HOST PATHOGEN INTERACTIONS IN THE CYSTIC FIBROSIS AIRWAY: *PSEUDOMONAS AERUGINOSA* IMPAIRS THE RESOLUTION OF PULMONARY INFLAMMATION BY MANIPULATING HOST LIPID SIGNALING

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Pseudomonas aeruginosa is a clinically important, opportunistic bacterial pathogen that can cause a wide range of severe infections. The bacterium is inherently resistant to many antibiotics and chronic infections are very difficult to treat. Patients with the genetic disease cystic fibrosis (CF) often have recurrent *P. aeruginosa* pulmonary infections resulting in robust inflammatory responses that lead to tissue destruction. Despite a hostile hyper-inflammatory environment and vigorous neutrophilic responses, *P. aeruginosa* can persist in the CF lung and eventually become the dominant bacterium in the airways.

We demonstrate that *P. aeruginosa* manipulates host lipid signaling and inflammatory response in the CF airway by secreting Cif, a virulence factor with epoxide hydrolase activity. Cif reduced the transcellular production of 15-epi lipoxin A_4 (15-epi LXA_4), a pro-resolving lipid mediator whose endogenous production is critical in limiting tissue inflammation. In the airway, neutrophil 15-epi LXA_4 production is stimulated by the epithelial-derived eicosanoid 14,15-epoxyeicosatrienoic acid (14,15-EET). Cif sabotaged the production of 15-epi LXA_4 by rapidly hydrolyzing 14,15-EET into its cognate diol, eliminating neutrophil generation of 15-epi LXA_4. Cif mediated inhibition of 15-epi LXA_4 eliminated the pro-resolving signal that potently suppresses IL-8-driven neutrophil trans-epithelial migration *in vitro*. Moreover, our retrospective analyses of CF patient samples supported the translational relevance of these preclinical findings. Elevated levels of Cif in bronchoalveolar lavage fluid correlated with

lower levels of 15-epi LXA_4, increased IL-8 concentrations and impaired lung function. These findings provide structural, biochemical and immunological evidence that the *P. aeruginosa* epoxide hydrolase Cif disrupts resolution pathways during bacterial lung infections in the CF airway. This study and the recent identification of Cif homologs in *Acinetobacter* and *Burkholderia* species suggest that bacterial epoxide hydrolases represent a novel virulence strategy shared by multiple respiratory pathogens.

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PREFACE

I would like to thank my parents David and Debra Flitter, my sister Jessica Hecht and my brother Ben Flitter for their love and support throughout all of life's adventures. To my husband Skylor Dinkelmann, you are my rock. I could not have completed this journey without you by my side. You are my everything, I am truly blessed to have you as my partner.

I dedicate my dissertation to my aunt Dr. Priscilla A. Campbell. Her research provided many significant contributions to the field of immunology, particularly in the areas of innate immunity and host pathogen interactions. She studied the immune response to *Listeria monocytogenes,* characterized multiple macrophage populations and postulated in 1990 that neutrophils respond to adaptive immune signals [1]. I was recently thrilled to discover that she was also contributing member of the research team that first described the exposure of neutrophil phosphatidylserine as an important signal that drives macrophage efferocytosis [2]. This seminal work continues to be a pertinent in the field as the article has been cited over 3000 times. The signals that promote the removal of apoptotic and necrotic neutrophils from inflamed tissues are of great interest to me, and a topic that I would like to continue to explore in the future. Pixie was a trailblazer, a strong advocate for women in science and is greatly missed. I think of her often and strive to follow in her footsteps.

1.0 PULMONARY INFECTION IN CYSTIC FIBROSIS

1.1 CYSTIC FIBROSIS: THE VICIOUS CYCLE

Cystic fibrosis (CF) is a life-threatening genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). There is no cure and it is estimated that almost 70,000 individuals worldwide and 30,000 in the United States suffer from CF. Although patients experience serious gastrointestinal, endocrine and fertility disorders, the majority of deaths associated with the disease are due to respiratory failure [3, 4]. Progressive lung deterioration is often the most challenging in the management of CF and is the major determinant of life span and quality of life in affected individuals. The loss of CFTR function results in poor innate epithelial defense and dysfunctional neutrophil responses, which are unable to eliminate bacterial pathogens from the CF airway. Substantial clinical data demonstrate *Pseudomonas aeruginosa* respiratory infections in the CF lung correlate with symptomatic lung disease, which is marked by excessive airway inflammation [4-6]. Persistent bacterial infections in the CF respiratory tract promote a perpetuating cycle of vigorous inflammation, resulting in extensive damage to tissue and eventually respiratory failure.

1.1.1 CFTR activity and airway epithelial host defense in the cystic fibrosis lung

The removal of foreign particles and pathogens from the lungs depend on the coordinated directional movement of the mucus secretions by ciliated cells that line the airways, termed mucociliary clearance [7]. In individuals with CF, mutations in CFTR cause thick mucus secretions to accumulate in the lung. CFTR is an anion channel that transports Cl^- and HCO_3^- ions across epithelial cell surfaces and whose function is essential for maintaining airway surface liquid (ASL), a critical component of innate host defense [8, 9]. ASL is comprised of two layered secretions, aqueous perciliary fluid coated by mucus secretions [10]. The periciliary fluid is directly above and in contact with airway epithelial cells (AEC), affording space for ciliary beat by distancing the mucus from the cell surface. Mucus secretions trap inhaled debris and microorganisms, which is then removed from the lung via mucociliary clearance. In CF, the loss of CFTR function results in sodium ion and water hyper-absorption by epithelial cells, resulting in the dehydrated periciliary fluid and dense mucous secretions [10].

In addition to sequestering and removing debris from the lung, ASL also contains numerous antimicrobial peptides, proteins, and lipids, which are important for innate mucosal defense [11]. The reduction of CFTR mediated HCO_3^- secretion by CF airway epithelia lowers the acidity of ASL, which has a negative impact on mucosal innate immunity [12-14]. Studies using CF pig models and primary CF epithelial cells demonstrate that the acidic environment in the CF lung reduces the activity of antimicrobial peptides and innate host defense [9, 14]. Acidic pH can reduce epithelial secretion of beta-defensins [15] and inhibit the functional activity of LL-37 [16]. Furthermore, HCO_3^- has also been shown to have pH independent effects on antimicrobial peptide function and is a critical ionic factor that can

directly enhance antimicrobial peptide activity [17]. As antimicrobial peptides in the ASL have synergistic interactions, a reduction of HCO_3^- secretion and pH in the CF lung may disrupt these cooperative relationships [18, 19].

Defective HCO_3^- secretion also disrupts rheological properties of mucins and is associated with abnormal mucus viscosity in CF [20]. The accumulation of dense and adhesive mucous, a reduction in normal mucociliary clearance and reduced antimicrobial peptide function produces conditions ideal for supporting robust bacterial growth in the CF airway. Despite repetitive antibiotic therapy, pulmonary bacterial infections still remain the primary cause of morbidity and mortality in CF patients. Thus, mutations in CFTR result in defective mucociliary clearance and poor innate antimicrobial activity in the CF airway, two very important features for normal lung function.

1.1.2 Dysfunctional neutrophil responses

Progressive bronchiectasis in CF is largely the result of persistent neutrophil accumulation and release of harmful proteases [21]. Neutrophil are often the first immune cells recruited to inflamed tissues and provide non-specific host defense through phagocytosis and the secretion of antimicrobial peptides and proteases [22]. However, in CF unrelenting neutrophil accumulation in the lung is ineffective at controlling pulmonary infections [21].

Although early work suggested that CFTR was not expressed on neutrophils [23], recent studies demonstrate not only is the anion channel important in neutrophil function, but loss of CFTR results in dysfunctional activity [24, 25]. Absent or defective CFTR results in abnormal phagolysosome chlorination of engulfed pathogens thus impairing neutrophil microbial killing activity [26-28]. One recent study demonstrated neutrophils from CF patient

sputum displayed functional exhaustion and reduced respiratory burst capacity compared to matched peripheral blood neutrophils [29]. CF neutrophils also have reduced surface expression of CD14, CD16 and CXCR1, which has been linked to poor phagocytic capacity [30, 31]. Interestingly, CF patients do not have difficulty containing bacterial infections outside of the lung and it remains unclear why abnormal neutrophil function is important for controlling infection in the airway, but not in other organs.

Many chemoattractants contribute to the neutrophil accumulation in the CF airway. The CF lung contains massive amounts of IL-8, LTB_4, C5a, IL-17 and bacterial products such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) [32-37]. The steep gradient of these chemoattractants in the lung, especially IL-8 is a hallmark of CF lung disease progression. However the relative contribution of these inflammatory mediators varies by individual patient [21]. The absence or dysfunction of CFTR does not appear to enhance the migration of CF neutrophils across epithelial cells [38]. However, AECs deficient in CFTR express elevated levels of adhesion molecules [39], which may increase neutrophil attachment to enhance transepithelial migration. Interestingly, studies have shown that purified blood neutrophils from CF patients migrate significantly faster through a Transwell filter compared to non-CF cells in response to IL-8 [40]. This suggests that CF neutrophils are already primed for activation in the periphery, most likely by the elevated production of soluble inflammatory molecules in CF patients.

The accumulation of dysfunctional neutrophils results in a large amount of proteases released into the airway, not only causing tissue damage, but also inactivating many host defense mechanisms. The concentration of proteases released in the CF airway environment also overwhelms host systems, particularly alpha-1-protease inhibitor and secretory

leukocyte protease inhibitor [41] which neutralize the toxic effects of neutrophil proteases. Cathepsin G, a protease released from primary neutrophil granules is found in large quantities in the CF lung and in a low pH environments can cleave and inactivate lactoferrin [42] and surfactant protein A [43] reducing their antimicrobial activity. The release of the neutrophil protease elastase has several deleterious effects and its concentrations measured in CF sputum correlate with pulmonary function [44, 45]. Elastase causes structural damage to the epithelial tight junctions promoting detachment [46] and also activates the apical epithelial sodium channel (ENaC) thereby increasing sodium hyperabsorption and further decreasing airway surface liquid height [47]. The abundance of elastase in CF lungs cleaves and impairs CXCR1 and complement receptors, which hinders bacterial phagocytosis and killing [46, 47]. Neutrophil elastase degrades macrophage phosphatidylserine receptors efferocytosis and inhibits normal resolution of inflammation [48]. Lastly, neutrophils also release large amounts of matrix metalloproteases into the CF airway [49], which digest the extracellular matrix [50] and lead to airway remodeling. The elevated release of neutrophil proteases has several harmful effects and greatly contributes to the immunopathology seen in the CF lung.

Frustrated CF neutrophils display delayed apoptosis and often undergo necrosis rather than clearance by normal apoptotic mechanisms [51]. Decomposing neutrophils release large amounts of intracellular contents, which fuel further neutrophil influx and contribute to the high viscosity of CF sputum [51-53]. This defect in normal neutrophil death could be due to low amount of several counter-regulatory molecules in the CF airway that are important for terminating or resolving inflammation [32, 54]. Thus, understanding how to reduce of

neutrophil dominated host response in the CF airway is key for designing future therapeutics to alleviate the progressive and damaging pulmonary inflammation.

