenic comparisons between the CF and the CFTR-corrected cells, including primary airway epithelial cells. They showed that different responses of normal, CF and CFTR-corrected airway epithelia were likely due to differences in cells, and were unrelated to the presence of CFTR. Pizurki et al. (130) used the adenoviral method to express CFTR in a CF cell line and similarly showed that inflammatory responses to cytokines were similar in CF and CFTR-corrected cells. Also, Becker et al. (10) showed that normal and CF primary airway epithelial cells exposed to bacterial supernatants caused equivalent activation of cytokine expression and secretion, though they did observe differences between CF and non-CF after 24 h of treatment. Joseph et al. (74) have also shown that long-term incubation with *P. aeruginosa* caused larger innate immune responses in CF cells compared with non-CF, consistent with previous work (90) showing that long-term bacterial exposure may magnify differences in inflammatory responses between CF and non-CF or CFTR-corrected epithelia.

Although a firm conclusion is presently impossible, we propose that subtle technical artifacts have contributed to the apparent proinflammatory phenotype observed by many investigators in studies of CF vs. non-CF or CFTR-corrected airway epithelia. *In vivo* experiments demonstrating inflammatory mediators in CF BAL fluids in the absence of bacterial infections may have suffered from undetected bacteria or bacterial products. Although it is clear that the CF cell line (IB3) exhibits a pro-inflammatory status compared with matched cells with integrated wild-type CFTR (C38) (e.g., 36, 41, 166, 167), there are no differences in constitutive or stimulated inflammatory responses among IB3 cells, IB3 cells that had been CFTR-corrected using an adenovirus and cells infected with adenovirus expressing a control transgene (3), indicating that there were non-CFTR-dependent differences between the IB3 and C38 cell lines. Similar problems may explain proinflammatory differences observed between other similar

pairings, including 9/HTEO-/pCEP-R ("CF") – 9/HTEO-/pCEP (non-CF) cells (e.g., 52).

In this regard, Babnigg et al. (6) have observed variability in store-operated Ca^{2+} influx into human embryonic kidney-293 cells, and they argue, based on a careful analysis of this variability, that isolating clones from a heterogeneous population can lead to clones with significantly different Ca^{2+} influx, even though they were isolated from the same parent population. They further argue that it is important to compare effects of gene expression based on transfections of many cells (>200). Growing only a few transfected cells can yield a biased population. This problem might contribute to the apparent differences in inflammatory properties of the IB3 vs. C38 cells or the pCEP vs. pCEPR cells. Further use of the adenoviral or similar method to make isogenic comparisons between CFTR and ΔF508 -CFTR-expressing cells would help settle such controversies because the method leads to expression of the CFTR in a high percentage of the cells and also permits comparison with vector controls.

In vitro studies of CF vs. normal primary cells may also suffer from subtle technical problems that contribute to apparent hyperinflammation in CF. Ribeiro et al. (144) have shown that ΔF508 CF primary bronchial epithelia exhibited a hyperinflammatory phenotype as defined by an increased basal and bradykinin-induced IL-8 secretion during the first 6–11 days of culture. However, this CF phenotype appeared to result from the chronic exposure in vivo to inflammatory conditions because this phenotype was lost in long-term (30 to 40 days old) cultures, and exposure of 30- to 40-day-old cultures of normal airway epithelia to supernatant from mucopurulent material from CF airways induced the hyperinflammatory phenotype in the normal cultures. These results showed that the hyperinflammatory phenotype, which also included dramatic changes in structure of the ER (144–146), was independent of mutant CFTR expression and that this phenotype was maintained for extended times in culture. Future studies of hyperinflammation in CF vs. normal primary cells will need to account for these prolonged effects of the in vivo inflammatory state on cells in culture.

The absence of a proinflammatory phenotype in CF airway epithelia would be consistent with the fact that CF epithelia like sweat duct (139) and intestine (117) that normally express CFTR at high levels do not apparently exhibit a proinflammatory phenotype. For example, CF mouse small intestine exhibits increased expression of several inflammatory markers (e.g., serum amyloid A and complement factors) and large influx of mast cells and neutrophils (117) compared with non-CF mice. These data were consistent with data obtained from CF humans showing increased levels of inflammatory markers (e.g., IL-1 β and IL-8) and nitric oxide, as well increased infiltration of monocytes (21, 133, 164). However, this inflammatory response in the intestine seems to have resulted solely from an overgrowth of luminal bacteria (116). Thus, when CF mice were treated with antibiotics, inflammatory markers and cells were reduced to those of the non-CF murine intestines (116). Interestingly, the exaggerated inflammation in CF mouse intestine was also reduced by treatment of patients with *Lactobacillus*, indicating that the specific bacterial flora were important determinants of the inflammation (116).

