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REVIEW

Cystic Fibrosis and *Pseudomonas aeruginosa*: the Host-Microbe Interface

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SUMMARY In human pathophysiology, the clash between microbial infection and host immunity contributes to multiple diseases. Cystic fibrosis (CF) is a classical example of this phenomenon, wherein a dysfunctional, hyperinflammatory immune response combined with chronic pulmonary infections wreak havoc upon the airway, leading to a disease course of substantial morbidity and shortened life span. *Pseudomonas aeruginosa* is an opportunistic pathogen that commonly infects the CF lung, promoting an accelerated decline of pulmonary function. Importantly, *P. aeruginosa* exhibits significant resistance to innate immune effectors and to antibiotics, in part, by expressing specific virulence factors (e.g., antioxidants and exopolysaccharides) and by acquiring adaptive mutations during chronic infection. In an effort to review our current understanding of the host-pathogen interface driving CF pulmonary disease, we

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discuss (i) the progression of disease within the primitive CF lung, specifically focusing on the role of host versus bacterial factors; (ii) critical, neutrophil-derived innate immune effectors that are implicated in CF pulmonary disease, including reactive oxygen species (ROS) and antimicrobial peptides (e.g., LL-37); (iii) *P. aeruginosa* virulence factors and adaptive mutations that enable evasion of the host response; and (iv) ongoing work examining the distribution and colocalization of host and bacterial factors within distinct anatomical niches of the CF lung.

KEYWORDS cystic fibrosis, *Pseudomonas aeruginosa*, airway, antimicrobial peptides, inflammation, innate immunity, lung infection, reactive oxygen species, ROS

INTRODUCTION

Cystic fibrosis (CF) remains one of the most prevalent, life-shortening genetic diseases in the Caucasian population (1). According to the Cystic Fibrosis Foundation, there are approximately 30,000 people living with CF in the United States and upwards of 70,000 worldwide (2). Incidence varies by country but remains approximately 1 in 3,000 live births in Caucasians in both North America and Europe (3, 4). In the 1950s, CF was an exclusively pediatric disease, as the vast majority of patients did not live beyond infancy (5). However, by the 1990s, life expectancy had improved to approximately 20 years (2). Today, with further advancement in early diagnostic and management strategies, the median life expectancy of a CF patient is 29.6 years in the United States and 31 years in the United Kingdom (2, 6). Moreover, the median predicted survival (i.e., the age beyond which 50% of CF patients born today are expected to live) is 47 years in both the United States and United Kingdom, resulting in an increasingly higher proportion of adult patients (2, 6). Nevertheless, CF patients continue to suffer profound morbidity throughout their lifetimes, necessitating steadfast research efforts to better understand CF pathophysiology and to spur development of novel therapeutics.

CF is caused by a mutation in a gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate ion transport channel that maintains osmotic balance across multiple epithelial surfaces in the body (7). CFTR mutations are inherited in an autosomal recessive pattern, and approximately 2,000 disease-causing mutations have been identified thus far; these mutations have been subsequently categorized into six classes based on the manner in which CFTR function is perturbed (5, 8). Deletion of a phenylalanine at position 508 (Phe508del or ΔF508) remains the most common mutation and is represented in over two-thirds of CF patients (9). Ultimately, CFTR dysfunction causes an inability to regulate chloride and bicarbonate ion transport across epithelia, resulting in multiple-organ dysfunction, wherein the major clinical manifestations of CF include high chloride content of sweat (which is the basis of the gold-standard diagnostic technique for the disease [i.e., sweat chloride test]), meconium ileus, distal intestinal obstruction, exocrine pancreatic insufficiency, growth disturbance, male infertility, and chronic and recurrent pulmonary infections due to opportunistic pathogens (10–12). Long-term complications of CF also include diabetes and mood disorders, such as depression and anxiety; unfortunately, pulmonary failure remains an inevitable fate for nearly all CF patients (13, 14).

PATHOPHYSIOLOGY OF CF LUNG DISEASE: THE HOST-PATHOGEN INTERFACE

Overview

Obstructive pulmonary disease is the substantial determinant of morbidity and the leading cause of mortality for CF patients (15). There are multiple working hypotheses to explain early events leading to the development of CF lung pathology, and each of these postulates lies at the interface of host-pathogen interactions. Broadly, deficits in the immune response due to CFTR dysfunction result in a predisposition to acute and, ultimately, chronic lung infections with opportunistic pathogens (16). Subsequently, a combination of inflammatory host immune products and bacterial virulence factors drives progressive lung damage throughout a CF patient's lifetime, resulting in respiratory failure, candidacy for lung transplantation, and, eventually, death (7).

Understanding the early events of CF disease development is essential, as CF patients can display signs of permanent changes to lung architecture within the first 5 years of life (5). By the age of 3 years, CF patients often already show air trapping, mucus obstruction, and bronchiectasis (i.e., enduring enlargement of airways) by computed tomography (CT) scanning; elevated levels of inflammatory markers (e.g., neutrophil elastase [NE], which is described in detail below); as well as polymicrobial infection of the lung with pathogens that persist into late stages of disease (e.g., *Staphylococcus aureus* and *Pseudomonas aeruginosa*) (17–20).

In the laboratory, many insights about early disease pathophysiology have been derived from both *in vitro* and *in vivo* studies. Wherein the latter have been augmented by the relatively recent development of the CF ferret and pig models (21–23), most animal work within the field has continued, primarily in mouse models (24). Additionally, translational work using bronchoalveolar lavage (BAL) fluid and immune cells obtained directly from CF patients has added significantly to the field's understanding of the host-microbe interface but, at times, also fueled controversy regarding the role of primary immune dysfunction in CF (25).

Defects in Host Immunity

The most common and generally well-accepted paradigm regarding the development of CF lung disease is the "low-volume hypothesis" (1, 7, 26). CFTR dysfunction results in an inability to secrete chloride and bicarbonate ions into the airway lumen, which normally balances sodium reabsorption via a different channel, the epithelial Na^+ channel (ENac); unopposed sodium reabsorption results in net water uptake by the respiratory epithelium, resulting in dehydration of the airway surface liquid (ASL) (27, 28). The ASL has multiple functions, but chief among these roles is hydration of mucus, a key component of the innate immune response as part of the mucociliary ladder (29). Dehydrated mucus ultimately compromises mucociliary and cough clearance of mucus, providing a nidus for colonization and infection by opportunistic pathogens (30).

Relatedly, a second hypothesis for the development of CF lung disease pertains to the altered pH of ASL. Diminished functionality of CFTR reduces bicarbonate secretion into the airway lumen, resulting in decreased pH of the ASL; indeed, some studies have shown that ASL from CF patients is more acidic than that of healthy patients (31–33). The more acidic ASL within the CF lung has multiple consequences. First, CF ASL (derived from the pig model of disease, which recapitulates acidic airway secretions better than the mouse model) exhibits reduced bacterial killing due to compromised function of cationic antimicrobial peptides (AMPs) (34, 35). AMPs are small innate immune proteins, present within epithelial and leukocyte secretions, with broad antimicrobial activity against bacterial and viral pathogens as well as immunomodulatory functions (36). The microbial killing activities of AMPs present within the CF airway, including human β-defensin-3 (hBD-3) as well as LL-37, are reduced under acidic pH conditions (37, 38). AMPs are also further discussed in greater detail below (see "LL-37, an Antimicrobial Peptide with Immunomodulatory Actions"). Second, in the CF pig model, independent of ASL volume, altered pH of ASL also causes mucus tethering and impaired mucus detachment from the lung epithelium (39). This effect also promotes mucus plugging and reduced mucociliary clearance, but the primary mechanism here is the acidic ASL pH rather than ASL dehydration (35, 39). Finally, CF ASL activates proteases, which can directly damage lung tissue and degrade innate immune effectors (40, 41).

Various studies also suggest that primary dysregulation of the immune system (i.e., due to abrogated CFTR function) contributes to CF lung disease, although this theory remains somewhat controversial; much of the disagreement appears to focus upon whether intrinsic immune defects promote a hyperinflammatory microenvironment within the CF lung or if bacterial infection represents the first event that incites early inflammation within the CF lung (42, 43).

Indeed, there are multiple lines of evidence that support a predilection toward hyperinflammation within CF tissues, independent of bacterial infection. Studies have

shown elevated concentrations of proinflammatory markers in the cell-free supernatants of CF epithelial cell cultures and in *ex vivo* CF tissue specimens that are free of infection (compared to healthy controls) (44–47). Research using CF mouse and ferret models demonstrates that newborn animals with CFTR mutations already have inflammation of the lung in the absence of detectable bacteria and fungi; the possibility of early viral infection, however, was not excluded in this work (23, 48, 49). Evidence of inflammation included early neutrophil and macrophage infiltration into the naive mouse lung, whereas elevated concentrations of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α] and interleukin-8 [IL-8]) were observed within BAL fluid of newborn ferrets with CF (compared to healthy animals) (23, 48, 49).

Congruent with these findings in animal models of CF lung disease, BAL fluid taken from 4-week-old CF patients who were culture negative for common CF pathogens contains higher concentrations of relevant proinflammatory cytokines (e.g., IL-6, IL-8, TNF-α, and arachidonic acid derivatives) than in BAL fluid from healthy controls (1, 24, 50–52). CF patients also show reduced concentrations of anti-inflammatory mediators (e.g., IL-10, lipoxin, and docosahexaenoic acid) within BAL fluid compared to healthy patients (1, 24, 50–52). The concentration of glutathione, a critical antioxidant, is reduced within CF airways, whereas CF immune cells overproduce reactive oxygen species (ROS), thereby predisposing patients to greater tissue damage due to oxidative stress (53–55). Given these findings, a critical balance between proinflammatory/anti-inflammatory mediators and oxidant/antioxidant molecules is dysregulated within the CF lung at baseline.

There are multiple proposed mechanisms for how basal inflammation in CF promotes subsequent bacterial infection. One potential explanation is that excess production of proinflammatory cytokines within the airway is responsible for robust, early recruitment of inflammatory cells, specifically neutrophils (further discussed in detail below) (56). Activated neutrophils release multiple products that drive CF lung pathology and inadvertently predispose patients to infection, including serine proteases (e.g., neutrophil elastase) (57). Neutrophil proteases can degrade vital innate immune antimicrobials, including AMPs such as defensins, and even components of the complement system, thus contributing to a secondary vulnerability to bacterial infection (41, 58).

Altered Effector Functions of Immune Cells: the Pathological Role of Neutrophils in the CF Lung

Intrinsically diminished or altered effector functions of both innate and adaptive immune cells in CF patients may also contribute to reduced clearance of opportunistic pathogens and early damage to CF lung tissue (59). Neutrophils normally represent the critical frontline innate immune response to infection: activation of neutrophils via stimulation of their pathogen recognition receptors (PRRs) results in these cells employing versatile effector functions, including phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation (NETosis), a form of programmed cell death that results in the release of DNA and other intracellular contents, to respond to intruders (59, 60). Neutrophil granules contain a wide variety of antimicrobials, including AMPs such as cathelicidins (e.g., LL-37), serine proteases, and enzymes such as myeloperoxidase (MPO), which aid in the synthesis of chlorine-containing ROS (e.g., hypochlorous acid [HOCl]) (61, 62). Neutrophil granular constituents (which can be deployed intracellularly within phagosomes or released extracellularly via degranulation) normally function to clear infection and modulate the immune response (61, 62). However, under chronic inflammatory conditions and when produced in excess, many of these same compounds can have directly toxic effects on host tissues (63).

In CF, neutrophils play a primary role in driving inflammation and tissue damage within the lung (64). Excessive influx of neutrophils into the CF lung (without the concomitant capacity to clear infection) is a histopathological hallmark of the disease; in fact, neutrophils represent the most abundant immune cells within CF lungs (65–67). The presence of neutrophilic inflammation has been noted in a study of CF fetal tissue,

suggesting that these immune cells may be recruited early and even influence the prenatal, sterile CF lung environment (68). Under *in vitro* conditions (in the absence of bacteria), with buffer control or lipopolysaccharide (LPS) stimulation, airway- and blood-derived neutrophils from children with CF secrete significantly more IL-8 than neutrophils from children with non-CF lung disease; given that IL-8 is a potent neutrophil chemokine, CF neutrophils likely contribute to their own abundant recruitment to the lung (69). Moreover, early elevation of IL-8 concentrations within the CF airway likely serves not only to recruit neutrophils but also to promote degranulation and production of ROS that can have deleterious consequences for lung tissue (as discussed below) (59).

Another critical factor which may promote profuse neutrophil migration to the CF lung is high levels of circulating selectins (compared to those in healthy patients); E- and P-selectins mediate the first step of transendothelial migration of neutrophils from circulation into the lung (70). Importantly, although elevated levels of cytokines and circulating selectins may mediate early recruitment of neutrophils to the CF lung, the presence of these cells within the lung throughout the course of disease is likely sustained by acute and chronic bacterial infection (i.e., via the release of pathogen-associated molecular patterns [PAMPs], which can directly stimulate inflammatory responses) (71).

A further complication is that CF neutrophils demonstrate reduced apoptosis (likely secondary to high levels of granulocyte-macrophage colony-stimulating factor [GM-CSF] and reduced levels of IL-10 within the airway) and release more damaging proteolytic compounds than healthy neutrophils, including neutrophil elastase (NE), a highly reliable prognostic indicator in CF patients (72–74). Levels of NE correlate strongly with a more rapid decline of pulmonary function as determined by pulmonary function tests (PFTs) as well as the development of bronchiectasis, the abnormal enlargement of airways that is a key manifestation of obstructive lung disease in CF (17, 18, 75). Intracellular NE breaks down phagocytosed proteins (e.g., bacteria and bacterial products) but, when released into the extracellular milieu, can also directly degrade lung connective tissue (e.g., collagen and elastin) in addition to innate immune effectors, such as complement proteins, as described above (64, 70). NE also directly stimulates the airway epithelium to release IL-8 (76).

Additionally, neutrophils undergo significant NET formation or NETosis within the CF airway, a type of programmed cell death that results in the extracellular release of chromatin (i.e., DNA and histones) as well granular antimicrobial contents (e.g., NE and MPO) (77, 78). NETosis can be stimulated by cytokines (e.g., IL-8 and TNF-α) and/or microbial infection (e.g., bacterial PAMPs such as LPS), and the ultimate manifestation of this process is thought to be a final, "frustrated," suicidal attempt by neutrophils to clear an infection that has otherwise proven to be resistant to phagocytosis (79). While the primary objective of NETosis in killing bacteria and clearing bacterial infections fails within the context of CF, due to the adaptable and diverse abilities of CF pathogens to evade the host response, secondary, harmful consequences of NETosis on host tissues likely occur (77, 79). For instance, the presence of abundant free DNA within the CF airway is associated with obstruction (i.e., more viscous airway secretions) and decline of pulmonary function (80, 81); accordingly, treatment of CF patients with dornase alpha (DNase) reduces DNA recovery from airway specimens as well as the rate of pulmonary function loss (82, 83). Levels of other NET-associated products, including NE and MPO, have also been associated with poor outcomes (17, 18, 75, 84). A recent study mechanistically linked greater production of NETs by CF neutrophils (than by healthy neutrophils) to reduced apoptosis/longer life spans of these cells (85).

Some have argued that compared to healthy neutrophils, CF neutrophils exhibit other aberrant effector functions, including reduced phagocytosis and degranulation as well as impaired chlorination (i.e., ROS production) of the phagolysosome (86–88). These CFTR-dependent defects in neutrophil function ultimately result in abrogated bacterial killing. While the presence of CFTR in neutrophils was once the subject of debate, the chloride channel has now been localized to phagolysosomes of neutrophils,

and its dysfunction has been directly implicated in reduced chlorination of phagocytosed bacterial products (88). In another elegant study, CFTR dysfunction was shown to disrupt sodium and chloride flux within the neutrophil, resulting in reduced degranulation of secondary and tertiary granules (87). Notably, neutrophils derived from patients treated with the novel CFTR potentiator ivacaftor (which improves chloride flux through CFTR in patients with a specific mutation, G551D) showed partial correction of the degranulation defect and improvement in bacterial killing; these data suggest that CFTR dysfunction is directly tied to reduced degranulation of neutrophils (87). In part, this set of work explains why, despite an excessive influx of neutrophils into the CF lung, the primary objectives of surveillance and clearance of pathogens by these cells remain unfulfilled.