1.2 *PSEUDOMONAS AERUGINOSA* PULMONARY INFECTIONS IN CYSTIC FIBROSIS

1.2.1 Adaptation and chronic colonization in the cystic fibrosis airway

P. aeruginosa is a Gram-negative bacteria that is able to utilize a wide variety of nutrient sources allowing it to live in the soil, water and in many tissue types in the human body [55]. Although *P. aeruginosa* is ubiquitous in the environment, it mainly causes disease in individuals who have compromised immune systems, thereby classifying it as an opportunistic pathogen. The bacterium can cause life threatening infections for individuals with severe burns, or implanted medical devices and CF patients, who starting early in life are particularly susceptible to *P. aeruginosa* pulmonary infections [55]. A high proportion of CF patients become infected at an early age and have recurrent respiratory infections throughout their lifetime [4]. Frequent *P. aeruginosa* infections and chronic dysregulated host responses combine to cause the majority of morbidity and mortality in CF patients [56].

The relatively large genome of *P. aeruginosa* provides enormous phenotypic flexibility and allows the bacteria to adapt and thrive in a variety of environments [8, 21]. By altering the production of virulence factors and switching to a biofilm mode of growth, the bacteria is able adjust to the CF lung environment and persist in the airway. This adaptation in the host by *P. aeruginosa* is demonstrated by the substantial differences in the phenotypes

between isolates from acute and chronic infections [57]. These phenotypic changes are thought to promote *P. aeruginosa* survival in the respiratory track by protecting the bacterium from host immune responses [56].

1.2.2 Bacterial pathogenesis and virulence factor production

P. aeruginosa is equipped with pili and monotrichous flagella, which are important for motility, surface attachment, bacterial aggregation and are essential for microcolony formation [57-59]. *P. aeruginosa* acute respiratory infections stimulate robust AEC inflammatory responses. AECs produce IL-8 upon TLR stimulation [60, 61]. Furthermore the bacteria stimulate the apical release of the eicosanoid hepoxilin A3 from AECs, which further promotes transepithelial migration of neutrophils into the lumen [62]. During initial acute infection, planktonic bacteria secrete and release multiple harmful proteins, which disrupt multiple innate immune responses further promoting the colonization of the CF airway [8].

P. aeruginosa produces an impressive array of secreted virulence factors controlled by complex regulatory circuits and multiple signaling systems [63]. *P. aeruginosa* secretes several proteases including LasA, LasB and PrpL that damage tissues and disrupt defense mechanisms. LasA can degrade host surfactant destroy elastin important for many immune functions at the epithelial surface and lung expansion and contraction [64, 65]. LasB has been shown to cleave collagen, human immunoglobulin and several complement components, [66-68] and PrpL targets the antimicrobial protein lactoferrin and the iron transporter transferrin [63].

P. aeruginosa produces lipases LipA and LipC, that target lipids in surfactant or cell membranes [69]. The phospholipases PlcH, PlcB and PlcN destroy host mucins [70] and work in concert with bacterial rhamnolipids break down host phospholipids. *P. aeruginosa* rhamnolipid has a detergent-like structure that has been proposed to solubilize host phospholipids making them more accessible for cleavage [71]. While rhamnolipid secretion is traditionally not categorized as a virulence mechanism, it can inhibit AEC ciliary function and reduce immune cell function and cause neutrophil necrosis, further promoting bacterial colonization [72].

Most *P. aeruginosa* strains secrete pyocyanin, and high concentrations are detected in CF patient secretions and negatively correlate with lung function [73-76]. Pyocyanin is proinflammatory, impairs ciliary function and macrophage efferocytosis [77], while also being antimicrobial to other bacterial species, thereby likely promoting *P. aeruginosa* ability to outcompete other organisms in the CF lung [78-80]. It also has a pigment that gives blue-green color to the bacterial colonies, making it fun for graduate students and technicians to plate delightful *P. aeruginosa* LB agar birthday cakes.

A Type III secretion system is a major determinant of *P. aeruginosa* virulence that through a needle like aparatus injects the exotoxins ExoS, ExoU, ExoT and ExoY directly into host cells [56, 81]. Nearly all strains express one of the two major exotoxins ExoU or ExoS but rarely both while the most strains express ExoT [82]. ExoS and ExoT work in concert to inhibit actin polymerization, prevent phagocytosis and promote apoptosis [83]. ExoY can increase membrane permeability and also target actin polymerization [84]. ExoU is a phospholipase that causes rapid death of host cells due to loss of plasma membrane integrity [85]. Together these *P. aeruginosa* virulence factors dramatically disrupt normal

mucosal function in the lung by damaging host cells and preventing bacterial clearance thereby allowing the microbe to establish infection in the airways [86].

Interestingly, many virulence mechanisms are reduced or turned off during chronic infection in the CF lung [70, 87, 88]. The reduction of virulence factor production does not result in enhancing host clearance of the bacteria instead these changes facilitate long-term colonization in the host. As the bacteria adapt to their environment they undergo many phenotypic modifications that save energy and allows for persistence in the CF lung [89]. Both the upregulation of antibiotic efflux pumps and the downregulating many metabolic pathways during chronic infection make *P. aeruginosa* refractory to host defenses and many antimicrobial treatments compared to their planktonic counterparts [90]. These phenotypic alterations further highlight how *P. aeruginosa* can sense the surrounding environment and adapt to different niches.

1.2.2.1 CFTR inhibitory factor (Cif)

The secreted protein Cif (PA2934) was first identified as a *P. aeruginosa* virulence factor due to its capability to decrease plasma membrane expression of CFTR on human AECs [91, 92]. *P. aeruginosa* can secrete the Cif protein either directly into the surrounding environment or in outer membrane vesicles (OMVs) [93]. The packaging of bacterial proteins into OMVs facilitates delivery across dense mucus and long distances in the airways to then fuse with host cells and deliver their cargo into the cytoplasm [94]. Cif also regulates epithelial TAP1 ubiquitination to reduce MHC class I antigen presentation and the apical expression of the drug efflux ABC transporter P-glycoprotein on a variety of cell types [92,

95]. These studies demonstrate that Cif acts to alter host defense in several ways during *P. aeruginosa* infection.

Cif is an α/β-hydrolase with epoxide hydrolase activity which is important for Cif mediated effects on CFTR [91, 96, 97]. The protein has an unusual active site and is the first epoxide hydrolase described in its class [97, 98]. Cif can catalyze the hydrolysis of environmental epoxides [99] and interestingly, the regulation of Cif packaging into OMVs can be altered when the *P. aeruginosa* is grown in the presence of specific epoxides [100]. However, Cif's epoxide hydrolase activity in CF pathogenesis and the identity of possible host epoxide substrates needs further investigation.

Cif is expressed by *P. aeruginosa* clinical isolates and is detected in CF patient sputum [91, 99]. Recently Cif homologs have been identified in other airway pathogens, including *Acinetobacter nosocomialis* and *Burkholderia cepacia*, which also display epoxide hydrolase activity and reduction of CFTR abundance in AECs [101]. Therefore, bacterial epoxide hydrolases maybe a class of secreted virulence factors that are shared by several pulmonary pathogens.

1.2.3 Chronic infection and biofilm production

In the CF lung, the conversion of *P. aeruginosa* acute infection to chronic colonization often involves the development of bacterial biofilms. Biofilms are bacterial communities that are attached to living or abiotic surfaces, surrounded by extracellular matrix produced by the bacteria and often include DNA, polysaccharides and proteins [97, 101]. These communities develop in a well-coordinated manner that involves sensing and responding to environmental cues such as bacterial cell density and nutrients [102]. *P. aeruginosa* biofilms reside in the

mucus layer overlying AECs in the CF airway causing further obstruction, respiratory failure and eventual loss of pulmonary function at a young age [103-105].

To build a biofilm, *P. aeruginosa* must first attach to the surface and form a microcolony [106, 107]. The development of a biofilm is intricately linked to population density and the bacteria produce small membrane diffusible molecules to sense critical mass known as quorum sensing [61]. The accumulation of these molecules signal to the bacteria to then undergo phenotypic alterations that include downregulation of flagella and virulence factors such as type 3 secretion systems [104]. The bacterial aggregates then produce abundant extracellular polymeric substances (EPS), mainly composed of polysaccharides, DNA, lipids and proteins, which imparts a physical shield and helps to protect the bacterial from mechanical forces [63]. The generation of EPS and unregulation of efflux pumps help to shelter the bacteria from antibiotics, host defense molecules and phagocytosis [108], making *P. aeruginosa* biofilms extremely difficult to treat [55, 109-111]. Accumulating evidence suggests that persistence of *P. aeruginosa* biofilms contribute to chronic inflammation, which causes extensive damage to the lung tissue and plays an important role in the decline of pulmonary function [8, 55, 112].

Frustrated neutrophil responses also contribute to the biofilm formation through the release of DNA, actin and granule proteins from necrotic cells [53, 113]. F-actin and DNA released from neutrophils enhance *P. aeruginosa* biofilm architecture. DNAse treatment reduces biofilm formation and the therapeutic Pulmozyme (rhDNase I) has been shown to be effective in reducing bacterial colonization and improving mucociliary clearance in the CF patient airways [114, 115].

1.3 VIRAL BACTERIAL CO-INFECTION IN CYSTIC FIBROSIS

Respiratory viral infections in CF patients have been shown to be associated with increased hospitalization and decline in pulmonary function [116-119]. However, few studies have investigated if CFTR deficiency affects viral clearance mechanisms. While the incidence of viral infection is not elevated CF patients, the duration and severity of the illness is intensified [120]. Respiratory syncytial virus (RSV), influenza, and rhinovirus are the most commonly detected viruses in CF patients and their occurrence significantly correlates with disease progression and pulmonary exacerbation [121, 122]. A prospective study over a five-year period showed 50% of CF respiratory exacerbations requiring hospitalization were associated with the isolation of a respiratory virus [121]. Furthermore, viral pathogens have been detected in 46% of children and 33% of adults with CF during pulmonary exacerbation [123]. RSV represents 9-58% of all reported viral infections in CF with the highest incidence in young children [124, 125]. Studies indicate that RSV infection in CF patients increases hospitalization rates up 14.6% and note a greater decline in lung function among infants with CF with a history of RSV-related hospitalization than those who did not require admission [123]. Although clinical studies demonstrate clear associations between viral infections and poor pulmonary outcomes, the underlying cellular mechanisms and immunopathology that contributes to morbidity in CF remain unclear.

Clinical studies show an association between respiratory viral infections in CF and the development of chronic *P. aeruginosa* infection [116, 123-128]. The first isolation of *P. aeruginosa* in CF patients occurs in higher frequency during the winter months, which temporally correlate with the peak of incidence of many respiratory viral infections [116, 123-128]. In addition, 85% of new *P. aeruginosa* colonization in CF patients followed a viral

infection within 3 weeks [116]. Moreover, during a respiratory viral infection, CF patients show an impaired ability to clear *P. aeruginosa.* Although a variety viruses that are isolated from CF patients, RSV is reported to be the most common respiratory virus associated with the development of chronic *P. aeruginosa* infection in CF patients [117].

