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Cystic fibrosis: a mucosal immunodeficiency syndrome

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR) functions as a channel that regulates the transport of ions and the movement of water across the epithelial barrier. Mutations in CFTR, which form the basis for the clinical manifestations of cystic fibrosis, affect the epithelial innate immune function in the lung, resulting in exaggerated and ineffective airway inflammation that fails to eradicate pulmonary pathogens. Compounding the effects of excessive neutrophil recruitment, the mutant CFTR channel does not transport antioxidants to counteract neutrophil-associated oxidative stress. Whereas mutant *CFTR* expression in leukocytes outside of the lung does not markedly impair their function, the expected regulation of inflammation in the airways is clearly deficient in cystic fibrosis. The resulting bacterial infections, which are caused by organisms that have substantial genetic and metabolic flexibility, can resist multiple classes of antibiotics and evade phagocytic clearance. The development of animal models that approximate the human pulmonary phenotypes—airway inflammation and spontaneous infection—may provide the much-needed tools to establish how *CFTR* regulates mucosal immunity and to test directly the effect of pharmacologic potentiation and correction of mutant CFTR function on bacterial clearance.

Cystic fibrosis, which affects approximately 30,000 individuals in the United States, is caused by mutations in CFTR, a cAMP-regulated epithelial chloride channel (Box 1). Cystic fibrosis was originally recognized in babies with pancreatic insufficiency who failed to thrive and often succumbed to pulmonary infection in infancy or early childhood[1, 2]. Although there has been enormous progress made in understanding the basic biology of the CFTR chloride channel, it remains enigmatic how CFTR mutations cause enhanced susceptibility to pulmonary infection and how this susceptibility might be prevented.

Cystic fibrosis pulmonary disease is the most challenging problem in the management of cystic fibrosis and is the major determinant of life span and quality of life in affected individuals. Substantial clinical data have linked the recognition of bacterial infection in the lung, usually caused by *Staphylococcus aureus* or *Pseudomonas aeruginosa*, with the onset of symptomatic lung disease, which is marked by excessive airway inflammation and the eventual loss of pulmonary function. Aggressive and even prophylactic antimicrobial treatment often eradicates these infections and slows the deterioration of pulmonary function. However, the adaptive responses of the infecting organisms eventually result in isolates that are highly resistant not only to antimicrobial agents but also to the immune response that would readily eradicate these bacteria in hosts. CFTR dysfunction has long been associated with viscid mucus that causes the entrapment of bacteria in airway secretions. The prevailing hypothesis is that CFTR dysfunction, a lack of transport of

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chloride and accompanying water across the airway epithelium and excessive sodium reabsorption lead to dehydrated airway surface fluid, impaired mucociliary clearance, infection and inflammation[3, 4]. However, as we discuss below, there is accumulating evidence to suggest that CFTR dysfunction affects several components of innate immunity and that the initial predisposition to infection in infants with cystic fibrosis may represent a primary defect in local mucosal immunity.

Cystic fibrosis pulmonary disease is initiated in airways that are essentially normal at birth but that later become obstructed with mucus plugs. Early pathological findings of cystic fibrosis pulmonary disease include goblet-cell and mucus-gland hyperplasia[5, 6]. Even in the absence of clinically apparent infection, either viral or bacterial, there is often evidence of inflammation in cystic fibrosis airways, as evidenced by polymorphonuclear neutrophil (PMN) accumulation and excessive concentrations of interleukin-8 (IL-8) and free proteases[7–11]. Airway colonization and infection occur with diverse flora, which is followed by clinically apparent infection with the typical pathogens *S. aureus, P. aeruginosa* or both, even in infants at a very young age. Bacterial shedding of immunostimulatory pathogen–associated molecular patterns (PAMPs), such as cell-wall components, lipopolysaccharide (LPS), flagella and DNA, activate a brisk proinflammatory response. Bacterial adaptation to the airway milieu ensues, with a shift from a planktonic to a biofilm mode of growth, followed by the selection of mutants with abundant exopolysaccharide production that are resistant to phagocytosis. The intense inflammatory reaction to this airway infection consists of chemokine and cytokine expression (IL-8 and tumor necrosis factor (TNF)) and mucin secretion[12], as well as PMN accumulation[13] and the associated release of serine proteases[14, 15], which are themselves proinflammatory stimulants[16, 17]. It is clear that increased airway inflammation does not result in enhanced bacterial clearance[18]. Airway obstruction results initially in hyperinflation, destruction of the airway walls and fibrosis, leading to decreased lung function as measured by forced expiratory volume and vital capacity.

Effects of CFTR mutation on epithelial innate immune function

The many roles of the airway epithelium in the host defense of the lung are well appreciated[19]. As CFTR is highly expressed in the airway epithelium, it is logical that defective CFTR function should affect the contribution of the epithelium to innate immunity. CFTR mutations have been associated with both constitutive activation of proinflammatory signaling in the absence of apparent microbial stimuli as well as exaggerated responses to bacterial products (Fig. 1). Endogenous activation of NF-κB (nuclear factor κ light-chain enhancer of activated B cells) and substantial sequestration of leukocytes has been observed in human cystic fibrosis fetal tracheal explants[10] obtained before exposure to microbial flora; these observations were confirmed in pathological studies of cystic fibrosis fetuses[11]. Analyses of bron-choalveolar lavage fluid indicated that the induction of IL-8 and TNF in the airways of infants with cystic fibrosis is higher, often in the absence of known pathogens and elevated out of proportion to the bacterial load in airways contaminated with potential pathogens[20–22] compared with normal infants. Long-term primary cultures of cystic fibrosis airway epithelial cells do not reproducibly show constitutive activation of proinflammatory signaling[23], although this may be a function of clonal selection *in vitro*. These cultures are also influenced by epigenetic factors that are specific to the individual patients and that may influence inflammatory signaling. Whether there is, in fact, constitutive activation of proinflammatory signaling directly linked to CFTR dysfunction in humans is still unresolved and is a difficult issue to address experimentally.