Relevant to this discussion, the primary impairment of neutrophil effector functions in CF is not completely established and remains an area of controversy (59). Some studies have directly contradicted the above-mentioned evidence, suggesting instead that CF neutrophils are not deficient in phagocytosis, the production of ROS, or even NET formation (which is an oxidative-burst-dependent process) (59, 89, 90). In other disorders with primary dysfunction of neutrophils, such as chronic granulomatous disease (CGD), there is an expected and observed predilection for invasive, systemic infections; in contrast, in CF, infecting bacterial populations remain localized to the lung, rarely causing disseminated infections (25). This clinical observation suggests that any defects of neutrophil function in CF are likely to be less overt than in other diseases.

Importantly, although neutrophils are understood to be key players in CF lung disease, altered effector functions of CF macrophages and even adaptive immune cells, such as T cells, illustrate that primary immune dysfunction in CF encompasses multiple components of the host defense apparatus (91–93). For instance, autophagy, which is a critical homeostatic and innate immune mechanism, is impaired in epithelial cells and macrophages derived from CF patients (94). In healthy cells, autophagy is induced upon starvation and results in the encapsulation of cytosolic contents within double-membrane compartments known as autophagosomes (95). Autophagosomes subsequently fuse with lysosomes, resulting in the enzymatic degradation of their cargo into substrates for central metabolism (e.g., free amino and fatty acids) (95). Importantly, autophagy also recycles damaged mitochondria (i.e., a source of endogenous oxidative stress), removes aggregated/nonfunctional proteins, and clears phagocytosed and/or intracellular pathogens as well (94–96). Dysfunctional autophagy has been implicated in various diseases in human pathophysiology, including CF (97).

Although the nexus between impairment of CFTR function and abrogated autophagy remains unclear, there are several working hypotheses, which include the following: (i) proteins of the autophagic machinery are sequestered within cytosolic aggregates consisting of misfolded CFTR proteins, and (ii) CF cells exhibit an accumulation of certain microRNAs, which specifically downregulate the expression of genes encoding autophagy proteins (94). Ultimately, dysregulated autophagy in CF results in an increased production of inflammatory cytokines, greater endogenous ROS production, as well as a reduced capacity to clear bacterial infection (as demonstrated in CF macrophages *in vitro* and CF mouse models *in vivo*) (94–96). As such, perturbation of autophagy represents another example of a CFTR-intrinsic "hit" to innate immune defenses, promoting both immunopathology and a predisposition to opportunistic infection.

In brief summary, within the hyperinflammatory milieu of the CF lung, the secretion of various neutrophil-derived antimicrobials, including the above-mentioned proteases (e.g., neutrophil elastase), cationic peptides, extracellular DNA (eDNA), and ROS, likely contributes to the progression and exacerbation of disease. Many of these neutrophil effectors are released in an unsuccessful attempt to clear bacterial infection but have secondary, inadvertently damaging effects on host tissues. In the following section, we focus on two neutrophil antimicrobials that are implicated in CF lung disease: LL-37, a cationic AMP, and reactive oxygen species, including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

LL-37, an Antimicrobial Peptide with Bactericidal and Immunomodulatory Actions

AMPs are defined as gene-encoded small peptides with antimicrobial activity; this definition specifically excludes innate immune proteins with hydrolytic or enzymatic activity (e.g., lysozyme and chitinases) (98). These small peptides, which have been conserved across eons of natural selection and across diverse taxa of organisms, play a vital role in the innate defense against microbial infection; multiple AMPs exhibit broad killing capacity against an assortment of microbes, including Gram-positive/Gram-negative bacteria, viruses, fungi, and protozoa (99). AMPs have been identified and studied in multiple plant, insect, and mammalian species: in plants and insects especially, AMPs are thought to play an essential role, given the lack of a complex adaptive immune system in these organisms (100). Various forms of AMPs have also been described in animals, in both mammalian and nonmammalian species (99).

Importantly, although bacteria are an often-studied target of AMPs that are produced by more-complex organisms, bacteria are also known to produce their own AMPs, known as bacteriocins (e.g., pyocins of *Pseudomonas aeruginosa*) (101, 102). Bacteria that express bacteriocins are able to kill and outcompete susceptible members of their own (or very closely related) species; as such, bacteriocins are tools for intraspecies competition within an ecological niche for limiting resources, e.g., space and nutrients, etc. (101, 102). The pyocins of *P. aeruginosa* may have additional functionality as well. The R/F-pyocin cluster of *P. aeruginosa* has recently been implicated in bacterial autolysis and eDNA release, which is important for bacterial adherence, biofilm formation, and antibiotic resistance (see below for further discussion of the *lys*-encoded endolysin) (103).

Human AMPs represent crucial components of the body's innate immune or rapid response to pathogens. These peptides are largely synthesized and secreted by the epithelia of multiple organ systems (e.g., skin, respiratory, ocular, and gastrointestinal epithelia, etc.) as well as by phagocytic cells, including neutrophils, wherein they are stored within cytoplasmic granules (104). The classification system for AMPs is loosely based on the amino acid composition and secondary structure of these peptides; for example, peptides may be classified by whether they have a linear, alpha-helical structure; the presence of disulfide bonds that maintain beta sheet conformation; or an overrepresentation of a single amino acid (99, 105). Nonetheless, there are some structural and charge commonalities among human AMPs: these peptides are typically cationic (net positive charge) with amphipathic regions (i.e., composed of both hydrophobic and hydrophilic amino acids), which aid in binding or embedding within the negatively charged membranes of pathogens (99). AMPs are also typically synthesized as propeptides, with a highly conserved N-terminal region (often containing a signal peptide for trafficking to the endoplasmic reticulum for secretion) and a variable C-terminal region that exhibits antimicrobial activity; the propeptides are often degraded by endogenous proteases into their final form prior to secretion (106).

Additionally, there are several proposed mechanisms for the antimicrobial activity of AMPs. Two commonly cited models for bacterial killing are the toroidal pore-forming model and the carpet model (107). Both mechanisms rely on the binding of cationic, amphipathic peptides to predominantly negatively charged membranes of bacteria, leading to membrane disruption, loss of osmotic balance, and cell lysis (99). The toroidal pore-forming model is initiated by aggregation of AMPs at the membrane, induction of membrane curvature, and subsequent formation of pores (with the peptides and hydrophilic phosphate heads of lipids within the membranes forming the channels of the pores); the pores promote leakage of intracellular contents and, ultimately, membrane rupture (108, 109). The carpet model manifests as significant local accumulation of AMPs ("carpeting") on the membrane, followed by disruption of the membrane in a detergent-like manner; the bacterial membrane disintegrates into micellar structures, covered with the peptides (107, 110). There is additional evidence that at sublethal concentrations, AMPs can traverse bacterial membranes and have toxic intracellular effects by disrupting macromolecular synthesis (111, 112).

To date, three main classes of AMPs have been described in humans: defensins, histatins, and LL-37, the only known human cathelicidin (113, 114). Defensins, which exhibit a beta sheet structure, cross-linked by disulfide bridges, are broken up into two subfamilies: α-defensins (including HNP1 to -4, HD5, and HD6) and β-defensins (hBD1 to -4) (114). The α-defensins HNP1 to -4 are predominantly synthesized and stored in neutrophil azurophilic granules, whereas HD5 and HD6 are secreted by the gastrointestinal epithelium (115, 116). β-Defensins (hBD1 to -4) are largely synthesized and secreted by the epithelia of multiple organs (e.g., respiratory, skin, gastrointestinal, and ocular epithelia) in response to infectious or inflammatory stimuli (115, 116). The histatins are histidine-rich peptides present in human saliva with significant antimicrobial activity against fungal species (117).

Although a detailed discussion of the roles of defensins and histatins in human biology is out of scope here, there are a few salient aspects of the regulation and importance of defensins in innate immunity that are worth mentioning in the context of CF. The α-defensins (HNP1 to -4) are constitutively expressed, released from neutrophils upon degranulation, and found in high concentrations under infectious/inflammatory conditions (113); within CF airway specimens, the concentrations of HNP1 to -3 are high enough not only to kill pathogens but also to be cytotoxic to mammalian cells (118). The release of β-defensins from the epithelium (specifically, hBD2 to -4) is induced by infectious and inflammatory (cytokine) stimuli, and the role of hBD3 in the early pathophysiology of CF lung disease, i.e., its presence and reduced antimicrobial efficacy in acidic ASL, has already been discussed above (37). The efficacy and expression of hBD1 and hBD2 are reduced within the CF airway, creating a vulnerability to opportunistic pathogens such as *P. aeruginosa* (119–121). Deletion or depletion of defensins in mouse models of other diseases (outside the context of CF) has also established the importance of these AMPs in protection from bacterial infection (98). Finally, apart from their antimicrobial functionality, defensins (like other AMPs) also have immunomodulatory actions, implicated in both pro- and anti-inflammatory contexts (122).

The cationic AMP that has been the focus of recent studies by our group is LL-37/hCAP18, the only known human cathelicidin. All cathelicidins, which have been identified in numerous mammalian species, have common structural hallmarks: each has a highly conserved N-terminal domain, which is composed of a signal sequence and a conserved region, termed "cathelin" (cathepsin L inhibitor), and a C-terminal domain, which is released after peptide cleavage, can be of various lengths, and exhibits the antimicrobial activity of the peptide (117). The gene encoding LL-37/hCAP18 is located on chromosome 3, in close proximity to genes encoding MyD88 and Toll-like receptor 9 (TLR9), suggesting potential coregulation of LL-37 expression with genes involved in proinflammatory responses (36). This gene directly encodes the precursor peptide hCAP18, which is subsequently cleaved to release the C-terminal AMP LL-37, so named because it consists of 37 amino acids, beginning with two leucine residues; cleavage occurs immediately before release from neutrophil granules, likely via an endogenous serine protease, proteinase-3 (123). Although LL-37 was first localized within neutrophil-specific granules as well as epithelia (e.g., respiratory and skin epithelia), it has now been found to originate from other immune cells as well, including monocytes, lymphocytes, and NK cells (36, 124).

LL-37 synthesis is induced by proinflammatory cytokines (e.g., TNF-α and IL-6), explaining its abundant presence at sites of infection and inflammatory responses (36, 125). The LL-37-encoding gene also contains vitamin D-responsive elements, theorized to connect the role of LL-37 to autoimmunity (which is correlated with vitamin D deficiency and predisposition to infections) (126); this finding also points to the role of LL-37 in modulation of immune responses (discussed further below).

As an antimicrobial, LL-37 is broadly efficacious against Gram-positive and Gram-negative bacteria, including CF-relevant and antibiotic-resistant pathogens (127); *in vitro* studies have also demonstrated the efficacy of LL-37 in inhibiting *P. aeruginosa* biofilm formation (128). Furthermore, LL-37 synergizes with other host-derived airway

antimicrobials in killing bacteria (e.g., defensins and lysozyme) (35, 124, 129, 130). There are multiple posited mechanisms by which LL-37 directly kills bacterial cells: there is evidence in the literature for the carpet model of bacterial killing (131) as well as for a modified, toroidal pore-forming model (132, 133), both of which compromise bacterial membrane integrity, resulting in lysis. There is additional evidence that LL-37 preferentially attacks septating (actively growing/dividing) bacterial cells, thereby inhibiting bacterial proliferation (134).

Apart from its role as an antimicrobial (and similar to other AMPs discussed above), LL-37 is also known to modulate the immune response. There are multiple known proinflammatory actions of LL-37. LL-37 treatment of epithelial cells results in the release of IL-8, likely contributing to neutrophil recruitment and subsequent inflammation (135). LL-37 also contributes to the recruitment of immune cells to sites of inflammation by directly binding to a common receptor on neutrophils, monocytes, and T cells called formyl peptide receptor-like 1 (FPRL1) (136). Furthermore, LL-37 directly stimulates neutrophils to release ROS, α-defensins, and IL-8 (137). Conversely, there are multiple anti-inflammatory roles of LL-37 as well. The most well studied is the capacity of LL-37 to bind LPS and thereby reduce LPS-stimulated proinflammatory cytokine secretion and ROS production by macrophages *in vitro* (138). By extension, the LPS-binding ability of LL-37 reduces mortality in a mouse model of endotoxic shock (i.e., systemic LPS injection coupled with LL-37 delivery) (139).

LL-37 is known to be overproduced within the CF airway: LL-37 has been quantitated within CF airway secretions at high, bactericidal levels, with concentrations of up to 30 μg/ml (119, 140). Studies have found that the LL-37 concentration in CF correlates with the level of inflammation (total cell number and total number of neutrophils present within BAL fluid) (119). However, despite the high concentration of LL-37 within CF patient BAL fluid, LL-37 bactericidal activity may be affected by proteolysis (due to neutrophil elastase and cathepsin D) and/or complexation of the peptide with polyanionic glycosaminoglycans (e.g., heparan sulfate and hyaluronic acid) within the airway (140). In addition to inhibiting the activity of hBD3, low pH has been shown to inhibit the antimicrobial activity of LL-37 and the synergism between LL-37 and other airway antimicrobials (37, 129); given that CF ASL pH tends to be acidic, this suggests that the antimicrobial activity of LL-37 may be inhibited by the airway pH in CF patients, compromising a key component of innate immune defense.

On a structural level, this inhibition is thought to be because the alpha-helicity of LL-37, which is directly correlated with its antimicrobial activity, is reduced under acidic conditions (38). However, this effect on LL-37 structure occurs at pH $<$5, and CF airway pH, based on the above-mentioned pig model and human studies, seldom reaches this threshold (32, 34). Furthermore, other work in the field, including a recent study by Schultz et al. employing a novel fiber-optic pH probe, showed no difference in the pH of ASL derived from healthy and CF children (141, 142). These results suggest that the impact of reduced pH on antimicrobials in CF lungs and perhaps the role of acidic pH in early pathogenesis of CF lung disease, a critical finding from fundamental studies in the pig model, remain a source of some debate (143). These conflicting findings could be explained by differences in the ages of CF patients enrolled in each study. Whereas CF ASL acidity was demonstrated using specimens from human neonates (or newborn pigs), Shultz et al. and others found no pH differences in ASL from older children or adults with CF compared to healthy donors (33, 34, 141, 142). Thus, it is possible that while the very primitive CF airway represents an acidic environment with a corresponding, early compromise of innate immunity (including AMPs such as LL-37), pH perturbation may be abolished with age, wherein other mechanisms would subsequently explain immune dysfunction later in life.

Regardless, the importance of LL-37 in host defense against CF-relevant pathogens has been demonstrated in mouse models of disease. Overexpression of LL-37 within the airway via an adenovirus (AdV) vector reduced the *P. aeruginosa* burden and the resultant proinflammatory response after bacterial challenge (139). Additionally, in a CF mouse tracheal xenograft model, overexpression of LL-37 within CF xenografts restored

killing of *P. aeruginosa* and *S. aureus* by ASL derived from the tracheal grafts (compared to ASL derived from healthy grafts) (144). Outside the context of CF, expression of the mouse analog of LL-37, CRAMP (cathelicidin-related antimicrobial peptide), is protective against invasive skin infections due to *Streptococcus pyogenes*, demonstrating the versatility and relevance of the peptide in innate defense against multiple infectious diseases (145).

Notably, while LL-37 may be present at bactericidal concentrations within the CF airway, bacteria have developed tools to evade AMP stress. AMPs were thought to attack a critical, conserved vulnerability, often called an "Achilles' heel," of prokaryotic species (i.e., the negatively charged outer leaflet of their cell membranes), thereby explaining broad efficacy against Gram-positive and Gram-negative species and making it unlikely that bacteria might develop resistance against AMPs (132). This theory has changed over time as numerous bacterial mechanisms of resistance against AMPs have been elucidated (146). These mechanisms include the bacterial capacity to alter the membrane charge (thereby reducing AMP binding) and the expression of AMP efflux systems (146). Moreover, LL-37 at sublethal concentrations has been shown to promote bacterial mutagenesis, thereby directly contributing to bacterial pathoadaptation within the host (147, 148). Here, we further explore bacterial defense against AMPs (and other innate antimicrobials) as well as the contribution of these antimicrobials to bacterial mutagenesis in the context of *P. aeruginosa* mucoid conversion (see below).