RSV preferentially infects ciliated epithelial cells and causes bronchiectasis and airway obstruction. Although the prevalence of the virus is widespread there is a failure to develop natural immunity to infection. In recent years Palivizumab prophylaxis for RSV infections has been developed and a studies have investigated its use in CF populations [129-131]. These inital small studies indicate that overall hospitalization rates are reduced following administration of Palivizumab in CF patients, however, *P. aeruginosa* colonization rates remained the same or in some cases, were reported to be higher [129-131]. This could be due to the Palivizumab targeting antigenic regions of the F protein that are preferentially expressed after virus has entered the cell and suggests that newer vaccine that target the pre-fusion F protein or nanobodies delivered intranasally could be more efficacious [132]. A different approach to combating immunopathology following respiratory viral infections is to target the host response to viral pathogens. Therefore studies directed towards understanding host inflammatory responses to respiratory viral infections and as well as developing therapeutics that target detrimental host responses, will continue to be essential line of investigation.

2.0 TRAFFIC CONTROL: THE INTERSECTION OF LIPID MEDIATORS, INFECTION AND CHRONIC INFLAMMATION IN THE LUNG

2.1 GENERATION AND ANTI-INFLAMMATORY FUNCTIONS OF EICOSANOIDS

Lipids have a crucial role in cellular function, forming membranes, as well as acting as cellular signaling molecules. Arachidonic acid (AA) is a major constituent of the mammalian phospholipids and together with its wide range of metabolites (termed eicosanoids), play a substantial role in lipid signaling, including modulation of the innate immune response [133]. When AA is released from membrane phospholipids it can then be metabolized by cyclooxygenases (COX), lipoxygenases (LOX), monooxygenases (CYP450), which results in the generation of a broad range of bioactive lipid mediators called eicosanoids. There are four main classes of eicosanoids: prostaglandins, prostacyclins, thromboxanes and leukotrienes.

Cyclooxygenases catalyze the insertion of two oxygen atoms in AA [134] and in mammalian cells, two isoforms exist, COX-1, which is constitutively expressed, and COX-2, which is inducible [133]. The initial reaction of AA oxidation, mediated by COX, yields prostaglandin G_2 (PGG_2). PGG_2 is reduced to prostaglandin H_2 (PGH_2), which serves as a

precursor for various other immunomodulatory compounds, including prostaglandins, thromboxanes and Prostaglandin E_2 (PGE_2) [133].

Lipoxygenases (LOX) are a large group of dioxygenases that catalyze oxygen insertion into polyunsaturated fatty acids in animals, plants as well as microorganisms [135, 136]. LOX catalyze the synthesis of leukotrienes, lipoxins and hepoxilins through the combination of various enzymatic activities [133, 135]. Various isoforms of LOX exist with different stereospecificity and activities that catalyze oxygenation and hydroperoxidation of PUFAs at the various carbon positions on the AA backbone.

Many LOX products have been implicated in both pro-inflammatory and anti-inflammatory responses. Both hydroperoxy-eicosatetraenoic acids (HpETEs) and hydroxy-eicosatetraenoic acids (HETEs) which are intermediate products can lead to the formation of either pro-inflammatory or pro-resolving lipid mediators [137]. The interaction of COX- and LOX-derived lipid mediators, as well as the combination of these two pathways, leads to the antagonisim of the inflammatory response [137]. PGE_2, a COX-derived eicosanoid has also been argued to induce the expression of both 12-LOX and 15-LOX in neutrophils. Furthermore, the non-steroidal anti-inflammatory drug aspirin, was shown to acetylate COX isozymes leading to the formation of 15(*R*)-HETE, which acts as LOX substrate for the formation of the specialized pro-resolving lipid mediators (SPM) lipoxins (LXA_4) [138]. Therefore, depending on the environmental cues, COX and LOX enzyme can produce lipid mediators that either promote or antagonize inflammation.

In addition to these pathways, cytochrome P450 enzymes (CYP450) can metabolize AA to form epoxyeicosatetraenoic acids (EETs). CYP450 are epoxygenases that can insert an oxygen atom on any of the four double bonds of arachidonic acid and the double bond is

reduced as the epoxide is formed. Four regioisomers, 5,6-, 8,9-, 11,12- and 14,15-EET can be formed by CYP450 epoxidation, and although many CYP450 enzymes can produce multiple regioisomers, one is usually predominates [139].

Much of the research regarding EETs has focused on their cardioprotective functions, including induction of the endothelium-derived hyperpolarizing factor that decreases blood pressure [140-142]. Studies indicate that EETs have additional potentially beneficial effects on the vascular system, heart, kidneys, and nervous system, and many current studies are directed at these actions [140-143]. Soluble epoxide hydrolases (sEH) converts EETs to dihydroxyeicosatrienoic acids (DHETs) and attenuates many of the functional effects of EETs [144]. Therefore, sEH inhibition or EET mimicry as a therapeutic strategy for increasing the beneficial effects of EETs is currently being investigated [140, 144, 145].

EETs also have anti-inflammatory properties [146]. 8,9-EET, 11,12-EET, and 14,15-EET inhibit endothelial apoptosis and maybe important in renal inflammation [147]. The addition of 8,9-EET or 11,12-EET inhibited basal TNFα secretion from monocytes (THP1) by about 90% and 40%, respectively [148]. 11,12-EET, but not 14,15-EET, was shown to inhibit adherence of monocytic cells to vascular endothelium in an *ex vivo* model and inhibition of adherent cells was comparable to that of treatment with a blocking VCAM-1 antibody [146]. The anti-inflammatory effect of all EET regioisomers in endothelial cells is mediated by to PPARγ inhibition of NF-κB activation [149]. 14,15-EET treatment reduces inflammation in human bronchi and has antiapoptotic actions in the endothelium [143, 150], and more recently has been implicated in promoting the generation of pro-resolving lipids [151].

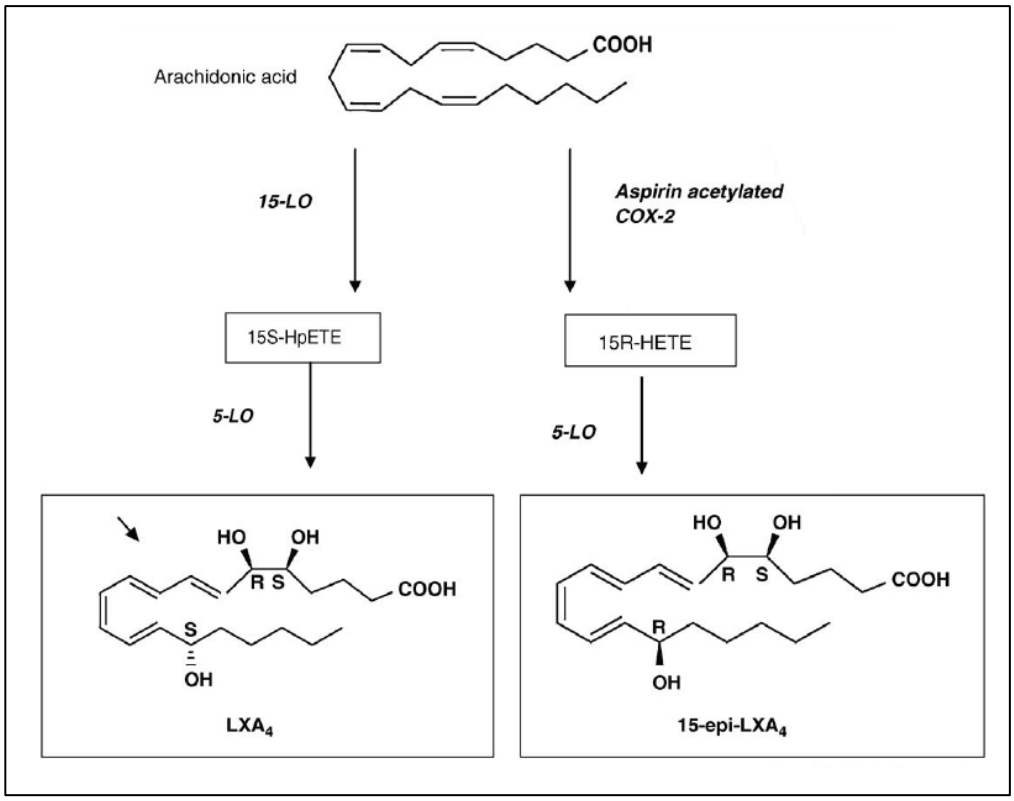
The effects of eicosanoids on mammalian cells are dependent on the type of target tissue and the physiological state of these tissues [137]. Considerable research is being done to determine the role eicosanoids play in the host during sterile, allergic and microbial-induced inflammation and more recently, whether they can enhance the clearance of pathogens.

2.2 PRO-RESOLVING EICOSANOID MEDIATORS AND PULMONARY DISEASE

Prolonged inflammation contributes to the pathogenesis of many diseases and pathology. Loss of tissue or organ function as a result of an inappropriate inflammatory response is also seen in various pulmonary diseases, such as chronic bronchitis, emphysema, asthma, and CF. Lipoxins (LXA_4) are potent anti-inflammatory molecules that promote the resolution of inflammation and the return to tissue homeostasis [152]. They are generated endogenously during what is called “class switch” when COX and LOX enzymes reduce the production of inflammatory lipid mediators and instead switch to generate pro-resolving lipid mediators [153].

LXA_4 is synthesized by the oxygenation of AA by 15-LOX which generates 15-SHpETE in epithelial cells. Neutrophils or monocytes, which carry 5-LOX, metabolize 15-HpETE into 5(6)-epoxytetraene which is rapidly converted by LTA hydrolase to generate LXA_4 [154]. 15-epi lipoxin A_4 (15-epi LXA_4), an epimer of LXA_4, has a different biosynthetic pathway. The acetylation of COX-2 oxygenates AA at the 15 position to generate 15-*R*HETE, which is then further metabolized by 5-LOX [154]. The acetylation of

COX2 by the addition of aspirin can occur in many cell types to produce 15-*R*HETE. However, immune cells such as neutrophils or monocytes that carry both COX2 and 5-LOX, can synthesize 15-epi LXA_4 without the addition of extracellular 15-HETE [154].



Modified from Ryan and Godson, 2010

The proper clearance of neutrophils is important for the resolution of inflammation. Both LXA_4 and 15-epi LXA_4 regulate many neutrophil functions that help promote the return to tissue homeostasis [152, 155, 156]. They have been found to inhibit neutrophil chemotaxis, degraulation and entry into inflamed tissues in animal models [157-159]. Also the generation of LXA_4 and 15-epi LXA_4 can inhibit LTB_4 formation and neutrophil adherence [156, 160]. Furthermore, binding to their receptor ALX/FPR2 on neutrophils,

LXA_4 and 15-epi LXA_4 oppose acute-phase protein serum amyloid A signaling, which prolongs neutrophil longevity by suppressing constitutive apoptosis [161, 162].

