Adverse Effects of *Pseudomonas aeruginosa* on CFTR Chloride Secretion and the Host Immune Response

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Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; COPD, chronic obstructive pulmonary disease; ENaC, epithelial sodium channel; OMV, outer membrane vesicle; sRNA, short interfering RNA; Cif, CFTR Inhibitory Factor; *Phe508del-CFTR*, deletion of the phenylalanine at the 508 amino acid position; *Gly551Asp*, substitution of a glycine with an aspartic acid at the 551 amino acid position; FEV$_1$, percentage of the predicted value for the forced expiratory volume in 1 second; G3BP1, Ras GTPase-activating protein-binding protein 1; USP10, ubiquitin specific peptidase 10; SGK1, Serum glucocorticoid kinase 1; c-Cbl, *Casitas B-lineage Lymphoma*; TAP1/TAP2, Transporter associated with antigen

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ABSTRACT

In the healthy lung the opportunistic pathogen, *P. aeruginosa*, is rapidly eliminated by mucociliary clearance, a process that is dependent on the activity of the CFTR anion channel that, in concert with a number of other transport proteins, regulates the volume and composition of the periciliary surface liquid. This fluid layer is essential to enable cilia to clear pathogens from the lungs. However, in cystic fibrosis (CF), mutations in the *CFTR* gene reduce Cl⁻ and HCO₃⁻ secretion, thereby decreasing periciliary surface liquid volume and mucociliary clearance of bacteria. In CF this leads to persistent infection with the opportunistic pathogen, *P. aeruginosa*, which is the cause of reduced lung function and death in ~95% of CF patients. Others and we have conducted studies to elucidate the effects of *P. aeruginosa* on wild type and Phe508del-CFTR Cl⁻ secretion as well as on the host immune response. These studies have demonstrated that Cif (CFTR Inhibitory Factor), a virulence factor secreted by *P. aeruginosa*, is associated with reduced lung function in CF, induces the ubiquitination and degradation of wt-CFTR as well as TAP1, which plays a key role in viral and bacterial antigen presentation. Cif also enhances the degradation of Phe508del-CFTR that has been rescued by ORKAMBI, a drug approved for CF patients homozygous for the Phe508del-CFTR mutation, thereby reducing drug efficacy. This review is based on the Hans Ussing Distinguished Lecture at the 2016 Experimental Biology Meeting given by the author.

Tribute to Professor Ussing

The development of the “short circuit” technique to measure electrogenic sodium (Na⁺) transport across frog skin, published in 1951 by Professor Hans Ussing, led to the first comprehensive and mechanistic understanding of Na⁺ transport across frog skin (39, 81). This approach to measure electrogenic Na⁺ as well as chloride (Cl⁻) transport across epithelial cell monolayers is highly relevant 66 years after its development. It has enabled numerous investigators to elucidate the ion transport properties of a number of epithelia, including epithelial cells in the lung in health and disease including Cystic Fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Two excellent and
comprehensive reviews of Professor Ussing’s career and the development of the short circuit current

technique have been published (44, 45).

**Introduction**

This article will first review what is currently known about electrogenic Na\(^+\) and anion (Cl\(^-\) and HCO\(_3^-\)) transport by airway epithelial cells, and how salt and water transport plays an essential role in mucociliary clearance of bacteria from the lungs. This is followed by a discussion of how mutations in the CFTR anion channel cause CF, which leads to chronic, unremitting colonization of the lungs by bacteria, the major cause of reduced lung function in CF patients. Subsequent sections of this review will focus on studies describing how the virulence factor Cif (CFTR Inhibitory Factor), secreted by *P. aeruginosa*, the dominant pathogen in late stage CF, inhibits wild type and ORKAMBI rescued Phe508del-CFTR anion secretion, reduces bacterial and viral antigen presentation, a key component of the adaptive immune response, and inhibits the generation of host proresolving lipids.