Reactive Oxygen Species

Reactive oxygen species (ROS) are a vital component of the oxygen-dependent microbial killing function of phagocytic cells such as neutrophils. Unfortunately, while the intended action of ROS production is clearance of bacteria (and other microbes), ROS can nonspecifically target host tissues and thus can also play a pathophysiological role in settings of chronic inflammation, including within the CF airway.

Endogenous sources of ROS include aerobic respiration (i.e., oxidative phosphorylation) in mitochondria. Electrons from the electron transport chain (ETC) can directly reduce oxygen (O_2) to superoxide ($O_2^{\cdot -}$), which can then undergo dismutation via superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2) (149). Typically, the very low concentration of superoxide and peroxide produced via the ETC can be sufficiently detoxified via intracellular antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase, and therefore do not play a pathophysiological role in most diseases (149). However, a more significant source of ROS within eukaryotic species is the oxidative/respiratory-burst response of phagocytes (150). Neutrophils have been prototypically studied to understand the origins of ROS as host-derived antimicrobials, although eosinophils and monocytes/macrophages also represent important sources of ROS within the innate immune apparatus of the host (29). Although not discussed here, via a mechanism different from ROS production, phagocytes also generate reactive nitrogen species (RNS), which are also potent free-radical antimicrobials (151).

The importance and mechanism of NADPH oxidase-dependent ROS generation within neutrophils are well established. While one isoform of the NADPH oxidase, Nox2, is present in neutrophils (and other phagocytes), other homologs of this system are expressed more broadly across tissues in the body, including Duox1 and Duox2, which are found on the respiratory epithelium (Duox1 and -2 are discussed below in the context of the CF lung) (152–154).

Nox2, a multiprotein complex, includes membrane-associated proteins (gp91phox and p22phox) as well as cytosolic protein components (p67phox, p47phox, p40phox, and RacGTP) (155, 156). In neutrophils at rest, the cytosolic components of Nox2 are present in inactive complexes within secondary granules (152). Neutrophil activation by the engagement of TLRs with microbial products (e.g., LPS and flagellin, etc.) directs opsonophagocytosis of microbes (via complement or Fc receptors), or exposure to proinflammatory cytokines can trigger the ROS burst response in these cells (63, 157). Upon activation of the neutrophil, secondary granules fuse with both the plasma and phagolysosomal membranes; the cytosolic Nox proteins subsequently undergo phos-

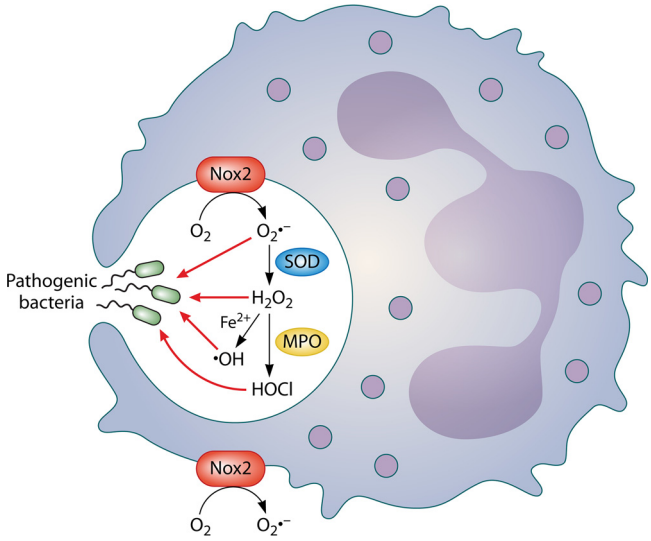

FIG 1 Simplified model for intracellular and extracellular generation of reactive oxygen species (ROS) by phagocytes. Molecular oxygen is converted by NADPH oxidase 2 (Nox2) to superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 may then be converted to either hydroxyl free radical ($^{\cdot}OH$) (via Fenton chemistry in the presence of ferrous iron [Fe^{2+}]) or halogenated ROS, such as hypochlorous acid (HOCl), via myeloperoxidase (MPO). Nox2 is localized within phagosomes as well as on the cell membrane (158).

phorylation and translocation to both membranes, where they bind to the membrane-associated proteins of Nox2, making the complex functionally active (152, 155, 156). Functional Nox2 then directly couples the oxidation of NADPH to the reduction of oxygen (O_2) to superoxide ($O_2^{\cdot-}$) (Fig. 1): stoichiometrically, the oxidation of one molecule of NADPH liberates two electrons, which can then generate two molecules of $O_2^{\cdot-}$ (158).The orientation of the complex (and, as such, the secretion of $O_2^{\cdot-}$) is aimed extracellularly at the plasma membrane and toward the internal contents of a phagolysosome (152). $O_2^{\cdot-}$ is then used as a substrate to generate multiple forms of ROS via a stepwise process, as discussed below.

Importantly, CGD is the inheritance of a nonfunctional NADPH oxidase (impairing ROS production and ROS-dependent processes such as NET formation) that leads to a predisposition for life-threatening, invasive bacterial and fungal infections (e.g., predominant causative agents include *S. aureus* and *Aspergillus fumigatus* [63]). As such, CGD indicates the essential role of the Nox complex and intact ROS production for innate immune defense against certain pathogens (159).

Multiple forms of ROS can be synthesized within the phagosome of neutrophils in a stepwise fashion (Fig. 1): $O_2^{\cdot-}$ generated by Nox2 can spontaneously dismutate to form H_2O_2 (via the consumption of 2 hydrogen ions under acidic conditions), or this reaction can be catalyzed by the host enzyme SOD (149). H_2O_2 formed in this manner can act directly on engulfed bacteria or be converted to other forms of ROS. MPO (which enters the phagosome via fusion with primary granules) catalyzes the conversion of H_2O_2 to halogenated ROS, represented predominantly by hypochlorite (HOCl) within the phagosome (150). Via Fenton chemistry, in the presence of iron (Fe^{2+}), H_2O_2 can also be converted to hydroxyl radical ($^{\cdot}OH^{-}$), a very potent antimicrobial (149).

The primary function of ROS production within neutrophils is killing of engulfed pathogens. There are oxidative and nonoxidative means by which ROS produced within the phagosome contribute to bacterial killing, and here, we primarily focus on H_2O_2 and HOCl, which were the subjects of our work. H_2O_2 exhibits a bimodal killing pattern, causing significant bacterial killing *in vitro* at very low (0.15 mM) and very high (25 mM) concentrations but reduced killing at intermediate concentrations (160). The primary mechanism of H_2O_2 killing at low concentrations is DNA damage (mode 1 killing): this mode of killing depends on the presence of iron (which generates

secondary ROS, likely via Fenton chemistry), promoting the oxidation of guanine bases (i.e., 8-hydroxyguanine production), which is likely to be mutagenic/toxic (161, 162).

In contrast, mode 2 killing by H_2O_2 (i.e., at high concentrations) can likely target any bacterial macromolecule (161). For instance, ROS can target bacterial proteins, resulting in denaturation and loss of function: iron/sulfur clusters, due to their abundance, rapidly react with ROS (149). Aromatic and sulfur residue-containing amino acids can also react with ROS to produce protein carbonyls, which serve as reactive intermediates that damage other targets (152, 158). Lipid peroxidation of bacterial cell membranes, resulting in membrane damage, has been observed following ingestion by phagocytes, suggesting that the bacterial cell membrane is an important site of ROS action within the phagosome (163). Lipid peroxidation can also produce ketones and aldehyde intermediates that can mediate further oxidative damage to secondary targets as well (149).

Despite being recognized as a potent form of ROS, the exact mechanism of bacterial killing via HOCl remains poorly defined. Proteins are likely to be the main target, as indicated by chlorination of amino acid residues (e.g., chlorination of tyrosine) in the presence of HOCl; sulfur-containing amino acids (e.g., cysteine and methionine) are thought to be other likely targets, based on high *in vitro* reaction rates (158). In *Escherichia coli*, HOCl causes lethal DNA damage as well (164).

Importantly, ROS produced within the phagosome facilitate nonoxidative means of bacterial killing as well. When superoxide is generated within the phagosome, there is a compensatory influx of potassium to balance the negative charge of superoxide; this potassium influx activates neutrophil enzymes (e.g., elastase) that are able to proteolytically attack phagocytosed bacteria (165). The ROS burst is also a vital precursor to NET formation, which facilitates trapping and killing of bacteria via extrusion of DNA and associated proteins (e.g., elastase, MPO, histones, and LL-37) (165).

For *in vitro* bacterial susceptibility testing, the use of ROS concentrations that are most physiologically representative remains a source of some debate within the literature. Although this is a controversial area, most agree that there are differences in ROS concentrations within the extracellular milieu of phagocytes versus those within the phagosome. Some studies have shown that bacteria *in vivo* are likely to be exposed to H_2O_2 concentrations ranging from 12 μM within the extracellular environment (released by stimulated neutrophils) to 100 mM within the phagolysosomal compartment (166). This is substantiated by other work that suggests that the rates of ROS generation within the phagosome for both superoxide, the direct precursor of peroxide, and HOCl are several millimoles per minute (167, 168). Indeed, most *in vitro* investigations of bacterial susceptibility to many forms of ROS, including H_2O_2, have been performed with ROS concentrations in the millimolar range, mimicking the intraphagosomal setting (161).

As stated above, the overabundance of ROS within the CF airway is attributed to the chronicity of the inflammatory process (i.e., driven by bacterial infection and a continuous influx of neutrophils) as well as an underlying imbalance between ROS and endogenous antioxidants (e.g., glutathione) (54). There is abundant evidence for overproduction of ROS in the CF lung, including multiple studies that have employed specimens derived directly from patients to study markers of oxidative stress (169–176). Studies seldom include the direct measurement of ROS but rather seek to measure oxidized macromolecules as a surrogate for oxidative stress. These markers include hallmarks of lipid peroxidation (e.g., lipid hydroperoxides, 8-isoprostane, 4-hydroxynonenal, and malondialdehyde), protein oxidation (e.g., protein carbonyls, glycophore formation, and chlorinated/halogenated amino acids), DNA oxidation (e.g., 8-hydroxydeoxyguanosine), and neutrophil enzymes that directly participate in ROS formation (e.g., MPO) (169–177).

Many of these studies have shed light on the role of ROS in CF pathogenesis. Compared to healthy patients, concentrations of ROS markers are significantly elevated in lung specimens (i.e., sputum or BAL fluid) from CF patients (169, 170, 173, 175, 176).

This elevation in oxidative stress within the airway can be noted in children as young as 2 to 3 years old, suggesting that ROS overproduction is part of the early events of disease development (175, 176). Concentrations of ROS markers are further elevated when CF patients are infected with *S. aureus* and/or *P. aeruginosa*, suggesting that bacterial infection is a key determinant of oxidative stress in CF (171, 175, 176). Furthermore, concentrations of systemic markers of oxidative stress (within venous blood) are significantly elevated during pulmonary exacerbations and reduced with treatment, indicating a clear connection with the changing inflammatory/infectious status of CF patients (174). Perhaps most importantly, there is an inverse correlation between markers of oxidative changes within the lung and the pulmonary function of CF patients, drawing a nexus between ROS and lung tissue damage (172).

There are three main sources of ROS production in the CF lung: neutrophils, the airway epithelium, and other immune cells, such as macrophages. Given the predominance of neutrophilic inflammation within the CF lung, neutrophils are thought to be the primary source of ROS, and there are two studies that support this argument. In both studies, the concentrations of markers used as a surrogate for oxidative stress (e.g., halogenated tyrosines and protein carbonyls) directly correlated with the number of neutrophils and neutrophil-associated proteins in young children with CF (i.e., NE and MPO); CF patients also had higher concentrations of ROS markers than non-CF controls (175, 176). While there was a correlation between ROS markers and neutrophils, there was also a strong relationship in both studies between ROS within the airway and infection, particularly with *P. aeruginosa* (175, 176). Both studies thus conclude that infection is a critical driver of neutrophil-derived ROS production within the CF airway.

The CF airway epithelium is another important source of ROS. Alveolar type II epithelial cells can directly produce ROS via Nox homologs, Duox1 and Duox2 (i.e., dual oxidases) (152, 153). These protein complexes are responsible for continuous H_2O_2 production (whereas the direct product of Nox is superoxide), even in the absence of infection in CF airways (152). H_2O_2 generated via Duox complexes can further be used as a substrate (along with thiocyanate [SCN^-]) by lipoperoxidase (LPO) to generate hypothiocyanite ($OSCN^-$), another form of antimicrobial ROS (153). In CF, it is theorized that before the establishment of chronic bacterial infection, H_2O_2 generated by the Duox complexes represents the primary source of ROS; however, following chronic infection, Nox-dependent generation of ROS by phagocytes (primarily polymorphonuclear leukocytes [PMNs]) predominates (54).

Other phagocytic cells within the airway represent the third main source of ROS in CF patients. Nox2 is also expressed in macrophages, which play a role in the phagocytic, innate immune response to bacterial infection within the CF lung (152, 178). Thus, macrophages are likely to be another source of ROS in CF.

A suggested primary defect in the transport of glutathione, a critical endogenous antioxidant, likely contributes to ROS stress within the CF airway. Glutathione exists in a reduced form (GSH) that is oxidized (GSSG) by oxidizing agents such as ROS; GSH is regenerated from GSSG via the pentose phosphate shunt (i.e., through NADPH oxidation) (179). CFTR is responsible for the release of reduced and oxidized forms of glutathione in *in vitro* studies using a kidney cell line (stably expressing normal CFTR) (180). Furthermore, CFTR mutation in airway epithelial cells (with the homozygous ΔF508 mutation) reduces the extracellular transport of glutathione without affecting the intracellular synthesis of glutathione; conversely, restoration of CFTR in the $CFTR^{-/-}$ cell line (via transfection) increases the extracellular release of GSH, suggesting that aberrant transport of GSH is specific to the CFTR protein (181). Additionally, pancreatic insufficiency in CF patients results in reduced intake/absorption of fat-soluble, dietary antioxidants (e.g., vitamin E and carotenoids), likely contributing to the primary oxidant/antioxidant imbalance in CF patients (54).

Although the primary target of ROS produced within the CF airway is pathogenic microbes, these organisms are adept at evading ROS stress. Specifically, bacteria that infect CF patients have developed multiple strategies to neutralize oxidative species,

including expression of antioxidants (e.g., catalase, superoxide dismutase, and peroxidases, etc.), induction of transcriptional stress responses (e.g., via global oxidative stress regulators such as *oxyR*), and activation of DNA repair machinery (i.e., to correct lesions due to free radicals that would otherwise prevent replication) (162, 182). Additionally, similar to LL-37, sublethal concentrations of ROS can promote bacterial mutagenesis, leading to adaptations that may also facilitate evasion of ROS stress (183). Many of these versatile pathways in bacteria to detoxify ROS are discussed in the context of *P. aeruginosa* mucoid conversion and evasion of innate immunity (see below).

However, one of the implications of bacteria thriving within an ROS-rich environment in CF is that chronic infection perpetuates the inflammatory response; this leads to an even greater production of ROS, which ultimately overwhelm endogenous host antioxidants (e.g., glutathione), resulting in off-target effects. Indeed, ROS can act as second messengers directly on the airway epithelium to facilitate proinflammatory responses. IL-1β activation of NF-κB depends on intracellular H_2O_2, which can accumulate within the extracellular milieu of the CF airway and freely diffuse across cell membranes (184). Relatedly, another study shows that CF epithelial cells amass intracellular H_2O_2 due to reduced expression of Nrf-2, a transcription factor that is central to the antioxidant response. As such, the CF epithelium shows intrinsically reduced expressions of several host antioxidants (e.g., thioredoxin, peroxiredoxins, catalase, and glutathione) but increased expression of superoxide dismutase, thus explaining the intracellular accumulation of H_2O_2. Increased intracellular H_2O_2 then directly increases IL-6 and IL-8 secretion by the CF epithelium, thereby linking excess ROS production, dysregulation of antioxidant responses, and the proinflammatory environment of the CF lung (185).

