Cystic Fibrosis and Defective Airway Innate Immunity

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Abstract Cystic fibrosis is a common autosomal recessive disease caused by mutations in the *CFTR* gene that encodes an anion channel expressed in epithelia and other cell types. While the disease affects multiple organ systems, it is progressive pulmonary disease, characterized by airway infection and inflammation, that is life limiting. The origins of the lung disease associated with loss of CFTR function are complex and likely multifactorial. Current research is defining how loss of CFTR anion channel activity alters the volume and composition of respiratory secretions and thereby impacts host defenses. Here we review the current understanding of the defect in innate immunity that characterizes the airway disease in cystic fibrosis. Advances in cystic fibrosis basic science research and the development of new animal models of disease are shedding new light on the causes of lung disease and may lead to new, more targeted therapies.

1 Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Rommens et al. 1989). *CFTR* encodes an anion channel regulated by nucleotides and phosphorylation. Over 1,500 disease-associated *CFTR* mutations have been reported. The most common mutation is a three-base deletion in exon 10 resulting in the loss of a phenylalanine residue at position 508 (ΔF508), present on ~70 % of mutant alleles (Kerem et al. 1989). In addition to primary or secondary CFTR-associated changes that lead to

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pulmonary disease manifestations, polymorphisms in other genetic loci may influence the CF phenotype (Garred et al. 1999; Henry et al. 2001; Salvatore et al. 2002). Several candidate modifier genes have been proposed for CF including mannose-binding protein (Garred et al. 1999), HBD-1 and HBD-2 (Salvatore et al. 2002), alpha 1-antitrypsin enhancer (Henry et al. 2001), and HLA class II (Aron et al. 1999). A recent genome-wide association study identified two new modifier loci for CF (Wright et al. 2011).

CF is a multiorgan system disease affecting the gastrointestinal tract (liver, gall bladder, small and large intestine, pancreas), sweat glands, reproductive system, sinuses, and respiratory tract. While the signs and symptoms of the disease have been recognized for centuries, it was only in the 1930s that Fanconi (Fanconi et al. 1936) and Andersen (1938) recognized and characterized the disorder as a distinct pathologic entity and noted its genetic basis. Although loss of CFTR function causes disease in many tissues and cell types, it is the effect on the respiratory tract that is most life limiting. More than 90 % of people with CF die of progressive lung disease associated with chronic bacterial infection and inflammation within the airways (Rowe et al. 2005). CF lung disease is associated with the eventual chronic colonization of the airways with large numbers of bacteria, notably *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Rosenfeld et al. 2001). It is increasingly recognized that CF-associated airway infections are polymicrobial and involve biofilm formation (Bjarnsholt et al. 2009; Sibley and Surette 2011; Singh et al. 2000a). Remarkably, these infections are confined to the respiratory tract and spread to other organs is extremely rare.

In this chapter, we will focus our attention on the host defense problem within the airways. In CF, bacteria grow in regions of the lung that are normally sterile. The clinical course of CF lung disease correlates with the acquisition of bacterial infection and its progression. Whatever the cause underlying this propensity for infection, it is lung specific. These features indicate that loss of CFTR activity impairs the innate defenses of the lung. The precise link between loss of CFTR function in the airways and the host defense defect remains an area of intense study and scientific debate. Here we review our current understanding of CF lung disease.

2 Overview of CF Lung Disease

The loss of CFTR anion channel activity has a profound impact on the function of many organs, most notably those lined by epithelia and involved in the elaboration of secretions at mucosal surfaces (Welsh et al. 2001). In addition, there is growing evidence that CFTR may be important to the function of non-epithelial cell types, including alveolar macrophages (Di et al. 2006; Zhang et al. 2010), neutrophils (Painter et al. 2006, 2008, 2010), lymphocytes (Bubien 2001; Bubien et al. 1990; Mueller et al. 2011), smooth muscle cells (Robert et al. 2004, 2005; Vandebrouck

et al. 2006), neurons (Rogan et al. 2010), and others. Here we will confine our focus to the impact of loss of CFTR function on the onset and progression of lung disease.

Lung disease in infants and preschool-aged children with CF can be remarkably asymptomatic at its earliest stages. A series of bronchoscopy and bronchoalveolar lavage (BAL) studies helped document that infants with CF may have significant inflammation and bacterial infection in the face of no respiratory symptoms or signs (Armstrong et al. 2005; Balough et al. 1995; Khan et al. 1995). Early laboratory evidence of lung disease includes the presence of neutrophils, proinflammatory cytokines, and culturable bacteria such as *H. influenzae* and *S. aureus* in BAL fluid (Khan et al. 1995; Muhlebach et al. 1999). High-resolution chest CT scans are among the most sensitive early radiologic measures of disease and may demonstrate inhomogeneity of aeration, subsegmental atelectasis, bronchial wall thickening, and airway obstruction in healthy-appearing young children. As the disease progresses, bronchial dilatation and bronchiectatic changes are observed in the airways. These findings indicated that the respiratory tract host defenses of children with CF are compromised early on in their ability to eradicate bacteria encountered by inhalation or microaspiration. While aggressive, early treatments have slowed the rate of progression of lung disease, CF remains a serious chronic disease. Lung transplant is currently the only option available for patients with advanced disease.

While studies of the host defense defect associated with CF usually focus on bacterial infections, there is evidence that people with CF may have problems in their ability to tolerate infections by respiratory viruses. One prospective study of infants and children with CF reported an increased morbidity associated with respiratory syncytial virus infections (Abman et al. 1988). Hiatt and coworkers found that compared to non-CF subjects, infants with CF were more likely to develop lower respiratory tract infections associated with hospitalization and reduction in lung function (Hiatt et al. 1999). In contrast, Ramsey and colleagues studied school-aged children with CF prospectively and did not identify any significant adverse effect of respiratory viral infections on pulmonary function compared with age-matched non-CF controls (Ramsey et al. 1989). In experiments using cultured primary CF and non-CF airway epithelia, Erzurum et al. noted an increase in parainfluenza type III replication in CF cells (Zheng et al. 2003). This increase in virus replication was associated with reduced nitric oxide synthase 2 (NOS2) and 2′,5′-oligoadenylate synthetase (OAS) 1 induction in response to virus or interferon gamma. The investigators linked these reductions in antiviral defenses to an impaired activation of signal transducer and activator of transcription (STAT)1. Recently, Sutanto and coworkers studied primary cells from CF and non-CF subjects and noted that human rhinovirus 1B replicated to higher levels in CF respiratory epithelia (Sutanto et al. 2011). This finding was also associated with a reduced apoptotic response and increased release of IL-8 in the CF epithelia. These studies are intriguing as some antimicrobial peptides and proteins exhibit antiviral properties (Daher et al. 1986), and signaling mediated by type I and type III interferons may also promote antibacterial defenses (Li et al. 2008). In addition, intracellular pathogen sensing mediated by NLRP1 and NLRP3