DOES ALTERED GOLGI pH IN CF CAUSE DEFECTS IN MEMBRANE AND MUCUS BIOCHEMISTRY AND BACTERIAL BINDING IN CF?

An early proposal (9), subsequently modified (131), was that absence of CFTR altered Golgi pH, which in turn reduced activity of Golgi enzymes leading to increased fucosylation and decreased sialylation of membrane surfaces (155, 175), including increased expression of asialoGM1 (9). *P. aeruginosa* produce lectins that bind to fucose moieties (65), and increased asialoGM1 expression and increased *P. aeruginosa* binding (12, 19, 53, 122) could increase inflammatory signaling. It has also been proposed that flagellin may activate airway epithelial cells by binding to asialoGM1 serving as co-receptor to TLR2 (1). In addition, some studies have shown altered cell surface glycosylation (135) and altered binding of some lectins in CF cells (38) or CFTR-expressing cells transfected with plasmids expressing either CFTR regulatory (R) domain or full-length ΔF508 CFTR (96). A related concept is that mucins secreted by CF airway epithelial cells could have similarly altered glycosylation and/or sulfation (e.g., 92, 143, 175) leading to bacterial binding.

However, several observations indicate that this altered Golgi pH-altered surface glycosylation hypothesis is likely to be incorrect. Seksek et al. (160) and Chandy et al. (26) showed that there were no CFTR-associated differences in Golgi pH (also see Ref. 49). Dunn et al. (39) similarly showed that pH of the endosomal compartment was not altered in CF. Instead, pH's of the Golgi and other organelles of the secretory (and perhaps endocytic) pathways appear to be regulated primarily by H^+ pumping into the organelle lumen by the well known H^+ v-ATPase balanced by a H^+ leak (26, 156, 195). The CFTR likely plays no role in controlling pH of the Golgi because the Golgi has its own K^+ and/or Cl^- conductances that dissipate the voltage associated with operation of the electrogenic H^+ v-ATPase (see Ref. 104). In this circumstance, Golgi pH will be determined by the activities and numbers of the H^+ v-ATPase and the H^+ leak and also by the cytosolic pH. Although CFTR conducts HCO_3^- and its activity affects cytosolic pH under some circumstances (132), there is no evidence that steady-state cell pH is affected by CFTR in airway epithelia.

In addition, CFTR expression in IB3 cells using an adenovirus had no effect on lectin binding (72), showing that previously measured differences were due to differences between the cell lines that were not related to CFTR expression. Furthermore, if Golgi pH and glycosylation and sulfation enzyme activities were altered in CF, it would be expected that mucins would exhibit altered glycosylation and sulfation. However, several mucins showed identical glycosylation and sulfation in CF and non-CF or CFTR-corrected cells (20, 96, 140, 150, 161).

DOES LOSS OF CFTR REDUCE BACTERIAL UPTAKE AND DISPOSAL IN CF?

Pier and colleagues have proposed that *P. aeruginosa* binds to CFTR at the first extracellular loop through interaction with the outer core oligosaccharide portion of bacterial LPS, and this binding leads to bacterial entry into the epithelial cell. Recent experiments (51, 86) have shown that CFTR may be located in lipid rafts. It is further proposed that bacterial uptake

into the cells has two beneficial effects: activation of apoptosis and cell sloughing aids in clearing bacteria from the airways, and activation of NF- κ B contributes to a subclinical, protective innate immune response and inflammation that resolves the infection (126–128). The absence of CFTR in the plasma membrane therefore reduces bacterial clearance and contributes to an overexuberant proinflammatory response (127).

Several observations indicate that this altered bacterial uptake hypothesis is likely to be incorrect. First, the hypothesis has been based partly on electron or light microscopic observations of in vivo lung specimens, and it is difficult to determine whether apparent bacterial uptake into epithelial cells was responsible for triggering apoptosis and desquamation or, alternatively, that the desquamating cells were particularly susceptible to bacterial binding and uptake, as has been observed in studies of bacterial binding on cultured airway epithelia (94). Second, the hypothesis conflicts with a number of observations. First, under normal conditions, bacterial binding to the apical surface, where CFTR is located, is infrequent (1 bacterium per 100 epithelial cells: see Refs. 128 and 129), and *P. aeruginosa* binding (94, 135) and uptake (46) occurs most prominently at the basolateral membrane of epithelia. Second, *P. aeruginosa* uptake may be negatively, not positively, correlated with CFTR expression (33). Finally, apical application of flagellin alone, even in the absence of bacteria, activates NF- κ B in all columnar cells lining the airway surface (179), showing that bacterial uptake is not required to induce a cellular innate immune response. Thus, although *P. aeruginosa* appear to be internalized by a small percentage of airway epithelial cells and internalized bacterial products could activate NF- κ B in these cells (178), it appears unlikely that CFTR plays a role in these processes.

DOES REMOVAL OF CFTR FROM SIGNALING COMPLEX TRIGGER INFLAMMATORY SIGNALING IN CF?