In vitro evidence of the effects of CFTR on NF-κB signaling was shown in Chinese hamster ovary cells expressing CFTR with a deletion of Phe508 (ΔF508 CFTR), which resulted in

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dose-dependent increases in NF-κB activation; this effect was not seen in cells expressing wild-type CTFR[8]. Numerous *in vitro* studies using human epithelial cystic fibrosis and control cell lines confirmed increased NF-κB signaling[8, 24–26] and the involvement of several proinflammatory cascades, including activation of Ca^{2+}-dependent signaling[27, 28], increased nuclear factor of activated T cells (NFAT) transcription[29] and mitogen-activated protein kinase (MAPK)-dependent activation of activator protein 1 (AP-1), in cystic fibrosis epithelia compared to control epithelia[30, 31]. It is crucial to recognize that the construction of wild-type and mutated cell lines with alterations in *CFTR* gene dosage and expression, as well as the use of CFTR inhibitors to mimic channel failure, could influence the outcome measurements[32].

Additional CFTR-dependent effects on epithelia influence bacterial clearance. Glycosylation of both surface-associated and secreted epithelial glycoconjugates are affected by CFTR[33, 34]. Glycosylation status influences bacteria-host interactions[34–36], mucus composition and rheology[37–39], all properties that are key in mucociliary clearance. Despite *in vitro* evidence that CFTR-mediated alterations in glycosylation affect bacterial attachment, it is clear that few intact bacteria actually adhere to airway epithelial cells *in vivo*. Instead, organisms enmeshed in airway mucin, growing within biofilms, shed PAMPs that are recognized either by epithelial receptors on the apical surface, such as Toll-like receptors (TLR2, TLR4 and TLR5), within the cytoplasm (nucleotide-binding oligomerization domains (NODs) and retinoic acid inducible gene (RIG)) or in endosomal compartments (TLR3, TLR4 and TLR9). CFTR-associated effects on the production or distribution of these pattern-recognition receptors could be crucial in the initial pathogenesis of pulmonary infection.

Cystic fibrosis–related increases in the amount of NF-κB–dependent gene products and decreases in the amount of Trif-dependent gene products have been well documented. Although several studies suggested that cystic fibrosis and non–cystic fibrosis epithelia have similar expression of TLRs[40, 41], alterations in receptor localization could result in key differences in TLR-initiated signaling between the two types of epithelia. Increased surface expression of TLR2 and TLR5 on human cystic fibrosis epithelial cells correlates with increased inflammatory responses to bacterial products[40, 41]. However, knockdown of myeloid differentiation primary response gene (88) (MyD88) in cystic fibrosis cells prevented bacterial-induced signaling but did not reduce baseline NF-κB signaling to the level seen in non–cystic fibrosis cells, which is consistent with some degree of constitutive activation of the NF-κB pathway in unstimulated cystic fibrosis cells. As this baseline proinflammatory status, with increased IL-8 production and neutrophil accumulation, is documented in clinical studies[20, 22, 42, 43], it is unclear exactly why bacteria present in the cystic fibrosis lung evade eradication. PMNs do not phagocytose as well when bacteria are suspended in fluid as when they are associated with the cell surface[44]. Thus, PMNs that have accumulated in excess airway mucin may not be optimal in pathogen elimination.

Intracellular innate immune signaling is also affected by dysfunctional CFTR. Activation of toll-like receptor adaptor molecule (Trif)-dependent effectors contributes to the resolution phase of an acute inflammatory response[45–47] and seems to be decreased in cystic fibrosis cells. TLR4 signals through MyD88 at the cell surface and, after internalization to the early endosome, activates Trif-dependent pathways that are linked to the type I interferons (IFNs)[48]. In cystic fibrosis epithelial cells, the surface expression of TLR4 is reduced[49], resulting in decreased activation of both MyD88 signaling (ref. 49) and Trif signaling[50]. These observations were corroborated in clinical studies showing reduced expression of Trif-dependent gene products in cystic fibrosis airway fluid[51–53]. Limited Trif signaling would interfere with the resolution of the epithelial NF-κB–dominated inflammatory

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response and minimize the dendritic-cell–mediated activation of the adaptive immune system[50, 54].

Epithelial cells are especially key in the activation of the type I IFN response (involving IFN-α and IFN-β), a signaling cascade that is central to the clearance of diverse airway pathogens[45–47, 55], including viruses and extracellular bacteria such as *Streptococcus pneumonia, P. aeruginosa* and *S. aureus*. There are limited data detailing how CFTR deficiency affects primary viral clearance mechanisms[56–58], which is in contrast to the abundant clinical data showing that viral infections exacerbate coexisting bacterial infections and cystic fibrosis lung disease[59–61]. Patients with cystic fibrosis seem to have no particular problems handling nonrespiratory viral infections, suggesting that the major mechanisms of viral immunity in these individuals are intact. However, cystic fibrosis epithelial cells produce reduced amounts of type I interferon in response to *P. aeruginosa* infection and, accordingly, are less capable of activating dendritic cell populations, which initiate the adaptive immune response[50]. Autocrine activation of signaling downstream of interferon production is subsequently reduced in cystic fibrosis epithelial cells in part because of increased concentrations of the protein inhibitor of activated signal transducer and activator of transcription 1 (PIAS1)[52, 62], further adding to the dysregulated immune response.

CFTR dysfunction contributes to oxidative stress in the airway

Whether or not the exaggerated signals for proinflammatory signaling are caused by exogenous (microbial) stimulation, CFTR-associated alterations in signal transduction or both, the net result is an excessive accumulation of dysfunctional PMNs and their products. Constitutive activation of NF-κB signaling results in increased amounts of reactive oxygen species being generated by neutrophils accumulated in the airway. Increased amounts of reactive oxygen species (O_2, H_2O_2 and HOCl) are associated with increased IL-8 production, increased IL-6 production in response to *P. aeruginosa*, defective autophagy and reduced CFTR expression[63–67]. Under normal conditions, CFTR contributes to the downregulation of NF-κB signaling during oxidative stress by controlling the degradation of nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, α (I-κB-α)[68], a response that is lacking in the cystic fibrosis lung (Fig. 2).