inflammasomes may influence both antiviral and antibacterial host defense responses (Cassel and Sutterwala 2010; Guarda et al. 2011; Poeck et al. 2010; Poeck and Ruland 2011; Strunk et al. 2011). Further studies are needed to determine if mutations in *CFTR* also cause a defect in antiviral innate immunity.

While our discussion focuses on how loss of CFTR function results in the primary manifestations of CF lung disease, it is important to understand that disease progression results in secondary complications that further compromise airway defenses. As airway bacterial infection in children with CF evolves from intermittent infection to chronic colonization, the host mounts an impressive response. Predominant findings include the release of proinflammatory cytokines by epithelia and immune effector cells that include IL-1, TNF-alpha, IL-6, 1L-17 (Decraene et al. 2010; Dubin and Kolls 2011; McAllister et al. 2005; Tan et al. 2011), and 1L-23 (Decraene et al. 2010; McAllister et al. 2005) and neutrophil chemoattractants such as IL-8, IL-17, and CCL20 (Armstrong et al. 2005; Balough et al. 1995; Khan et al. 1995; McAllister et al. 2005; Muhlebach et al. 1999) and the secretion of pathogen-specific antibodies (Doring et al. 1988). Intense neutrophilic infiltration of the airways ensues, and the associated inflammatory responses include the release of enzymes such as neutrophil elastase, myeloperoxidase, cathepsins, and others. In addition, airway pathogens can release proteases and other inflammatory stimuli that gradually lead to disease progression. These secondary inflammatory responses contribute to the destruction of airway tissue and stimulate proliferative responses in the airway epithelium (Leigh et al. 1995) with associated remodeling, such as goblet cell metaplasia (Bedrossian et al. 1976; Davis and Dickey 2008; Groneberg et al. 2002). This burden of proteases causes further secondary compromise in antimicrobial defenses by directly degrading host defense proteins such as lactoferrin (Britigan et al. 1993), defensins (Taggart et al. 2003), SLPI (Weldon et al. 2009), and elafin (Guyot et al. 2008). In addition, the protease-rich environment of the CF airways can cleave TLR-2 and TLR-4, which may further impair innate immune signaling (Greene et al. 2004). These persistent host responses to polymicrobial infection add to the difficulties in defining the host defense defects directly linked to loss of CFTR function and distinguishing them from those that arise as a consequence of the host inflammatory response.

3   Innate Immune Defenses in the Airways

The airways face a daily burden of inhaled or aspirated bacteria, viruses, and other potentially damaging particulates. To protect against these threats, the epithelium of the respiratory tract has evolved multiple mechanisms to prevent microbial infection and minimize tissue damage in response to infectious agents. At the most basic level, the epithelium protects the airways from such insults by serving as a physical barrier between the environment and the underlying tissue. However, the defensive capacity of this mucosal surface extends far beyond its simple barrier function. The conducting airways are lined by a pseudostratified columnar
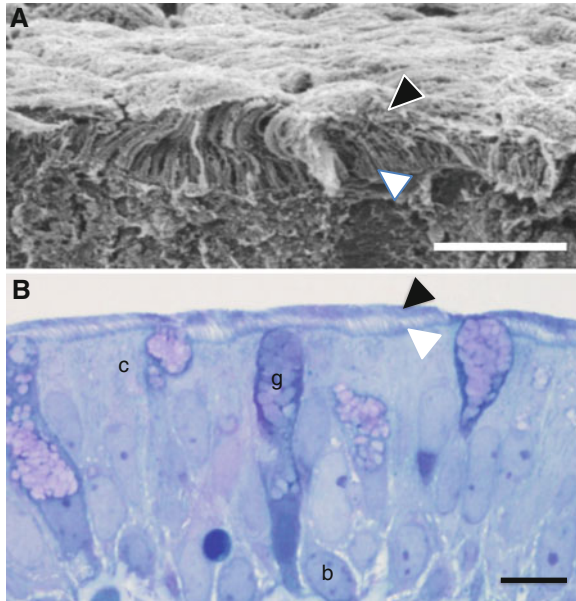
Fig. 1 The airway surface liquid (sol) and mucus (gel) layers provide an optimal environment for the function of secreted host defense factors. (**A**) Scanning electron microscope image of osmium and perfluorocarbon fixed cultured well-differentiated non-CF primary human airway epithelial cells. *Black arrowhead indicates gel layer; white arrowhead indicates sol layer*. This sentence refers to fig. 1B. (**B**) Note ciliated cells (c), goblet cells (g), and basal cells (b). Light microscopy image of osmium and perfluorocarbon fixed newborn non-CF pig tracheal epithelium stained with *toluidine blue*. *Black arrowhead* indicates gel layer; *white arrowhead* indicates sol layer. Scale bars in both images indicate 10 μm

epithelium consisting of ciliated and non-ciliated surface cells, mucin-producing goblet cells, and a progenitor cell type termed basal cells. The conducting airway epithelium also possesses submucosal glands, which supply bulk liquid secretion, additional mucins, and other molecules with antimicrobial, anti-inflammatory, or other host defense functions to the airway surface. Together, the surface airway epithelia and submucosal glands are responsible for generating the airway surface liquid (ASL)—a mixture of secreted proteins and peptides with innate immune functions, as well as lipids, surfactants, and electrolytes important for ASL volume homeostasis. The ASL is organized into two compartments: an aqueous phase near the cell surface that bathes the cilia (the *sol* or periciliary layer) and a layer of hydrated mucus (the *gel* layer) that rests atop the sol phase (Fig. 1). Inhaled microbes and other particles are trapped in this mucus layer, which, propelled by the coordinated beating of the cilia, slides along the top of the periciliary fluid layer in a process known as mucociliary clearance. In this way, particles are swept up and out of the airways to the nasopharynx, where they are eliminated by swallowing. Similarly, cough clearance also removes particles from the airway lumen (Bartlett et al. 2008a).