In contrast to their inhibitory effects on neutrophil and eosinophil recruitment, LXA_4 are potent chemoattractants for monocytes [163]. LXA_4 also promote the phagocytic clearance of apoptotic cells by macrophages, which might contribute further to the resolution of inflammation. LXA_4 stimulates monocyte adherence to vascular endothelium and chemotaxis [164]. Interestingly, LXA_4 recruited monocytes do not generate superoxide anions or degranulate in the presence of LXA_4. Therefore, both 15-epi LXA_4 and LXA_4 have a crucial role in resolving inflammation by regulating neutrophil accumulation and recruiting monocytes to clear the inflamed site of necrotic and apoptotic neutrophils [163].

Patients with chronic pulmonary inflammatory diseases have higher levels of pro-inflammatory mediators and lower levels of mediators that contribute to the resolution of inflammation [152]. For example asthma patients have reduced LXA_4 in their airways, which correlate with sEH activity [151]. Administering 15-epi LXA_4 to asthma animal models has shown a significant decrease in airway hyper-responsiveness and inflammation [165]. Chronic obstructive pulmonary disease is another airway inflammatory disease associated with long-term exposure of the lungs to irritants like cigarette smoke and other pollutants found in the air. In chronic obstructive pulmonary disease, there is an overexpression of C reactive protein and serum amyloid A, which may be alleviated with the administration of LXA_4, [152, 166, 167]. CF patients also have reduced levels of LXA_4 in their airway secretions [54]. However, when IV antibiotics was administered for two weeks CF patients saw an increase LXA_4 in sputum and reduced IL-8 in serum [168]. These results indicate that in CF, infection status might play a role in decreasing LXA_4 generation in the airways.

2.3 *P. AERUGINOSA* AND INTERACTIONS WITH HOST LIPIDS

P. aeruginosa pulmonary infection is associated with an overproduction of PGE_2. This increase in PGE_2 is due to the large amount of AA released during *P. aeruginosa* infection, mediated by ExoU, an intracellular phospholipase [169, 170]. The elimination of ExoU diminished severity of inflammation and PGE_2 production in a mouse model and on epithelial cells [170, 171]. Furthermore, the *P. aeruginosa* quorum sensing molecule, 3-oxo-HSL, induces COX-2 and PGE_2 production in human lung fibroblasts [172]. This demonstrates that the bacteria can release PGE_2 by two methods to further enhance and manipulating inflammatory pathways in the host. Interestingly, a recent study showed that LXA_4 is an inhibitor of the quorum sensing receptor LasR and decreases the bacteria's release of pyocyanin [173]. This study suggest that treatment with LXA_4 could not only disrupt *P. aeruginosa* bacterial cell communication, but also prevent the bacterial biofilm growth and chronic infection [174].

Although the presence of LOX in plants and animals has long been known, their presence in lower eukaryotes and prokaryotes has only recently been established. *P. aeruginosa* possesses a secretable 15-LOX (LoxA) capable of producing 15-HETE [175]. It has been postulated that the generation of 15-HETE by LoxA increases the biosynthesis of LXA_4 and promotes an anti-inflammatory state. The biosynthetic pathway for LXA_4 generation derived from 15-HETE requires an additional two enzymes 5-LOX and LTA/B hydrolase to complete the synthesis [154]. *P. aeruginosa* does not produce either of those enzymes and would have to rely on the host neutrophils or monocytes to produce either LXA_4 (or LXB_4). Currently there is no direct evidence that supports *P. aeruginosa* enhances LXA_4 production. Moreover, a recent study demonstrated that LoxA does not interact

directly with 15-HETE or 5-HETE, suggesting that it is unlikely that LoxA regulates lipoxin production [176]. The production of 15-HETE, while important for generating SPMs, is also linked to the promotion of airway inflammation and mucus production [177]. It is possible that *P. aeruginosa* has evolved the ability to generate 15-HETE to increase inflammation in the lung or manipulate the environment to promote chronic infection (See Appendix A).

2.4 PRO-RESOLVING LIPID MEDIATORS AS THERAPIES FOR INFECTIOUS INFLAMMATION

Understanding how pro-resolving lipid mediators play a role in the acute inflammatory response to microorganisms is an expanding area of interest. Current drug regiments for infectious disease are either antibiotics that target a particular pathogen or immunosuppressive therapies for chronic inflammatory conditions. Treatment with SPMs could be a novel host protective approach to resolve aggressive infectious inflammation rather than the traditional therapies that dampen immune responses.

Several studies have demonstrated that SPM can stimulate antimicrobial activities at epithelial surfaces. Bactericidal permeability increasing protein (BPI) production in intestinal epithelium was enhanced following treatment with a 15-epi LXA_4 analogue and was shown to decrease *S. typhimurium* bacterial burden [178]. Resolvin E1 treatment of gut epithelial cells induces intestinal alkaline phosphatase, which detoxifies bacterial LPS in the gut [179]. These studies show that SPM treatment can enhance natural host defense against bacterial pathogens.

A few investigations demonstrate that utilizing LXA_4 analogues can reduce bacterial

burden and pathogen associated inflammation. Periodontitis is caused by an overgrowth of resident Gram-negative bacteria in the oral cavity and mucosal inflammation. In *Porphyromanas gingivalis* infection models the introduction of stable analogues of LXA_4 result in a reduction of neutrophil recruitment to the site of infection [180]. Also administration of a LXA_4 analog in mice challenged intratracheally with *P. aeruginosa* reduced neutrophil infiltration, weight loss, and bacterial burden [54]. These studies indicate that SPM treatment could be helpful in targeting bacterial pathogens and lessening of disease severity. Furthermore, supplementing current therapies with SPMs might be a one-two punch for bacterial organisms. For example, the SPM resolvin D1, was shown to protect mice in an *Escherichia coli* infection model by enhancing host antimicrobial responses and accelerated the resolution and bacterial killing, as compared to antibiotics alone [132].

More recently, SPM administration has been explored as a treatment for viral infection. The SPM protectin D1 has been shown to inhibit influenza infection in mice and improve survival even when administered as late as 48 hours after infection [107]. Another SPM, resolvin D1, reduced herpes associated ocular stromal keratitis pathology [181]. Some of these studies postulate that SPM treatment inhibits viral replication however, more work is needed to confirm these observations.

Unfortunately, SPM treatment may not always be beneficial for resolving all infectious disease. For example the pathogens *Mycobacterium tuberculosis* and *Toxoplasma gondii* favor the generation of LXA_4, most likely to promote immune evasion [182, 183]. The fungal pathogen *Candida albicans* can biosynthesize its own resolvin E1 that limits IL-8-mediated neutrophil infiltration in the host enabling colonization [184]. These examples further illustrate the complicated relationship between the host response and microbial

adaptation and survival in an inflammatory environment. Further work is needed to understand how either exogenous administration or host SPM production controls immune responses to pathogens.

3.0 *PSEUDOMONAS AERUGINOSA* SABOTAGES THE GENERATION OF HOST PRO-RESOLVING LIPID MEDIATORS

Adapted from:

Pseudomonas aeruginosa sabotages the generation of host pro-resolving lipid mediators

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3.1 INTRODUCTION

Recurrent *P. aeruginosa* infections coupled with robust, damaging neutrophilic inflammation are characteristic of the chronic lung disease cystic fibrosis (CF) [21]. CF is caused by mutations that impair the function of the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel that controls epithelial fluid and ion homeostasis. The resulting failure of mucociliary clearance in the CF lung allows microorganisms to repeatedly infect the respiratory tract [8]. These bacterial infections incite robust inflammatory responses, dominated by elevated pro-inflammatory cytokines and continued accumulation of neutrophils in the CF airway [21]. However, these responses are ineffective at clearing pathogenic microbes in the CF lung [185], instead creating a hyper-inflammatory cycle that leads to host-tissue damage, respiratory failure, transplant or death.

Most adult CF patient airways are chronically infected by the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, which is a major cause of morbidity and mortality. *P. aeruginosa* thrives in the hyper-inflammatory CF lung, forming biofilms that are mechanically robust and resistant to clinically achievable levels of antibiotics [8]. *P. aeruginosa* also persists in the airways by interfering with host defense via secreted bacterial virulence factors and small molecules [8]. We recently showed that *P. aeruginosa* secretes the CFTR inhibitory factor (Cif), an epoxide hydrolase that triggers the degradation of ABC transporter family members, including CFTR [91, 92, 95, 97, 98]. Cif transcripts have been observed in CF patient sputum and clinical isolates from CF patients confirm that *P. aeruginosa* expresses Cif during pulmonary infection [91, 100]. Nonetheless, a role for Cif's epoxide hydrolase activity in CF pathogenesis and the identity of possible host epoxide substrates remains unclear.

Following a pathogenic insult, the host rapidly releases polyunsaturated fatty acids from cell membranes and converts them into various lipid mediators that either stimulate or inhibit inflammation. The correct balance of these signals is required to optimize pathogen clearance while minimizing collateral damage to host tissues, and perturbations in either direction can be deleterious [137]. Among these lipid mediators, arachidonic acid-derived eicosanoids, including epoxides, play important roles. Although many eicosanoids induce pro-inflammatory cascades, recent studies have also identified immunomodulatory and pro-resolving functions [167, 186]. In particular, lipoxins decrease neutrophil extravasation and enhance macrophage efferocytosis, thus promoting the resolution of inflammation and a return to tissue homeostasis [164, 187]. In the CF lung, the concentration of lipoxin A_4 (LXA_4) is significantly reduced, suggesting that a failure to activate pro-resolving mechanisms contributes to excessive inflammation in the airway [54].

In this study, we show that Cif selectively converted an endogenous epoxide-containing eicosanoid 14,15-epoxyeicosatrienoic acid (14,15-EET) to its corresponding diol, destroying the signal that triggers increased biosynthesis of the specialized pro-resolving mediator 15-epi LXA_4. The translational relevance of this unexpected biochemical virulence activity to clinical CF was investigated by retrospective analysis of bronchoalveolar lavage fluid (BALF) samples and suggested that Cif contributes to the hyper-inflammatory environment of the chronically infected CF lung.

3.2 METHODS

Epithelial cell culture. The immortalized human CF bronchial epithelial cell line CFBE41o− (referred to here as CFBE cells) were a gift from Dr. J.P. Clancy (University of Cincinnati). CFBE cells were seeded on 0.4μM polyester Transwell filters (Corning) and coated with collagen and fibronectin and grown at air-liquid interface for at least 7-10 days, as previously described [93]. Primary human CF AECs (CF HBEs) acquired from the University of Pittsburgh Airway Cell and Tissue Core were cultured from CF patient explanted lungs using an Institutional Review Board approved protocol at the University of Pittsburgh (IRB# 11070367), described in [188]. CF HBEs were cultured on collagen-coated Transwell filters (Corning) for 4-6 weeks at air-liquid interface (ALI) prior to use in experiments.