Epithelial cells control oxidative damage through the production of antioxidants, such as glutathione (GSH) and thiocyanate (SCN^-), that are trafficked into the epithelial-lining fluid[69] and may help to modify mucus viscosity[70–72]. GSH secretion is markedly reduced in patients with cystic fibrosis[73] (as well as in the cystic fibrosis mouse[74]) as a result of a protein trafficking defect in the cystic fibrosis epithelial cells[75]. CFTR channel dysfunction impairs the trafficking of GSH into the airway surface fluid from epithelial cells but does not impair the production of GSH[76, 77]. Increased oxidative stress further contributes to alterations in IL-8 gene expression[64] and defects in bacterial killing. Diminished trafficking of SCN^-, a potent antioxidant[78], which is also associated with mutations in CFTR, similarly contributes to the diminished killing of the cystic fibrosis pathogens *P. aeruginosa* and *S. aureus*[79]. Thus, deficient CFTR function in the epithelium results in a diminished ability to counter the oxidative stress that is induced by both constitutive and exogenously activated inflammation.

CFTR affects immune cell function

Effective mucosal immunity is the result of the coordinated participation of epithelial cells and resident and recruited immune cells[80]. Particularly in the airway, in which excessive PMN accumulation interferes with pulmonary function, proinflammatory signaling is tightly regulated. The influx of immune cells, dendritic cells, macrophages and T cells is crucial in

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this process. Immunohistological and physiological data indicate that CFTR is both transcribed and functional in several different types of leukocytes[81, 82]. How CFTR dysfunction in immune cells might interfere with the regulation of innate immunity has not been well studied. Whether or not CFTR dysfunction in these cells has any biological role is unclear given the large amount of clinical data indicating that patients with cystic fibrosis have normal immune cell function at sites other than the lung. There are no clinically apparent defects in the function of cystic fibrosis macrophages or T cells, as would be suggested by the increased prevalence of infection caused by organisms usually controlled by T cells, such as *Salmonella* or *Mycobacterium tuberculosis*, in individuals with cystic fibrosis. There is also no increased incidence of abscesses or granulomatous reactions to organisms that would be suggestive of a primary defect in PMN killing in cystic fibrosis. Murine models, although lacking characteristic cystic fibrosis lung disease, nonetheless have been shown to have alterations in macrophage signaling that contribute to the exaggerated inflammatory response in the cystic fibrosis lung[83]. Excessive proinflammatory signaling by murine and human cystic fibrosis macrophages may caused in part by prolonged TLR4 signaling from the early endosome, resulting in the elevated activation of the NF-κB and interferon pathways[84].

A phagocytic defect associated with CFTR mutations that would explain the failure to clear inhaled bacteria has long been sought[85–87]. Unfortunately, analyses of PMN function in cells harvested from the peripheral blood of patients with cystic fibrosis are complicated by the exposure of these patients to circulating concentrations of LPS and to activated lymphocytes and their products. PMNs[85, 86] and macrophages[87] isolated from patients with cystic fibrosis have a modest reduction in the ability to kill phagocytosed bacteria *in vitro*, but such data have not been uniformly reproduced[88]. In contrast, a recent study of PMNs harvested from newborns with cystic fibrosis identified by neonatal screening studies did not find appreciable differences in PMN phagocytic function between these newborns and normal controls[89]. Less controversial are studies showing that PMNs isolated from patients with cystic fibrosis have a slower rate of apoptosis than PMNs from control individuals without cystic fibrosis[90–92] as a result of a resistance to the proapoptotic cytokine TNF-α and the effects of anti-apoptotic granulocyte-macrophage colony-stimulating factor (GM-CSF), an NF-κB–dependent cytokine that is produced in greater quantity by cystic fibrosis epithelial cells than in non–cystic fibrosis cells[93]. As a result, cystic fibrosis PMNs persist in the airway, further contributing to airway inflammation. The abundance of PMN-derived proteases also thwarts efficient phagocytosis[94, 95], resulting in the cleavage of macrophage receptors and causing inefficient opsonization and impaired bacterial killing[96]. Thus, even if CFTR does not directly contribute to phagocytic function, it may influence the kinetics of PMN degradation, protease and DNA release to further exacerbate the hyperinflammatory state in the cystic fibrosis airway.

T cell function is also affected by defects in CFTR, although the specific mechanisms involved in this effect have not been defined. Diminished recruitment of T cells in response to *S. aureus*[9] by cystic fibrosis epithelial cells, as well as altered T cell responses to *Aspergillus fumigatus* in both human[97] and murine[98] infections, have been documented. Several studies have suggested that CFTR dysfunction results in a T helper type 2 (T_H2) cell bias in response to respiratory pathogens[99, 100], although more recent data reflect the key role of T_H17 signaling in the host response to extracellular pathogens in the lung, including *P. aeruginosa*[101]. IL-17 participates in the regulation of neutrophil recruitment by several mechanisms, including inducing the expression of IL-8, and is necessary for an effective host response to *P. aeruginosa*[102–107]. Increased T_H17 signaling contributes to cystic fibrosis lung pathology, and cystic fibrosis sputa have elevated amounts of IL-17, further enhancing neutrophil recruitment in the cystic fibrosis lung[108, 109]. Both T cell and neutrophil production of IL-17 are increased in cystic fibrosis compared to control lung

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specimens[110, 111], but there is no evidence that increased production of IL-17 is a direct consequence of CFTR dysfunction.

Effective clearance of inhaled bacteria from the respiratory tract involves the integrated activities of both the epithelium and the immune cells. Clinical observations suggest that induction of the PMN-dominated inflammatory responses in the cystic fibrosis airway is not normally regulated; there is a relative lack of IL-10 production[112], diminished lipoxin production[113–115] and a failure of the normal progression from PMN predominance to monocytes with the expected resolution phase of acute inflammation in the cystic fibrosis airway[95, 116]. Thus, even if the primary CFTR-dependent alteration in innate immunity is primarily an epithelial defect, there is a failure of the recruited and resident leukocytes to adequately control the hyperinflammatory state in the airway, and there is ample documentation that the presence of activated PMNs in the airway does not correlate with bacterial eradication.