In addition to promoting inflammation within the lung, ROS can directly damage host tissues via the oxidation of macromolecules: lipids, DNA, and proteins (i.e., the same ways in which these oxidants are toxic to pathogens) (54). Prolonged ROS exposure can induce lipid peroxidation, compromising membrane integrity and causing DNA strand breaks, resulting in mutagenesis or cell death and alteration of protein structure/function (186). Different forms of ROS affect the host by distinct mechanisms; for example, H_2O_2 has been linked to ATP depletion in the airway epithelium, whereas HOCl is implicated in intracellular protein damage via sulfhydryl group oxidation and protein carbonyl formation (187).

Although the collateral effects of ROS illustrate how immune dysregulation (i.e., hyperinflammation) can damage the CF airway, infection is also a critical driver of lung pathology in CF patients. In the following section, we discuss the role of bacterial infection in CF pathogenesis, specifically in the context of early events of disease manifestation.

The "Chicken-and-Egg" Counterargument: Bacterial Infection Precedes Inflammation

The "chicken-and-egg" question of what comes first in the CF lung, inflammation (i.e., primary dysregulation of the immune response) or bacterial infection, remains a source of debate. Thus far, we have discussed studies wherein defects in the immune response, particularly in innate immunity, predispose patients to early inflammation and secondary bacterial infection. However, in direct contrast to the above-mentioned evidence, other elegant work with CF patient specimens as well as the pig model of disease emphasizes the importance of bacterial infection as the first event that drives subsequent inflammation within the CF lung.

Indeed, one landmark study of fetal CF lung tissue suggests that the early CF lung is histopathologically normal, i.e., indistinguishable from non-CF fetal tissue and free of neutrophilic inflammation in the absence of infection (188). The same study also showed that staining for proinflammatory cytokines such as IL-6 and IL-8 is similar between CF and non-CF fetal tissues, indicating the absence of intrinsic inflammation in the prenatal stage of disease (188). Additionally, multiple prospective clinical studies done by Armstrong and colleagues suggest that concentrations of markers of inflam-

mation (e.g., proinflammatory cytokines, total number of neutrophils, and free neutrophil elastase) within BAL fluid from infants diagnosed with CF (aged $<$1 year) are elevated only directly in response to bacterial infection and resolve with bacterial eradication via antibiotic therapy; infants with CF who are free of infection (either never infected and asymptomatic or cleared infection via antimicrobial treatment) do not show significant elevations in concentrations of inflammatory markers compared to non-CF controls (189–191). Conversely, young patients who become unable to clear their bacterial infection show the highest elevation in concentrations of inflammatory markers. These clinical studies demonstrate that infection not only initiates inflammation within the CF lung but likely also maintains inflammation (189–191).

Similarly, the pig model of CF ($CFTR^{-/-}$), developed by the Welsh laboratory, substantiates the above-mentioned clinical studies that indicate that infection precedes inflammation within the CF lung. The pig has been validated as a reasonable model of disease, as it exhibits multiple prototypical characteristics of early CF presentation, particularly within the gastrointestinal tract: intestinal obstruction (meconium ileus), pancreatic and hepatobiliary changes, as well as failure to thrive (21, 22). Additionally, there are parallels between early anatomical abnormalities, such as reduced lumen of the trachea, in both human and pig CF lungs (192). In a well-cited publication from the Welsh group, the laboratory demonstrated that in newborn CF pigs free of bacterial infection (although viral infection was not excluded), the CF lung appears to be histopathologically normal compared to healthy swine controls (193). They also found no elevation in levels of proinflammatory cytokines and immune cells within the CF pig lung at this early time point compared to their control animals. However, shortly after birth, the CF pigs become colonized with bacteria, after which there are noticeable inflammatory changes within the airway; the predisposition to bacterial infection in CF pigs but not in non-CF controls is likely due to defects in innate immunity (e.g., acidic pH of ASL or impaired mucociliary clearance), which have been investigated by the Welsh group and are discussed at length above (34, 35, 39). As such, these fundamental, controlled animal studies also suggest that early pathophysiology of CF lung disease is best characterized by lung hyperinflammation that is secondary to bacterial infection.

Progression of Events following Lung Infection and End-Stage Lung Disease in CF

Importantly, CF lung pathobiology is likely to be multifactorial, and each of the above-mentioned chicken-and-egg hypotheses may contribute to the disease process. Based on all studies described above, we speculate that the early events of CF lung disease proceed as follows: (i) CFTR-dependent defects in innate immunity predispose a patient to initial infection; (ii) bacterial infection then incites inflammation, characterized by the production of proinflammatory cytokines and the influx of neutrophils (which produce antimicrobials such as LL-37 and ROS in abundance); and (iii) the inflammatory response is overly exuberant and ineffective in clearing infection (due to bacterial adaptations and the capacity to evade immunity) and inflicts collateral damage on host tissue due to intrinsic deficits in endogenous anti-inflammatory and antioxidant molecules (e.g., IL-10 and glutathione). While these events may initiate the early manifestations of disease, the persistence of infectious and inflammatory factors throughout the disease course drives progression toward chronicity and the ultimate loss of pulmonary function. In other words, CF invariably remains a disease that is borne out of the bacterium-host interface.

Indeed, regardless of the temporal sequence of early events (inflammation and then bacterial infection or vice versa), the later progression, beginning with acute and subsequently chronic bacterial infections of the lung, appears to be well established. Unsuccessful clearance of infection promotes a destructive cycle of inflammation (i.e., neutrophil recruitment, frustrated phagocytosis, and the production of neutrophil-derived antimicrobials) (7). Patients may suffer symptomatic complications of these infectious/inflammatory cycles, often manifested as "pulmonary exacerbations," characterized by increased coughing, dyspnea, change in sputum volume/color, weight loss, and reduced pulmonary function (194–196). Within a state of chronic pulmonary

inflammation, both bacterial and host factors can damage lung tissue, leading to greater inflammation and eventual gross, irreversible changes to airway and parenchymal anatomy: bronchiectasis, cystic changes, mucus plugging, and emphysema (197, 198). The ultimate endpoint of disease is pulmonary failure, leading to candidacy for lung transplantation or death (7). While transplantation remains a palliative life-extending intervention for CF patients who are considered appropriate transplant candidates, it is not curative; there are significant associated complications, and the 5- and 10-year survival rates posttransplantation for children with CF remain at 57% and 45%, respectively (199–201). Notably, expresses andadult CF patients who receive lung transplants have superior outcomes, with 5- and 10-year posttransplantation survival rates of 67% and 55%, respectively (199).

Given the importance of infection as a driver of the disease process, the following discussion turns to the pathogenic microbiome of the CF lung and how the microbial composition changes throughout a CF patient's lifetime.

THE CF LUNG MICROBIOME

Despite past dogma, it is now a well-accepted paradigm that the healthy lung is not sterile (202–204). The healthy lung microbiome is composed primarily of members of the bacterial phyla *Bacteroidetes* and *Firmicutes*, which migrate into the lung from the oropharynx via aspiration of saliva (204). Furthermore, dysbiosis of the healthy lung microbiome is linked to pulmonary as well as systemic diseases (202–204).

Early dysbiosis of the CF lung and colonization with pathogenic bacterial species are attributed to defective or dysregulated immunity, including altered mucociliary clearance (as discussed above) (205). The predominant, "conventional" pathogenic bacterial species that colonize/infect the early CF lung include *S. aureus* (including methicillin-resistant *S. aureus* [MRSA]) and *Haemophilus influenzae*, whereas *P. aeruginosa* and the *Burkholderia cepacia* complex dominate in the later stages of disease; whereas early infections are intermittent, separated by periods of clearance and remittance of respiratory symptoms, the CF airways eventually become chronically infected with bacteria (206). Factors that influence the composition of the CF microbiome include patient age, antibiotic treatment, and disease modifier therapy (i.e., ivacaftor) (207–209). In general, as the patient ages, the microbial diversity of the CF lung declines, and chronic infection (i.e., resistant to antibiotic therapy) due to only one or two pathogenic bacterial species, most commonly *P. aeruginosa*, is established (210).

Other isolated or detected opportunistic pathogens in CF include bacteria such as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and nontuberculous mycobacteria (i.e., *Mycobacterium abscessus*); fungi such as *Aspergillus* and *Candida* spp.; and viruses (e.g., rhinovirus, influenza virus, and respiratory syncytial virus [RSV]) (211, 212). Although an in-depth discussion of the vast majority of these pathogens is out of scope here, their presence within the CF lung indicates that these species, both individually and via complex polymicrobial interactions, likely influence disease pathobiology.

To that end, there is ample evidence within the clinical literature of the consequences of early and chronic bacterial infection upon CF pulmonary function (and other outcome measures). We have already discussed evidence here that early bacterial colonization of infants with CF promotes inflammation, permanent changes to lung architecture, and decline of pulmonary function (19, 20, 213, 214). In children monitored from infancy to 6 years of age, the loss of diversity of the lung microbiota and the predominance of conventional pathogenic species (e.g., *S. aureus* and *P. aeruginosa*) are independently correlated with elevated inflammatory indices within BAL fluid (215). Chronic infection with Gram-negative pathogens of CF, *P. aeruginosa* and *B. cepacia*, is associated with a rapid decline in pulmonary function, more frequent exacerbations, and higher rates of mortality (216). Ultimately, 90% of deaths in CF patients are attributed to pulmonary dysfunction directly associated with chronic infection, underscoring the absolute importance of studying the pathogenesis of CF-relevant microbes

(212). Given the central role of *P. aeruginosa* in CF pulmonary disease, the following sections focus on this opportunistic pathogen.

PSEUDOMONAS AERUGINOSA: A CRITICAL PLAYER IN CF LUNG DISEASE

P. aeruginosa is a Gram-negative aerobe/facultative anaerobe that is found ubiquitously in soil and aquatic environments (217). Metabolically, *P. aeruginosa* is oxidase positive (i.e., preferring to grow in aerobic or microaerobic environments) and nonlactose fermenting but also capable of using nitrite or nitrate as a terminal electron acceptor under anoxic conditions (including within the CF lung) (218). The genomes of multiple strains of *P. aeruginosa* have been sequenced, demonstrating the versatility and adaptability of this organism: the total genome size is between 5.5 and 7 Mbp and GC rich (i.e., >65% GC content), with an accessory genome of up to 200 kbp, indicating the capacity of *P. aeruginosa* to acquire genetic elements via horizontal gene transfer: transformation, conjugation, and transduction (219). *P. aeruginosa* is also capable of three forms of motility, which include flagellum-dependent swimming, flagellum- and pilus-dependent swarming, and pilus-dependent twitching (220).

In the clinical setting, *P. aeruginosa* was first described by Gessard in 1882 in the article entitled *On the Blue and Green Coloration of Bandages*, thus documenting the elaboration of a blue-green pigment (pyocyanin) by the bacterium within skin wound pus (221). As an opportunistic pathogen of humans, *P. aeruginosa* causes both acute and chronic infections in immunocompromised and hospitalized patients. *P. aeruginosa* has been implicated as a causative agent of endocarditis/bacteremia, skin and soft tissue infections (e.g., burn wounds and surgical-site infections), urinary tract infections, gastrointestinal infections, meningitis, ocular infections, ear infections, and ventilator-associated pneumonia (VAP) (222–224). Among the causative agents of VAP, *P. aeruginosa* still carries the highest mortality rate (223). Disturbingly, within the hospital environment, *P. aeruginosa* has been found growing within disinfectants and distilled water and on other inanimate surfaces, including sinks and mops (221, 225–227). Although the nosocomial transmission of *P. aeruginosa* remains an area of debate, it may be spread via direct contact with contaminated surfaces or hospital equipment (e.g., mechanical ventilators and stethoscopes) or aerosolization or by contaminated health care workers (228–231). In the community, important reservoirs of this bacterium include hot tubs/whirlpools and freshwater lakes, wherein acquisition/infection can cause folliculitis and otitis externa, respectively (232, 233).

P. aeruginosa poses multiple challenges to effective treatment, such as significant antibiotic resistance. Multidrug-resistant (MDR) strains of *P. aeruginosa* have been documented with greater frequency in the clinical setting and are associated with very poor clinical outcomes; MDR strains employ multiple mechanisms of antibiotic resistance, including overexpression of efflux pumps and beta-lactamases (221, 234, 235). *P. aeruginosa* has also been studied extensively as a paradigm-defining, biofilm-forming bacterium (236–238). Based on the often-cited Parsek-Singh and Stoodley/Hall-Stoodley criteria, bacterial biofilms are defined as communities of bacteria (i.e., clusters or microcolonies of bacterial cells), adherent to a surface, that elaborate an extracellular matrix composed of bacterial and host materials and exhibit enhanced resistance to antibiotics compared to planktonic bacteria; "planktonic" refers to free-floating, non-surface-adherent bacterial cells (239, 240). Indeed, biofilm formation confers enhanced resistance not only to antibiotics (i.e., up to 1,000-fold greater resistance than for planktonic bacteria) but also to effectors of innate and adaptive immunity (236–238). Additional mechanisms of *P. aeruginosa* evasion of immune clearance are discussed further below (see *P. aeruginosa* Virulence Factors, Adaptations, and Host Responses during Chronic Infection).

In the study of human biology and pathophysiology, *P. aeruginosa* is inextricably linked with CF, wherein it is a prominent causative agent of chronic pulmonary infection. *P. aeruginosa* is the predominant pathogen infecting adult CF patients; approximately three-quarters of CF adults are infected with this organism (206, 241). Multiple studies also find that *P. aeruginosa* infection is a determinant of lower/

declining lung function and a leading cause of respiratory failure and mortality in CF patients (242–246). In a study examining multiple infecting microbial species as independent correlates of pulmonary outcome measures in CF patients, only *P. aeruginosa* correlated with reduced pulmonary function, increased concentrations of serum C-reactive protein (CRP) (a systemic marker of inflammation), and higher concentrations of neutrophil elastase in sputum (247). This study, among others mentioned above, suggests the primary importance of studying *P. aeruginosa* pathogenesis in the context of CF pulmonary disease, despite the diversity of microbial species that may well infect the CF lung throughout a patient's lifetime.

While the ultimate effects of *P. aeruginosa* in the chronic/end stages of disease are well established, its impacts on CF patients at an early age are also important. In one study, the median age of *P. aeruginosa* acquisition was 1.0 year; however, approximately 30% of patients in this cohort were culture positive for *P. aeruginosa* by as early as 6 months of age (248). In another study, 70% of CF patients were culture positive for *P. aeruginosa* by 3 years of age, but using a combination of serology and cultures to detect the bacterium, approximately 98% were positive for *P. aeruginosa* (249). These data suggest that although *P. aeruginosa* infection may be intermittent at an early age (i.e., prior to the onset of chronic infection), this bacterium is capable of infecting CF patients in infancy, thus influencing the disease course throughout a patient's lifetime. Infection with *P. aeruginosa* at infancy is associated with early decline in lung function, elevated concentrations of markers of pulmonary inflammation, and irreversible changes to the airway architecture upon imaging (19, 20, 213, 214). These findings emphasize why many intervention efforts in pediatric CF patients focus upon aggressive antibiotic treatment of *P. aeruginosa* infections to eradicate the bacterium from the airways (250–252).

Unfortunately, despite improved surveillance and therapeutic regimens, these eradication efforts fail in the long run, as most CF patients become chronically infected with *P. aeruginosa* (250–252). Moreover, flourishing communities of *P. aeruginosa* are often isolated from CF patient lungs postmortem or posttransplantation (i.e., within the diseased, explanted lung), suggesting that these bacteria not only are driving the disease process but also are able to outlive the infected tissue and host (253–255). As such, the questions that are central to ongoing investigation in the field pertain to the staying power of *P. aeruginosa* within the unique, hyperinflammatory environment of the CF lung. What allows *P. aeruginosa* to endure, proliferate, and profoundly impact all stages of CF pulmonary disease? Importantly, by identifying the tools that *P. aeruginosa* uses to survive within the CF lung, can we illuminate targets for the development of novel, antimicrobial therapeutics?