Bacterial strains and culture conditions. *Pseudomonas aeruginosa* strains PA14 and PA14Δ*cif* (gifts from Dr. George O'Toole, Geisel School of Medicine at Dartmouth) [91, 189] were used in this study. Dr. Jane Burns at the University of Washington provided pulmonary *Pseudomonas aeruginosa* clinical isolates from CF patients [57]. Overnight cultures grown in LB were washed and diluted in minimal essential medium (Gibco) supplemented with 2mM glutamine to an OD_{600} of 0.5. Epithelial cells were inoculated with ~$7x10^6$ bacteria, corresponding to a multiplicity of infection of 25.

Protein Expression and Purification. Wild type Cif protein (Cif-WT) and the Cif-D129S and Cif-E153Q mutants were expressed as described previously [96, 190, 191]. Briefly, 6-His tagged Cif, Cif-D129S, and Cif-E153Q were expressed from an arabinose-inducible

vector in TOP10 *E. coli* cells. The proteins were isolated using nickel-affinity purification, concentrated, and dialyzed into PBS.

Cif secretion by clinical isolates. *P. aeruginosa* paired clinical isolates [57] grown overnight in LB were washed and diluted in minimal essential medium supplemented with 2mM glutamine to an OD_{600} of 0.5. Polarized CFBE cells were inoculated with ~$7x10^6$ bacteria and incubated 37˚C. After 5 hours, apical supernatants were collected, spun down to remove planktonic bacteria and the supernatants were transferred to a new tube. The samples were frozen on dry ice and stored at -80˚C.

High binding 96 well ELISA plates (Costar) were coated with 100µl of anti-Cif (used in earlier studies) polyclonal antisera diluted 1:12,000 in PBS containing 35mM sodium carbonate and 70mM bicarbonate overnight at 4˚C. The plate was blocked with PBS, 1% BSA for 1.5 hours at room temperature and washed three times with PBS, 0.05% Tween 20 prior to the addition of sample. 100µl of either unknown sample was added to the plate along side purified Cif-WT which serially diluted and utilized as a standard curve. The plate was incubated at room temperature for 2 hours. After removing unbound sample with three subsequent wash steps, 100µl of detection of recently generated anti-Cif DM163 antibody diluted 1:4000 in PBS was added to the plate and incubated for 2 hours at room temperature. The detection antibody, anti-Cif HRP, was conjugated prior to the day of the assay to HRP using a conjugation kit (Abcam). The plate was developed with 15-20 minutes incubation with TMB substrate followed by the addition of 2M sulfuric acid stop the reaction. The plate was read at 450nM on a SpectraMax M2 plate reader and the concentrations were determined using a standard curve of purified Cif.

***In vitro* Hydrolysis Assay.** Hydrolysis of the epoxyeicosatrienoic acids by Cif was measured using an adrenochrome reporter assay [101, 192]. Briefly, 40 μM Cif protein was incubated separately with 1 mM of each epoxyeicosatrienoic acid regioisomer (Cayman Chemical) in 2% DMSO in PBS at 37˚C for 60 minutes. The reactions were quenched with $NaIO_4$ in 90% acetonitrile to a final concentration equimolar to initial EET concentrations and incubated at room temperature for 30 minutes. Epinephrine was added in excess to react with the residual $NaIO_4$. A_{490} values were measured and compared to a standard curve generated with 14,15-DHET.

Crystallographic structure of Cif with 14,15-EET. Cif-E153Q:14,15-EET co-crystals were obtained by vapor diffusion against 400 μL of reservoir solution in a 4 μL hanging drop at 291 K [190, 191]. 5 mg/mL Cif-E153Q protein in PBS was incubated with neat 14,15-EET at 277 K overnight. The mixture was added in a 1:1 ratio with reservoir solution consisting of 12% (*w/v*) polyethylene glycol 8000, 200 mM $CaCl_2$, and 100 mM sodium acetate (pH 5). Prior to data collection, crystals were washed in cryoprotectant solution consisting of 12% (*w/v*) polyethylene glycol 8000, 200 mM $CaCl_2$, 100 mM sodium acetate (pH 5), and 20% (*w/v*) glycerol and flash cooled by plunging into a liquid nitrogen bath. Oscillation data were collected at 100 K at the X6A beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. Diffraction images were processed and scaled with the XDS package (v December 6th, 2010) [193]. Molecular replacement with the Phenix suite (v 1.10.2055) used the apo-Cif-WT structure (PDB ID 3KD2) as the search model and revealed two dimers in the asymmetric unit [194-196]. Iterative rounds of automated and manual

refinement were carried out with Phenix and WinCoot (v 0.7), respectively. The adducted ligand was included in the model after two rounds of automated and manual refinement, and before the placement of waters. Final coordinates were deposited to the Protein Data Bank (PDB ID 5JYC). Pymol (www.pymol.org) was used to render structure images of the final model of chain D, in which the active-site structures are best defined.

14,15-EET quantification from airway epithelial cell secretions. Polarized epithelial cells were exposed to 1 ng/ml of TNFα (R&D systems) in minimal essential medium (Gibco) supplemented with 2 mM glutamine for 24 hours prior to apical treatment with either 1 μM of purified Cif protein 45 minutes or $7x10^6$ bacteria for 5 hours at 37˚C with 5% CO_2. The apical supernatants were collected and immediately added to 3 volumes of cold HPLC-grade methanol (Fisher Chemical), layered with N_2, and stored at -80˚C. To identify 14,15-EET concentrations in the apical secretions, the samples were acidified to a pH of 4 and solid-phase lipid extracted using C18 Sep-Pak cartridges (Waters) as previously described [151, 197, 198]. The methyl formate (Sigma) fraction was collected, brought to dryness under a gentle stream of N_2 and resuspended in 1 ml of methanol prior to quantification by ELISA (Detroit R&D). Prostaglandin B2 (100 ng) was added to each sample as an internal standard and monitored by HPLC to control for variances in extraction recovery. Additionally, 14,15-EET concentrations in apical supernatants were confirmed by liquid chromatography mass spectrometry using previously described techniques [199, 200].

Neutrophil isolation. Neutrophil (polymorphonuclear leukocytes or neutrophils) were obtained by venipuncture from volunteers who had given written informed consent to a

protocol approved by University of Pittsburgh IRB Committee (IRB# PRO14070447). Neutrophils were isolated from heparinized peripheral blood, by dextran (Sigma) sedimentation followed by a histopaque (Sigma-Aldrich) gradient [201]. Following RBC hypotonic water lysis neutrophils were washed, counted and resuspended to $2x10^7$ cells/ml in phosphate buffered saline without calcium and magnesium (referred to here as PBS-/-). Only neutrophil preparations that exceeded 90% viability by trypan blue exclusion were used in experiments.

Quantification of neutrophil generation of LXA_4 and 15-epi LXA_4. $1x10^6$ neutrophils were resuspended in 0.15 ml of phosphate buffered saline with 1mM calcium chloride and 0.5 mM magnesium chloride (PBS+/+) (Gibco) and incubated for 15 minutes at 37˚C with 1 µM 14,15-EET (Cayman Chemical) in the presence of 5 µM A23187 (Sigma). In some conditions 14,15-EET was previously incubated with 1 µM Cif-WT or Cif-D129S at 37˚C for 30 minutes prior to neutrophil treatment. The reactions were stopped with 3 volumes of ice cold methanol, layered with N_2, and stored at -80˚C until solid phase extraction and quantification of 15-epi LXA_4 by ELISA (Neogen) as previously described [202]. Prostaglandin B2 (100 ng) was added to each sample as an internal standard and monitored by HPLC to control for variances in extraction recovery. For neutrophil-airway epithelial cell co-culture experiments, $2.5x10^5$ polarized CFBE cells were treated with TNFα (1 ng/ml) for 24 hours and apically exposed to 1 µM Cif for 45 minutes prior to the addition of $1.25x10^6$ neutrophils for 15 minutes. The apical supernatants were collected, added to 3 volumes of cold HPLC grade methanol, layered with N_2, and stored at -80˚C until further processed for quantification of both LXA_4 and 15-epi LXA_4 by ELISA.

Neutrophil transepithelial migration. Polycarbonate Transwell filters with 3 μm pores were inverted, coated with collagen and fibronectin, seeded with $1x10^5$ CF AECs, and allowed to adhere overnight at 37˚C [203]. The following day the filters were moved to a sterile 24-well tissue culture plate with 0.5 ml of medium added to the apical chamber and 0.1 ml to the basolateral compartment. After allowing the cells to grow to confluency for 3 days, apical medium was removed and the cells were grown at air liquid interface for 7 days. CF AECs were washed with PBS+/+ and allowed to calibrate for 1 hour prior to the application of neutrophils on the basolateral surface. Transepithelial electrical resistance (TEER) measurements were conducted to confirm polarization of monolayers.

Isolated neutrophils were labeled with 3 μM Calcein AM (Life Technologies) in PBS without calcium and magnesium (PBS -/-) (Gibco) for 30 minutes at 37˚C in 5% CO_2. The labeled neutrophils were washed twice and resuspended in PBS+/+ before incubation in the presence or absence of 14,15-EET (100 nM), Cif-WT (100 nM) or Cif-D129S (100 nM) for 45 minutes at 37˚C with 5% CO_2. In some conditions neutrophils were incubated in parallel with 15-epi LXA_4 (100 nM) for 15 minutes at 37˚C with 5% CO_2. IL-8 (100 ng/ml) in PBS+/+ was applied to the apical chamber 10 minutes prior to the addition of $5x10^5$ of the treated neutrophils to the basolateral compartment [204]. After incubating for 2 hours at 37˚C the filters were removed and the plate was gently spun at 300 x g for 5 minutes. Neutrophils that had migrated to the apical chamber were measured by Calcein AM fluorescence detected by a SpectraMax M2 plate reader.

Murine *P. aeruginosa* pneumonia model. The protocol for animal infection and respiratory physiology measurements was approved by the Institutional Animal Care and Use Committee of the Geisel School of Medicine at Dartmouth (Hanover, NH) and done in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Mouse experiments were carried out as described previously, with modification [205]. Overnight LB cultures of *P. aeruginosa* were measured by OD_{600}, pelleted and washed twice with PBS. *P. aeruginosa* strains were resuspended to give 1×10^6 viable CFUs in 40 μl PBS. Actual inoculum was determined by serial dilution of the input bacterial suspension on *Pseudomonas* isolation agar (Difco). Adult male C57Bl/6J mice, 8–12 weeks old (Jackson Laboratories, Detroit, MI), were inoculated with 40 μl (1×10^6 CFU) of *P. aeruginosa* PA14 or isogenic PA14 *cif*-D129S via oropharyngeal aspiration following brief anesthesia with isoflourane. At 24 hours after infection, mice were anesthetized with intraperitoneal sodium pentobarbital, tracheas were cannulated, and bronchoalveolar lavage fluid (BALF) was collected and stored on ice. A portion of the BALF was immediately diluted 1:4 in ice-cold methanol and snap frozen for lipid extraction. Lungs were excised, minced, and immediately placed either into 1 ml of cold PBS followed by homogenization or stored on ice. Viable bacterial counts in lungs were determined by plating serial dilutions of organ homogenate onto *Pseudomonas* isolation agar plates followed by incubation at 37°C for 24 hours.