Bacterial adaptation to the host

Having blamed the failure to eradicate inhaled bacteria on a presumed CFTR-associated defect in mucosal immunity, it is also crucial to recognize the prodigious capabilities of typical cystic fibrosis pathogens to adapt to and flourish within the cystic fibrosis lung. The vast majority of patients with cystic fibrosis eventually become infected with opportunistic pathogens, often *P. aeruginosa* and *S. aureus*, in addition to a growing list of both cultivatable and non-cultivatable bacterial species, including anaerobes[117, 118]. Infection is caused not only by host defects in innate immunity but also by the selection of bacteria that are able to evade immune clearance. The successful cystic fibrosis pathogens share a genetic flexibility and an ability to adapt to the pressures imposed by mucosal immunity (Fig. 3). Even within individual clones from one patient, there is tremendous heterogeneity, such that numerous different gene expression profiles are expressed by the bacteria colonizing the lung, allowing for the selection of optimally fit clones[119, 120]. Planktonic organisms aspirated from the environment upregulate gene expression for motility, proteolytic activity and carbon utilization, whereas the pathways involved in immune evasion and iron scavenging are increasingly expressed during infection *in vivo*. Sequential *P. aeruginosa* strains isolated from chronically infected patients with cystic fibrosis have alterations in the expression of numerous virulence factors (O-antigen biosynthesis, type III secretion and mobility) and of multidrug-resistance genes[121], as well as of genes required for survival within the nitrogen-rich, nutrient-deficient cystic fibrosis lung[122–124].

Changes in LPS structure contribute to the proinflammatory milieu in the cystic fibrosis lung. LPS from *P. aeruginosa* isolated from chronically infected patients with cystic fibrosis (cystic fibrosis LPS) is structurally distinct from the environmental strains that initiate infection. The lipid A portion of cystic fibrosis LPS is typically penta-acylated, as opposed to the more common hexa-acylated structure, and contains substantially more palmitate and aminoarabinose than non–cystic fibrosis LPS[125, 126]. These alterations correlate with increased induction of IL-8 from human endothelial cells. Worth noting is that human TLR4 responds to these changes in LPS structure, whereas murine TLR4 does not[127], a factor that may be relevant to the failure of the murine model of cystic fibrosis to reflect human pulmonary pathology.

Once a critical mass of bacteria is present in the airways, *in vivo* data indicate that they form biofilms[128, 129]. This then facilitates the coordinate expression of numerous genes throughout the microbial population through secretion of highly soluble quorum sensors, such as the *Pseudomonas* homoserine lactones and the quinolones, which act in concert with specific transcriptional activators[130]. The switch to the biofilm phenotype does not

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necessarily indicate a reduction of proinflammatory stimulation. *P. aeruginosa* in biofilms is associated with the expression of more inflammatory LPS[131], as mentioned above, and the enrichment of extracellular DNA[132], both of which contribute to the activation of TLR-mediated proinflammatory signaling in the airways. When serially isolated cystic fibrosis strains of *P. aeruginosa* were instilled into mice, isolates from late-stage infection did not cause lethal infection, as the early isolates did, but were equally capable of inducing excessive lung inflammation and establishing infection[133]. Thus, the adaptation to the host does not necessarily imply a failure to activate local mucosal signaling but does suggest a combined failure of first the innate, and then the adaptive, immune response to clear infection.

From within this large bacterial population there is also the spontaneous selection of MucA mutants that overexpress the exopolysaccharide alginate[134, 135]. These mucoid organisms elude phagocytosis and have been virtually pathognomonic for cystic fibrosis[136]. Although alginate is immunogenic and elicits antibody production, this amplification of the inflammatory immune response further contributes to oxidative stress without resulting in the clearance of the organisms[137, 138]. An indication of well established *P. aeruginosa* infection, the predominance of alginate-producing organisms represents the end result of failed innate immunity and an effective bacterial adaptation to the host.

Bacterial products further contribute to the adaptation of organisms to the milieu within the cystic fibrosis airways. Pyocyanin, a phenazine pigment produced by all *P. aeruginosa*, particularly those growing in biofilms[139], activates proinflammatory signaling[140] and inhibits the activity of the antioxidants GSH and *N*-acetylcysteinie by blocking the dual-oxidase–based antimicrobial system[141]. Pyocyanin may also affect CFTR function by blocking Cl^- transport in human bronchial cells[142]. The *P. aeruginosa* toxin CFTR inhibitory factor (Cif) redirects CFTR from recycling endosomes to the lysosome, where it is degraded[143], compounding the deficiency associated with the major CFTR mutations; however, the *in vivo* role of this activity in cells that already have mutant CFTR targeted for degradation is not clear. These direct modifications of CFTR function in the host further potentiate the local immune defects associated with cystic fibrosis.

S. aureus has long been recognized as a major cystic fibrosis pathogen, and infection with the epidemic community-acquired methicillin-resistant *S. aureus* strains has been associated with decreased survival in individuals with cystic fibrosis[144]. Staphylococci adapt to the cystic fibrosis lung, proliferate as a biofilm and modulate proinflammatory activity, as monitored by cytokine and MAPK activation[145]. Similar to *P. aeruginosa, S. aureus* adapt their gene expression during colonization to enhance their fitness in the cystic fibrosis lung[146]. Within the *S. aureus* biofilm, the slower growing small-colony variants are more persistent or become resistant to antibiotics and evade host recognition by downregulating numerous virulence factors[147–149]. Subpopulations of the biomass revert to planktonic growth, further stimulating inflammation in the airways. Culture-independent as well as traditional methodologies have identified numerous other cystic fibrosis pathogens, especially streptococci and anaerobes that have successfully adapted to the cystic fibrosis lung and whose presence has been correlated with clinical symptomatology[150]. Thus, the major cystic fibrosis pathogens share key characteristics, one of which is the genetic flexibility to adapt to the milieu within the human airway, including resistance to antibiotic pressure, metabolic versatility and the ability to evade innate immune clearance mechanisms. These properties help to explain why the cystic fibrosis lung is infected by a relatively discrete group of organisms.

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Implications for therapy

Whether cystic fibrosis is an innate or an acquired immune deficiency, the clinical benefits of targeting both the pathological immune response as well as the infecting organisms in this disease have been well documented in controlled clinical trials[151, 152]. Longitudinal studies of infants with cystic fibrosis in the first 2 years of life have documented loss of lung function, even in the absence of *P. aeruginosa* or *S. aureus* infection, both of which accelerate such a decline[153]. Antimicrobial therapy, in addition to the mechanical loosening of secretions and improved nutrition, have long been the mainstays of cystic fibrosis therapy, and longevity has paralleled the development of highly active antibiotics to *P. aeruginosa* (http://www.cff.org/). Recognition that young infants have airway inflammation that is out of proportion to the recovery of bacterial pathogens[13, 22] led to clinical trials of anti-inflammatory agents such as systemic and inhaled steroids[154, 155] and ibuprofen[156], but each trial was limited by substantial toxicities. Azithromycin, a macrolide antibiotic that has a major anti-inflammatory activity, is effective in preventing loss of lung function in patients with cystic fibrosis[157], even in those individuals who are not infected with susceptible bacteria, and is now a part of routine cystic fibrosis care at most centers. However, the accumulation of azithromycin within macrophages may also contribute to an increasing susceptibility to atypical mycobacterial infection in patients with cystic fibrosis who are treated with this drug[158].