From measurements of regulated on activation of normal T-expressed and presumably secreted production in both CF and CFTR-corrected primary cells and cell lines IB3 cells transfected with a variety of different CFTR mutants, Schwiebert and colleagues (43, 159) concluded that CFTR expression in the plasma membrane served to inhibit AP-1 and NF- κ B signaling through interactions with EBP50 (also termed Na⁺/H⁺ exchange regulatory factor), the cytoskeleton and associated inflammatory activator proteins. In the absence of CFTR in the apical plasma membrane, this inhibition would be lifted, contributing to increased inflammatory signaling in CF. Others have similarly proposed that absence of CFTR may alter interactions with AMP kinase (AMPK) or annexin 1, either of which could play roles in controlling inflammation. AMPK is located in a similar cellular location as CFTR (54) and appears to interact with regulate its channel activity (55–57), indicating that a CFTR-AMPK “signaling complex” might exist. In addition, CF airway epithelial cell lines and primary cells expressed less AMPK and larger secretion of IL- and IL-8 than non-CF cells, and the apparent proinflammatory phenotype was reduced by treating CF cells with a chemical activator of AMPK (54). The colon, pancreatic ducts, and lung airways also express annexin 1 in similar location as

CFTR, and annexin 1 expression was reduced in CF (12). Because annexin 1 regulates phospholipase A2 and may serve an anti-inflammatory function in cells, it was argued that hyperinflammatory responses of CF airways resulted from the loss of annexin 1 (12).

Although the COOH-terminus of CFTR associates with EBP50 and other PDZ-related proteins (e.g., 53, 97, 170, 189) and could therefore serve as an organizer of a macromolecular signaling complex in or near the apical membrane of airway epithelia, it seems likely that there will be many more EBP50, AMPK, and annexin 1 molecules than CFTRs in airway epithelial cells, so the absence of CFTR may not alter the distribution and organizational function of the potential signaling partners. The modulation of AMPK through CF-induced changes in cellular [Ca²⁺] (or other signaling events) was also proposed as a connection between CFTR and AMPK (54). The potential role of CFTR in affecting or controlling cellular [Ca²⁺] will be discussed below. Alteration of annexin 1 function by cellular [Cl⁻] has been proposed to explain the different annexin function in CF vs. non-CF cells (12), although experiments on cultured nasal cells indicate that there is no difference in cell [Cl⁻] between CF and non-CF (193). Overall, it seems likely that if there is a role for CFTR in controlling proinflammatory signaling, this will be mediated not through direct molecular interactions with a signaling complex but through some indirect effect of CFTR on the cellular environment.

DOES HYPOXIA IN CF TRIGGER ROS PRODUCTION AND HYPERINFLAMMATION?

The potential roles of hypoxia and ROS-regulated signaling in controlling inflammatory processes in CF have not been considered previously, but CF could alter oxidative status of both cells and ASL through changes in oxygen use by the airway epithelial cells (Fig. 1). Using O₂-sensitive microelectrodes, Wortlisch et al. (194) found Po₂ >150 mmHg in the fluids 700–800 μ m above the surface of cultured airway epithelia. Po₂ decreased in a curvilinear manner to values <50 mmHg as the electrodes reached the surface of non-CF cells, and this hypoxia was even more pronounced in CF, with Po₂ reaching 5–15 mmHg. It was hypothesized that the lower Po₂ values in CF were due to increased Na⁺ absorption that occurs in CF cultures, leading to increased ATP consumption by the Na⁺-K⁺-ATPases in the basolateral membranes of the cells, resulting in increased O₂ consumption (18, 123, 169). On the basis of data in other cell types, hypoxia could activate MAPK and/or NF- κ B-signaling pathways leading to intrinsic inflammation even in the absence of bacteria. Thus the hypoxia-inducing factors-1 or HIF-2 of many cells are tightly controlled by cellular oxygen tension (157, 188) through reactions controlled by enzymes whose activities are dependent on [O₂] (60, 93). When [O₂] <5% (i.e., when Po₂ = 38 mmHg, close to values observed in the fluids above airway epithelial cells, Ref. 194), production of ROS by mitochondria increased (24, 25), leading to activation of signaling pathways, including p38 MAPK (25), which has been implicated (e.g., 83 and 196) as an integral downstream component of the MyD88-dependent branch of the TLR pathway as well as other pathways likely involved in the response to pathogens.