In addition to these physical and mechanical defense mechanisms, the airway epithelium actively interfaces with the environment and constitutively or inducibly secretes an array of innate immune effector molecules into the ASL that sense and respond to microbial threats (Table 1). This "chemical shield" includes cationic peptides with broad-spectrum antimicrobial activity, such as the beta-defensins (Pazgier et al. 2006; Schutte and McCray 2002; Singh et al. 1998), CCL20 (Starner et al. 2003), and the human cathelicidin LL-37 (Bals et al. 1998b), as well as the prototypic antimicrobial protein lysozyme (Fleming 1922; Fleming and Allison 1922), which kills bacteria by degrading peptidoglycan in the bacterial cell wall. There are also innate immune molecules that combat bacteria in ways that do not involve direct killing, such as iron sequestration by lactoferrin (Masson et al. 1966; Oram and Reiter 1968) or binding of bacterial siderophores (iron-chelating molecules) by the neutrophil gelatinase-associated lipocalin (NGAL, or lipocalin-2) (Goetz et al. 2002). Many of these molecules are multifunctional. For example, the collectins surfactant protein A (SP-A) and surfactant protein D (SP-D) bind microbes and act as opsonins (Kuan et al. 1992; Tenner et al. 1989; van Iwaarden et al. 1990, 1991, 1994) and also help to modulate inflammation through their interactions with phagocytes and inflammatory-signaling molecules (Murakami et al. 2002; Sano et al. 1999; Sato et al. 2003). The abundant secreted protein PLUNC (palate, lung, nasal epithelium clone) possesses potent surfactant activity and is proposed to contribute to airway epithelial defenses through antimicrobial as well as anti-biofilm effects (Chu et al. 2007; Gakhar et al. 2010; Lukinskiene et al. 2011; McGillivary and Bakaletz 2010; Zhou et al. 2008). Airway secretions also contain proteins with anti-inflammatory functions, such as the protease inhibitors elafin and secretory leukocyte protease inhibitor (SLPI) (Butler et al. 2006; Henriksen et al. 2004; Sallenave 2010). Porter and coworkers demonstrated that airway epithelia secrete lipids that exert direct antimicrobial activity and synergize with host defense proteins (Do et al. 2008).

In addition to polypeptide- and lipid-based defenses, the secreted enzyme lactoperoxidase (LPO) contributes to an oxidative host defense system. In this system, LPO uses H_2O_2 produced by the dual oxidases DUOX1 and DUOX2 (members of the NOX gene family) to catalyze the oxidation of the secreted anion thiocyanate (SCN^-) in the ASL. This reaction generates the antibacterial product hypothiocyanate ($OSCN^-$), which is toxic to several relevant airway pathogens (Conner et al. 2002; Forteza et al. 2005; Gerson et al. 2000; Moskwa et al. 2007; Wijkstrom-Frei et al. 2003). In all cases, these defensive molecules may be either constitutively expressed or induced in response to various inflammatory stimuli. This redundancy in the number of host defense factors that act by multiple mechanisms is further augmented by their ability to act together in synergistic or additive manners (Singh et al. 2000b).

A third arm of lung host defense involves the resident phagocytic cells, including alveolar macrophages and neutrophils that may be recruited to sites of airway infection and inflammation. In addition to engulfing and destroying bacteria, both macrophages and neutrophils release a variety of potent antimicrobial factors to augment the epithelial responses to invading microorganisms. Macrophages and

Table 1 ASL host defense factors

Product	Relative concentration in ASL	Cellular source	Function(s)	References
Lysozyme	μg-mg/mL	Epithelia, neutrophils	Antibacterial	Fleming (1922), Fleming and Allison (1922), Hiatt et al. (1952)
Lactoferrin	μg/mL	Epithelia, neutrophils	Antibacterial, antiviral, antifungal, inhibition of microbial growth through iron sequestration	Arnold et al. (1980), Masson et al. (1965, 1966), Oram and Reiter (1968), Sano et al. (2003), Xu et al. (1999)
Surfactant protein A (SP-A)	ng-μg/mL	Epithelia	Microbial aggregation, opsonization, modulation of inflammation	Hartshorn et al. (1998), Korfhagen et al. (1996), LeVine et al. (1999a, b, 2000), Murakami et al. (2002), Sano et al. (1999), Sato et al. (2003), Tenner et al. (1989), van Iwaarden et al. (1990, 1991)
Surfactant protein D (SP-D)	ng-μg/mL	Epithelia	Microbial aggregation, opsonization, modulation of inflammation	Hartshorn et al. (1998, 1994), Kuan et al. (1992), LeVine et al. (2000), Yoshida et al. (2001)
Secretory leukocyte protease inhibitor (SLPI)	μg/mL	Epithelia, neutrophils, macrophages	Protease inhibition, anti-inflammatory, antimicrobial	Henriksen et al. (2004), Hiemstra et al. (1996), Mihaila and Tremblay (2001), Saitoh et al. (2001), Sallenave et al. (1994, 1997)
Peptidase inhibitor 3 (elafin)	ng/mL	Epithelia, neutrophils, macrophages	Protease inhibition, anti-inflammatory, antimicrobial, opsonization	Henriksen et al. (2004), King et al. (2003), Mihaila and Tremblay (2001), Sallenave et al. (1994), Simpson et al. (1999), Wilkinson et al. (2009)
Cystatin S	*Unknown*	Epithelia	Cysteine protease inhibitor	Isemura et al. (1984), Lindahl et al. (1999)
Lipocalin-1 (tear lipocalin)	*Unknown*	Epithelia	Cysteine protease inhibitor, iron sequestration through siderophore binding	Fluckinger et al. (2004), Lindahl et al. (1999), Redl et al. (1998), van't Hof et al. (1997)