An alternative approach to cystic fibrosis therapy is based on targeting the dehydrated airway surface fluid present in individuals with the disease. Such strategies to counteract the physiological consequences of defective CFTR seem physiologically valid but have not been optimal thus far in controlled clinical trials. One approach to correct the defect in Cl^- and water transport across the airway epithelium using a purinergic receptor P2Y purinoceptor 2 (P2Y2) receptor agonist to activate alternative Cl^- channels was expected to result in hydrated airway surface fluid and improved mucociliary clearance. Despite promising initial results[159], a large clinical trial using this drug failed to ameliorate lung disease in patients with a mild cystic fibrosis[160]. Whether these results were caused by a failure to target the innate immune defect, which is presumably CFTR dependent, is not clear. Similarly, the use of osmotic agents such as hypertonic saline or mannitol (Bronchitol) have had mixed results in clinical trials[161–164].

The pharmacological potentiation of mutant CFTR function or the correction of mutant CFTR trafficking is a more desirable and realistic goal that, if achieved early enough in childhood, might prevent chronic infection and inflammation and irreversible airway damage. *In vitro* characterization of such compounds is currently in progress[165]. As clinical trials in human neonates are fraught with ethical and technical hazards, the newly developed *CFTR*$^{-/-}$ pig and *CFTR*$^{-/-}$ ferret models may be especially crucial in establishing whether pharmacologic therapy early in life can prevent cystic fibrosis lung disease (Box 2). Sequential bronchial biopsies could be done to ascertain whether endogenous upregulation of NF-κB activity is normalized, the amount of IL-8 and TNF in the airway is corrected and the numbers of recruited immune cells in the bronchoalveolar lavage fluid are normalized. Drugs to correct specific defects in CFTR biology, either by increasing the trafficking of mutant CFTR to the apical surface of the cell or by increasing Cl^- transport through the mutant channel or both (by the use of two drugs in combination) are currently in clinical trials in patients with cystic fibrosis with specific genotypes[166–168]. However, there have not been any data published thus far that show that pharmacologic correction of channel function *in vivo* corrects abnormalities in innate immunity or prevents infection. This outcome should be a reasonable expectation, as *in vitro* correction of mutant CFTR decreases proinflammatory signaling in numerous model systems[169, 170]. Ideally, treatment of infants with cystic fibrosis with drugs that target specific *CFTR* genotypes would not only

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correct the defect in electrolyte transport in these infants but would also restore normal epithelial innate immune function, diminish endogenous proinflammatory signaling inflammation and prevent infection by correcting the many abnormalities detailed above. Such therapy has recently become available with the licensing of Kalydeco (ivacaftor) in January 2012, a CFTR potentiator that targets the CFTR G551D mutant, which has been found in approximately 1,200 people in the United States. Phase 3 clinical trials showed that the patients with cystic fibrosis carrying the G551D mutation (>6 years of age) who received this drug had improved pulmonary function. In the presence of established infection, as is the case in many patients with cystic fibrosis who are old enough to participate in clinical trials, a realistic goal of CFTR correctors and potentiators would be to stabilize lung function and decrease proinflammatory signaling. The availability of the porcine and, perhaps, the ferret models of cystic fibrosis should greatly expedite the evaluation of these new CFTR pharmacotherapeutics and facilitate the identification of optimal drug combinations, doses and methods of delivery.

Concluding remarks

A tremendous amount of progress has been made in understanding the protean manifestations of cystic fibrosis: the genetic basis for cystic fibrosis and the influence of modifier genes in the disease has been established, the structure of the CFTR Cl^- channel has been defined, and much of the complex physiology of CFTR and its central role in epithelial electrolyte transport has been explained. Cystic fibrosis pigs and ferrets are being developed that seem to reproduce human disease, providing model systems to explore the pathogenesis of cystic fibrosis and potential therapeutics, even in neonates. Clinical trials are ongoing to develop potent therapies based on this information to correct the multiple CFTR-associated abnormalities in epithelial function that together result in such a major defect in mucosal immune function. The involvement of CFTR in so many epithelial functions that are relevant to the host-pathogen interaction (constitutive activation of NF-κB, dysregulated TLR4 trafficking and signaling, associated defects in type I IFN regulation, impaired epithelial antioxidant activity and the failure of appropriate immune regulation) indicate that there is not a single ‘cause’ of the defective mucosal immunity associated with cystic fibrosis.

However, major questions remain unanswered. (i) Exactly how does CFTR participate in innate immune signaling? Is CFTR function involved in pathogen recognition, PAMP trafficking or the afferent limbs of signal transduction? (ii) Why do activated neutrophils in the cystic fibrosis airway fail to clear the initial *P. aeruginosa* infection? (iii) Does CFTR dysfunction affect dendritic cells, T cells or the development of adaptive immunity in the lung? (iv) What amount of CFTR ‘correction’ or ‘potentiation’ is necessary to restore normal innate immune function? (v) How early must therapy to correct CFTR function commence to prevent infection?

We anticipate that pharmacological correctors and potentiators of CFTR function, currently defined by restoration of chloride channel activity, will also ameliorate defects in mucosal immunity and diminish proinflamatory signaling and infection and the subsequent pulmonary pathology. Nonetheless, there are many unresolved issues that merit intensive investigation.