Indeed, part of the answer to these questions must include an understanding of *P. aeruginosa* virulence factors and the remarkable capacity of this bacterium to adapt via genotypic/phenotypic alterations. These pathoadaptive processes are central to the evasion of antimicrobials as well as the immune system and, thus, are essential for establishing chronic infection.

P. AERUGINOSA VIRULENCE FACTORS, ADAPTATIONS, AND HOST RESPONSES DURING CHRONIC INFECTION

P. aeruginosa Evasion of Innate Immunity

The premise that CF is a disease borne out of host-pathogen interactions is well established. Within this paradigm, the survival of *P. aeruginosa* within the CF lung is predicated upon a clash between bacterial virulence factors (which often arise via genetic/phenotypic adaptations) and the immune response. Unfortunately, the patient is caught in the cross fire between these bacterial/host elements, experiencing all of the deleterious effects on pulmonary function due to chronic *P. aeruginosa* infection (as described in detail above) and the immunopathological response elicited. In part, *P. aeruginosa* survival within the lung depends upon successful evasion of the innate immune response (256). Therefore, it is important to understand some of the bacterial factors that shield *P. aeruginosa* from the hyperinflammatory, neutrophil-rich CF lung

microenvironment, thus enabling long-term infection and perpetuation of the disease process.

P. aeruginosa expresses multiple virulence factors during infection that enable evasion of the host response; among these are lipases, proteases, rhamnolipids, pyocyanin, quorum sensing (QS) molecules, catalases, and exopolysaccharides (257). Some of these factors directly damage host tissue and subvert immune cell functions. However, the factors that specifically affect neutrophils are important to consider, as neutrophil recruitment, phagocytosis, and the production of neutrophil-derived antimicrobials (i.e., granular contents) are thought to be essential for *P. aeruginosa* clearance. Neutropenic mice have been shown to be vulnerable to fatal pulmonary infection with very-low-dose inocula ($<$100 CFU/mouse) of *P. aeruginosa* compared to nonneutropenic mice (10^7 to 10^8 CFU/mouse) (258). Correspondingly, patients with neutropenia, due to either HIV or cancer chemotherapy treatment, also demonstrate susceptibility to *P. aeruginosa* pneumonia (259, 260). Furthermore, patients with inherited disorders of neutrophil functions, including leukocyte adhesion deficiency (LAD) and specific granule deficiency, also show a predilection for *P. aeruginosa* infection (261). Neutrophil-specific granules are known to contain the cathelicidin LL-37; mouse models of LL-37 depletion (in parallel with specific granule deficiency in humans) show reduced clearance of *P. aeruginosa*, suggesting the importance of LL-37 (and neutrophils) in controlling *P. aeruginosa* infection *in vivo* (262, 263).

Despite a profuse influx of neutrophils into the CF lung, CFTR-dependent neutrophil impairment (e.g., reduced chlorination of the phagosome), as discussed above, may contribute to the vulnerability of CF patients to *P. aeruginosa* (88). However, *P. aeruginosa* virulence factors also directly interfere with neutrophil-mediated bacterial clearance in multiple ways. For instance, exotoxins produced via the *P. aeruginosa* type III secretion system directly lyse neutrophils (264); pyocyanin, the blue-green pigment of *P. aeruginosa*, induces neutrophil apoptosis (265, 266); and rhamnolipids, which are powerful detergents, cause neutrophil necrosis (267). *P. aeruginosa* alkaline protease has been shown to degrade complement proteins, thus inhibiting complement-mediated opsonophagocytosis by neutrophils (268). The expression of all of the above-mentioned virulence factors is regulated by QS, a mechanism of transcriptional control activated by bacterial density via the production of diffusible signaling molecules (i.e., autoinducers); QS is known to be induced within the CF airway, and QS-deficient *P. aeruginosa* has been shown to exhibit attenuated virulence (269). In this manner, several *P. aeruginosa* virulence factors directly eliminate phagocytic cells or impair effector functions that are critical for bacterial clearance *in vivo*.

Pathoadaptation during Chronic Infection

Importantly, during chronic infection of the airway, *P. aeruginosa* is confronted with multiple environmental stresses: intra/interspecies competition for nutrients and space, an anaerobic environment caused by mucus plugging, high concentrations of antibiotics used to aggressively treat infection, and an abundance of neutrophils and neutrophil-derived antimicrobials (e.g., AMPs and ROS) (270). These significant pressures within the airway drive *P. aeruginosa* microevolution, characterized by the acquisition of spontaneous mutations followed by the selection of genotypically/phenotypically distinct variants better suited for long-term colonization of the airway (i.e., variants exhibiting reduced virulence but greater resistance to antimicrobials and host immunity) (269). This evolutionary process facilitates the transition of *P. aeruginosa* from an "early/acute" to a "late/chronic" pulmonary pathogen in CF. Some phenotypic characteristics associated with this transition include the downregulation of some virulence factors (i.e., motility appendages [pilus and flagellum] and the above-mentioned proteases, rhamnolipids, and pigments, etc.), increased biofilm formation (i.e., exhibiting characteristics of a sessile, multicellular lifestyle as opposed to planktonic behavior), and upregulation of exopolysaccharide expression (Psl/Pel and alginate) (271).

There are significant benefits conferred by the genotypic/phenotypic conversion of *P. aeruginosa* into a more chronically adapted CF pathogen. For instance, although *P. aeruginosa* flagellar motility is necessary for initial colonization of a host, the flagellum is a PAMP (i.e., TLR5 ligand) recognized by the epithelium and phagocytes; as such, downregulation of the flagellum during chronic infection enables evasion of immune cell recognition and phagocytosis while simultaneously diminishing the production of proinflammatory cytokines/chemokines (272–275). The loss of flagellar motility also inhibits *P. aeruginosa* stimulation of superoxide production and NET formation by neutrophils (276); late isolates of *P. aeruginosa* from the CF lung, which tend to exhibit reduced motility, are also more resistant to NET-mediated killing (although the precise mechanism of this resistance is not well understood) (90). Similarly, *P. aeruginosa* LPS, which is also a PAMP (i.e., TLR4 ligand), exhibits lipid A modifications in late CF isolates, causing reduced phagocyte recruitment and lower proinflammatory cytokine production in an animal model of disease (277).

P. aeruginosa within the chronically infected CF lung also demonstrates biofilm characteristics. These bacterial communities appear as microcolonies or aggregates in close association with the airway epithelium and surrounded by neutrophils (253, 254, 278). As stated above, while *P. aeruginosa* biofilms exhibit significantly enhanced resistance to antibiotics (relative to planktonic bacteria), these communities also confer resistance to innate immune effectors (279). For example, biofilms of *P. aeruginosa* exhibit reduced activation of the complement system compared to planktonic cultures (280). Furthermore, when neutrophils interact with *P. aeruginosa* biofilms, the immune cells become immobilized at the upper layers of the microcolonies, engorged with bacteria, and unable to substantively clear the bacterial communities (281). Moreover, while NETs are still induced by *P. aeruginosa* within biofilms, NET components, including DNA, not only are unable to trap or kill the bacteria but also become incorporated into the extracellular polymeric substances (EPSs) of the biofilm itself (282). As such, *P. aeruginosa* biofilms can inhibit or coopt the innate immune response to thrive within the CF lung.

Mechanisms of Bacterial Mutagenesis *In Vivo* and "Insurance Effects"

Pathoadaptation of *P. aeruginosa* within the CF lung is primarily driven by genetic mutations, often attributed to direct exposure to mutagenic host antimicrobials, in particular, ROS (e.g., H_2O_2) (269, 283–285). *In vitro* investigations have supported this premise, as exposure of bacteria to neutrophils or H_2O_2 promotes an increased frequency of spontaneous mutations (147, 183, 285–287). There is also evidence that AMPs (e.g., LL-37) can induce bacterial mutagenesis at sublethal concentrations, and these data are discussed further in the context of *P. aeruginosa* mucoid conversion (see below) (147, 148). Host-derived mutagens can damage bacterial DNA, inducing DNA repair mechanisms (e.g., RecA-mediated SOS response, mismatch repair [MMR], translesion repair by polymerase IV [Pol IV] [DinB], and 8-oxo-2-deoxyguanosine [GO] repair by Pol V) (288–290). If these repair mechanisms are overwhelmed, defective due to loss-of-function mutations, or error prone (e.g., translesion repair) or otherwise fail to correct nonlethal DNA lesions, stable mutations may arise in a subpopulation of bacteria (288, 289). Mutational variants may then be selected and maintained within the CF lung if they are better suited to withstand challenging host and antimicrobial pressures. Interestingly, loss-of-function mutations in the MMR apparatus-encoding genes (e.g., *mutS* or *mutL*) lead to the emergence of "hypermutator" populations of *P. aeruginosa*; these hypermutators are isolated in abundance from CF patients in the late stages of disease and exhibit a loss of virulence and significantly heightened resistance to antibiotics (290–293).

Multiple genetic variants of *P. aeruginosa*, including hypermutators and nonmutators (among others), are often maintained together within the CF lung; indeed, *P. aeruginosa* isolates from chronically infected CF patients exhibit significant genetic/phenotypic heterogeneity, illustrating the "insurance hypothesis" (269, 294–296). This principle posits that genetic diversity within a group of organisms can confer selective

advantages to the entire population (297). As discussed further below, the diversification of *P. aeruginosa in vivo* likely contributes not only to antibiotic resistance but also to host immunity.

In the following section, three additional *P. aeruginosa* virulence factors and their roles in combatting host responses are examined in more detail. Some of these factors are overexpressed due to adaptive mutations (e.g., Psl/Pel in the rugose small-colony variants [RSCVs] and alginate in mucoid variants), whereas other factors are either constitutively or inducibly expressed by *P. aeruginosa* in direct response to host effectors (i.e., catalases and genes regulating autolysis/extracellular DNA [eDNA] release in response to H_2O_2 exposure).

Bacterial Catalases

Pathogenic bacteria are frequently confronted by oxidative stress *in vivo* (182). ROS may be generated by bacteria endogenously as a by-product of aerobic respiration or may represent an exogenous pressure from the infected host (162). The genesis of different forms of ROS as part of the innate immune response (i.e., via Nox/Duox complexes) is discussed in detail above. Here, the evolution, mechanism of action, and regulation of the bacterial antioxidant catalase are examined further, illuminating how evasion of ROS stress is critical for *P. aeruginosa* survival and virulence.

The catalase protein was first biochemically characterized in 1900 and was one of the first enzymes to be crystallized in 1937 (298). Based on functionality and structure, catalases are split into two main groups: heme-containing and nonheme catalases (299). Within the heme-containing catalases, there are two subgroups: monofunctional catalases and bifunctional catalase-peroxidases (300). Nonheme catalases are expressed only by prokaryotes (300). In contrast, monofunctional catalases are widely expressed and demonstrate significant homology across prokaryotes, archaea, and eukaryotes, suggesting that these proteins have been conserved through natural selection and coevolution of bacterial pathogens/humans (298, 300, 301). Both monofunctional catalase and catalase-peroxidases use heme iron as an intermediate to convert H_2O_2 to nontoxic products, water and oxygen. Heme iron (Fe^{3+}) is first oxidized to Fe^{4+} by H_2O_2 and then reduced by a second molecule of H_2O_2 to recover the original ferric form (299). As such, H_2O_2 acts first as an oxidizing agent and then as a reducing agent in the process of being detoxified by monofunctional catalases. The stepwise and overall reactions are as follows:

$$\text{Por-Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \left[\cdot\text{Por-Fe}^{4+}{=}\text{O}\right]^{+} + \text{H}_2\text{O} \qquad \text{(reaction 1)}$$

$$\left[\cdot\text{Por-Fe}^{4+}{=}\text{O}\right]^{+} + \text{H}_2\text{O}_2 \rightarrow \text{Por-Fe}^{3+} + \text{H}_2\text{O} + \text{O}_2 \qquad \text{(reaction 2)}$$

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \qquad \text{(reaction 3 [overall])}$$

where Por is the porphyrin complex of catalase containing heme iron.

Catalase-peroxidases are also ubiquitously expressed across the three domains and subtly differ from monofunctional catalases both structurally and functionally (298, 300, 301). Monofunctional enzymes have only catalase activity and are homotetrameric. In contrast, catalase-peroxidases exhibit both catalase and peroxidase activities and are dimeric (302). Peroxidases catalyze an overall reaction that is similar to that of monofunctional catalase (reaction 3); both types of enzymes are capable of cleaving the peroxidic bond in H_2O_2 (H-O-O-H) and in organic peroxides such as peroxyacetic acid (R-O-O-H) (302). However, peroxidases can use organic electron donors (instead of a second molecule of H_2O_2) to recover the ferric form of heme iron in the second reaction. As such, peroxidase activity to detoxify H_2O_2 would be expressed as follows (298, 300–303):

$$\text{Por-Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \left[\cdot\text{Por-Fe}^{4+}{=}\text{O}\right]^{+} + \text{H}_2\text{O} \qquad \text{(reaction 1)}$$

$$\left[\cdot\text{Por-Fe}^{4+}{=}\text{O}\right]^{+} + 2\text{AH}_2 \rightarrow \text{Por-Fe}^{3+} + 2\text{AH}^{\cdot} + \text{H}_2\text{O} \qquad \text{(reaction 2)}$$

Given that H_2O_2 is bactericidal (via mechanisms described in detail above), catalase expression protects pathogenic bacteria from the toxic effects of this ROS, whether

generated endogenously or by host phagocytes (182, 304). Additionally, catalase also enables circumvention of free radicals that would be formed using H_2O_2 as a substrate (e.g., HOCl via the enzyme MPO and $^{\cdot}OH$ via Fenton chemistry), for which bacteria lack specific detoxifying enzymes (289).

P. aeruginosa expresses an impressive arsenal of antioxidants, including three monofunctional catalases (KatA, KatB, and KatC), at least two alkyl hydroperoxide reductases (i.e., peroxidases AhpCF and AhpB), as well as two superoxide dismutases (SodA and SodB) (305, 306). As stated above, superoxide dismutases (in both bacteria and humans) act upstream of catalase by mediating the conversion of superoxide into H_2O_2. To date, the functionality of KatC is not well understood (307). *P. aeruginosa* catalase (KatA) expression occurs during infection of the CF lung as patients develop antibodies (within BAL fluid and blood) that are KatA reactive (308). Furthermore, under laboratory conditions, KatA is universally expressed by tested *P. aeruginosa* clinical isolates, regardless of the site of infection/isolation (i.e., blood isolates for bacteremia infection and sputum isolates for respiratory tract infection); this is not true for all *P. aeruginosa* antioxidants, as SodA is not expressed in all isolates (305). In total, these findings suggest that catalase likely plays a critical role during *P. aeruginosa* infection of the CF airway.

KatA is the constitutive, heterotrimeric, stationary-phase/"housekeeping" catalase produced by *P. aeruginosa* (309, 310). The level of constitutive expression of *katA* is very high, and exposure to millimolar concentrations of H_2O_2 does not induce more than a 2-fold change in expression (311). In contrast, *katB* is inducibly expressed only upon exposure to H_2O_2 (309, 311). Both catalases are transcriptionally regulated by multiple factors, including OxyR, RpoS, and quorum sensing systems. *P. aeruginosa* OxyR is a global sensor of H_2O_2 stress and is homologous to the OxyR protein of *E. coli* (312). Conserved cysteine residues of OxyR are oxidized in the presence of H_2O_2, leading to the generation of a disulfide bond, followed by a conformational shift of the protein to its active form; this form of OxyR can then bind to DNA and regulate gene expression (312). Initially, it was thought that only *katB* expression is regulated by OxyR; in light of the H_2O_2-inducible transcription of *katB*, this seemed logical (166, 309). However, further work in the field has now shown that OxyR can promote the transcription of both *katA* and *katB* as well as the transcription of other antioxidant-encoding genes (e.g., *ahpCF* and *ahpB*) (313–315). Consequently, *oxyR* mutants exhibit heightened sensitivity to ROS- and neutrophil-mediated killing as well as reduced virulence in animal models of infection (306, 309, 316).