**Overview of Cl\(^-\) and HCO\(_3^-\) secretion by airway epithelial cells: role of wt-CFTR**

Ussing chamber and patch clamp studies have demonstrated that Cl\(^-\) and HCO\(_3^-\) secretion by wt-CFTR plays an essential role in the secretion of isotonic fluid onto the airway surface, which is essential for the mucociliary clearance from the lungs of inhaled bacterial and other pathogenic organisms as well as environmental contaminants (8, 21, 22, 32, 34, 46, 61, 69, 72, 73). A model of the transport proteins that mediate isotonic fluid secretion by airway epithelia cells is depicted in Figure 1. Cl\(^-\) enters airway epithelial cells across the basolateral membrane via the NKCC1 transporter that has a stochiometry of 1K\(^+\)/2Cl\(^-\)/1Na\(^+\). The K\(^+\) that enters the cell via NKCC1 recycles across the basolateral membrane into blood via a K\(^+\) channel. The Na\(^+\) that enters the cell via NKCC1 also recycles across the basolateral membrane back into the blood via the Na\(^+\)-K\(^+\)-ATPase. Cl\(^-\) exits the cell across the apical membrane via the CFTR anion channel, as well as Anoctamine 1 (ANO1, also called TMEM16A) a calcium activated Cl\(^-\) channel. Cl\(^-\) secretion by CFTR and ANO1 generates an apical-negative transepithelial voltage that provides the driving force for the paracellular diffusion of Na\(^+\) across the tight junctions between cells. Although the epithelial Na\(^+\) channel, ENaC, is present in the apical membrane, several studies, but not all, have shown that CFTR suppresses ENaC activity, such that normally little Na\(^+\) enters the cell across
the apical membrane (21, 34, 67).

Airway epithelial cells also secrete HCO$_3^-$ (Figure 1). NBC1 (SLC4A2), a Na$^+$-HCO$_3^-$ cotransporter, mediates HCO$_3^-$ uptake across the basolateral membrane. The HCO$_3^-$ that enters the cell is secreted across the apical membrane by CFTR and SLC26A4 (pendrin), a Cl$^-$/HCO$_3^-$ exchanger (34, 69). The Na$^+$ that enters the cell across the basolateral membrane with HCO$_3^-$ is recycled across the membrane by the Na$^+$-K$^+$-ATPase. Some Cl$^-$ that is secreted by CFTR recycles across the apical membrane back into the cells to drive HCO$_3^-$ secretion by SLC26A4. HCO$_3^-$ secretion by CFTR and SLC26A4 plays a key role in establishing a pH of the periciliary fluid of ~7.4, a value that is important for the optimal antimicrobial properties of the periciliary fluid (34, 60, 69).

The secretion of NaCl and NaHCO$_3$ into the airway surface establishes a small osmotic gradient that drives the paracellular and transcellular movement of water into the airway surface via aquaporin water channels, which hydrates the overlying mucus, thereby decreasing its viscosity. In the presence of wt-CFTR the transport processes depicted in Figure 1 maintain the periciliary fluid depth at ~7 μm, which is the length of the cilia, and is the optimal depth to allow cilia to beat (52). This clears bacteria from the airways that have been trapped in the mucus overlying the periciliary fluid (Figure 2).

**Mutations in the CFTR gene cause CF**

Mutations in the CFTR gene cause CF, the most common fatal genetic disease in Caucasians (34, 69). Worldwide ~80,000 people have CF and ~95% die from respiratory failure subsequent to chronic bacterial infections of the lungs at a mean age of 38 (24, 26, 75). However, recent studies in the United States based on a ten year period from 2000 to 2010 have shown that survival has improved by 1.8%/year due to advances in treatment (49). If this rate continues it would predict a mean age of survival for children born in 2016 at 56 years (49).