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BOX 1 CFTR deficiency in cystic fibrosis

CFTR is a large glycoprotein consisting of two membrane-spanning regions and a cytoplasmic regulatory R domain[178] and is expressed primarily in epithelial cells but also in many other cell types, including lymphocytes and PMNs[81, 82]. In addition to its role as a Cl^- channel, CFTR is crucial in the regulation of ion transport, particularly Na^+ and HCO_3^- (ref. 179). A lack of CFTR function results in sodium absorption through ENaC[180], and mice with overexpression of ENaC develop lung pathology that, in some ways, mimics cystic fibrosis[173, 181], although these mice do not spontaneously develop infection. There are several classes of CFTR mutations[182] that correlate well with pancreatic disease, which is also a key component of cystic fibrosis, but these mutations are associated with more variable pulmonary phenotypes[183]. The most common CFTR mutation, ΔF508/ΔF508, results in a misfolded protein that is improperly glycosylated, is targeted for endosomal degradation and fails to reach the apical surface of the epithelium. Other CFTR mutations, such as G551D, form a partially functional channel whose activity can be potentiated[165]. The expression of a large number of modifier genes markedly affects the clinical manifestations of the disease[184]. For example, mannose-binding lectin 2 (MBL2) protein concentrations, especially in combination with high amounts of transforming growth factor β (TGF-β) production, is associated with severe pulmonary disease[185]. The central role of CFTR in regulating the hydration of the airways has become the focus of therapies, which seek to potentiate partially functional CFTR as well as correct the defective CFTR function attributed to specific mutations[166–168].

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BOX 2 Animal models of cystic fibrosis

A tractable animal model of cystic fibrosis would be a tremendous tool in understanding how CFTR is linked to infection and for assessing the efficacy of therapy to correct CFTR dysfunction, particularly in young infants. The ideal animal model would accurately reflect the salient characteristics of human lung disease, namely excessive airway inflammation, spontaneous development of bacterial infection and progression to chronic infection with characteristic biofilm formation (Table 1). Although CFTR correction is typically monitored by the restoration of a cAMP-mediated Cl^- current[186], an animal model could be used to establish the clinically relevant outcome measurement, namely, the amount of CFTR correction that is necessary to prevent inflammation and infection. The much heralded *Cftr*-deficient mouse that was developed in 1992 (refs. 174, 187) unfortunately did not fulfill these goals; cystic fibrosis mice did not develop spontaneous lung disease, and an accurate reflection of cystic fibrosis pancreatic disease was dependent on the strain of mouse used[175]. Although these mice and, later, gut-corrected ($Cftr^{tm1Unc}$-TgN(FABPCFTR) mice did show excessive proinflammatory signaling with increased chemokine (C-X-C motif) ligand 1 (CXCL1 or KC), TNF and PMN recruitment into the airways and increased weight loss during the course of infection, they spontaneously cleared even large inocula of typical cystic fibrosis pathogens. Thus, these mice have not been useful for testing the efficacy of antimicrobial regimens or of anti-inflammatory therapy or for monitoring effects of pharmacological correction of CFTR channel activity on inflammation or infection.

Recently developed *CFTR*-deficient[171] and ΔF508/ΔF508 pigs[188] spontaneously develop lung disease that is characterized by inflammation, mucus overproduction, airway obstruction and infection[189]. These features, which are typical of human disease, develop very early in life in these pigs. The *CFTR*-deficient pig fails to clear staphylococcal infection, a characteristic that may help to clarify the roles of *CFTR* expression in innate immunity. Physiological studies have suggested that the biology of porcine and human airway surface fluids are similar[176]. Notably, the antimicrobial activity of the airway surface fluid from cystic fibrosis pigs is not impaired[172]. *CFTR*-deficient pigs manifest the predicted defect in chloride and bicarbonate transport that is typical of human CFTR mutations, but they do not hyperabsorb sodium nor do they show diminished amounts of airway surface fluid. These results suggest that decreased hydration of airway surface fluid may not be central to the development of infection and inflammation[190]. Similarly, the cystic fibrosis ferret[177] also develops lung infection very early in life, which is severe enough to require antibiotic treatment[191]. These models support the hypothesis that there is a direct role of CFTR in mucosal immunity beyond its contribution to the hydration of the airway surface fluids. These animal models may provide a useful model system to test the pharmacologic agents under development to correct specific CFTR mutations.

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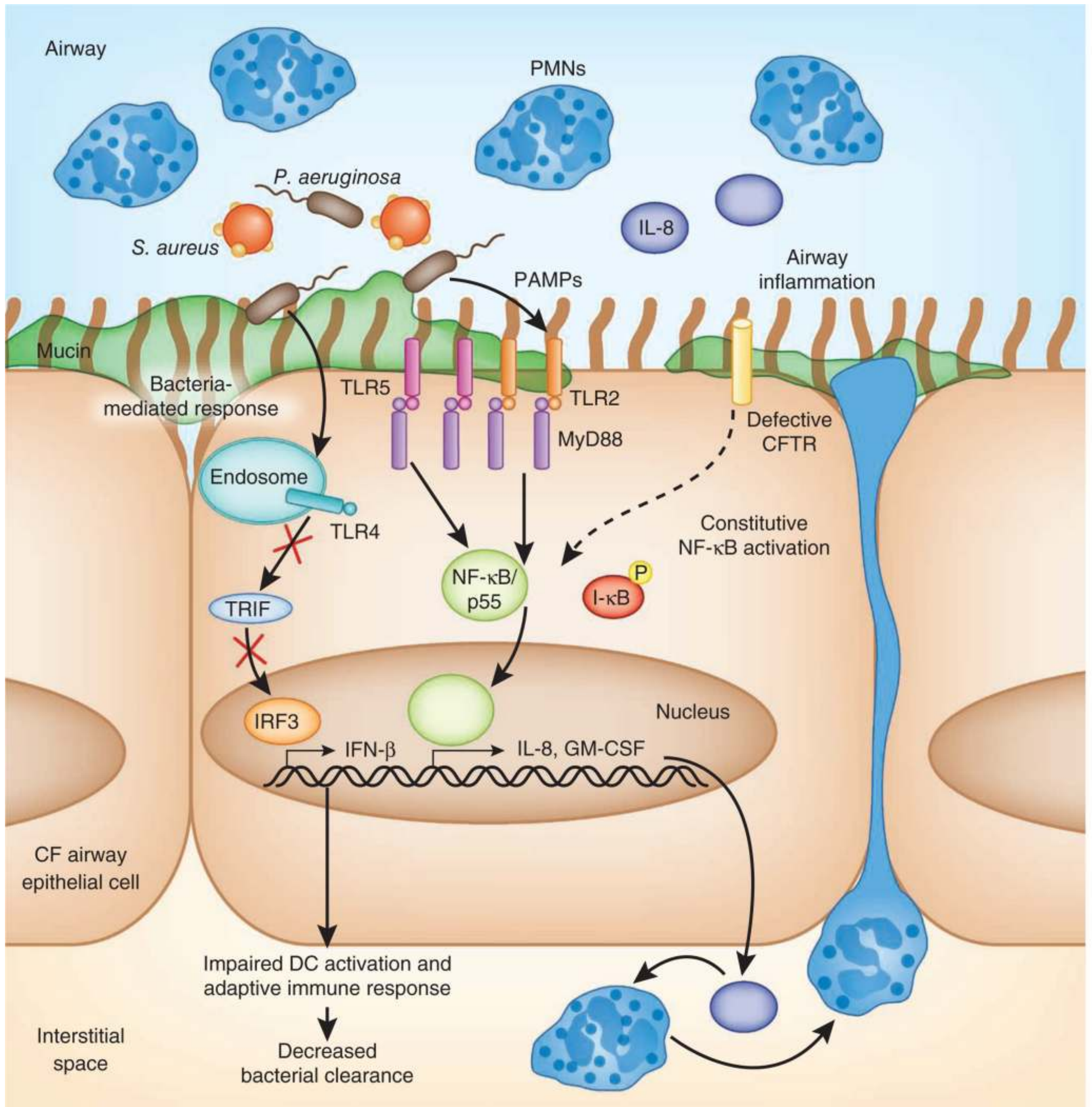