The induction of RpoS (a sigma factor responsive to stress/nutrient limitation) and/or the collaborating stringent response (activated upon amino acid starvation) also upregulates the transcription of both *katA* and *katB* (317). This is important because the host presents a nutrient-limiting, stressful environment, wherein we may infer that the expression of catalases is induced via these pathways (318). Indeed, loss-of-function mutations in stress response genes result in increased susceptibility of *P. aeruginosa* to oxidative stress, decreased catalase expression, and loss of virulence in animal models of disease (317–321). Additionally, two quorum sensing systems in *P. aeruginosa* also regulate *katA*, but not *katB*, transcription; deletion of autoinducer genes of the LasRI/RhlRI systems reduces *katA* transcription and correspondingly increases the H_2O_2 susceptibility of the mutants (322). This finding, combined with the role of RpoS in regulating *katA* transcription, helps to explain why *katA* expression is maximal at stationary phase (i.e., at a high bacterial cell density).

Although the transcriptional regulation networks of *katA* and *katB* appear to be somewhat interconnected, there are notable differences between the two catalase proteins in the degree of protection that each affords against H_2O_2 exposure, the intracellular localization of both proteins, and extracellular release mechanisms. KatB is not produced during the normal growth cycle (i.e., it exhibits only inducible expression), whereas KatA is the housekeeping catalase and expressed across all phases of growth (323). In the vast majority of studies, KatA has been shown to be essential for protection against bactericidal, millimolar concentrations of H_2O_2, whereas deletion of

katB results in only marginally increased sensitivity of *P. aeruginosa* to lower doses of H_2O_2 (323–327). Correspondingly, in a transposon mutant screen seeking to identify *P. aeruginosa* genes essential for defense against H_2O_2, both *katA* and *oxyR* mutants were identified as highly H_2O_2 sensitive, although this was not true for *katB* (316).

In addition, while KatB is localized only within the cytoplasm, KatA is present within both the cytoplasm as well as the periplasm, suggesting that KatA is a released or secreted protein (323). In further investigating the potential extracellular role of KatA, one study showed that cell-free supernatants taken from a *P. aeruginosa katA* mutant were unable to protect a highly sensitive *oxyR* mutant from H_2O_2 stress, whereas supernatants from a *katB* mutant were able to rescue the *oxyR* mutant (309). This study speculated that KatA may be released extracellularly via bacterial cell lysis, although direct evidence of this mechanism was not shown. Furthermore, the presence of KatA (not KatB) appears to be indispensable to shield *P. aeruginosa* biofilms from H_2O_2 penetration/killing (324, 325). For many of these reasons, it is unsurprising that *katA* mutants of *P. aeruginosa* demonstrate significantly reduced virulence in animal models of infection, while this is not true for *katB* mutants (326, 327). Interestingly, mucosal immunization with KatA increases pulmonary clearance of *P. aeruginosa* in a rat model of acute infection (328).

Finally, research has identified other important roles for KatA in *P. aeruginosa* biology. A recent study showed that KatA protects *P. aeruginosa* from nitric oxide stress under anaerobic conditions, suggesting that KatA is a versatile protein that can detoxify both ROS and RNS (329). Additionally, some studies suggest that *katA* expression is reduced in *P. aeruginosa* biofilms compared to planktonic cultures *in vitro* (although paradoxically, biofilms still exhibit enhanced resistance to H_2O_2) (330, 331). *P. aeruginosa* mutability is also increased in biofilms compared to planktonic growth, and indeed, *katA* mutants demonstrate higher rates of spontaneous mutations than wild-type bacteria (285, 331). As such, the expression of *katA* (or lack thereof) may influence the capacity of *P. aeruginosa* to undergo pathoadaptation by detoxifying endogenous or exogenous sources of mutagenic ROS.

Autolysis/eDNA Release

Autolysis, as a form of programmed cell death, is a recognized phenomenon in prokaryotic species and thought to be analogous to apoptosis in eukaryotes (332–334). At first glance, the tendency of bacteria to commit suicide in direct response to certain forms of stress seems counterproductive. However, this behavior, when exhibited by a subset of bacterial cells within a population, is thought to confer significant advantages for the survival of the larger bacterial community during infection (332–334). Using superresolution microscopy and standard bacterial genetics, the mechanism and regulation of *P. aeruginosa* autolysis were recently elucidated by Turnbull and colleagues in a landmark publication (103).

The stepwise progression of this mechanism, called "explosive cell lysis," is described as follows (103). Upon exposure of *P. aeruginosa* to cell wall or genomic stress (i.e., due to antibiotic/ROS treatment), the RecA-mediated SOS response against DNA damage is triggered. As part of the global transcriptional effect of this response, transcription of the R/F-pyocin gene cluster is upregulated (Fig. 2A); this set of genes represents a prophage-derived fraction of the *P. aeruginosa* accessory genome that is well conserved across multiple clinical and environmental isolates (335, 336). While the role of pyocins in the intraspecies interactions of *P. aeruginosa* communities is discussed above (in the context of antimicrobial peptides), none of the structural pyocin genes in the R/F-pyocin cluster are required for autolysis. Instead, *P. aeruginosa* lysis depends upon only one gene within the cluster, *lys* (PA0629), which encodes a phage-derived endolysin (Fig. 2A). This endolysin traverses the inner membrane and degrades *P. aeruginosa* peptidoglycan, thereby destabilizing the bacterial cell wall and causing cell lysis (Fig. 2B).

Access of Lys to the periplasmic space is likely mediated by three holin proteins, which are inserted into the inner membrane: CidA, AplB, and a putative holin, Hol

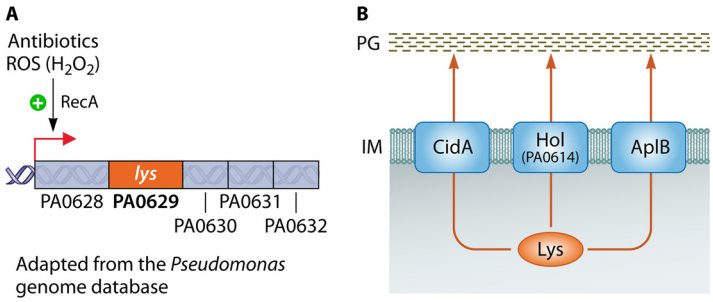

FIG 2 Working model for Lys-mediated autolysis in *P. aeruginosa*. (A) Part of the R/F-pyocin gene cluster (PA0628-PA0632), a putative operon containing *lys* (PA0629), which encodes an endolysin implicated in *P. aeruginosa* autolysis ("explosive cell lysis") and eDNA release. As part of the RecA-dependent SOS response, *lys* transcription is upregulated by cell membrane and genotoxic stresses (caused by exposure to antibiotics or reactive oxygen species [e.g., H_2O_2]) (103, 336). (B) Lys traverses the inner membrane (IM) via holin proteins (CidB, AplB, and a putative holin, Hol [PA0614]) and degrades peptidoglycan (PG), thereby destabilizing the cell wall and prompting cell lysis (103).

(PA0614) (also encoded within the R/F-pyocin cluster) (103, 337, 338) (Fig. 2B). The role of CidA and AlpB in *P. aeruginosa* autolysis was established in publications that preceded the identification of *lys* (103, 337, 338). However, Turnbull et al. found that the expression of both *lys* and *hol* in *trans* was necessary to induce cell lysis in *E. coli*, indicating that the expression of either the endolysin or holins individually is not sufficient to mediate this process; they further posit that in *P. aeruginosa*, all three holins could potentially partner with Lys, thereby hypothesizing that deletion of one holin-encoding gene may not be sufficient to abrogate autolysis (103). To that end, their laboratory recently showed that deletion of all three holins is required to completely abolish cell lysis or to phenocopy the single deletion of *lys* (Cynthia Whitchurch, presented at the 16th International Conference on Pseudomonas, Liverpool, United Kingdom, 2017).

There are few additional nuances of *P. aeruginosa* autolysis that are worth acknowledging. First, *P. aeruginosa* autolysis was shown to occur in both biofilm and planktonic cultures (103). Autolysis does not occur in every cell within a *P. aeruginosa* population: Turnbull et. al showed via microscopy that the frequency of cell lysis in different *P. aeruginosa* strains can vary from 1 in 3,000 to 1 in 100,000 cells (also confirming that the phenotype is conserved across multiple laboratory and clinical strains) (103). Second, other work within the field suggests that the *Pseudomonas* quinolone signal (PQS) quorum sensing system, which is distinct from the above-mentioned RhlRI/LasRI systems, is required for cell lysis (although Turnbull et al. find that PQS is dispensable for autolysis) (339, 340). One PQS-dependent mechanism occurs via upregulated synthesis of pyocyanin, a phenazine compound that is the blue-green pigment of *P. aeruginosa* (341). Multiple roles of pyocyanin in virulence and innate immune evasion are discussed above. However, pyocyanin can additionally act as an electron acceptor from molecules such as NADH and as an electron donor to molecular oxygen, thereby participating in the endogenous generation of H_2O_2 (ROS) by *P. aeruginosa* (340, 341). H_2O_2 is then predicted to cause genomic DNA stress, resulting in the *recA*-dependent autolytic pathway discussed above. Endogenous or exogenous ROS stress has been implicated as a trigger for autolysis in *E. coli* as well (342).

Additionally, the LasRI system has also been implicated in autolysis: loss-of-function *lasR* mutants, which exhibit an autolytic phenotype, are isolated in abundance from the chronically infected CF lung (343, 344). While any direct link between LasRI gene regulation and *lys*-mediated autolysis has not been established, the fact that multiple late-adapted *P. aeruginosa* isolates exhibit this phenomenon suggests that autolysis likely confers advantages during chronic infection of the host.

Indeed, the "altruistic suicide" of a proportion of *P. aeruginosa* populations has been shown to be principally responsible for eDNA release (345–347). eDNA is a vital component of the extracellular polymeric substances of *P. aeruginosa* biofilms and has

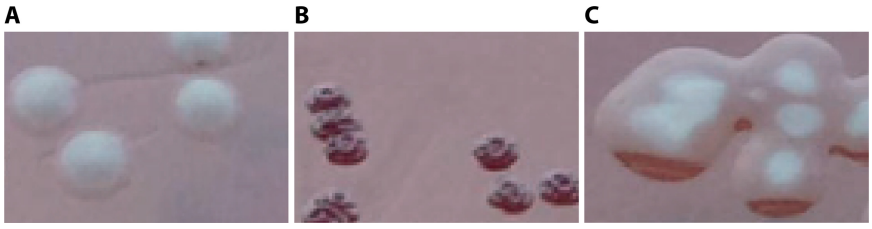

FIG 3 Predominant colony morphologies of *P. aeruginosa* variants within the cystic fibrosis lung. *P. aeruginosa* strains were grown and imaged on Vogel-Bonner minimal medium (VBMM) with Congo Red. (A) Wild type (nonmucoid); (B) rugose small-colony variant (RSCV) (nonmucoid); (C) mucoid variant. RSCVs overproduce Psl and Pel exopolysaccharides. Mucoid variants overproduce the alginate exopolysaccharide. The red color of RSCV colonies is due to positive staining with Congo Red. All colonies were imaged at the same magnification. (Adapted from reference 238 with permission.)

been shown to have essential roles in *P. aeruginosa* early attachment/biofilm formation and resistance to antimicrobials such as tobramycin (346, 347). *lys* mutants are severely compromised in eDNA release as well as microcolony formation (103). Correspondingly, deletion of *alpB*, which encodes one of the holins involved in autolysis/eDNA release, reduces the capacity of *P. aeruginosa* to colonize the murine lung (337). Under conditions of host stress (i.e., ROS/antibiotic exposure), autolysis can also facilitate dispersal of *P. aeruginosa* from established biofilms, enabling colonization of a different niche with more favorable microenvironmental conditions for survival (348, 349). As discussed above, abundant free DNA within the CF lung (likely to include bacterial eDNA as well as host DNA from neutrophils) correlates with viscosity of airway secretions and decline in pulmonary function (80, 81).

Another consequence of bacterial cell lysis is the generation of membrane vesicles (MVs), thought to form during *lys*-mediated autolysis via the passive enclosure of outer membrane fragments (103). These MVs are distinct from outer membrane vesicles (OMVs), which can form and be released independent of cell lysis/bacterial cell death (350, 351). Similar to OMVs, however, MVs have been shown to colocalize with eDNA and are predicted to encapsulate additional cargo, including "moonlighting" (intracellular) proteins or virulence factors, which may serve as public goods for the benefit of multicellular bacterial populations (103, 269).

Exopolysaccharides: Psl, Pel, and Alginate

The acquisition of adaptive mutations by *P. aeruginosa* during chronic infection of the CF lung has been well documented (as described above) (269). One set of commonly encountered mutations in chronically adapted variants results in the overproduction of *P. aeruginosa* exopolysaccharides: Psl, Pel, and alginate (352) (Fig. 3A to C). These polysaccharides form part of the extracellular polymeric substances of *P. aeruginosa* and represent multifunctional virulence factors, conferring properties of biofilm formation and enhanced resistance to environmental stressors such as antibiotics and host effectors (352–354). The overproduction of alginate, which results in the mucoid colony phenotype of *P. aeruginosa*, is described in detail below (Fig. 3C). Here, we briefly describe the roles of Psl and Pel in the antibiotic tolerance and immune evasion characteristics of the rugose small-colony variant (RSCV) (Fig. 3B).

The *P. aeruginosa* RSCV, which describes a colony morphotype of small, wrinkled appearance, is often isolated from the late, chronically infected CF lung (355–357). RSCVs exhibit increased production of Psl and Pel, two of the *P. aeruginosa* exopolysaccharides (352, 358, 359). Psl and Pel are encoded by 7- and 15-gene operons, respectively, both of which are transcriptionally regulated (in part) by the intracellular second messenger cyclic di-GMP; the acquisition of gene mutations that result in elevated intracellular levels of c-di-GMP promotes the upregulation of Psl/Pel synthesis, thereby producing the RSCV phenotype (352, 358, 359). RSCVs demonstrate hyperaggregation and significantly enhanced biofilm formation compared to isogenic wild-type bacteria; here, "wild type" refers to environmental isolates of the pathogen that are

initial or early colonizers of the CF lung but may subsequently undergo adaptive mutagenesis/differentiation (358–360). Given that the expression of either Psl or Pel is required for biofilm formation in *P. aeruginosa*, the augmented "biofilm phenotype" of RSCVs is attributed to the overproduction of these polysaccharides (352).

Additionally, RSCVs show increased resistance to antipseudomonal antibiotics (e.g., aminoglycosides) and to innate immune effectors, including ROS and AMPs (361, 362). Accumulation of c-di-GMP in these variants contributes to the downregulation of flagella, likely mediating evasion of immune recognition (per the TLR-dependent mechanisms discussed above) (358). Furthermore, in animal models of disease, RSCVs cause more persistent infections than wild-type bacteria and are associated with greater lung histopathology (361). Likely due to a combination of all of these properties, the emergence of RSCVs within the CF lung is associated with poor clinical outcomes, including loss of pulmonary function and prolonged hospitalization (356, 363).

ALGINATE AND THE MUCOID *P. AERUGINOSA* PHENOTYPE IN CF

There are multiple similarities between the properties of the *P. aeruginosa* RSCV and the mucoid phenotype. Parallel to the overproduction of Psl/Pel in the RSCV, overproduction of alginate by mucoid variants of *P. aeruginosa* (Fig. 3C) also confers significant advantages to the bacterium and simultaneously proves disastrous for the CF patient. The mucoid phenotype of *P. aeruginosa* was first documented by Sonnenschein in 1937, and this phenomenon was subsequently (although imprecisely) characterized as a "capsulated" *P. aeruginosa* variant based on microscopy (364). Since then, mucoid variants of *P. aeruginosa* have also been reported in multiple clinical settings of *P. aeruginosa* infection and across organ systems, including urinary tract infections, non-CF bronchiectasis (e.g., chronic obstructive pulmonary disease [COPD]), and ocular infections (365–367). Nonetheless, within the biomedical literature, the mucoid *P. aeruginosa* phenotype carries its most significant and common association with the CF lung, leading some to suggest that the phenotype be considered pathognomonic for the disease (367). During intermittent infection of the CF airway, nonmucoid (i.e., environmental, wild-type) *P. aeruginosa* predominates; at this stage, it is possible to achieve eradication of nonmucoid bacteria with aggressive antibiotic therapy (252, 368). However, the transition to the chronically infected stage of disease, wherein bacteria are recalcitrant to antimicrobials, is associated with the emergence of mucoid *P. aeruginosa* variants, which profoundly impact the clinical status of the CF patient (369).