Mutations in the CFTR gene reduce the abundance of functional CFTR anion channels in the apical membrane of airway epithelial cells and submucosal glands, leading to a dramatic reduction in Cl$^-$ and HCO$_3^-$ secretion, a decrease in periciliary depth and pH, and an increase in the amount of mucus secreted by submucosal glands, resulting in a layer of thick mucus that sticks to cilia inducing ciliastasis and an inability to clear bacteria from the airways (52)(Figure 3 and 4). At the time this review was written
2,008 mutations had been identified in the CFTR gene (http://www.genet.sickkids.on.ca/StatisticsPage.html). However, only about 150 mutations are known to cause CF, and these mutations have been divided into six classes based on the effects that the mutations have on CFTR location and function (23, 25, 26, 63, 75). Class I mutations lead to an absence of CFTR protein due to lack of mRNA transcripts and stop-codon mutations that lead to degradation of mRNA. Recently, it has been suggested that Class I mutations can be subdivided into two categories based on the effect on CFTR mRNA (24, 25, 51, 76). Class II mutations, the most common (e.g., Phe508del), lead to premature degradation or incomplete maturation of CFTR such that the channel is degraded in the proteasome. As a result it does not reach the apical plasma membrane resulting in a dramatic reduction of CFTR mediated Cl⁻ and HCO₃⁻ secretion. The Phe508del mutation also reduces channel activity and the residence time of chemically rescued CFTR in the apical plasma membrane (82, 83). Approximately fifty percent of CF patients are homozygous for the Phe508del mutation. Class III mutations lead to defects in CFTR channel regulation and gating. The most common mutation in this category is Gly551Asp, which is present in ~4-5% of CF patients. Although Gly551Asp-CFTR is present in the apical membrane of airway epithelial cells, it does not secrete anions. Class IV mutations, present in 1-2% of CF patients, lead to defective Cl⁻ and HCO₃⁻ conductance of CFTR. Class V mutations cause a major reduction in the levels of functionally normal CFTR protein due to mutations in the promoter region of CFTR or abnormal mRNA splicing. Finally, Class VI mutations reduce the stability, and therefore the amount of CFTR in the apical plasma membrane, thereby reducing the amount of CFTR mediated Cl⁻ and HCO₃⁻ secretion.

In addition to a progressive decline in lung function, assessed by measuring FEV₁ (percentage of the predicted value for the forced expiratory volume in 1 second), CF is also characterized by exocrine pancreatic insufficiency resulting in diabetes, gastrointestinal malabsorption that results in malnutrition and impaired growth, sinusitis and male infertility due to a congenital bilateral malformation of the vas deferens (63). Mutations in CFTR also severely reduce the ability of neutrophils and macrophages to kill bacteria and dramatically increase the levels of proinflammatory cytokines in the lungs (63).

Although CF is caused by mutations in the CFTR gene, there is considerable phenotypic variation
even among individuals with the same mutation (63). Although some of the variation has been attributed to modifier genes, including SLC26A9, SLC9A3, SLC6A14, TNF, and TGFβ1, environmental factors including second-hand smoke, pollutants, and infectious agents such as bacteria, fungi and viruses also influence disease severity (30, 63).

**Drug Discovery and CF: KALYDECO and ORKAMBI**

In 2012 KALYDECO (Ivacaftor, VX-770) was approved by the FDA for CF patients who have at least one Gly551Asp allele, and more recently was approved for other mutations in class III (2, 62, 82, 83). KALYDECO increases the open probability of Gly551Asp-CFTR channels and increases Cl− and HCO3− secretion. KALYDECO also decreases mucus viscosity (9). Importantly, KALYDECO in the short term also reduces the number of Gly551Asp-CFTR patients who are culture positive for *P. aeruginosa* and Aspergillus, but not *Staphylococcus aureus* or other common CF pathogens (35, 66). In 2015 the FDA approved ORKAMBI, a combination of Ivacaftor (VX-770) and Lumicaftor (VX-809), which increases the export of misfolded Phe508del-CFTR from the endoplasmic reticulum to the apical plasma membrane, for patients homozygous for the Phe508del mutation (82, 83, 85). Although ORKAMBI does not reduce the bacterial load of *P. aeruginosa* in the CF lung, and only increases FEV1 by 2.6 to 4.0%, it reduces the rate of pulmonary exacerbations by 30%-39% (85). It has been suggested that the modest effect of ORKAMBI on FEV1 is due to an inhibitory effect of Ivacaftor (VX-770) on the ability of Lumicaftor (VX-809) to increase plasma membrane Phe508del-CFTR (20, 77, 84). However, a recent study suggests that the inhibitory effect on Lumicaftor only occurs at levels of Ivacaftor above pharmacologically relevant levels (53).