Figure 1.

Altered TLR expression and signaling in the cystic fibrosis (CF) epithelium. Expression of TLR2 and TLR5 at the apical surface is increased, whereas TLR4 expression is restricted to the endosome. NF-κB in cystic fibrosis airway epithelial cells is constitutively activated, resulting in the production of inflammatory cytokines, such as IL-8 and GM-CSF, and the recruitment of PMNs independently of TLR's interaction with the adaptor protein MyD88. After infection, bacterial PAMPs further increase NF-κB signaling through activation of TLR-MyD88 signaling. Intracellular TLR4 activation of Trif from the endosome is impaired, preventing interferon regulatory factor 3 (IRF3) translocation to the nucleus and

the activation of type I IFN gene products, which are required for the activation of dendritic cells (DCs) and the clearance of some cystic fibrosis–related pathogens.

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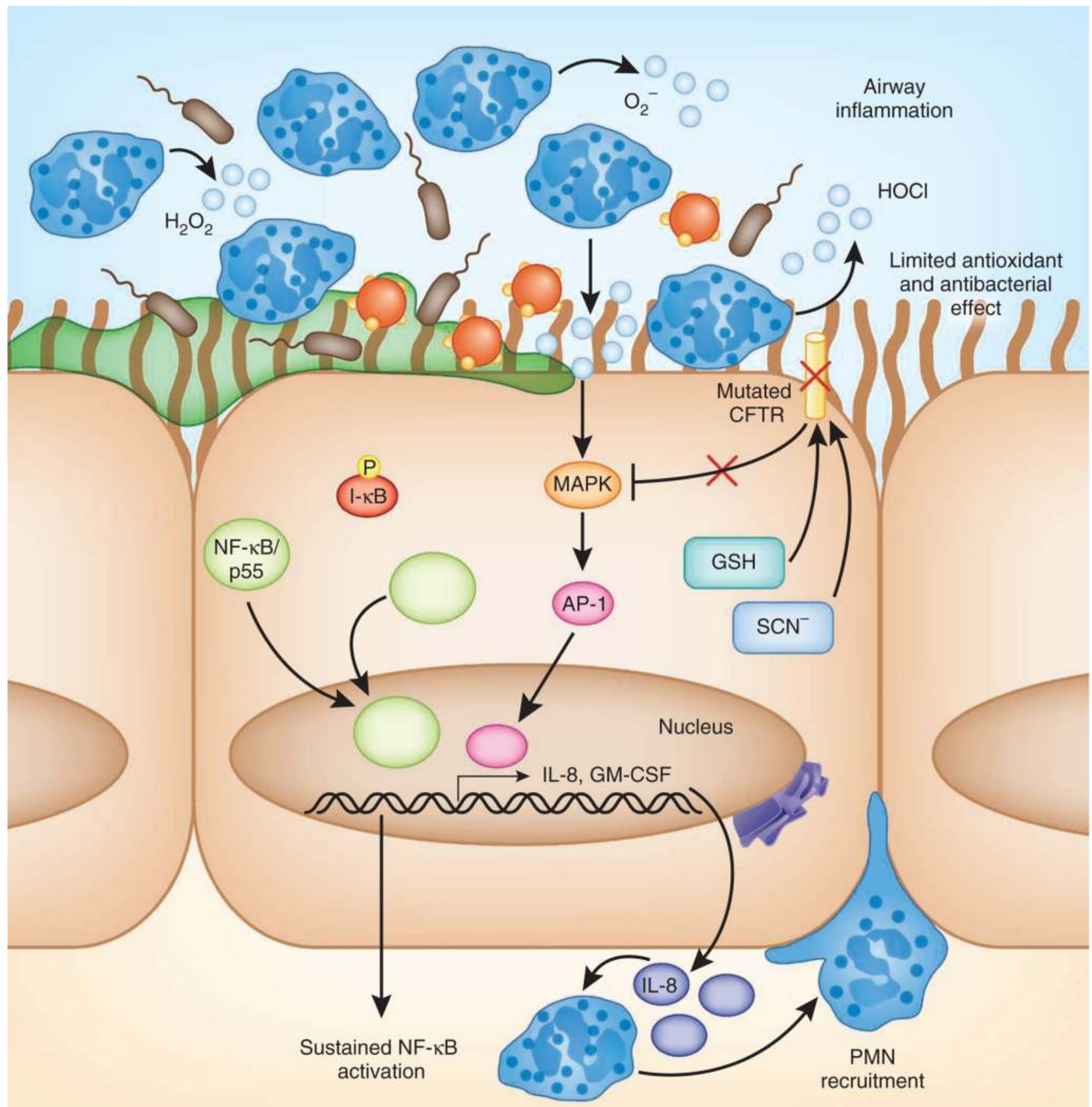

Figure 2.