(continued)

Table 1 (continued)

Product	Relative concentration in ASL	Cellular source	Function(s)	References
Neutrophil gelatinase-associated lipocalin (NGAL, or lipocalin-2)	ng/mL	Epithelia, neutrophils	Iron sequestration through siderophore binding	Cowland et al. (2003), Goetz et al. (2002), Kjeldsen et al. (1993), Triebel et al. (1992)
Bactericidal/permeability-increasing protein (BPI)	Unknown	Neutrophils	Antibacterial, anti-inflammatory, endotoxin neutralization, opsonization	Iovine et al. (1997), Marra et al. (1990), Weiss et al. (1978)
PLUNC (or SPLUNC1, LUNX, NASG, SPURT, BPIFA1)	μg/mL	Epithelia, neutrophils	Antimicrobial, biofilm inhibition	Bartlett et al. (2008b), Bingle and Bingle (2000), Chu et al. (2007), Gakhar et al. (2010), Lindahl et al. (2001), Lukinskiene et al. (2011), McGillivary and Bakaletz (2010), Zhou et al. (2008)
Phospholipase A2	μg-mg/mL	Epithelia, neutrophils	Antibacterial, proinflammatory	Elsbach et al. (1979), Rosenthal et al. (1995), Weinrauch et al. (1996), Zallen et al. (1998)
IgA secretory component	μg/mL	Epithelia	Prevents bacterial adhesion to mucosal surfaces	Fiedler et al. (1991), Phalipon et al. (2002)
Complement factor C3	Unknown	Epithelia	Complement signaling, antibacterial, antifungal	Candiano et al. (2007), Nordahl et al. (2004), Sonesson et al. (2007)
S100A8 (calgranulin A) and S100A9 (calgranulin B) (together known as calprotectin)	Unknown	Epithelia, neutrophils, macrophages, monocytes	Antibacterial, antifungal effects through sequestration of divalent cations, neutrophil chemotaxis	Corbin et al. (2008), Cornish et al. (1996), Murthy et al. (1993), Nisapakultorn et al. (2001a, b), Ryckman et al. (2003), Sohnle et al. (1991, 1996), Steinbakk et al. (1990), Vandal et al. (2003), Xu et al. (1999)
S100A7 (psoriasin)	Unknown	Epithelia, macrophages	Antibacterial	Andresen et al. (2011), Glaser et al. (2005), Lee and Eckert (2007)
S100A12 (calgranulin C)	Unknown	Neutrophils, monocytes	Antibacterial, antifilarial	Cole et al. (2001), Gottsch et al. (1999), Guignard et al. (1995)
Defensins (alpha and beta)	ng-mg/mL	Epithelia, neutrophils, macrophages	Antibacterial, antifungal, antiviral, chemotactic for T cells, monocytes, dendritic cells	Bals et al. (1998a), Chertov et al. (1996), Daher et al. (1986), Ganz et al. (1985), Garcia et al. (2001a, b), Goldman et al.

				(1997), Harder et al. (2001), Jia et al. (2001), Lehrer et al. (1988), McCray and Bentley (1997), Singh et al. (1998), Territo et al. (1989), Yang et al. (1999)
CCL20	ng/mL	Epithelia, neutrophils, macrophages	Chemokine, antibacterial, antifungal	Starner et al. (2003), Yang et al. (2003)
LL-37	*Unknown*	Epithelia, neutrophils	Antibacterial, endotoxin neutralization, wound repair, angiogenesis, chemotactic for mast cells, neutrophils, monocytes, T cells	Agerberth et al. (2000), Bals et al. (1998b), Cowland et al. (1995), Heilborn et al. (2003), Koczulla et al. (2003), Larrick et al. (1995), Niyonsaba et al. (2002), Sorensen et al. (1997), Tjabringa et al. (2006), Turner et al. (1998), Yang et al. (2000)
Cholesteryl esters (cholesteryl linoleate, cholesteryl arachidonate)	μg/mL	Epithelia	Antibacterial	Do et al. (2008)
Lactoperoxidase (LPO)	μg/mL	Submucosal gland epithelia	Catalyzes oxidation of SCN^- to form $OSCN^-$	Conner et al. (2002), Gerson et al. (2000), Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)
H_2O_2 (via DUOX)	0.1 μM	Epithelia	Oxidizes SCN^- to form antibacterial $OSCN^-$	Conner et al. (2002), Forteza et al. (2005), Gerson et al. (2000), Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)
SCN^-	μM	Epithelia	Antibacterial in oxidized form ($OSCN^-$)	Conner et al. (2002), Gerson et al. (2000), Lorentzen et al. (2011), Moskwa et al. (2007), Wijkstrom-Frei et al. (2003)

neutrophils store and secrete many of the same innate immune factors released by airway epithelia, such as alpha- and beta-defensins (Ganz et al. 1985; Garcia et al. 2001a), LL-37 (Cowland et al. 1995), lysozyme (Hiatt et al. 1952), lactoferrin (Masson et al. 1969), NGAL (Kjeldsen et al. 1993), and PLUNC (Bartlett et al. 2008b), as well as phagocyte-specific products such as the anti-inflammatory bactericidal/permeability-increasing protein (BPI) (Weiss et al. 1978). Macrophages and neutrophils actively communicate with airway epithelia by releasing and responding to cytokines and other molecules involved in amplification and/or regulation of inflammatory responses.

In airway epithelia, as well as in macrophages and neutrophils, many innate immune responses are mediated by Toll-like receptors (TLRs), a family of pattern recognition receptors that evolved to recognize a variety of pathogen-associated molecular patterns (PAMPs). Engagement of a TLR typically initiates a signaling cascade that results in an immune response tailored to the invading organism. TLRs respond by activating the transcription of numerous cytokine genes, including TNF, IL-6, and IL-12 as well as a variety of chemokine genes (Akira and Takeda 2004). TLRs also induce proforms of IL-1β and IL-18, which are then processed and secreted by NOD-like receptor-mediated caspase-1 activity. Additional intracellular receptors also coordinate epithelial innate immune responses to pathogens, including the NOD-like receptors, RIG-I, and MDA5 (Andrejeva et al. 2004; Yoneyama et al. 2004). Reactive oxygen species (ROS) are among the most evolutionarily conserved pathway of responses to infection or injury and are triggered by all danger-associated molecular patterns (DAMPs) and PAMPs. A ROS-dependent pathway triggers the inflammasome complex formation in myeloid cells like macrophages (Cruz et al. 2007).