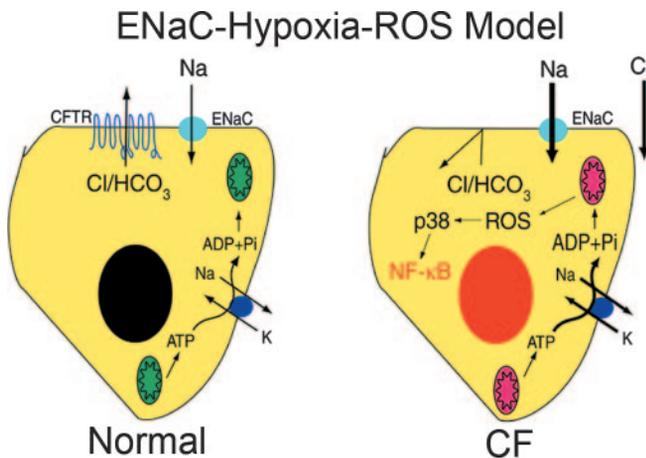


Fig. 1. Increased Na⁺ transport in cystic fibrosis (CF) causes hypoxia and production of reactive oxygen species (ROS)? It has been proposed (see Ref. 158) that the absence of CFTR increases Na⁺ absorption (shown by larger arrow) through epithelial Na⁺ channels (ENaC) (electroneutrality provided by paracellular flux of Cl⁻), leading to increased ATP utilization by basolateral Na⁺-K⁺-ATPase. The increased energy demand increases consumption of O₂ [thereby depleting P_{O2} in the airway surface liquid (ASL)] and increased production of ROS by mitochondria (shown as green in normal and red in CF). ROS could activate NF-κB signaling and contribute to inflammation.

DOES HYPERINFLAMMATION RESULT INDIRECTLY FROM CFTR'S ROLE IN CONTROLLING VOLUME, PH, AND GSH OF ASL?

CFTR is expressed in both the glands (42) and the surface epithelium (87) of the airways, and it is likely that both glands and surface cells participate in controlling the ionic composition and volume of the ASL. The glands (8, 67), primarily the acinar cells (73), appear to serve an important secretory function, with CFTR conducting both Cl⁻ and HCO₃⁻ from the cells into the lumen (64, 67, 89, 187, 191). In contrast, the surface cells appear to be capable of both secretion and absorption (182), with absorption dominating in the quiescent state in the absence of activation of cAMP/PKA signaling. Secretion by both glands and surface cells is reduced in CF (73).

Although the effects of the absence of CFTR on the composition and volume of the ASL in CF are still being debated (see Refs. 29, 70, 165), it seems likely that the absence of CFTR decreases volume of the ASL (73). This reduction in volume of the ASL would be expected to increase the concentration of other secreted products that may be proinflammatory, even in the absence of bacterial infections. For example, bradykinin and the purinergic agonists ATP, ADP, and adenosine activate inflammatory signaling (99, 106–108, 121, 144, 145), and these would all be expected to be present in increased concentrations if the volume of the ASL is reduced in CF. In addition, airway epithelial cells secrete small, but significant amounts of proinflammatory cytokines into the ASL (179), and the cells have apical receptors for these cytokines (179) that could then activate or synergize with inflammatory signaling triggered by bacteria. The magnitude of this proinflammatory, concentrating effect of reducing ASL volume will depend on the amounts of these inflammatory mediators that are secreted into the lumen and the concentration dependence of the effects of these agonists on epithelial inflammatory signaling, which remain to be tested.

CFTR may be involved in maintaining redox status of the ASL and mutations in CFTR could impair lung antioxidant defenses, thereby increasing oxidative stress in the ASL in CF airways (14, 47, 62, 151). WT-CFTR conducts reduced GSH (see Ref. 101), a key redox buffer in cells. Cells containing defective CFTR secrete less GSH than control cells containing functional CFTR, and transfection with functional CFTR restores GSH secretion (47). Furthermore, bronchioalveolar lavage fluids from CFTR-knockout mice had decreased concentrations of GSH and increased concentrations of thiobarbituric acid-reactive substances and 8-hydroxy-2-deoxyguanosine, two indicators of oxidative stress. However, tissue concentrations of GSH were similar, and the activities of GSH reductase and GSH peroxidase were increased, whereas the activity of γ-glutamyltransferase was unchanged (185), indicating that changes in redox may not always occur in CF. It remains to be determined whether increased ASL oxidation in CF results directly from the absence of CFTR or indirectly from the infiltration of leucocytes that produce ROS and also whether increased ASL oxidation affects cytosolic redox. However, no matter how the oxidative stress in CF originates, such oxidation could potentially activate NF-κB (75) and p38 MAP kinase (81, 98, 105) and hyperinflammatory responses (185) (Fig. 2).

The acidity of ASL appears to increase in CF, and this could similarly affect cellular signaling. The ASL of both normal and CF airways is slightly more acidic than plasma (29, 69, 70, 91), and nongastric H⁺-K⁺-ATPase (29), v-type H⁺-ATPase (67), and Zn²⁺-sensitive H⁺ conductance (45) in the apical membranes of the epithelial cells may all contribute to this acidity.