Increased oxidative stress in the cystic fibrosis airway. Constitutive NF-κB–mediated production of chemokines, including IL-8, leads to PMN recruitment, which persist in the airway, increasing the oxidative burden in the lung. Oxidative stress activates MAPK signaling pathways in the cystic fibrosis epithelium, amplifying the production of IL-8 and, therefore, recruiting additional PMNs. Mutant CFTR in the epithelial cells is unable to channel the antioxidants GSH and SCN^- into the airway, limiting its ability to counteract the oxidative stress. Because SCN^- also has antimicrobial properties, bacterial killing in the airway is diminished as well.

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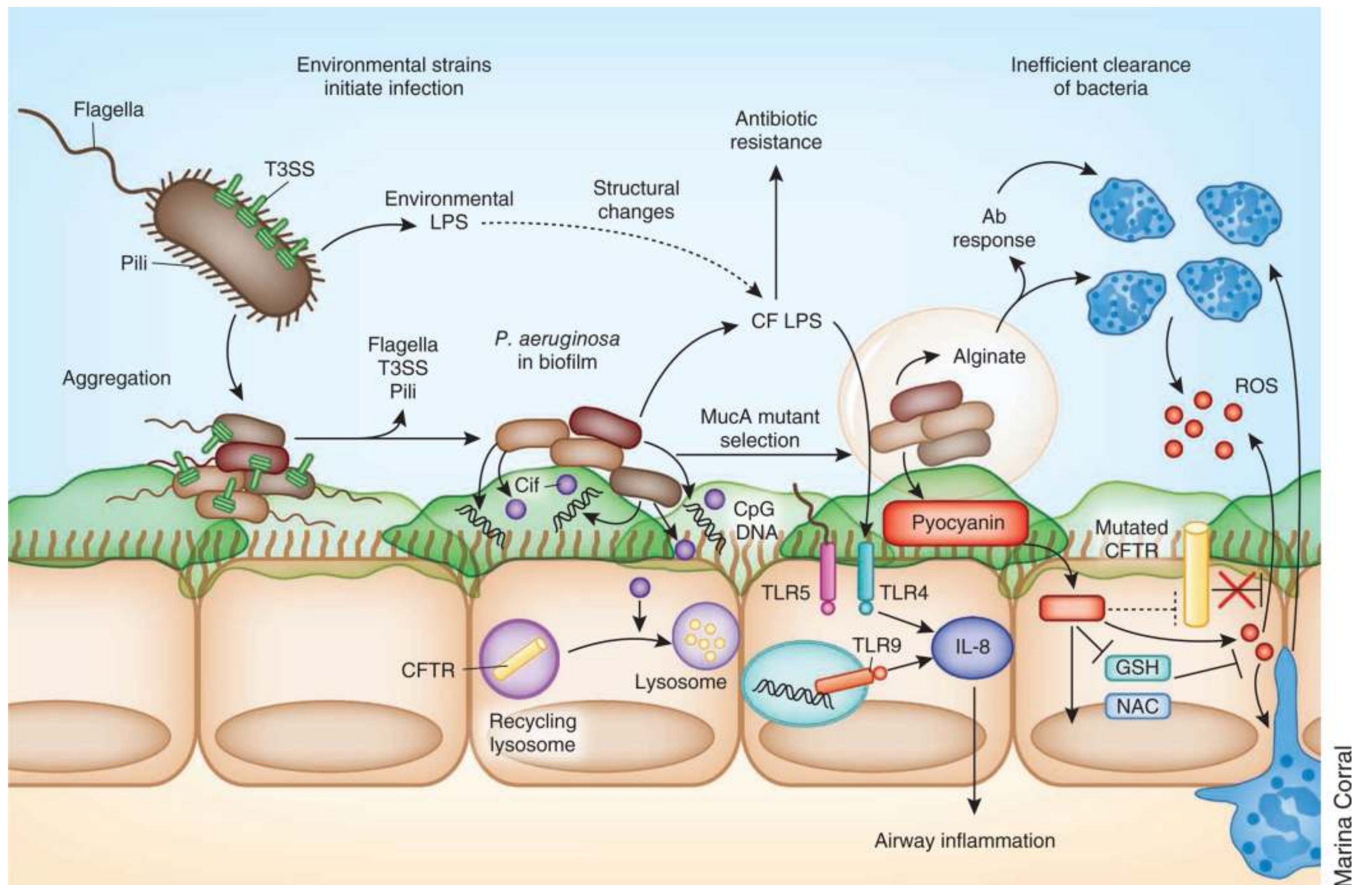

Figure 3.

Adaptation of inhaled bacteria to the cystic fibrosis airway. Inhaled bacteria expressing flagella, pili and a type 3 secretion system (T3SS) aggregate within the cystic fibrosis lung, resulting in the formation of biofilm. Within the biofilm, bacteria lose flagella, pili and the T3SS, increase alginate production, release CpG DNA and express a diverse range of virulence factors promoting evasion of the host immune system. *P. aeruginosa* also releases outer membrane vesicles containing Cif, a protein that inhibits the recycling of CFTR in the host. Furthermore, the lipid A structure of the LPS is altered through the addition of palmitate and aminoarabinose, resulting in increased antibiotic (Ab) resistance and increased induction of IL-8 production by host cells.

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Table 1

Characteristics of cystic fibrosis in human disease and animal models

		Mouse				
	Human	$CFTR^{-/-}$ and $CFTR^{\Delta F508/\Delta F505}$	ENaC overexpression	Ferret	Pig	References
Spontaneous infection	*P. aeruginosa; S. aureus;* streptococci; anaerobes	–	–	Streptococci; staphylococci enterococci	Streptococci; staphylococci	116, 117 171, 172
Airway inflammation	PMNs; NF-κB activation; IL-8; TNF-α	Macrophage recruitment (strain dependent)	PMNs; NF-κB activation	Uncharacterized	PMNs	7–12, 173–176
Mucus accumulation in airway	Submucosal gland secretion; mucus dehydration	–	Mucus plugs	Uncharacterized lesions in newborn lungs	Mucus (hydrated); expanded submucosal glands	5, 6, 173, 172, 176 177

ENaC, epithelial sodium channel.