The nonmucoid-to-mucoid transition (i.e., mucoid conversion) occurs at a median age of 13 years, although mucoid variants may be noticed by as early as 18 months of age in CF patients; in contrast, the median age of the first (nonmucoid) *P. aeruginosa* infection is 1 year (248). In the United States, approximately 70% of CF patients become infected with mucoid *P. aeruginosa* at some point during their disease course (292). As discussed above, there are deleterious effects of early *P. aeruginosa* colonization on the CF lung (i.e., worsening inflammatory status, architectural changes, and decline of pulmonary function) (19, 20, 213, 214). Unfortunately, mucoid conversion heralds even greater damage to the CF airway: infection with mucoid *P. aeruginosa* is associated with severe bronchiectasis, accelerated decline in patient lung function, and increased mortality, explaining why mucoid conversion remains a feared complication in the management of CF (242, 248, 252, 370–373).

Alginate Biosynthesis and Regulation

Mucoidy is defined by the overproduction of alginate, which is arguably the most extensively studied and well-characterized exopolysaccharide of *P. aeruginosa* (352). Alginate, a polyanionic polymer of β-1,4-linked D-mannuronic and L-guluronic acids, is synthesized by both *P. aeruginosa* and *Azotobacter vinelandii*, which is a Gram-negative, nonpathogenic, soil-dwelling bacterium (374) (Fig. 4). Various species of brown algae (seaweeds) also produce alginate, which is extracted and sold commercially as alginic

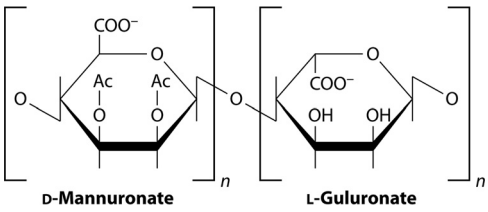

FIG 4 Chemical structure of alginate. Alginate is a polyanionic exopolysaccharide, composed of β-1,4-linked D-mannuronic and L-guluronic acids. (Reproduced from reference 238 with permission.)

acid; the only difference between bacterial and seaweed alginates is that only bacterial alginate is acetylated, although both polysaccharides are composed of the same constituent monosaccharides (374, 375).

Alginate biosynthesis is energetically costly and, as such, is carefully controlled via a complex regulatory network (367) (Fig. 5). The alginate biosynthetic operon consists of 12 genes (*algD* [PA3540] through *algA* [PA3551] [*algD-algA*]) and is regulated via the *algD* promoter (367, 376, 377). The expression of this operon requires the activity of a sigma factor encoded by *algT* (also known as *algU* and σ^{22}); AlgT is encoded in a separate operon (*algT-mucA-mucB-mucC-mucD*), which also includes the gene products that ultimately regulate AlgT activity (374, 375). Notably, AlgT is a homolog of *E. coli* σ^{E}, which is responsive to multiple forms of envelope stress; as such, not only does AlgT act as a positive regulator of alginate biosynthesis, it also is predicted to regulate more than 290 open reading frames in *P. aeruginosa* as part of the global stress response (i.e., to heat, osmotic, and oxidative stresses) (378–380).

In nonmucoid *P. aeruginosa* and under conditions of limited environmental stress, AlgT is sequestered by its cognate anti-sigma factor, MucA, which is an inner membrane protein (381–383) (Fig. 5). However, during chronic infection of the CF lung, *P.*

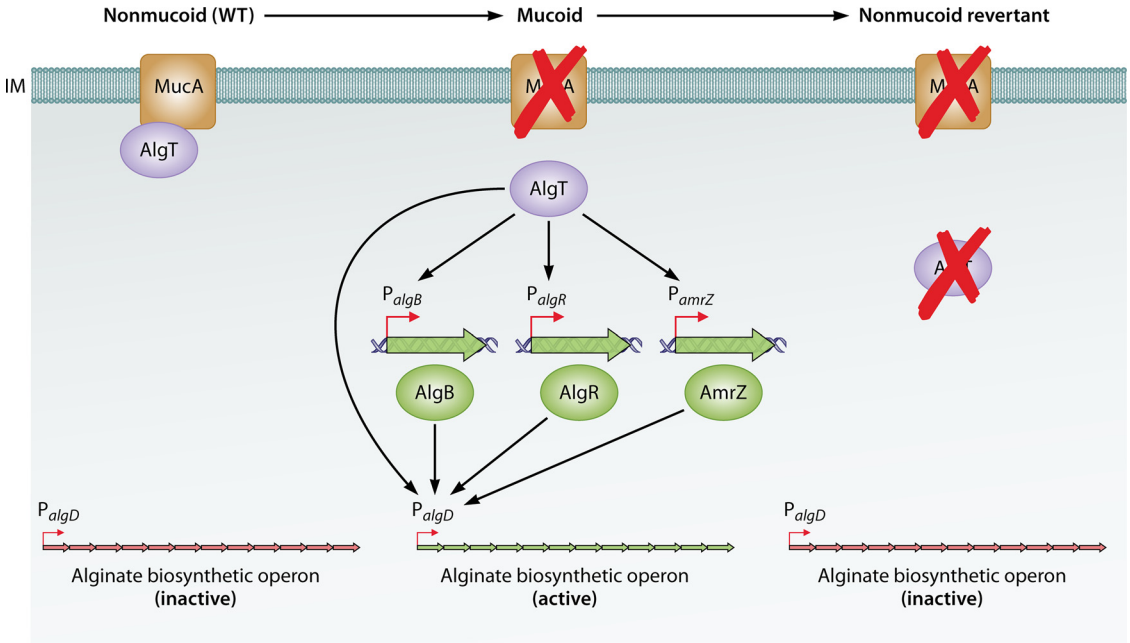

FIG 5 Regulation of alginate biosynthesis: mucoid conversion and reversion. In wild-type, nonmucoid *P. aeruginosa*, the alginate biosynthetic operon (*algD-algA*) is inactive, as a sigma factor, AlgT, is sequestered at the inner membrane (IM) by its cognate anti-sigma factor, MucA. Acquisition of a *mucA* mutation results in a truncated MucA protein that is no longer able to bind AlgT. AlgT can then activate alginate biosynthesis in 2 principal ways: (i) by activating the transcription of the alginate biosynthetic operon at the *algD* promoter (P*algD*) and (ii) by activating the transcription of genes encoding three ancillary transcription factors, AlgB, AlgR, and AmrZ, which also bind to P*algD* and are essential for alginate production. Mucoid variants of *P. aeruginosa* can revert back to a nonmucoid phenotype via the acquisition of a secondary (suppressor) mutation in *algT*, which inactivates alginate biosynthesis (375, 425).

aeruginosa can spontaneously acquire mutations in *mucA*, resulting in a truncated, nonfunctional MucA protein that is no longer able to sequester AlgT; as such, AlgT is then free to bind to RNA polymerase and direct the transcription of the alginate biosynthesis genes and other downstream targets (381–383). Mutations in *mucB* and *mucD* have also been shown to destabilize the MucA-AlgT interaction and result in mucoid conversion; however, 80% of mucoid isolates from CF patients show mutations in *mucA*, suggesting its primary relevance to the phenomenon of mucoidy in the clinical setting (382–385).

AlgT is also known to act as a positive transcriptional regulator of its own operon and genes encoding ancillary transcription factors that are also necessary for alginate biosynthesis, including *algB*, *algR*, and *amrZ* (377, 386–388) (Fig. 5). AlgB and AlgR are the response regulator proteins of two distinct two-component regulatory systems in *P. aeruginosa* (389, 390). Although the cognate sensor kinases for both proteins (i.e., KinB and FimS, respectively, and their phosphorylation activities) are not required for alginate biosynthesis, both AlgB and AlgR are required (391). AmrZ (formerly AlgZ) is a ribbon-helix-helix DNA-binding protein that is also essential for alginate biosynthesis (387). Importantly, there are other transcription factors that are also known to bind to the *algD* promoter and mediate alginate transcription, including integration host factor (IHF), AlgP, and AlgQ; however, to date, AlgT is specifically known to upregulate the transcription of genes encoding the factors AlgB, AlgR, and AmrZ only (377).

Binding sites for AlgB, AlgR, and AmrZ have been demonstrated within the *algD* promoter region, and similar to AlgT, all of these transcription factors also regulate multiple downstream targets (i.e., virulence factors), independent of alginate biosynthesis (392–394) (Fig. 5). Indeed, AlgR has been shown to be a positive regulator of biofilm formation via quorum sensing and a negative regulator of type III secretion (395, 396). Additionally, AlgR is important for *P. aeruginosa* defense against some host antimicrobials, and deletion of *algR* (in a nonmucoid, wild-type strain background) attenuates virulence in a murine model of pulmonary infection (397, 398). Similarly, AlgB is a global regulator of multiple virulence factors, including motility, pyocyanin, and elastase (375). Among its many targets, AmrZ is known to positively regulate twitching motility (via the type IV pilus) and repress Psl synthesis (399).

It is worth noting that although the AlgT sigma factor canonically acts as a positive regulator of transcription, it has been shown to repress genes via its downstream transcription factors (399–402). For instance, in mucoid variants of *P. aeruginosa*, wherein AlgT and, correspondingly, AmrZ activities are upregulated, Psl and flagellum synthesis is repressed; conversely, mutation of both *algT* and *amrZ* results in derepression of Psl/flagellum synthesis, and AmrZ has been shown to directly bind to the promoters of operons encoding Psl/flagella (399, 400). Similarly, repression of type III secretion in mucoid *P. aeruginosa* is both AlgT and AlgR dependent (401).

Mutagenic Host Immune Effectors Induce Mucoid Conversion

The acquisition of mutations and their contributions to the adaptive evolution of *P. aeruginosa* are discussed at length above. However, in the context of mucoid conversion, several landmark publications have studied potential host factors (i.e., mutagens) that may promote or increase the frequency of *mucA* mutations, thereby triggering mucoidy *in vivo*.

Indeed, the first detailed mechanism for mucoid conversion via host factors was provided by Mathee et al., who showed that exposure of *P. aeruginosa* biofilms to sublethal concentrations of H_2O_2 or to activated human neutrophils resulted in the isolation of mucoid variants after 3 to 8 days (183). These mucoid isolates demonstrated the same *mucA* mutation allele as the one commonly observed in clinical isolates (i.e., *mucA22*, a deletion of a guanine residue resulting in a frameshift and premature termination of translation) (384). Thus, those authors conclude that exposure to oxidative stress may actually be beneficial to *P. aeruginosa*, as ROS can promote adaptive mutagenesis of the bacterium. To that end, Mathee et al. propose two possible

mechanisms: ROS exposure either directly generates the *mucA* mutations or selects spontaneous *mucA* variants more capable of evading oxidative stress.

The nexus between ROS exposure and induced mutagenesis in bacteria has since been better understood; as described above, ROS can directly cause DNA damage, which can either activate error-prone repair mechanisms or overwhelm these systems altogether (147, 183, 285–287). The result is genetic variants, which subsequently undergo selection based on microenvironmental pressures within the host. DNA repair mechanisms implicated in ROS-dependent mutagenesis include MMR (e.g., mutations in *mutS-mutL*, common in the "hypermutator" phenotype of *P. aeruginosa*) and translesion repair (i.e., the activity of DinB, an error-prone polymerase) (288–290). Studies have shown that deletion of *mutS* promotes mucoid conversion of *P. aeruginosa* upon ROS treatment *in vitro*, whereas *dinB* mutation abrogates mucoid conversion (403–405). These findings suggest that the link between exposure to host effectors and the resultant mucoid conversion in *P. aeruginosa* depends upon DNA damage/repair.

Furthermore, Limoli et al. also elegantly demonstrated that exposure to sublethal amounts of ROS, neutrophils, and LL-37 directly induces *mucA* mutagenesis in *P. aeruginosa*, independent of selection (147). This phenomenon of LL-37-induced mutagenesis was also shown to be DinB dependent. The genetic construct used in this study was a chloramphenicol resistance cassette that was placed under the control of an *algD* promoter in a wild-type, nonmucoid strain that was chloramphenicol sensitive. Thus, mucoid conversion would be detectable upon growth of bacteria on chloramphenicol-containing medium. The nonmucoid strain was then exposed to various host effectors and subsequently plated on both nonselective media (i.e., to quantitate the total number of bacteria) and selective media (i.e., to quantitate spontaneous mucoid variants). There was no statistically significant change in the total number of bacteria across the various treatments, but the frequency of mucoid variants (i.e., number of mucoid colonies/total colonies) increased upon exposure to H_2O_2, neutrophils, and LL-37, suggesting that mutations were induced and not selected in these *P. aeruginosa* populations (147).

Fascinatingly, Limoli et al. also demonstrated not only that sublethal concentrations of LL-37 induce mucoid conversion but also that the resulting mucoid variants are more resistant to lethal concentrations of LL-37 (compared to their wild-type, nonmucoid parent) (147). Therefore, a host response mechanism, which is supposed to clear bacteria, instead backfires spectacularly, contributing to bacterial mutation and to resistance.

The Fitness Advantages of Mucoidy (and Associated Controversies)

Indeed, many of the drastic, detrimental clinical manifestations of mucoid conversion are attributed to the phenotypic advantages conferred by alginate overproduction. Although alginate is not required for biofilm formation (406), mucoid variants of *P. aeruginosa* demonstrate multiple biofilm-like characteristics, including enhanced resistance to antibiotics and to host-derived antimicrobials: several studies show that mucoid variants are more resistant to conventional, antipseudomonal antibiotics than are isogenic, nonmucoid variants (407–410). Additionally, mucoidy confers advantages in evading the immune response. Mucoid variants are more resistant to complement-mediated killing, phagocytic killing by macrophages and neutrophils, and NET-mediated killing (90, 147, 411, 412). Mucoid variants also downregulate the production of flagella, which likely enables evasion of immune detection by the mechanisms specified above (413).

However, despite these observed advantages of mucoidy *in vitro* and *in vivo*, which correlate with the clinical observations of chronicity and recalcitrance to therapeutics, there are contradictory findings within the literature as well. Some studies demonstrate that mucoid variants are more susceptible to antibiotics than are paired nonmucoid isolates; these findings suggest that alginate production alone is not a reliable predictor of antibiotic susceptibility in clinical isolates (414, 415). Moreover, whereas some have found that alginate acts as a scavenger of ROS from phagocytic cells (without affecting

the viability or oxygen consumption of the phagocytes), others suggest that alginate stimulates a very robust ROS burst response (416–418).

Animal studies with paired mucoid and nonmucoid variants are informative regarding this important topic as well, although they offer similar paradoxes. Mouse models of pulmonary infection with *P. aeruginosa* generally show that mucoid strains cause more persistent infection than nonmucoid strains (384, 419, 420). However, there are notable exceptions, wherein infection of either a mouse or a seedling model showed an attenuated mucoid phenotype with a significantly higher 50% lethal dose (LD_{50}) (i.e., lower lethality) than for nonmucoid variants (421, 422). The latter studies argue that as part of their chronic adaption, mucoid strains likely stimulate a reduced inflammatory response to escape immune clearance. However, where directly studied, the impact of mucoid versus nonmucoid strains on the immunopathology of the CF lung is still debated. Some have shown no difference between mucoid and nonmucoid strain infections in causing tissue histopathological changes in the animal lung, whereas other studies show greater tissue damage attributed to mucoidy (384, 419, 420, 423). More recent work argues that regardless of colony morphotype, both mucoid and nonmucoid strains that are "late adapted" (i.e., isolated from chronically infected patients) cause reduced mortality and inflammation during murine pulmonary infection compared to "early" nonmucoid isolates (i.e., from initial *P. aeruginosa* infection) (423, 424). Some of the differences across these studies may be explainable due to variations in infection strategies (i.e., acute versus chronic infection) and how these infections are achieved (e.g., with or without intratracheal inoculation with agarose beads, which are sometimes used to establish chronic *P. aeruginosa* infection).