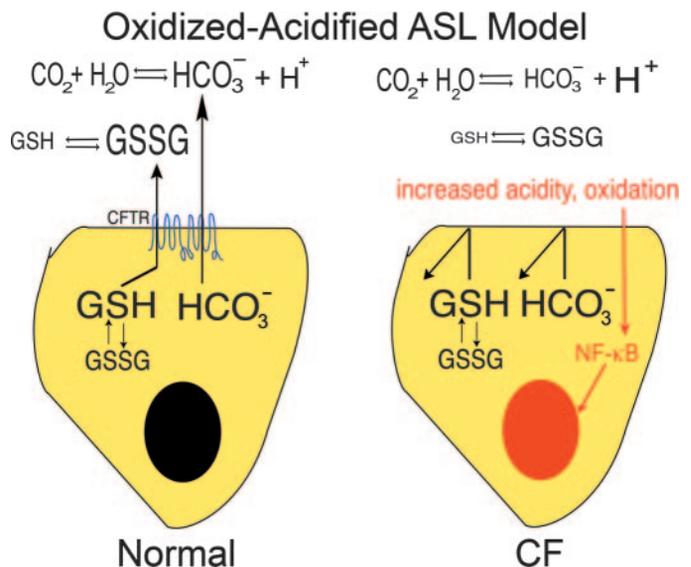


Fig. 2. ASL redox and/or pH controls inflammatory signaling in CF? According to this model, normal airway epithelial cells (left) conduct reduced glutathione (GSH) and HCO₃⁻ from the cell cytosol to the ASL through CF transmembrane conductance regulator (CFTR), and these transport activities are reduced in CF (left). GSH is in equilibrium with oxidized GSH (GSSG), and HCO₃⁻ and H⁺ are in equilibrium according to the well known reaction shown. Relative concentrations of GSH, GSSG, HCO₃⁻, and H⁺ in normal and CF are shown by the type sizes. CFTR thereby helps maintain a less oxidized and less acidic ASL in normal airways than in CF. Reduced GSH and HCO₃⁻ transport in CF leads to increased oxidation and acidity of the ASL, which then act on the cytosol to activate NF-κB and contributes to inflammatory signaling in CF.

CFTR conducts HCO_3^- (64, 132), and both submucosal glands (8, 67, 165, 177; also see Refs. 89 and 95) and surface epithelium (29, 120) secrete HCO_3^- into the ASL. The absence of CFTR is expected to reduce HCO_3^- secretion and because H^+ secretion is likely to be unaffected in CF (29), this will increase ASL acidity in CF (29, but also see Ref. 69). Because extracellular pH can influence intracellular pH, increased ASL acidity in CF could alter cell signaling leading to inflammation (Fig. 2). Such an effect of luminal pH on epithelial signaling has been observed in the CF mouse intestine (77): the duodenum is abnormally more acidic in CF than in non-CF due to decreased HCO_3^- secretion through CFTR, and this increased acidity in the intestinal lumen triggers the intestine to signal the exocrine pancreas (likely through secretin) to increase HCO_3^- secretion. Normalizing duodenal pH of CF mice corrected these effects.

Even in the absence of effects of reduced volume and increased oxidation and/or acidification ASL on inflammatory signaling, the altered ASL is expected to have secondary, proinflammatory effects, e.g., reduced clearance and increased accumulation of bacteria. For example, altered ASL may lead to increased mucin cross-linking and viscosity and reduced ciliary beating and mucociliary clearance (125). However, it should be noted that mucociliary clearance in vivo is reduced by <50% in CF (Ref. 11; see also Refs. 100 and 109), whereas there is a much larger percentage increase in accumulation in bacteria and subsequent activation of inflammation in CF. A possible explanation for these apparently contradictory data is that small reductions in mucociliary transport may accumulate over time, leading to the bacterial accumulation characteristic of the disease (see also Refs. 31 and 82).

INCREASED INTRACELLULAR Ca_i AND INFLAMMATORY SIGNALING RESULTING FROM ER "STRESS" IN CF?

The role of Ca_i^{2+} in inflammatory processes has been controversial. Some studies showed that both intact *P. aeruginosa* (107, 137) and flagellin (1, 108) increased cytosolic $[\text{Ca}_i^{2+}]$, intracellular Ca_i^{2+} (Ca_i), and activation of NF- κ B or other inflammatory signaling (1, 106–108, 137). In addition, increased NF- κ B signaling was reproduced by thapsigargin, the Ca_i^{2+} -ATPase/SERCA pump blocker, which increases Ca_i in cells, and blocked by the cellular Ca_i^{2+} -buffer BAPTA-AM (137). Ca_i -elevating agonists like bradykinin and ATP also increase cytokine expression and secretion (145, 147). However, there is also evidence that elevations of Ca_i are not involved in activating innate immune responses triggered by *P. aeruginosa*. Strains PAO1 and PAK activate NF- κ B and IL-8 expression and secretion in JME/CF15 and Calu-3 cells without affecting Ca_i (63, 68, 103). Flagellin also activates NF- κ B and IL8 secretion in Calu-3 and JME/CF15 cells without affecting Ca_i (Z. Fu and T. Machen, unpublished observations), and TLR signaling is not known to trigger increases in Ca_i in other cell types (2). The discrepancies among studies which found *P. aeruginosa*- or flagellin-induced increases in Ca_i and those which did not may result from subtle differences in bacterial preparations or epithelial cells, or in amounts of ATP released into the extracellular fluid (which would trigger Ca_i^{2+} signaling; see Refs. 106–108) during addition of the bacteria to the epithelial cells. Overall, it appears that elevating Ca_i by