**Chronic lung infections in CF**

The lungs of individuals with CF become chronically colonized with bacteria early in life, in part due to decreased mucociliary clearance and increased mucus viscosity due to periciliary dehydration, the release of DNA by neutrophils, acidification of the periciliary layer that reduces the activity of antimicrobial peptides secreted by airway epithelial cells, and defects in the ability of macrophages and neutrophils to clear pathogens (7, 34, 60, 63) (Figure 4). Although there is considerable variability among CF patients regarding the composition of the lung microbiome, the most common pathogens include *P.*
aeruginosa, Staphylococcus aureus, and Aspergillus species. Respiratory exacerbations caused by P. aeruginosa are associated with an elevated inflammatory response in CF that leads to precipitous declines in FEV₁, which often does not recover following hospitalization and antibiotic treatment (7, 63). In addition, methicillin-resistant S. aureus (MRSA) lung infections are present in as many as 30% of CF patients in some populations. Acute viral infections also induce pulmonary exacerbations and decrease FEV₁ (7, 63).

A variety of virulence factors secreted by P. aeruginosa have adverse effects on mucociliary clearance. Rhamnolipids promote ciliastasis (64), and alginate increases mucus production, thereby reducing immune recognition and mucociliary clearance of bacteria (7). Ciliary beating and mucociliary transport are also reduced by pyocyanin, which also decreases CFTR Cl⁻ secretion (71). Alkaline proteases also contribute to lung infection by proteolytically activating ENaC, which increases Na⁺ absorption, thereby reducing periciliary fluid volume and mucociliary clearance (18, 19).

**P. aeruginosa inhibits CFTR Cl⁻ secretion and mucociliary clearance**

In a series of studies others and we have demonstrated that P. aeruginosa (PA14 and six clinical isolates, including three mucoid and three non-mucoid isolates) reduces both wt-CFTR and ORKAMBI rescued Phe508del-CFTR Cl⁻ secretion by airway epithelial cells (3, 6, 16, 48, 68, 77, 79, 80). This work began several years ago when we initiated a series of studies examining the effect of P. aeruginosa on CFTR Cl⁻ secretion by human airway epithelial cells. The initial hypothesis was that P. aeruginosa would stimulate wt-CFTR Cl⁻ secretion and thereby enhance NaCl and fluid secretion and promote mucociliary clearance of bacteria to protect the lungs from infection. Intuitively, this hypothesis made sense from a homeostasis perspective. However, to our surprise the opposite was observed. Addition of P. aeruginosa to the apical side of a variety of polarized airway epithelial cells lines as well as primary airway epithelia cells, revealed that P. aeruginosa dramatically reduced wt-CFTR Cl⁻ secretion (3, 6, 16, 48, 77, 79). In addition, P. aeruginosa also inhibited ORKAMBI (VX-809+VX-770) rescued Phe508del-CFTR Cl⁻ secretion (3, 6, 16, 48, 77, 79).

By contrast, P. aeruginosa (PAK) has been shown to stimulate fluid secretion by pig submucosal glands (47). The discrepancy between this observation and those by others and us may reflect a
difference in the pig versus human, a difference in the effects of P. aeruginosa on surface epithelial cells versus submucosal glands and/or a unique feature of PAK, a laboratory strain of Pseudomonas. Since LPS, homoserine lactone and flagellin stimulate CFTR Cl⁻ secretion whereas phospholipase C (PLcH), sphingomylenases and β-lactamase inhibit CFTR Cl⁻ secretion by airway epithelia cells, it is possible that the effect of variants of P. aeruginosa on CFTR Cl⁻ secretion may also depend on the relative abundance of factors that stimulate versus those that inhibit CFTR Cl⁻ secretion (6, 15-17, 36, 77).

The inhibition of wt-CFTR and ORKAMBI rescued Phe508del-CFTR Cl⁻ secretion by P. aeruginosa is mediated by outer membrane vesicles (OMVs) (6, 15, 16, 77). P. aeruginosa resides primarily in the mucus layer and secreted OMVs diffuse through the mucus layer and fuse with lipid rafts in the apical membrane of airway epithelial cells. OMVs, spheroid proteoliposomes 10 to 300 nm in diameter secreted by Gram-negative bacteria, including P. aeruginosa, contain a number of toxins and virulence factors and deliver virulence factors directly into host cell cytoplasm by fusion with lipid rafts in the plasma membrane (17, 36, 43). Others and we have shown that OMVs secreted by P. aeruginosa contain alkaline phosphatase, hemolytic phospholipase C (PLcH), sphingomylenases and β-lactamase (6, 15-17, 36, 77).