Nonetheless, these sometimes conflicting findings point toward an overall, incomplete understanding of the advantages of mucoid conversion. Furthermore, as discussed below, mucoid variants of *P. aeruginosa* are often found to coexist with other genetic/phenotypic variants within the CF lung, suggesting that intraspecies interactions among these variants may influence the true impact of mucoid conversion on the CF patient.

REVERSION: INSTABILITY OF THE MUCOID PHENOTYPE

P. aeruginosa mucoid variants (*mucA* mutant and *algT*$^+$) often revert back to a nonmucoid phenotype both *in vitro* and *in vivo* (423, 425–428) (Fig. 5 and 6). The most common pathway for reversion occurs via the acquisition of a second-site, suppressor mutation in *algT* (*mucA* and *algT* mutant) (425–427). A loss-of-function mutation in *algT* would shut off alginate production, despite a *mucA* mutation, as AlgT activity is required for transcription of the alginate biosynthesis operon (376). The vast majority (70%) of nonmucoid variants isolated from the chronically infected CF lung have a *mucA* mutation, suggesting that these populations are predominantly revertants rather than wild-type (*mucA*$^+$ *algT*$^+$) bacteria (425). While a second-site mutation in *algT* is the most commonly identified pathway for reversion *in vitro*, some revertants isolated both *in vitro* and *in vivo* have been shown to lack an *algT* mutation and thus cannot be complemented back to mucoidy via the expression of *algT* in *trans* (425, 426, 428). Although alternative, *algT*-independent pathways for reversion have not been well characterized, one publication shows that a second-site mutation in PA3257 (*algO*) can cause reversion *in vitro*; the function of AlgO is not understood, although the *algO* mutation was shown to reduce *algT* transcription, suggesting that either direct or indirect effects on AlgT function may be necessary for reversion (428). In theory, however, secondary mutations in any gene essential for alginate biosynthesis (e.g., *algD*, *algR*, *algB*, and *amrZ*, etc.) could be found to be responsible for *algT*-independent phenomena of reversion.

There are multiple hypotheses within the literature to explain the instability of the mucoid phenotype. Growth of *P. aeruginosa* in the absence of *in vivo* selective pressures is postulated to promote reversion *in vitro*; in part, this is attributed to the high energetic cost of alginate overproduction, which may not be advantageous under laboratory conditions (427). Additionally, certain growth modalities *in vitro* demonstrate

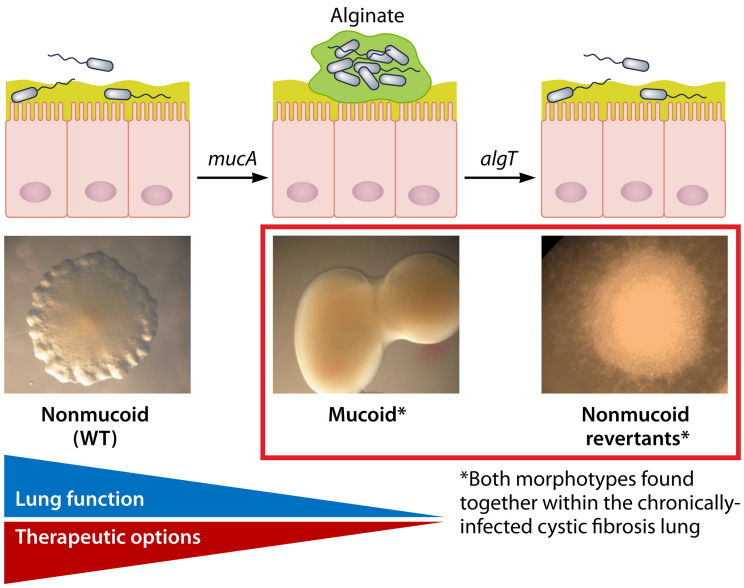

FIG 6 Paradigm for mucoid conversion and reversion within the cystic fibrosis (CF) lung. CF patients are initially infected by environmental (wild-type [WT]) isolates of *P. aeruginosa*. During chronic infection of the CF lung, nonmucoid variants of *P. aeruginosa* commonly acquire a *mucA* mutation, leading to a phenotypic switch to mucoidy (i.e., alginate overproduction). Mucoid conversion portends a decline in patient lung function, and mucoid variants exhibit increased recalcitrance to antibiotic therapy. However, via the acquisition of second-site, suppressor mutations (e.g., *algT*), mucoid variants commonly revert back to a nonmucoid phenotype both *in vitro* and *in vivo*. Mixed populations of mucoid and nonmucoid revertants of *P. aeruginosa* are commonly observed within the CF lung, suggesting a selective advantage for mucoid/nonmucoid coinfection. (Adapted from reference 377 with permission.)

a propensity for causing reversion, including growth of mucoid strains under static, aerobic conditions in liquid medium or within biofilms (425, 429, 430). Nonetheless, the triggers or microenvironmental cues that promote reversion of mucoid *P. aeruginosa* within the CF lung remain unknown.

However, in a longitudinal study of nonmucoid and mucoid isolates of *P. aeruginosa* from a cohort of patients in Denmark, it was found that nonmucoid revertants (*mucA* and *algT* mutant) can acquire third-site mutations, which restore mucoidy, and fourth-site mutations that cause reversion once again (431). These findings suggest that with regard to alginate production, *P. aeruginosa* isolates exhibit phenotypic switching within the CF lung throughout the course of disease. The mechanism of this phenomenon is attributed to sigma factor competition between AlgT and other *P. aeruginosa* sigma factors, including RpoN and RpoD; briefly, a mutation in *algT* that results in reversion may not cause a complete loss of function but rather may cause a reduced affinity of AlgT for the RNA polymerase; in this case, transcriptional regulation due to competing transcription factors (e.g., RpoD) may predominate. However, upon the acquisition of a subsequent *rpoD* mutation, AlgT may be able to more favorably compete for binding to the RNA polymerase and thus upregulate alginate biosynthesis once again (431, 432).

The above-mentioned study and numerous additional reports within the literature indicate that both mucoid and nonmucoid variants coexist within the chronically infected CF lung (253, 254, 269, 425, 431, 433–438) (Fig. 6). If alginate overproduction alone presented a substantial and permanent advantage for *P. aeruginosa*, we might expect all nonmucoid variants within the lung to be outcompeted. Indeed, the copresence of mucoid strains and nonmucoid revertants within the hyperinflammatory CF lung suggests a broader advantage for mixed-variant *P. aeruginosa* communities.

To that end, our group recently demonstrated that mixed-variant *P. aeruginosa* populations exhibit enhanced resistance to innate antimicrobials *in vitro* (439). Cocultures of mucoid and nonmucoid variants show greater resistance to both LL-37 and

H_2O_2 than monocultures of either variant. In mixed populations, each colony variant contributes to immune evasion via the sharing of public goods: mucoid strains protect nonmucoid strains from LL-37 via the production of alginate. In contrast, nonmucoid revertants (i.e., *algT* mutants) shield mucoid strains from H_2O_2 via the overproduction of catalase (KatA). We also demonstrate that the expression of *katA* is negatively regulated in a *mucA* background via AlgT and a downstream transcription factor, AlgR. Furthermore, in nonmucoid revertants, KatA release into the extracellular milieu is mediated by the *lys*-encoded endolysin (i.e., explosive cell lysis) (439).

These findings support the premise that genetically/phenotypically diverse *P. aeruginosa* populations *in vivo* may exhibit selective advantages in circumventing host immunity (and other environmental pressures). Further examination of the interactions between different *P. aeruginosa* colony morphotypes (e.g., mucoid and RSCV) that coinfect the CF airway is ongoing in our laboratory and others. However, one quickly emerging area of interest pertains to studying the host-microbe interface within spatially distinct niches of the CF lung.

CF LUNGS EXHIBIT INTERLOBAR VARIABILITY IN DISEASE: MICROENVIRONMENTAL STUDIES OF THE HOST-PATHOGEN INTERFACE

Clinical imaging studies and histopathological analyses of distinct anatomical regions of CF lungs demonstrate interlobar differences in tissue damage: the upper lobes and right side of CF lungs often show more severe histopathology and signs of permanent architectural damage (i.e., bronchiectasis, bullous emphysema, and air trapping, etc.) upon radiological imaging than the lower lobes and left side of the lungs (197, 198, 440–445). Additionally, to the extent that techniques have measured regional lung function and ventilation/perfusion defects, the upper lobes are more compromised than the lower lobes (446, 447). This interlobar heterogeneity has even prompted surgeons to perform lobectomies to selectively isolate and remove those regions of CF lungs that are most profoundly affected by the disease process (448, 449). Over time, these findings have led both scientists and clinicians to ask whether this interlobar variability is driven by intrinsically anatomical, immune, or pathogen-associated factors.

Recent evidence suggests that there are regional (i.e., lobar) differences in the distribution of bacterial and host factors across CF lungs. Multiple publications have examined the diversification/clonality of bacterial variants arising in different parts of the airway using explanted CF lungs. Willner et al. performed 16S sequencing of tissue sections from CF lung explants and found that bacterial populations present, in terms of diversity and abundance of species, were significantly different in each lobe of the lungs (450). These findings were confirmed and extended by seminal work in this field performed by Jorth et al. (451). In examining interlobar variations among *P. aeruginosa* isolates from CF lung explants, Jorth and colleagues demonstrated the presence of regionally distinct lineages of variants that were compartmentalized by lung lobe; that is, clonal relatedness of bacteria could be stratified spatially within the lungs, and there was limited intermixing among variants from one lobe to another (451).

Furthermore, variants derived from a given lung lobe shared not only genotypic but also phenotypic characteristics, including metabolic profiles, antibiotic resistance, and virulence factor expression. Most strikingly, *P. aeruginosa* isolates derived from a more damaged (upper lobe) region of the lungs exhibited greater resistance to antibiotics and immune effectors (i.e., to serum and neutrophil killing) and were also more virulent in an animal model of infection than isolates derived from a less damaged (lower lobe) region of the lungs (451). These paradigm-defining data suggest that the microenvironmental factors that select for genotypically/phenotypically distinct bacterial strains within the CF lungs likely vary by lung lobe; furthermore, the bacterial variants ultimately selected may differentially influence focal tissue damage within the organ.

Jorth et al. did not investigate variants of bacteria other than *P. aeruginosa*, perhaps because previous work by the same group found that in terms of sheer bacterial abundance, tested CF lung explants were often dominated by *P. aeruginosa* infection (451, 452). Nonetheless, other major coinfecting species also included *Burkholderia*

cenocepacia, *S. aureus*, *S. maltophilia*, and *A. xylosoxidans* (452). Indeed, the influence of these other organisms as well as their genetic/phenotypic diversity across different regions of the CF airway merit further investigation. To that end, a prodigious new approach to 3-dimensionally map the colocalization of multiple bacterial species (e.g., *P. aeruginosa* and *S. aureus*) within CF lung explants could yield exciting insights regarding bacterium-bacterium interactions *in vivo* (453).

Additional work in the field has sought to build on the above-mentioned findings. For instance, Hogan and colleagues examined regional BAL fluid and protected airway brush samples from the lungs of adult CF patients with stable disease (454). This study showed that measures of microbial diversity and abundance, accounting for *P. aeruginosa* and other bacterial organisms, were generally homogenous across different lung lobes of a given patient; furthermore, lobar tissue damage did not correlate with the regional microbes identified therein. Hogan et al. argue that their data may not agree with those of other studies because their work specifically examined specimens from patients with stable disease, whereas the studies by Jorth et al. and Willner et al. both used tissue from lung explants (i.e., end-stage disease) (450, 451, 454). This argument is partly substantiated by a recent publication that shows lobe-specific diversification of *S. maltophilia* lineages within a CF lung explant; however, there is also some evidence of global selective pressures within the lung as well, given how some *S. maltophilia* variants were found across multiple lung lobes (455). Regardless of some incongruencies within the literature, Hogan et al. aptly postulate that interlobar differences in tissue damage within CF lungs may not be the result of bacterial factors alone but rather may be the consequence of bacterium-host interactions.

There has been limited investigation of interlobar variations in host factors within CF lungs in direct relation to bacterial infection. Indeed, two studies using regional BAL fluid from CF patients find that while total bacterial density may differ between lung lobes, it does not correlate with markers of inflammation (e.g., proinflammatory cytokines [IL-8] and number of inflammatory cells) (456, 457). Independent of bacterial infection, however, the upper lobes of the lungs show greater numbers of neutrophils and higher concentrations of neutrophil-derived products (e.g., elastase) than the lower lobes (457).

These results are somewhat perplexing in the context of some above-mentioned studies wherein lavage of whole CF lungs demonstrated correlations between infecting bacterial populations (i.e., the presence of certain bacterial species as well as total bacterial density) and inflammatory indices (189–191). This discrepancy indicates that lobe-specific BAL and whole-lung BAL fluids may provide different answers about host-pathogen interactions. Nonetheless, given that there is some evidence that bacterial infection drives inflammation within CF lungs and conflicting data that suggest that patterns of local inflammation may not correlate with pathogens, more work is needed to uncover regional host-pathogen dynamics within the CF airway. These studies may ultimately enable a better understanding of focal, lobe-specific tissue damage, which remains a hallmark of CF pulmonary disease.

CONCLUSIONS

Scientific advancements have significantly affected the lives of CF patients, including revolutionary work in genetic therapy, but a cure for this devastating disease eludes us. Until a cure is discovered, investments continue in studying the pathogenesis of CF pulmonary disease in both the laboratory and clinical arenas. Indeed, a common thread uniting much of this work is the interaction between a dysfunctional immune response and persistent infection with highly adaptable, opportunistic pathogens, including *P. aeruginosa*. Although the temporal sequence of early events within the CF lung remains a source of some debate, both host immune effectors and bacterial virulence factors ultimately cause end-organ damage, leading to pulmonary failure and patient death (Fig. 7). Importantly, the genetic plasticity of *P. aeruginosa* makes it a particularly dangerous pathogen in CF, capable of evading diverse antimicrobial and innate immune stresses. Although *P. aeruginosa* acquisition is independently associated with

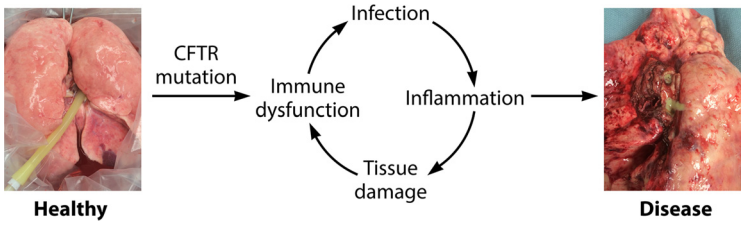

FIG 7 The pathogenesis of cystic fibrosis pulmonary disease: a vicious cycle driven by immune dysfunction and bacterial infection. Although the newborn CF lung is histopathologically comparable to a healthy lung, the CFTR mutation causes significant, primary defects in innate immunity (e.g., airway surface liquid [ASL] dehydration and acidification). These gaps in the airway's basic defenses predispose to early infection with bacterial pathogens, including *P. aeruginosa*. Bacteria within CF lungs trigger an exuberant, neutrophilic immune response, resulting in excessive inflammation. Bacterial virulence factors enable evasion of host inflammatory products, including reactive oxygen species, elastases, and extracellular DNA, which have collateral, damaging effects on healthy lung tissue. Compromised tissue integrity further contributes to immune dysfunction, initiating a cycle of recurrent and eventually chronic polymicrobial infections within a milieu of pathological inflammation. This disastrous series of events causes permanent destruction of airway architecture, manifesting as bronchiectasis, mucus plugging, and, ultimately, pulmonary failure.

poor outcomes, the emergence of specific phenotypic variants (e.g., RSCV or mucoid) further accelerates patient decline. Future work in this area will continue to examine the titanic struggle between bacterial infection and host responses to illuminate how changes in local microenvironments within the CF airway contribute to the overall disease process. In doing so, we continue to strive toward the development of novel antimicrobials, immune modulators, and other disease-modifying therapeutics that could profoundly impact the lives of CF patients.

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