Analysis of CF subject BALF. Bronchoalveolar lavage fluid (BALF) was collected prospectively over time in a pediatric population followed for management of CF at Children's Hospital of Pittsburgh, in accordance with a protocol approved by University of

Pittsburgh IRB Committee (IRB#504067). Documentation of a CF diagnosis as evidenced by one or more clinical features consistent with the CF phenotype and one or more of the following criteria; sweat chloride equal to or greater than 60 mEq/L by quantitative pilocarpine iontophoresis test or two well-characterized mutations in the CFTR gene. The seventeen samples analyzed in the current study tested positive for *P. aeruginosa* infection by dot blot using an anti-*Pseudomonas* antibody (Pierce). Patient BALF was methanol trapped and lipid extracted prior to quantification of 15-epi LXA_4 by ELISA (Neogen). IL-8 concentrations were determined using sandwich ELISA (R&D Systems). Cif protein quantification was determined by Western blot analysis. BALF samples were separated by SDS-PAGE, transferred to PVDF membrane, and detected by Cif-specific rabbit antisera. Cif and IL-8 values were normalized and expressed per mg of total BALF protein. As a result, overall changes in total protein concentration were factored out, providing a more stringent threshold for detection of differences. Pulmonary function testing (PFT) was performed on all patients as part of their CF management and the most recent PFT data in relation to the bronchoscopy was collected.

Cif Antisera. Cocalico Biologicals, Inc. provided test bleed sera for initial screening of rabbits as a host species for the development of a Cif-specific polyclonal antibody. We selected two rabbits with the lowest background sera reaction as determined by Western blot against multiple *P. aeruginosa* strains. Purified protein (500 µl at 4.3 mg/ml) was submitted to Cocalico Biologicals, Inc. Animals were initially inoculated at day 0, and boosted on days 14, 21, 49, 97, 140, and 170. Test bleeds were taken on days 35, 56, 111, and 154. The desired level of Cif detection was reached at day 154. Sera were harvested via two

production bleeds (days 177 and 184) followed by exsanguination on day 191. The sera that was demonstrated to have the most specific activity to purified Cif was DM163 which was was aliquoted and stored at -80°.

Statistics. Experimental differences for in vitro assays were evaluated for statistical significance by one-way analysis of variance (ANOVA) with a Tukey's post hoc test (GraphPad 6.0 Software). All in vitro data are shown as Mean ± SD. $P < 0.05$ was considered statistically significant. For the human studies, a Pearson correlation matrix was created using the cor package in R from measurements of Cif, 15-epi LXA_4, IL8 and spirometry values for the 16 patients for whom a complete set of values was available. The corrplot package in R was utilized to visualize the results. For statistical analyses pertaining to serum antibody titers and the differences between high verses low Cif cohorts, significance was determined using the nonparametric Wilcoxon Rank-Sum Test (GraphPad 6.0 Software).

3.3 RESULTS

3.3.1 *P. aeruginosa* CF clinical isolates secrete Cif when grown in the presence of airway epithelial cells

P. aeruginosa secretes several virulence factors that diminish host immune responses and facilitate bacterial colonization of the airway [206]. However, once *P. aeruginosa* inhabits the CF lung, the bacteria undergo substantial changes as it adapts to the host environment, including the loss of several virulence related molecules [57, 63, 88, 207]. Previous studies demonstrated that *P. aeruginosa* produces the virulence protein Cif in the CF respiratory tract, as the protein is detected in patient sputum [99]. To further investigate the temporal expression of Cif in the CF host and provide a better understanding of its role in either the initial establishment of acute infection or during chronic colonization, we assess Cif protein expression. Cif gene expression has been reported to be elevated in non-muciod compared to mucoid isolates, suggesting that Cif might important during initial infection and colonization [91]. Ten clinical paired pulmonary isolates from CF patients were studied to investigate if *P. aeruginosa* alters Cif protein production overtime in the CF lung [57]. Paired clinical isolates were grown parallel with *P. aeruginosa* laboratory strains in the presence of polarized CFBE cells and Cif secretion was determined by sandwich ELISA developed in our laboratory in the apical supernatants (**Figure 1**). As expected, the laboratory strain PA14 secreted abundant amounts of Cif compared to the laboratory strain PA01, which other studies have reported produce lower amounts of Cif [208]. Moreover, the PA14Δ*Cif* mutant secreted undetectable levels of Cif, further validating our assay. The majority of the clinical isolates tested secreted detectable levels of Cif when cultured in the presence of airway

epithelial cells, demonstrating that Cif is a highly conserved virulence factor among bacterial isolates from CF patients (**Figure 1B**). However, analysis of the paired pulmonary isolates tested showed no appreciable difference in Cif secretion between the early or late isolate cohorts. Our data indicate that although longitudinal Cif expression may vary in each individual patient, overall a majority of *P. aeruginosa* isolates maintain the capability to secrete Cif in the CF lung during chronic infection.

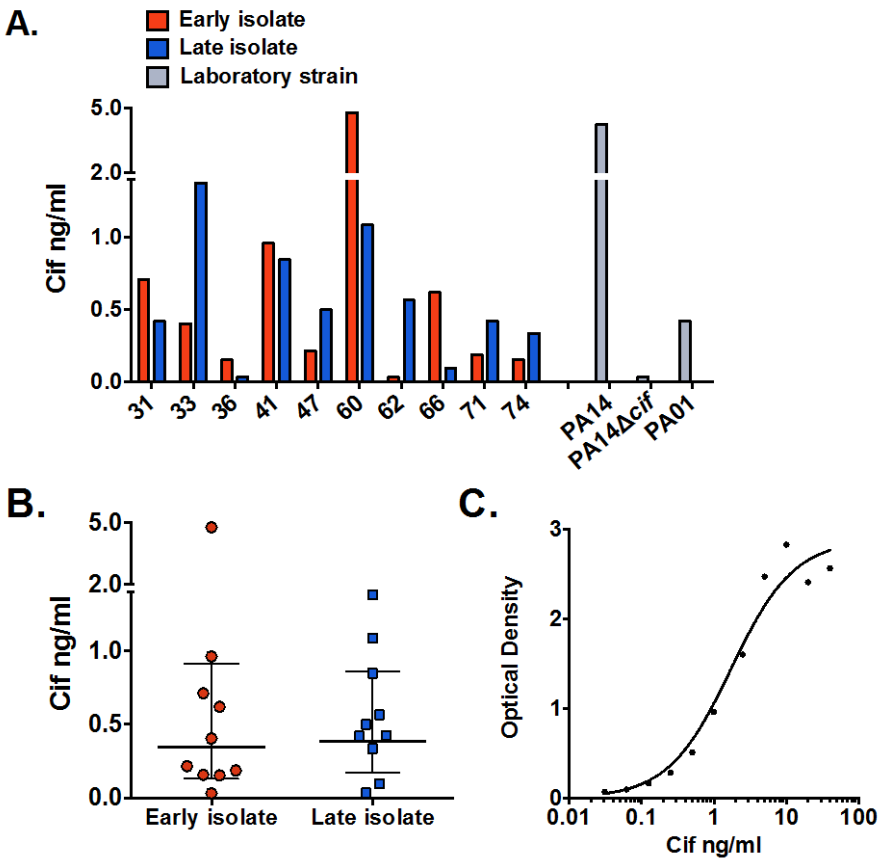


Figure 1. CF clinical isolates grown in the presence of AECs cells secrete Cif. (A) Quantification of Cif protein secretion by *P. aeruginosa* laboratory strains (grey bars) or paired clinical isolates from CF patients (early isolates red bars, late isolate blue bars) following inoculation of CFBE cells. (B) No significant difference in Cif secretion is detected among the CF patient isolates when separated into early and late cohorts (C) Standard curve of recombinant Cif used to determine protein concentration in a sandwich ELISA.

3.3.2 Cif specifically hydrolyzed the mammalian epoxyeicosatrienoic acid 14,15-EET

AECs produce numerous lipid mediators. Via a major cytochrome P450 monooxygenase-catalyzed pathway, they convert arachidonic acid into epoxide-containing eicosanoids called epoxyeicosatrienoic acids (EETs) (Figure 2A) **[137]**. To assess the ability of Cif to hydrolyze these candidate epoxide substrates, we incubated the enzyme with each of the four mammalian EET regioisomers, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET and assayed for production of a vicinal diol through a colorimetric assay **[98]**. Cif hydrolyzed 14,15-EET, but did not exhibit detectable epoxide hydrolase activity for any of the other three regioisomers (Figure 2C), suggesting selective conversion of 14,15-EET by Cif to the corresponding vicinal diol 14,15-dihydroxyeicosatrienoic acid (DHET) (Figure 2B). An immunoassay selective for the diol product confirmed conversion of 14,15-EET to 14,15-DHET by Cif, but not by the structurally conserved mutant Cif-D129S (Figure 2D), which is enzymatically inactive **[96]**.