treatment with purinergic agonists, bradykinin or thapsigargin is sufficient to increase activation of NF- κ B, but elevations in Ca_i are not required to activate inflammatory signaling in response to *P. aeruginosa* or flagellin in airway epithelia. Ca_i may play an important role, though, because during *P. aeruginosa* treatment, purinergic agonists elicit synergistic activation of NF- κ B mediated through increases in Ca_i (82).

A model linking mutation in CFTR to altered Ca_i signaling and inflammation is that ER stress resulting from accumulation of excessive amounts of misfolded Δ F508 CFTR in the ER lumen increases Ca_i , perhaps due to increased Ca_i^{2+} leakage from the ER (190) (Fig. 3). The increased Ca_i might then activate NF- κ B (3), contributing to inflammation. Δ F508 CFTR (~70% of patients) is a processing mutant that exhibits abnormal folding in the ER, leading to its retention (27, 191) and subsequent removal and degradation by proteosomes (48). The ER also stores Ca_i^{2+} , and altered Ca_i^{2+} handling by the ER has been observed in cells treated with adenoviruses that lead to ER accumulation of misfolded proteins (118, 119; also see Ref. 122). ER stress can also activate NF- κ B (122), and it has been proposed that ER accumulation of Δ F508 CFTR leads to activation of NF- κ B in the absence of bacterial stimulus (190; also see Ref. 7).

There are, however, inconsistencies with the ER stress: hyperinflammation hypothesis. First, CFTR is expressed at relatively low copy numbers (<5,000 channels in the apical plasma membrane of epithelial cells, e.g., see Ref. 110), and, though Δ F508CFTR is ~100% degraded in the ER, WT-CFTR is 75% degraded (only 25% reaches the plasma membrane) (27, 85), and it seems unlikely that a 25% difference in ER retention of this low abundance protein could trigger a stress response. A similar argument is relevant for Δ F508CFTR/WTCFTR heterozygotes, which exhibit normal Ca_i signaling but likely experience ER retention of Δ F508CFTR that is not much different from CF individuals. Second, measurements of Ca_i that supported the ER stress- Ca_i hypothesis (190) were based on small (20%) differences in fluo-3 fluorescence, which is difficult to quantitate because it is a nonratiometric dye. Measurements of Ca_i using the ratiometric dye fura-2 could help settle this issue (see Ref. 113). Because recent experiments on Calu-3 cells indicate that CFTR processing in cells that express "normal," as opposed to overexpressed, levels of CFTR may be different (184), it would be useful to compare a variety of CF vs. non-CF vs. CFTR-corrected cells.

INCREASED Ca_i RESULTING FROM HYPERPOLARIZED MEMBRANE POTENTIALS IN CF?

Another model that could connect CFTR to Ca_i signaling and inflammation is through effects of CFTR on cell membrane potentials, which will alter the electrical driving force for Ca_i^{2+} entry into the cells from the ASL or serosal fluid (Fig. 4). An early study (142) showed that Ca_i responses to histamine and prostaglandin E1, but not to carbachol, were reduced in CF cell lines compared with non-CF cells. In addition, adding the purinergic agonists ATP or UTP to the apical surface of primary airway epithelia elicited larger responses in CF than in non-CF, though Ca_i responses to basolateral ATP or UTP were similar in CF and non-CF cells (121). The different responses

ER Stress-Ca²⁺ Model

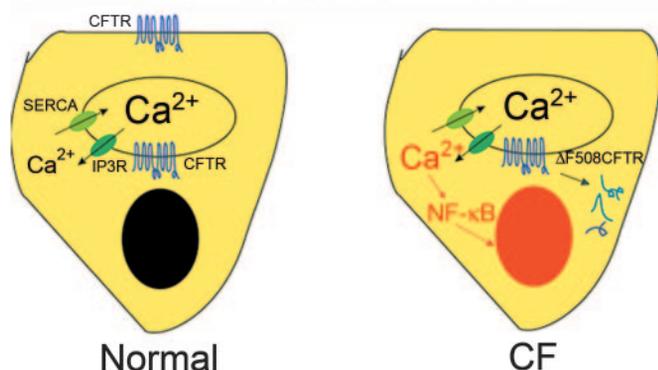


Fig. 3. Endoplasmic reticulum (ER) stress in CF increases cytosolic [Ca²⁺]. Retention and degradation of ΔF508CFTR in the ER and associated proteasomes (not shown) in CF might alter intracellular Ca²⁺ (Ca_i) through effects on ER Ca²⁺ accumulation by ATPase (SERCA) or leak (inositol triphosphate receptor, IP₃R). Increased Ca_i might then activate NF-κB and contribute to inflammation in CF.