4   How Does Loss of CFTR Function Alter Airway Host Defenses?

It has been challenging to explain the complex pathogenesis of CF lung disease. While it is well established that CF is caused by absent or reduced CFTR anion channel activity, it has been difficult to draw a direct line from this molecular defect to the varied CF disease manifestations. In the case of CF lung disease, the critical question is: How does the loss of anion channel activity cause defective innate immunity in the airways? This question has given rise to a number of hypotheses, which touch on multiple components of mucosal innate immunity. We stress that these hypotheses are not necessarily mutually exclusive and that it is possible, even likely, that the primary pathogenesis of CF lung disease is multifactorial.

4.1 Altered Na$^+$ and Cl$^-$ Transport in CF

CFTR is a Cl$^-$ channel that also conducts HCO$_3$$^-$, SCN$^-$, I$^-$, and other anions (Anderson et al. 1991a, b). For this reason, considerable effort has focused on identifying links between altered electrolyte transport by airway epithelia and the functions of the innate immune system. As epithelia use Cl$^-$ secretion and Na$^+$ absorption to coordinately regulate the volume of secretions, it is important to understand both Cl$^-$ and Na$^+$ transport and osmotically coupled liquid movement across the airway epithelium in CF patients. Several groups have investigated how CF may alter the volume or composition of respiratory secretions.

One hypothesis posits that CFTR activity is essential for the regulation of ASL volume and mucus hydration and that absence of CFTR causes dehydration of the ASL and reduced mucociliary clearance (Boucher 2004, 2007). This model rests on the concept that the depth of the ASL periciliary layer is tightly regulated to ensure a height of ~7 μm (Matsui et al. 1998). This ~7 μm depth, the approximate length of a cilium, is thought to be optimal for efficient ciliary beating and movement of the mucus layer. Early studies assessing the nasal and airway transepithelial voltage (Vt) in CF subjects indicated that the CF epithelium had a lower (lumen-negative) Vt and also exhibited a greater reduction in nasal Vt after application of amiloride, an inhibitor of the epithelial Na$^+$ channel (ENaC), relative to non-CF epithelia (Knowles et al. 1981, 1983a, b). Based on this observation, it was suggested that Na$^+$ hyperabsorption and concomitant liquid absorption reduces ASL volume in the CF airways. This depletion of ASL would then lead to dehydration of the mucus layer, resulting in thick, sticky, adherent mucus that is difficult to move, ultimately impairing mucociliary clearance in the CF airways. In support of this hypothesis, evidence for altered ASL volume homeostasis, thickened mucus, and reduced mucociliary transport has been reported using cultured airway epithelia from human CF and non-CF patients (Matsui et al. 1998). In a study of bronchial tissue biopsies from human CF and non-CF subjects, CF samples showed a trend toward reduced ASL depth, although this did not reach statistical significance (Griesenbach et al. 2011). In the same study, a comparison of the periciliary liquid height of the nasal epithelium in wild-type and CFTR-null mice revealed a significant decrease in samples from the CFTR-null animals (Griesenbach et al. 2011).

Data from recent experiments in cultured CF and non-CF epithelia provide an alternative interpretation for the increased nasal Vt observed in people with CF. Itani and coworkers studied cultured primary well-differentiated human airway epithelia and failed to find evidence for increased Na$^+$ conductance in CF epithelia (Itani et al. 2011). Under basal or cAMP-stimulated conditions, the transepithelial conductance of CF epithelia was reduced compared with non-CF epithelia, and the reduction could be accounted for solely by the loss of the CFTR channel conductance. The addition of amiloride resulted in greater decreases in Vt and short-circuit current in CF epithelia than non-CF epithelia. However, amiloride caused similar reductions in conductance and Na$^+$ absorption in cells from both genotypes, indicating that the effects of amiloride were due to a loss of Cl$^-$ conductance. These results suggest that loss of anion conductance is the critical defect in electrolyte transport in CF epithelia.

Inhibition of Na^+ transport in the respiratory tract as a therapy has been investigated in CF subjects given the ENaC inhibitor amiloride by inhalation. A multicenter randomized double-blind placebo-controlled clinical trial investigated the efficacy of thrice-daily inhaled amiloride on pulmonary function of CF patients over a 6-month period. The study failed to demonstrate significant benefit (Pons et al. 2000).

A second hypothesis proposes that changes in ASL ionic strength might adversely affect the activity of innate immune effector proteins. In this model, loss of CFTR-dependent Cl^- transport results in increased ASL NaCl concentrations due to an inability of the surface epithelium to modify ASL composition by absorption of Cl^- and Na^+. A predicted outcome of this scenario is a reduction in the activity of salt-sensitive antimicrobial factors. This hypothesis emphasizes the central importance of antimicrobials, particularly cationic peptides and proteins, in protecting the airways from microbes (Cole et al. 1999, 2002). It is known that the activity levels of numerous antimicrobial factors, including lysozyme, lactoferrin, alpha- and beta-defensins, and others, are diminished in solutions of increased ionic strength (Goldman et al. 1997; Porter et al. 1997; Singh et al. 1998, 2000b; Travis et al. 1999; Valore et al. 1998). In experiments using cultured well-differentiated human primary airway epithelia derived from CF and non-CF donors, Smith and colleagues found that CF epithelia exhibited a killing defect when a small bacterial inoculum was applied directly to the apical surface (Smith et al. 1996). However, when in vitro killing assays were performed using ASL that had been removed from the apical surface of the cultures using water as a diluent, there was no significant difference in killing between CF and non-CF-derived samples. This result implied that it was unlikely that CF ASL lacked a critical bactericidal factor, and suggested instead that the activity of a bactericidal factor or factor(s) was inhibited by some aspect of the ASL environment in the CF epithelia. To test the hypothesis that the NaCl concentration influences antimicrobial activity in CF ASL, the authors replaced the ASL of both CF and non-CF cultures with solutions containing either high (182 mM) or low (92 mM) Cl^- concentrations and repeated the bacterial killing assays. Bacterial killing was significantly reduced in non-CF cells that received the high salt solution, while killing activity was restored in CF cells under the low salt conditions (Smith et al. 1996).