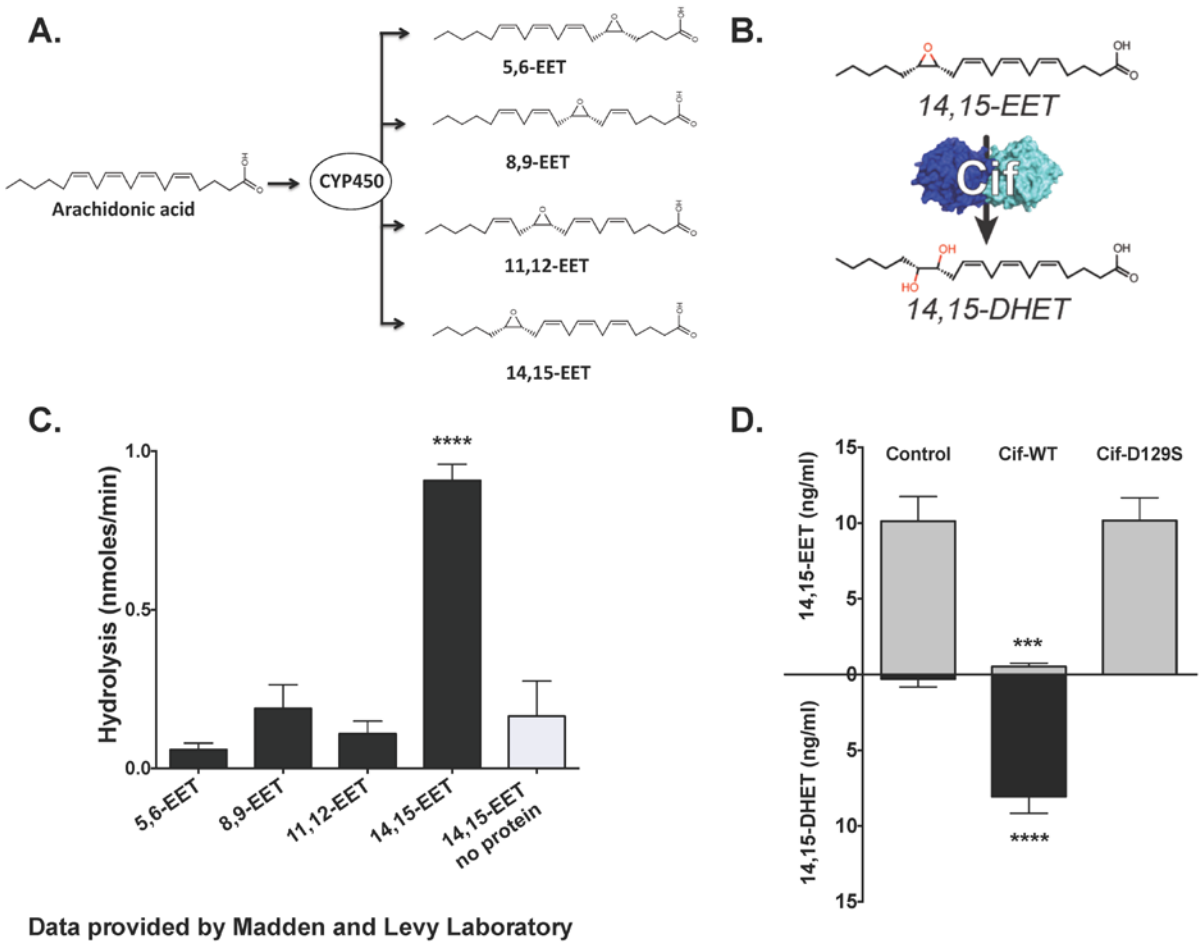


Figure 2. Cif selectively hydrolyzes the epoxyeicosatrienoic acid 14,15-EET. (A) Cellular cytochrome P450 enzymes can catalyze the epoxidation of arachidonic acid, a 20-carbon, ω-6, polyunsaturated fatty acid, resulting in the biosynthesis of four epoxyeicosatrienoic acid (EET) regioisomers. (B) Cif hydrolyzes the epoxide moiety of 14,15-EET converting it to the vicinal diol 14,15-DHET. (C) Incubation of recombinant Cif with each of the EET regioisomers demonstrates its ability to selectively hydrolyze 14,15-EET (black bars). **** $P < 0.0001$ for 14,15-EET compared to all other experimental conditions, one-way ANOVA with Tukey's post-hoc test; N≥3; Mean ± SD. (D) 14,15-EET (grey bars) and is hydrolyzed by Cif-WT to 14,15-DHET (black bars), but not by catalytic mutant Cif-D129S. *** $P < 0.001$ and **** $P < 0.0001$; one-way ANOVA Tukey's post hoc test; N≥3; Mean ±SD.

Previous crystal structures show a limited steric volume for the Cif active site [98], and almost all previously reported Cif substrates are terminal epoxides, which can enter deep into the active site. In contrast, lipid mediators typically carry epoxide groups in the middle of an extended carbon chain, and were thus thought unlikely to serve as Cif substrates. To

investigate how the enzyme nevertheless catalyzes 14,15-EET hydrolysis, we incubated the compound with the Cif-E153Q mutant, which can attack substrates with its active-site nucleophile, but cannot catalyze the secondary attack required for product release [190]. We then crystallized the resulting complex for X-ray diffraction analysis. The refined structure showed the expected adduct of the catalytic Asp^{129} (D129) nucleophile with the 20-carbon EET chain attached at the C^{15} moiety **(Figure 3A).** Unexpectedly, it also showed that the active site could expand at both ends, opening a tunnel through the enzyme to accommodate the extended eicosanoid substrate **(Figure 3B).** The strong preference for 14,15-EET presumably reflects the shorter distance in the other regioisomers between the epoxide moiety and the terminal carboxylate, which would thus have to be unfavorably sequestered within the active site during hydrolysis.

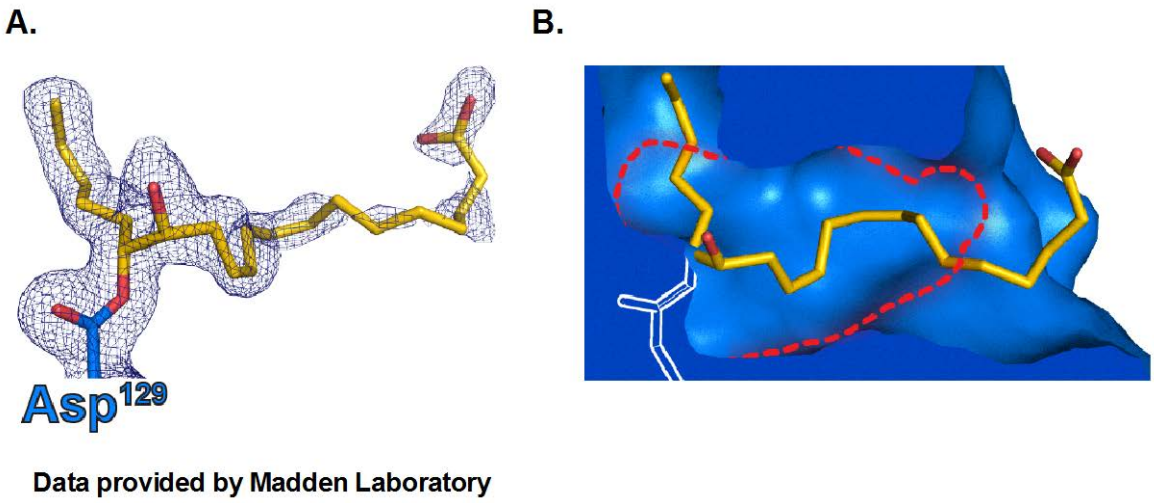


Figure 3. The Cif active site can expand at both ends providing adequate capacity to hold the extended eicosanoid 14,15-EET. (A) Experimental electron density ($2mF_O$-DF_C; blue mesh, contoured at 1σ) shows the refined position of the covalent enzyme-substrate intermediate formed by nucleophilic attack of Asp^{129} (D129; blue carbons; chain D) on 14,15-EET (yellow carbons). (B) In the covalently linked structure, several residues shift, opening a tunnel through the active site (blue surface area representation) allowing for accommodation of 14,15-EET (yellow carbons). The active site of the apo enzyme (PDB ID 3KD2) does not contain this tunnel. Its boundaries are shown as red dotted lines. Non-carbon atoms are colored by atom type (N=blue; O=red).

3.3.3 Cif-WT can hydrolyze of epithelial derived 14,15-EET produced in response to inflammation

To determine whether Cif's regioselectivity matches the biological epoxides present in the airway, we first tested whether polarized immortalized CF airway epithelial cells (CFBE41o- hereafter called CFBE cells) produce 14,15-EET in response to inflammatory signals. We treated CFBE cells with tumor necrosis factor-α (TNFα) [156] and performed lipid extractions on apical supernatants. Eicosanoid levels were determined in parallel by immunoassay (**Figure 4A**) and by mass spectrometry (**Figure 4B**). Supernatants from CFBE cells exposed to TNFα showed a substantial increase in 14,15-EET levels, compared to untreated cells. Furthermore, when CFBE cells were exposed to TNFα in the presence of purified Cif-WT protein, the apical level of 14,15-EET was reduced to that of unstimulated controls, whereas the catalytically inactive Cif-D129S protein had no significant impact on 14,15-EET levels.

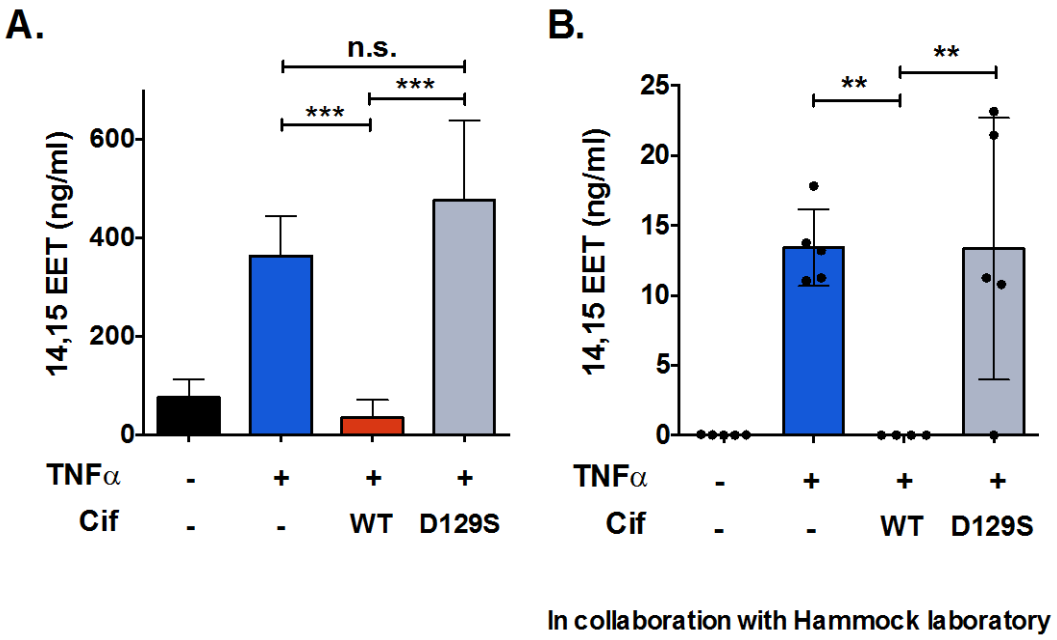


Figure 4. Cif-WT hydrolyzed 14,15-EET derived from CF AECs. (A) Recombinant Cif protein hydrolyzes 14,15-EET produced by TNFα-treated CFBE cells. Treatment of polarized CF AECs with TNFα (1 ng/ml) for 24 hours stimulates increased apical secretion of 14,15-EET (blue bars or symbols) compared to untreated cells (black bars or symbols). CFBE cells were apically treated with either recombinant Cif-WT or Cif-D129S (1 μM) for 45 minutes (red or grey bars respectively). Apical secretions were collected in cold MeOH, solid-phase lipid extracted and analyzed for 14,15-EET concentrations by ELISA. *** $P < 0.001$; one-way ANOVA with Tukey's post hoc test; N≥3; Mean ±SD. (B) Cif mediated hydrolysis of 14,15-EET derived from CF AECs confirmed by LC/MS Mass Spectrometry. CFBE cells were treated with TNFα (1 ng/ml) for 24 hours (blue bars) and then exposed to Cif-WT or Cif-D129S (red and grey bars respectively) for 45 minutes. Apical supernatants were collected in methanol, lipid extracted and 14,15-EET was measured by LC/MS Mass Spectrometry. Each data point represents one replicate, ** $P < 0.01$; one-way ANOVA with Tukey's post hoc test; N≥4; Mean ±SD.