to apical vs. basolateral agonists appears (144–146) to result from the fact that unidentified factors that accumulate in the mucopurulent material of the CF ASL cause dramatic structural and functional changes of the airway epithelia. The ER adjacent to the apical membranes expanded, leading to increased capability for Ca²⁺ storage and release, whereas ER adjacent to the basolateral membrane was unaffected in CF (144–146). The apical- and basolateral-localized ER operated independently from each other due to the presence of surrounding mitochondria that prevented apical or basolateral Ca_i changes from being propagated to the rest of the cell (146). Thus responses to apical agonists were larger than responses to basolateral agonists. These mucopurulent material-induced changes in cell structure and Ca_i signaling were accompanied

Cell Voltage-Ca²⁺ Entry Model

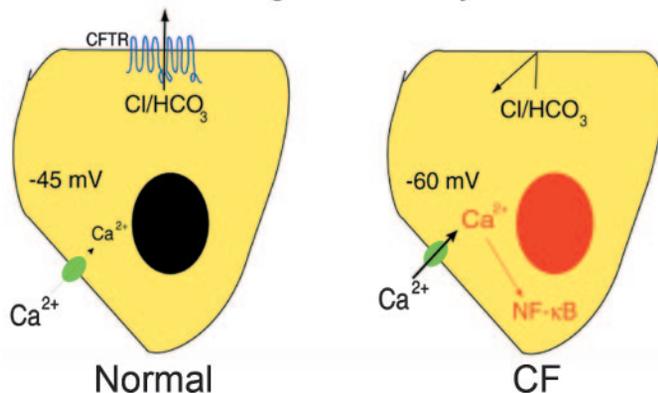


Fig. 4. Cell voltage hyperpolarization in CF increase cytosolic [Ca²⁺]. Loss of CFTR leads to a hyperpolarization of the basolateral membrane potential of airway epithelial cells from about -45 to -60 mV, and this hyperpolarization is expected to increase Ca²⁺ entry into the cells through voltage-independent calcium release-activated Ca²⁺ channels (CRAC) (green), resulting in increased Ca_i and activation of NF-κB. According to this model, differences in Ca²⁺ entry and Ca_i (and therefore in NF-κB activation) between normal and CF would be most apparent manifest during conditions in which CFTR and CRAC channels were both active. In this condition, CFTR would have its most profound effect on membrane voltage, and Ca²⁺ entry pathways will be operating.

by increases in production and release of IL-8, consistent with a potential role for Ca_i in a hyperinflammatory response.

In addition to identifying a potential role for factors in the CF ASL controlling epithelial cell structure and function, these results raise the issue of the potential interactive roles of CFTR and Ca_i in controlling or synergizing innate immune responses of airway epithelia. Ca²⁺ entry into airway epithelia will likely be required to sustain elevated Ca_i over extended periods, and a potential link among CFTR, Ca_i, and inflammation is through CFTR's effects on membrane potentials (Fig. 4). A relationship among Ca²⁺ entry, membrane voltage and inflammation was discovered first in lymphocytes by Cahalan and colleagues (40, 114), who found that membrane voltage was regulating Ca²⁺ entry into the cells through voltage-insensitive Ca²⁺ channels (28) (store-operated or transient receptor potential, TRP) by changes in electrical driving force on Ca²⁺. The resulting oscillations in Ca_i controlled inflammatory signaling and gene expression. Although there have been no studies of the effects of membrane potential on gene expression in airway epithelia, previous studies (44) in CFTR-expressing T84 intestinal epithelial cells showed that changes in membrane potential caused expected changes in Ca_i during agonist-induced activation of Ca²⁺ entry pathways. It therefore seems possible that differences in apical and/or basolateral membrane potentials (V_{ap} and/or V_{bl}) in CF vs. non-CF airway epithelia could lead to differences in apical vs. basolateral Ca²⁺ entry and Ca_i signaling and, consequently, increased activation of NF-κB (see Ref. 37) and innate host responses in CF vs. non-CF airway epithelia. These proposed effects of membrane potential on Ca²⁺ entry into CF and non-CF airway epithelial cells remain to be tested.