While this hypothesis remains a subject of debate, the idea that loss of CFTR function alters the ASL milieu in a manner that impairs the activity of host defense factors remains attractive. A confounding factor has been the difficulty of accurately measuring ASL salt concentrations in vivo. Some investigators reported that Cl^- concentrations are indeed elevated in CF (Gilljam et al. 1989; Joris et al. 1993; Kozlova et al. 2006a, b; Vanthanouvong et al. 2006; Zabner et al. 1998), while others found no significant difference in NaCl concentrations between CF and non-CF (Caldwell et al. 2002; Grubb et al. 2002; Jayaraman et al. 2001a, b; Knowles et al. 1997). If a difference in the concentration of ASL NaCl is not validated in CF, the experimental findings of the study of Smith and colleagues (1996) continue to point to a compositional change in CF ASL that negatively impacts the function of host defense factors.

Fig. 2 Model of the oxidative host defense system at the apical side of airway epithelia. DUOX enzymes are the H_2O_2-generating cytochromes in the apical membrane of airway epithelia. LPO is secreted by submucosal glands. SCN^- is utilized by LPO for $OSCN^-$ generation
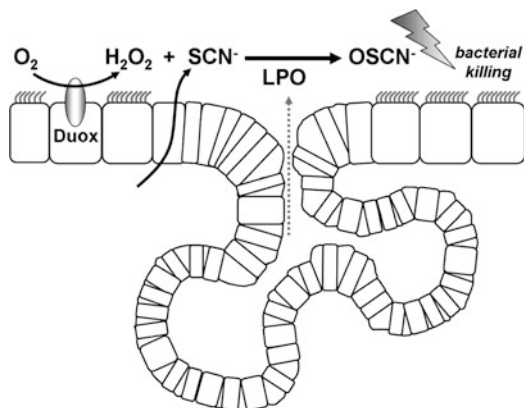
4.2 Altered SCN^- Transport in CF

This hypothesis builds from the observation that CFTR conducts thiocyanate (SCN^-) (Tabcharani et al. 1993). While many secreted host defense proteins and peptides are well characterized, the recognition of an airway epithelial oxidative microbicidal system is recent. This oxidative system consists of two H_2O_2-generating enzymes of airway epithelia, dual oxidases (DUOX)1 and 2, along with a pseudohalide anion (thiocyanate, SCN^-), and the enzyme lactoperoxidase (LPO) (Conner et al. 2002; Forteza et al. 2005; Gerson et al. 2000; Moskwa et al. 2007; Wijkstrom-Frei et al. 2003). The DUOX enzymes generate H_2O_2 into the apical extracellular space where H_2O_2 reacts with SCN^- in a LPO-catalyzed reaction to form the antibacterial molecule $OSCN^-$ ($H_2O_2 + SCN^- \rightarrow OSCN^-$) (Fig. 2). Both LPO and SCN^- are highly concentrated in the airway surface liquid. SCN^- is secreted apically by airway epithelia and accumulates in the ASL in concentrations of approximately 400–460 μM (Lorentzen et al. 2011; Wijkstrom-Frei et al. 2003). The DUOX/LPO/SCN^- system can generate sufficient $OSCN^-$ to eliminate bacteria in vitro and in vivo (Conner et al. 2007; Moskwa et al. 2007). SCN^- secretion is reduced in CF cells and tissues (Conner et al. 2007; Moskwa et al. 2007), leading to the hypothesis that diminished SCN^- (and therefore reduced $OSCN^-$) availability impairs defenses against airway pathogens. In support of this, Moskwa and coworkers reported that $OSCN^-$-mediated killing of *Staphylococcus aureus* was inhibited on the apical surface of cultured CF airway epithelia (Moskwa et al. 2007). The relative contribution of this system to normal airway defenses in vivo, and how relevant its inactivation may be to CF pathogenesis, is currently unresolved. In a recent study, Lorentzen and colleagues found that the concentrations of SCN^- were variable, and not significantly different, in the nasal secretions of humans with and without CF, suggesting that the bacterial killing activity of $OSCN^-$ was unlikely to be different between genotypes (Lorentzen et al. 2011). However, lung function correlated positively with SCN^- levels in the CF

subjects in this study. These findings suggest that while oxidative defenses are important for overall lung health, CFTR activity may not be the only determinant of ASL SCN^- levels; the authors cite SCN^- transport by alternative ion channels as potential compensating mechanisms in the CF airways (Lorentzen et al. 2011). One candidate for alternative SCN^- transport, the sodium-independent chloride/iodide transporter pendrin (also known as SLC26A4), is expressed by human airway epithelia and is upregulated in response to IL-4 (Pedemonte et al. 2007) and to viral infections (Nakagami et al. 2008). Therefore, it is possible that, in some individuals, increased pendrin expression might partially compensate for loss of CFTR activity in inflamed CF airways.

4.3 Altered HCO_3^- Transport in CF

It has been long recognized that loss of CFTR function results in the acidification of pancreatic secretions and that CF pancreatic secretions fail to properly alkalinize in response to secretagogues (Gaskin et al. 1982; Kopelman et al. 1988). CFTR expressed within the airways also transports bicarbonate (HCO_3^-) and thereby helps buffer ASL (Fischer and Widdicombe 2006; Smith and Welsh 1992). In the airways, loss of HCO_3^- secretion via CFTR is predicted to result in a diminished capacity to alkalinize respiratory secretions. A number of studies have assessed ASL pH using in vitro systems and in vivo models. There is evidence that secretions from submucosal glands derived from human CF nasal tissues are hyperacidified relative to non-CF secretions (Song et al. 2006) and that cultured CF human bronchial epithelia acidify their ASL more rapidly than do non-CF cells (Coakley et al. 2003). In studies comparing CFTR-null and wild-type mice, Jayaraman and coworkers noted no significant differences in the in vivo airway pH as measured using a tracheal window preparation (Jayaraman et al. 2001b). Reductions in ASL pH could negatively influence innate immunity by several mechanisms [reviewed in (Coakley and Boucher 2001; Poschet et al. 2002)] including inhibiting the activity of antimicrobials (Dorschner et al. 2006; Lehrer et al. 1983; Selsted et al. 1985), altering the viscosity of secretions (Bhaskar et al. 1991; Holma 1985), and decreasing ciliary beat frequency (Clary-Meinesz et al. 1998).