SMAse and PlcH hydrolyze sphingomyelin, which reduces the phosphorylation of the regulatory domain of CFTR, thereby decreasing Cl⁻ secretion. P. aeruginosa packages β-lactamase into the lumen of OMVs, which degrades β-lactam antibiotics including Tobramycin, the most commonly prescribed antibiotic for CF patients, and thereby increases antibiotic resistance of P. aeruginosa (42).

By western blot of P. aeruginosa OMVs and a mass spectrometry, proteomic approach we demonstrated that P. aeruginosa OMVs also contain a protein, PA2934, which codes for a novel 29 kDa epoxide hydrolase that enhances the ubiquitination and degradation of wt-CFTR (3, 5, 6, 13, 15, 16, 48, 77, 79, 87). Accordingly, we named PA2934 Cif, for CFTR Inhibitory Factor. Cif reduces wt-CFTR Cl⁻ secretion in a variety of airway epithelial cell lines and primary airway epithelial cells (3, 6, 15, 16).

Importantly, Cif is expressed in both mucoid and non-mucoid clinical isolates of P. aeruginosa obtained from CF lungs, and Cif homologs are expressed in Acinetobacter nosocomialis and Burkholderia cepacia that also infect CF lungs (4). Moreover, Cif also enhances the ubiquitination and degradation of Phe508del-CFTR (68). These observations led us to the conclusion that Cif, as well as the other
virulence factors noted above, could facilitate the colonization of the CF lung by *P. aeruginosa* by reducing ORKAMBI stimulated Phe508del-CFTR abundance and activity, thereby reducing mucociliary clearance of bacteria. If so, Cif may play a major role in reducing the efficacy of ORKAMBI resulting in the inability to reduce the *P. aeruginosa* load in the CF lung (85).

**Cellular mechanism of Cif inhibition of CFTR Cl− secretion**

The primary mechanism of action of Cif is that is disrupts the endocytic trafficking of CFTR and thereby reduces the amount of CFTR in the apical plasma membrane (1, 3, 5-7, 10, 15, 16, 28, 38, 40, 48, 50, 78, 86, 87)(Figure 5). In non-infected airway epithelial cells CFTR in the apical plasma membrane is endocytosed from the plasma membrane by a process that is regulated by c-Cbl (named after Casitas B-lineage Lymphoma), SGK1 (Serum glucocorticoid kinase 1) and a variety of other adaptor proteins (11, 27, 29, 50, 70, 74, 87). Whereas SGK1 inhibits endocytosis of CFTR (11), c-Cbl facilitates CFTR endocytosis by two mechanisms (12, 29, 87). First, c-Cbl is an adaptor protein that facilitates CFTR endocytosis by a ubiquitin-independent mechanism, and second c-Cbl ubiquitinates CFTR in early endosomes thereby facilitating its lysosomal degradation (12, 87). If CFTR in endosomes is deubiquitinated by the deubiquitinating enzyme, USP10 (ubiquitin specific peptidase 10), CFTR returns to the apical cell membrane in recycling endosomes, thereby maintaining CFTR anion secretion (12, 16). By contrast, if CFTR is not deubiquitinated by USP10, it is not recycled back to the apical membrane, and is delivered to the lysosome for degradation, resulting in a reduction in CFTR abundance and thus, anion secretion. During infection with *P. aeruginosa*, secreted OMVs fuse with the apical cell membrane of airway epithelia cells and deliver the virulence factors described above, as well as Cif, into the cytoplasm of airway epithelial cells where Cif’s activity stabilizes G3BP1:USP10 binding, thereby inhibiting USP10 activity (12, 13, 16). This in turn decreases the USP10-mediated deubiquitination of CFTR and increases the degradation of ubiquitinated CFTR in lysosomes (12, 13, 16).