V_{ap} and V_{bl} are determined by the dominant ion conductances (i.e., to Na⁺, K⁺, and Cl⁻), the respective ion concentration gradients across the membranes, and the transepithelial resistance. Microelectrode measurements of V_{ap} and V_{bl} in intact epithelial sheets of CF and non-CF human airway and sweat duct epithelia, both of which express apical CFTR and ENaC, have been summarized in Table 1. Data for other

Table 1. V_{ap} and V_{bl} in non-CF and CF epithelia

	V _{ap} , mV	V _{bl} , mV	Reference No.
Nasal (human)			192, 193
Non-CF	-23	-38	
CF	-16	-52	
Sweat duct (human)			139
Non-CF	-25	-35	
CF	+26	-50	
Tracheal (bovine)			182
Activated CFTR (+fsk)	-12	-57	
Inactive CFTR (-fsk)	+2	-72	
Airway Calu-3 Line (human)			173
Activated CFTR (+fsk)	-22	-44	
Inactive CFTR (-fsk)	-48	-60	
Mammary Line (mouse)			15
Activated CFTR (+fsk)	-47	-57	
Block CFTR (+fsk+NPPB)	-61	-67	

CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; V_{ap}, apical membrane potential; V_{bl}, basolateral membrane potential. The table summarized membrane potentials measured across the apical or basolateral membranes of CFTR-expressing epithelia. V_{ap} is referenced cell vs. apical solution and V_{bl} is referenced cell vs. basolateral solution. Data for human nasal epithelia were average values taken from Refs. 192 and 193.

epithelia, in which there were comparisons of cells where CFTR was either inactive or active have also been included (Table 1). When CFTR is inactive (i.e., in unstimulated non-CF epithelia or in CF epithelia), V_{ap} and V_{bl} are largely determined by the activity of apical ENaC, which depolarizes both membranes, and basolateral K^+ conductances, which hyperpolarize both membranes. Expression and activation of CFTR in the apical membrane is expected to move V_{ap} toward the Cl^- equilibrium potential, which is approximately -22 mV (assuming an intracellular Cl^- activity of 43 mM, see Ref. 173). The data in Table 1 show that activated CFTR hyperpolarizes V_{ap} in nasal epithelia, sweat duct, and bovine trachea, tissues that have depolarized V_{ap} (likely owing to ENaC activity) in the basal state. In Calu-3 and mouse mammary epithelium, which express little apical ENaC and have hyperpolarized V_{ap} in the basal state, activation of CFTR depolarized both V_{ap} and V_{bl} (Table 1). Thus reduction of apical Cl^- permeability through the loss of functional CFTR in CF either hyperpolarizes or depolarizes V_{ap} but consistently hyperpolarizes V_{bl} (Table 1). It is therefore predicted that Ca^{2+} entry across the basolateral membrane will increase in CF (see Refs. 44 and 154), especially during treatments with agonists that activate Ca^{2+} entry channels in the basolateral membrane. Although the CF-dependent changes in V_{bl} appear small (Table 1), they could be important if, as preliminary data suggest (103), Ca_i plays an important synergistic role in flagellin-TLR-NF- κ B signaling. Synergistic interactions among NF- κ B and Ca_i -signaling pathways could become especially important during extended infections because inflammatory signaling may alter expression of gene products, which will affect Ca_i signaling, giving rise to a positive feedback situation of inflammation enhancing inflammation.

In summary, although there is an exaggerated innate immune response in CF airways, available data indicate there is likely to be little difference in intrinsic inflammatory properties between normal and CF airway epithelia. However, this issue may not be resolved until experiments have been performed with properly paired CF and CFTR-corrected cells or CFTR-expressing cells treated with a specific CFTR blocker (102, 110, 172) during exposure to *P. aeruginosa* and to agonists that activate CFTR. The most likely models to explain altered inflammatory signaling in CF involve the effects of the absence of CFTR's anion channel function on ASL composition and volume, which secondarily alter cellular signaling. A smaller ASL volume in CF would concentrate proinflammatory factors like ATP, bradykinin, and epithelial-derived cytokines that could activate or synergize with TLR-MAPK-NF- κ B signaling. An acidic and/or oxidized ASL in CF resulting from reduced secretion of HCO_3^- or reduced GSH could also affect cell pH and/or redox, thereby altering cell signaling. Cytosolic redox might also be altered by increased O_2 consumption by CF cells, and the resulting hypoxia and altered redox could regulate signaling. The absence of CFTR hyperpolarizes V_{bl} , and this effect may increase Ca^{2+} entry into the cells. Increases in Ca_i alone appear to be sufficient to weakly activate NF- κ B, but larger Ca_i -induced activations of NF- κ B occur when the cells are simultaneously exposed to flagellin. The hyperinflammatory effect of CF on Ca_i and NF- κ B signaling would be most prominently expressed during exposure to both *P. aeruginosa* and also endocrine, paracrine or nervous agonists that activate Ca^{2+} signaling in the airway epithelia.

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