4.4 CF-Associated Changes in Airway Submucosal Gland Function and Secreted Mucins

Alterations in airway submucosal gland physiology are also implicated in the innate immune defects in CF. Submucosal glands are responsible for the secretion of liquid and mucins, such as the gel-forming mucin MUC5B (Groneberg et al. 2002), as well as numerous proteins and peptides with antimicrobial, anti-inflammatory,

and other host defense functions (Wine and Joo 2004). A submucosal gland is formed by invagination of the airway epithelial surface to form a single collecting duct, into which multiple mucous tubules empty (Wine and Joo 2004). The more proximal portions of these tubules are lined by mucous cells, responsible for mucin production, while the distal acini of the tubules contain serous cells that secrete liquid, electrolytes, and various protein and peptide components of ASL. CFTR is expressed primarily in the serous acini (Engelhardt et al. 1992), where its anion channel activity is thought to play a key role in liquid secretion. As major sites of mucus production, the submucosal glands contribute to the quantity and quality of mucus in the airways and are studied for their possible involvement in generating the abnormal mucus that is a hallmark of CF lung disease.

Several investigators hypothesize that liquid secretion by serous cells is reduced in CFTR-deficient submucosal glands, leading to altered hydration of secreted mucins and the ASL. The resulting mucus is predicted to be unusually viscous and would impede the efficient mechanical clearance of particles from the airways. Such changes in mucus rheologic properties could also lead to obstruction of submucosal gland ducts. In support of this hypothesis, there is abundant evidence that liquid secretion in response to secretory signals is reduced when CFTR function is impaired. A number of studies confirmed that CF glands fail to secrete in response to forskolin and vasoactive intestinal peptide (VIP), agonists that stimulate secretion by elevating intracellular cAMP levels (Choi et al. 2009; Joo et al. 2002, 2006, 2010; Lee and Foskett 2010). Similarly, secretion in CF glands is impaired in response to the neuropeptide substance P (Choi et al. 2009; Joo et al. 2010) and to carbachol when administered in combination with other agonists (Choi et al. 2007). In keeping with these data, studies of secretions collected from individual submucosal glands suggest that viscosity is significantly elevated in secretions from CF individuals (Jayaraman et al. 2001a; Salinas et al. 2005).

Defective HCO_3^- secretion is also hypothesized to impact mucin release and viscosity (Quinton 2008). One model posits that, early in the process of mucus gel formation and extrusion from the submucosal glands, polyanionic mucin molecules are packed together into condensed mucin granules. To maintain this structure, the mucin molecules are highly cross-linked and their charges neutralized by a "shield" of Ca^{2+} ions. HCO_3^-, which effectively chelates Ca^{2+}, can sequester these Ca^{2+} ions, thereby releasing the cross-links between mucins and allowing the mucins to rapidly expand as they are released from submucosal glands and/or goblet cells. In this way, HCO_3^- may be intimately involved in the swelling and hydration of the ASL mucus layer. This model is supported by the demonstration that adding HCO_3^- to mucus gels increases mucus dispersal in vitro (Chen et al. 2010a). Therefore, in CF glands and tissues, the absence of CFTR-mediated HCO_3^- secretion may result in mucus granules that de-condense inappropriately, giving rise to viscous, under-hydrated mucus that impairs mucociliary clearance (Chen et al. 2010a).

In addition to the impact of impaired secretory responses of CF glands on the hydration status of mucins, it is suggested that this phenomenon could affect the abundance of secreted antimicrobials and other host defense factors from gland serous cells. While there is currently little direct evidence for this, Wine and

colleagues reported that a number of known innate immune molecules, including lysozyme, NGAL (lipocalin-2), HSC-71, and the protease inhibitors alpha 1-antitrypsin and alpha 1-antichymotrypsin, are secreted by Calu-3 cells (a serous cell model) in response to forskolin stimulation (Joo et al. 2004). This observation leads to the prediction that secretion of antimicrobials may be reduced in the absence of CFTR. Additionally, when this observation is considered with the above-mentioned defects in liquid secretion and mucus hydration, it is tempting to speculate that in CF glands, secreted innate immune molecules may become "trapped" within viscous polyanionic mucus and are therefore less likely to be properly presented to the airway lumen.

Increased mucin production by the surface epithelium may also contribute to impaired mucociliary clearance in CF. Airway surface goblet cells secrete the gel-forming mucin MUC5AC and, to a lesser extent, MUC5B (Groneberg et al. 2002; Hovenberg et al. 1996). Goblet cell metaplasia is a response to chronic inflammation and is commonly seen in CF lung disease (Bedrossian et al. 1976; Davis and Dickey 2008; Groneberg et al. 2002). Therefore, the increased viscous secretions associated with CF are derived in part from the surface epithelium. In keeping with this, Derichs and colleagues reported that the viscosity was increased in both the mucus and the periciliary liquid layers of the ASL from cultured CF bronchial epithelia, which do not possess submucosal glands (Derichs et al. 2011). This raises the possibility that increased mucin production from goblet cells may also impact innate immunity by providing more binding sites for cationic antimicrobials and other molecules that normally associate with the polyanionic mucin polymers in the airways (Felgentreff et al. 2006), making those antimicrobial factors less available to interact with their target pathogens.