**P. aeruginosa reduces viral antigen presentation by MHC Class I**

Cif also has a negative effect on bacterial and viral antigen presentation by airway epithelial cells (13, 33)(Figure 6). TAP1, like CFTR, is a member of the ABC transporter family. The TAP1/TAP2 complex transports antigens across the membrane of the endoplasmic reticulum where they combine with MHC
Class I, whereupon the antigen loaded MHC Class I complex is transported to the plasma membrane where the complex is recognized by CD8^+ T lymphocytes, the activation of which is a key component of the adaptive immune response (13, 33). Like CFTR, TAP1 is ubiquitinated by an E3-ligase and deubiquitinated by USP10. When the lungs are infected with *P. aeruginosa* Cif activity stabilizes G3BP1:USP10 binding and inhibits the deubiquitinating activity of USP10, thereby increasing the amount of polyubiquitinated TAP1, which is degraded in the proteasome. The reduction in TAP1 abundance decreases antigen translocation into the endoplasmic reticulum, an effect that reduces antigen available to MHC class I molecules for presentation at the plasma membrane of airway epithelial cells and recognition by CD8^+ T lymphocytes. Cif is the first bacterial factor identified that inhibits TAP function and MHC class I antigen presentation (13).

**Cif inhibits host proresolving lipid mediators**

A recent study has identified an unexpected effect of Cif (28). Cif promotes the hydrolysis of airway epithelial cell derived 14,15-epoxyeicosatrienoic acid, thereby disrupting the production of the proresolving lipid 15-epi lipoxin A4, which suppresses IL-8 stimulated transmigration of neutrophils. Thus, Cif promotes excessive transmigration into the lungs of neutrophils, which release elastase and DNA, the proximate cause of lung damage and reduced lung function in CF (63). Importantly, it was also shown that levels of Cif in brochoalveolar lavage fluid obtained from CF patients correlated with reduced levels of 15-epi lipoxin A4, elevated IL-8 and reduced lung function (28). These exciting observations suggest that inhibition of Cif activity with a small molecule may be an effective therapy to reduce excessive inflammation and neutrophil transmigration in the CF lungs, and will also block the adverse effects of Cif to reduce ORKAMBI stimulated Phe508del-CFTR anion secretion (3, 28, 38, 77).

**Mutations in the CFTR gene and viral infection enhance the formation of antibiotic resistant P. aeruginosa biofilms by increasing iron availability**

In addition to Cif several other factors enhance the ability of *P. aeruginosa* to chronically infect the CF lung. In CF increased levels of iron in the airway surface fluid correlates with the frequency of bacterial exacerbations; thus, increased iron in the airway surface liquid has been proposed to play a role in the development and maintenance of chronic antibiotic resistant bacterial lung infections in CF (31, 65).
Several years ago we developed a co-culture model of *P. aeruginosa* growing on the apical side of polarized CF human airway epithelial cells to elucidate the mechanism(s) whereby the *Phe508del* mutation in CFTR enhances the formation of antibiotic resistance biofilms (14, 55-59). We observed that CF airway cells, compared to cells expressing wt-CFTR, enhance the development of Tobramycin resistant *P. aeruginosa* biofilms, in part, by releasing iron into the apical fluid. Chelation of iron reduced biofilm formation on airway cells, whereas iron supplementation enhanced the formation of *P. aeruginosa* biofilms (14, 55-59).

Because pulmonary exacerbations caused by viral infections in CF are associated with increased severity of bacterial infections, a recent study was conducted to elucidated the mechanism whereby viral infection increases bacterial infection by *P. aeruginosa* (37). Infection of airway epithelial cells with respiratory syncytial virus (RSV) increased biofilm formation via an increase in antiviral interferon (IFN) secretion, which stimulates the release of the iron-binding protein transferrin onto the airway surface fluid. Addition of an iron chelator blocked the effect of RSV on the formation of antibiotic resistant *P. aeruginosa* biofilms and addition of excess iron reversed the effect of the chelator. The effect of RSV on biofilm formation by *P. aeruginosa* was also observed in a mouse lung infection model (37, 54). Thus, in CF, disruption of iron homeostasis and viral stimulated iron secretion into the periciliary fluid enhances the development of antibiotic resistant biofilms of *P. aeruginosa*.

**OMVs contain sRNAs that mitigate the immune response to *P. aeruginosa*, thereby allowing *P. aeruginosa* to thrive**

*P. aeruginosa* also enhances its ability to chronically infect CF lungs by suppressing the innate immune response to infection (41). Several recent studies have shown that OMVs contain short RNAs that have the potential to target host mRNA function and/or stability (41). To examine the role of sRNAs in OMVs secreted by *P. aeruginosa* we used RNA-Seq to identify sRNAs in OMVs, and we observed that many sRNAs are selective packaged in OMVs and that OMVs deliver sRNAs into the cytoplasm of airway epithelial cells (41). One sRNA (sRNA52320) was abundant in OMVs and reduced LPS-mediated activation of the MAPK pathway and IL-8 secretion by primary human airway epithelial cells. Deletion of sRNA52320 enhanced both LPS and OMV-induced IL-8 secretion. sRNA52320 also reduced OMV-
stimulated secretion of KC (the mouse analog of IL-8) and neutrophil infiltration in mouse lung. Taken together, these findings demonstrate that P. aeruginosa secretes OMVs that deliver sRNAs into lung epithelial cells to reduce the host innate immune response, and thereby reduces the ability of the host to reduce and/or eliminate P. aeruginosa from the lungs.

**Future prospects**

This review has described a number of approaches that are utilized by P. aeruginosa to establish and maintain a chronic infection in the CF lungs. These include secretion of a number of virulence factors that have adverse effects on CFTR function, including Cif packaged in OMVs, that reduces ORKAMBI stimulated Phe508del-CFTR Cl⁻ and HCO₃⁻ secretion. Cif also suppresses MHC Class I bacterial and viral antigen presentation, a key component of the adaptive immune response. In addition, Cif promotes the hydrolysis of airway epithelial cell derived 14,15-epoxyeicosatrienoic acid, thereby disrupting the production of the proresolving lipid 15-epi lipoxin A4. This results in excessive neutrophil migration into the CF lungs and release of DNA, which increases mucus viscosity, and elastase that causes lung damage. Moreover, P. aeruginosa also secretes OMVs containing sRNA52320, which suppresses the innate immune response to bacterial infection. Thus, identification of novel strategies to block the fusion of OMVs with lipid drafts in host cells to block the delivery of Cif into CF epithelial cells, and/or a small molecule to inhibit Cif activity, are likely to lead to an increase in the efficacy of ORKAMBI and a dramatic improvement in lung function in CF.

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GRANTS
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AUTHOR CONTRIBUTIONS
B.A.S. wrote the manuscript.

FIGURE LEGENDS
Figure 1. Cell model of the transport proteins in airway epithelial cells that mediate NaCl and water secretion into the periciliary layer in cells expressing wt-CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). wt-CFTR activates SLC26A4 (Cl-/HCO₃⁻ exchanger, also called pendrin) and ANO1 (Anoctamine 1, also called TMEM16A) and inhibits ENaC (Epithelial Na⁺ Channel). NKCC1 (Na⁺-K⁺-2Cl⁻ cotransporter, also know as SLC12A2), ATP (Na⁺-K⁺-ATPase) and NBC1 (Na⁺-HCO₃⁻ cotransporter, also know as SLC4A2) are located in the basolateral membrane. Na⁺ diffuses across the tight junctions down and electrochemical gradient into the blood. Water is secreted into the periciliary space across the tight junctions and across cells via aquaporin water channels (not shown), due to the osmotic gradient across epithelial cells established by NaCl secretion into the periciliary fluid.

Figure 2. In the non-CF airway wt-CFTR mediates anion secretion that results in a periciliary layer that is ~7 μm deep, which allows cilia to beat and clear bacteria and environmental contaminants from the lungs (i.e., mucociliary transport). The yellow hexagons represent antimicrobial factors secreted by epithelial cells. The green circles represent mucins.
Figure 3. Cell model of the transport proteins in CF airway epithelial cells. Mutations in CFTR, in particular \textit{Phe508del}, cause the retention of Phe508del-CFTR in the endoplasmic reticulum, and its degradation in the proteasome (this is indicated by CFTR inside the cell and its absence in the apical plasma membrane). Because CFTR, which inactivates ENaC (Epithelial Na$^{+}$ Channel), is not present in the apical plasma membrane, ENaC is active and mediates Na$^{+}$ reabsorption. Moreover, the absence of CFTR, which activates ANO1 and SLC26A4, results in inactivation of ANO1 and SLC26A4. This is illustrated by the lack of ion movement across the apical plasma membrane via these transporters. NKCC1 and NBC1 do not contribute to NaCl reabsorption. ATP (Na$^{+}$-K$^{+}$-ATPase).