5   New Animal Models of CF Lung Disease

A limitation in advancing knowledge of the molecular basis of CF lung disease has been the lack of animal models that recapitulate key features of lung and other organ disease pathology. The mouse models with CFTR-null alleles and human CFTR mutations available since the early 1990s have contributed greatly to disease understanding but do not develop spontaneous lung disease similar to humans with CF. Recently, several groups used somatic cell targeting of the *CFTR* gene, followed by nuclear transfer and cloning to develop novel models in pigs (Klymiuk et al. 2011; Rogers et al. 2008a, b) and ferrets (Sun et al. 2008, 2010). These new animal models recapitulate key features of CF disease (Klymiuk et al. 2011; Rogers et al. 2008b; Sun et al. 2010). At birth, the airways of *CFTR* targeted pigs are free of inflammation but manifest a bacterial host defense defect without the secondary consequences of infection (Rogers et al. 2008b; Stoltz et al. 2010). Pigs with targeted *CFTR* genes spontaneously develop hallmark features of CF including airway inflammation, remodeling, mucus, and infection within months of birth (Fig. 3). Their lungs contain multiple bacterial species (Gram-negative and
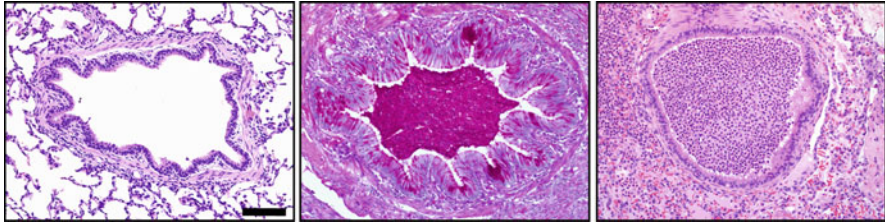
Fig. 3 Airway disease in a pig model of cystic fibrosis. Airways from a CFTR$^{+/+}$ (*left panel*, H&E stain) and a CFTR$^{-/-}$ pig (*middle* and *right*, PAS and H&E, respectively), bar = 90 μm. CFTR$^{-/-}$ pig is infected with the Gram-negative organism *Bordetella bronchiseptica*. Note the increased mucosal thickening and mucin expression in the epithelium with mucocellular lumen obstruction (*middle panel*). A CF pig airway is obstructed with neutrophils and mucopurulent debris (*right panel*)

Gram-positive), suggesting an equal opportunity host defense defect. While the lungs of newborn pigs show no inflammation, they are less often sterile than wild-type littermate controls. Moreover, after intrapulmonary bacterial challenge with *Staphylococcus aureus*, CF pigs fail to eradicate bacteria as effectively as wild-type pigs (Stoltz et al. 2010). These results suggest that impaired bacterial elimination is the pathogenic event initiating a cascade of inflammation and pathology.

Studies of CF pig submucosal glands have shown that the model exhibits responses to agonists qualitatively similar to glands from human patients with CF (Joo et al. 2010). CF pig glands produce almost no liquid in response to cAMP agonists and reduced volumes in response to all other stimuli except carbachol. Furthermore, glands from newborn CF pigs, like human CF glands, exhibit a reduced secretory response to substance P (Joo et al. 2010). Thus, CF glands have a reduced ability to respond to important secretagogues. These findings, combined with the hypoplastic submucosal glands of the newborn CF airways (Meyerholz et al. 2010), raise the possibility that the gland contributions to ASL are altered in their volume and composition in CF.

Recent studies in newborn CF pigs provide further support for the idea that an altered ASL environment is a primary cause of impaired innate immunity in the lung. In vitro and in vivo experiments showed that ASL pH was lower in CF pigs, and this reduction in pH reduced the antimicrobial activity of ASL (Pezzulo et al. 2012). Interestingly, a more acidic ASL pH also diminished bacterial killing in wild-type pigs, while increasing ASL pH increased antimicrobial activity in the ASL of pigs with CF. These results in newborn animals support the concept that reductions in ASL pH due to loss of CFTR-dependent HCO_3^- secretion impair the activity of endogenous antimicrobial proteins and peptides, allowing for the onset of airway infection.

Experiments in the CF pig model also yielded some unexpected findings. *CFTR* targeted pigs exhibit morphological abnormalities of the newborn trachea and large airways, including tracheal cartilage alterations, a decrease in submucosal gland mass, and more noticeable smooth muscle bundles in the posterior trachea

(Meyerholz et al. 2010). Interestingly, retrospective analysis of chest CT scans from children with CF revealed that the tracheas of CF subjects were less circular than the non-CF control individuals (Meyerholz et al. 2010), echoing the tracheal abnormalities observed in the CF piglets. These results are contrary to the dogma that the "lungs of children with CF are normal at birth," and raise the possibility that loss of CFTR function perturbs a developmental program in the lung resulting in structural abnormalities. Such changes could also contribute to the lung disease phenotype.

In a series of experiments using cultured airway epithelia and freshly excised nasal and tracheal tissues from $CFTR^{-/-}$ pigs, as well as in vivo electrophysiological measurements, Chen et al. reported that airway epithelia from newborn CF pigs displayed a reduced Cl^- conductance without an increase in Na^+ conductance (Chen et al. 2010b). The authors implicate the loss of the CFTR-dependent Cl^- conductance, rather than increased Na^+ conductance, as the explanation for the greater reduction in Vt in response to amiloride observed in CF epithelia relative to non-CF epithelia. Measurements of the periciliary liquid depth in fixed tracheal tissue from pigs indicated no significant difference in ASL depth between the CF and non-CF airways at birth. These data suggest that ASL/mucus dehydration due to Na^+ hyperabsorption in CF airways may not be a primary event in CF pathogenesis, although they do not rule out the possibility that perturbations in Na^+ transport may affect airway immunity as the disease progresses.

CFTR-null ferrets also develop multiorgan system disease, and neonatal animals manifest a pulmonary host defense defect in the airways associated with colonization by bacteria (Sun et al. 2010). Early results also indicate that adult CF ferrets develop a lung disease phenotype with similarities to human CF, including bacterial colonization (John Engelhardt, personal communication). Continued studies of animal models that reproduce key phenotypic features of human CF lung disease are likely to provide further insights into the links between loss of CFTR function and the initial pathologic events.

6 Future Directions

With recent advances in CF basic science research and the development of new animal models, the field is poised to make further breakthroughs in the understanding of disease pathogenesis, including a better grasp of the molecular basis of the airway host defense defect. Advancements in these areas are critical to the development of new pulmonary specific or systemic therapies to prevent the onset or slow the rate of disease progression.

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