Figure 4. In the CF airway the lack of CFTR anion secretion results in a dramatic reduction in the depth of the periciliary fluid layer, which reduces the ability of cilia to beat and clear bacteria, viruses and environmental contaminants from the lungs (i.e., mucociliary transport). Moreover, increased mucin secretion and the presence of DNA in mucus due to lysis of neutrophils increases mucus viscosity, which also decreases mucociliary transport. The green circles represent mucins. The red oblongs with tails (flagella) represent \textit{P. aeruginosa}.

Figure 5. Schematic representation of the intracellular trafficking of wt-CFTR in healthy, non-infected airway epithelial cells (left) and in airway epithelial cells infected with \textit{P. aeruginosa} (right). In non-infected cells CFTR is ubiquitinated and removed from the plasma membrane by endocytosis by a process that is regulated by c-Cbl and SGK1. Whereas SGK1 inhibits the endocytosis of CFTR, c-Cbl increases the endocytosis of CFTR. If deubiquitinatated by USP10, CFTR recycles back to the apical cell membrane. By contrast, if CFTR is not deubiquitinated it is delivered to the lysosome for degradation. During infections with \textit{P. aeruginosa}, OMVs secreted by the bacterium fuse with lipid rafts in the apical cell membrane of airway epithelia cells and deliver Cif to the cytoplasm where Cif activity stabilizes G3BP1:USP10 interaction. This inhibits USP10 activity and reduces USP10-mediated deubiquitination of CFTR. This increases the degradation of CFTR in lysosomes and reduces the amount of CFTR in the apical plasma membrane.
Figure 6. Schematic representation of antigen presentation by MHC class I in airway epithelial cells and the effect of Cif on antigen presentation. Left, viral and bacterial proteins in the cytoplasm of airway epithelial cells are degraded in the lysosome, transported into the lumen of the endoplasmic reticulum by the TAP1/TAP2 complex, whereupon antigen is presented to MHC class I, which transports the antigen from the endoplasmic reticulum to the plasma membrane. The antigen is recognized by CD8+ T lymphocytes, an important component of the adaptive immune response to infection. When the lungs are infected with *P. aeruginosa* secreted OMVs fuse with epithelial cells and deliver Cif into the cytoplasm (right side of figure). Cif activity stabilizes G3BP1:USP10 interaction. This inhibits USP10 activity and reduces USP10-mediated deubiquitination of TAP1. This results in increased degradation of ubiquitinated TAP1 in the proteasome, thereby reducing the ability of the TAP1/TAP2 complex to transport antigen into the lumen of the endoplasmic reticulum for antigen presentation by MHC Class I. This effect of Cif attenuates the adaptive immune response to infection by *P. aeruginosa*.

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P. aeruginosa releases OMVs containing virulence factors.

**HEALTHY**

- c-Cbl
- SGK
- Ubiquitinated CFTR is incorporated into early endosome
- USP10 deubiquitinates CFTR
- Recycling endosome returns CFTR to plasma membrane
- Ubiquitinated CFTR traffics to lysosome

**INFECTION**

- c-Cbl
- SGK
- Ubiquitinated CFTR is incorporated into early endosome
- E3-ligase ubiquitinates CFTR
- Cif enters cell
- Ubiquitinated CFTR traffics to lysosome
- Cif activity stabilizes G3BP1:USP10 binding and inhibits USP10 activity
- CFTR is degraded in lysosome
Ubiquitination and TAP1 Trafficking

**Healthy**
- Antigen is presented at the plasma membrane
- Viral protein is degraded by proteasome
- TAP1 presents viral peptide to MHC class 1 molecule
- E3-ligase ubiquitinates TAP1
- USP10 deubiquitinates TAP1
- Ubiquitinated TAP1 is degraded by proteasome

**Infection**
- Antigen is NOT presented at the plasma membrane
- Cif enters cell
- Cif activity stabilizes G3BP1:USP10 binding & inhibits USP10 activity
- Viral peptide is NOT presented to MHC class 1 molecule due to reduced TAP1
- TAP1 is degraded by proteasome
- P. aeruginosa releases OMVs containing virulence factors

Endoplasmic reticulum