We next sought to determine whether *P. aeruginosa* could utilize Cif activity to hydrolyze CFBE-derived 14,15-EET. CFBE cells were treated with TNFα and exposed to strain PA14, or to the corresponding *cif* deletion strain PA14Δ*cif* [91]. Apical supernatant concentrations of 14,15-EET were significantly reduced in the presence of the Cif-producing strain PA14, as compared to the TNFα-only control **(Figure 5A)**. To test for reduced 14,15-EET due to *P. aeruginosa* cytotoxicity, CF AECs were treated with the *P. aeruginosa* strains used in the aforementioned experiments. Both strains exhibited minimal cytotoxicity

following bacterial inoculation during the 5-hour experimental time point also used in the above assays (**Figure 5C**). In each case, the *cif* deletion mutant (PA14Δ*cif*) had no significant effect on the 14,15-EET levels. Similar results were observed for well-differentiated primary CF bronchial epithelial cells (CF HBEs) (**Figure 5B**) and also for the non-CF Calu3 cell line (**Figure 5D**). Interestingly, we detected comparable levels of 14,15-EET secreted by CF and non-CF AECs following TNFα treatment, suggesting that both CF and non-CF epithelial cells were similarly capable of secreting 14,15-EET in response to an inflammatory stimulus.

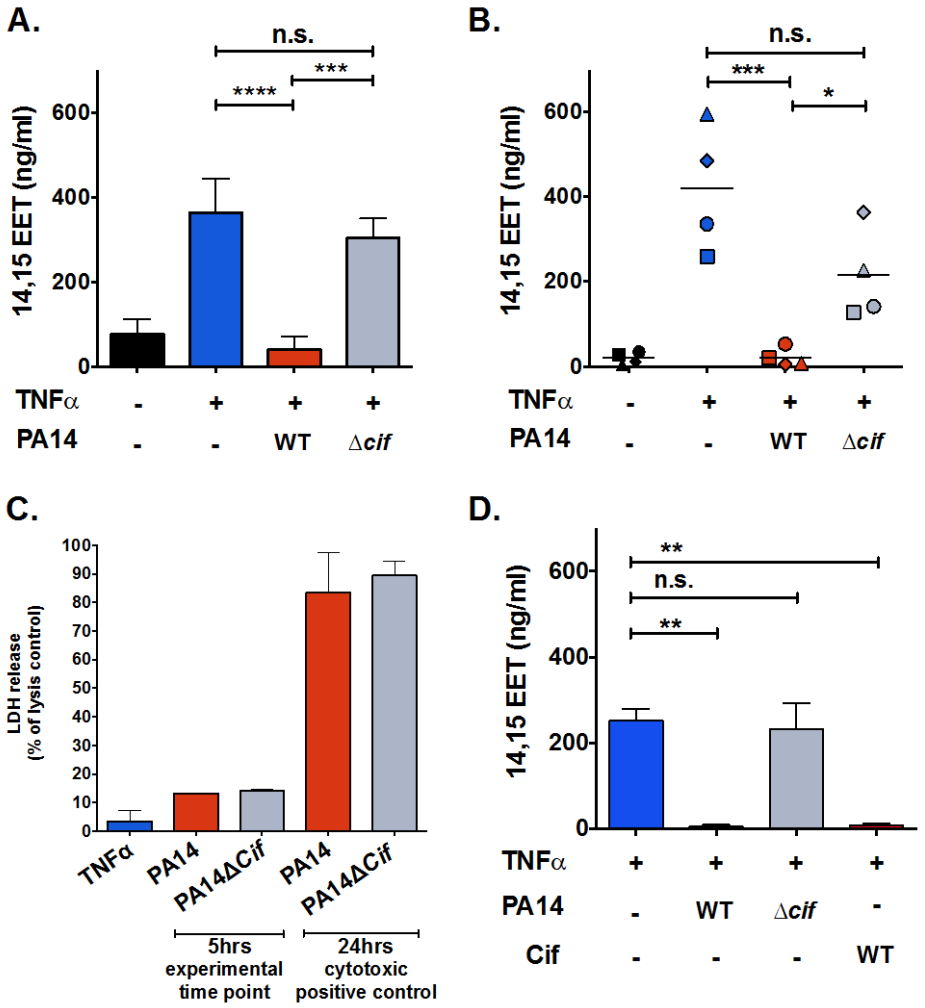


Figure 5. Cif secreted by *P. aeruginosa* hydrolyzed 14,15-EET derived from CF and non-CF AECs. (A) Cif secreted by *P. aeruginosa* hydrolyzes 14,15-EET produced by TNFα-treated CFBE cells. CFBE cells were inoculated apically with either PA14 or deletion mutant PA14Δ*cif* (MOI=25) for 5 hours (red bars and grey bars respectively) and analyzed for 14,15-EET concentrations by ELISA. *** $P < 0.001$ and **** $P < 0.0001$; one-way ANOVA with Tukey's post hoc test; N≥3; Mean ±SD. (B) *P. aeruginosa* hydrolyzes 14,15-EET produced by primary CF airway epithelial cells. Primary CF HBE cells were apically exposed to either PA14 or deletion mutant PA14Δ*cif* (MOI=25) for 5 hours (red and grey symbols). Apical secretions were collected and analyzed for 14,15-EET concentrations by ELISA. Each symbol represents one individual primary donor. * $P < 0.05$ and *** $P < 0.001$; one-way ANOVA with Tukey's post-hoc test; N≥3; Mean ±SD.(C) 5 hour apical treatment of PA14 and PA14Δ*cif* displays limited cytotoxicity against CF AECs at 5 hours. Polarized epithelial cells were exposed either 1 ng/ml of TNFα for 24 hours or apical treatment with PA14 or PA14Δ*cif* for 5 hours or for 24hrs. Cell supernatants and whole cell lysates were collected and LDH levels were measured. Data represented as percent LDH release compared to untreated cells. (D) Cif hydrolyzes 14,15-EET produced by non-CF AECs. Polarized immortalized Calu3 were stimulated with TNFα (1 ng/ml) for 24 hours to generate the apical secretion of 14,15-EET (blue bar). The Calu3 cells were subsequently apically treated with either PA14 (red bar) or PA14Δ*cif* (grey bar) for 5 hours or 1 µM Cif-WT (red bar) for 45 minutes. Apical secretions were collected in cold methanol, lipid extracted and analyzed for 14,15-EET concentrations by ELISA. ** $P < 0.01$; one-way ANOVA with Tukey's post hoc test; Data are represented as mean ±SD.

These results lead us to ask whether the presence of 14,15-EET would affect the growth rate of *P. aeruginosa* strain PA14, which expresses Cif (**Figure 6**). The supplementation of minimal media with 14,15-EET did not enhance or reduce the growth rate of PA14, indicating the bacteria do not utilize 14,15-EET as carbon source or conversely that 14,15-EET inhibits bacterial growth. Our data demonstrate that the *P. aeruginosa* hydrolase Cif, can efficiently hydrolyze 14,15-EET secreted by either CF or non-CF AECs.

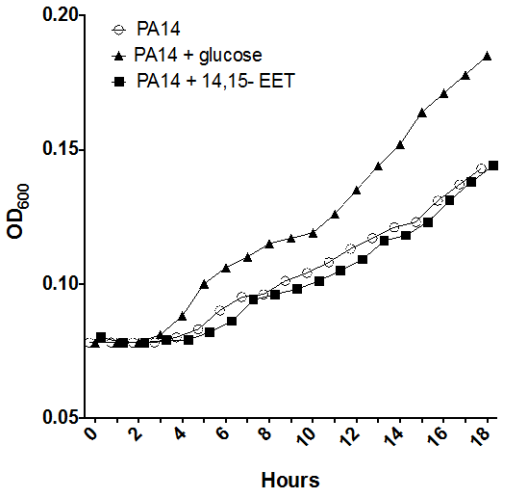
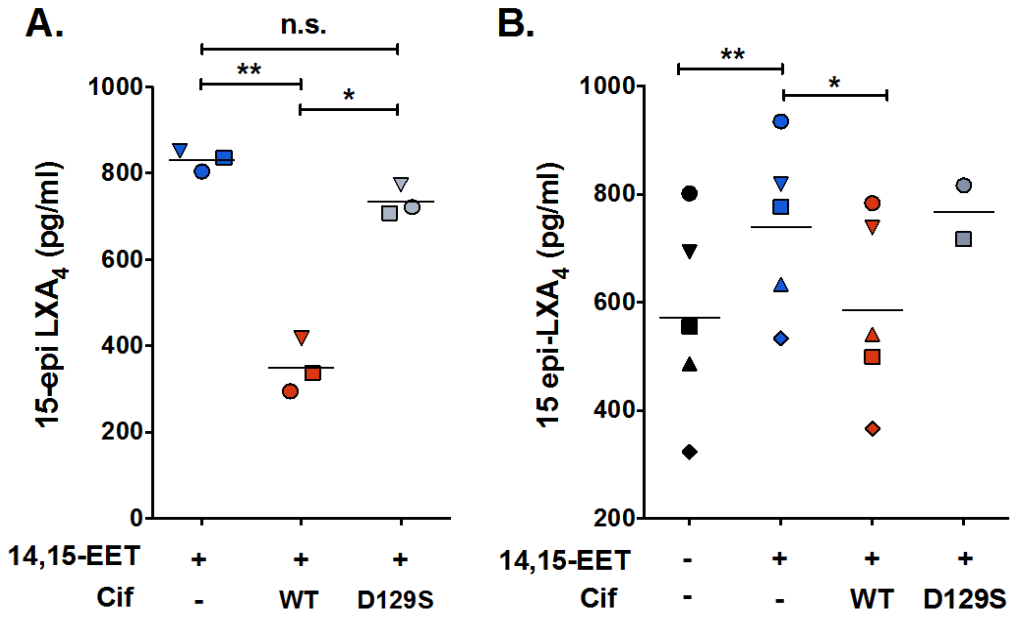


Figure 6. 14,15-EET does not enhance *P. aeruginosa* growth in minimal media. *P. aeruginosa* grown in minimal media supplemented with 14,15-EET does not alter growth kinetics. An overnight culture of PA14 was subcultured in M63 minimal media without a carbon source at a starting OD_{600} of 0.08. The bacterial suspension was supplemented with 0.5% glucose (as a positive control for growth) or 14,15-EET (1 µM) and bacterial growth was measured at one-hour intervals for 18 hours.

3.3.4 Cif-mediated 14,15-EET hydrolysis reduced 15-epi LXA_4 production and disinhibited transepithelial migration

Since AEC-derived 14,15-EET can trigger neutrophil generation of the pro-resolving mediator 15-epi LXA_4 [151, 156], we next examined if Cif-mediated hydrolysis of 14,15-EET impacted 15-epi LXA_4 biosynthesis. Neutrophils obtained from healthy donors or CF subjects were exposed to 14,15-EET alone or in combination with either Cif-WT or Cif-

D129S. Following incubation, lipids were extracted from supernatants and quantified by ELISA for 15-epi LXA_4. As expected, neutrophils generated 15-epi LXA_4 when incubated with 14,15-EET, and 15-epi LXA_4 levels were significantly reduced in the presence of Cif-WT **(Figure 7A)**. Similar results were also observed for neutrophils from CF donors **(Figure 7B)**.



in colaboration with Levy laboratory

Figure 7. Cif hydrolysis of 14,15-EET reduced 15-epi LXA_4 production by healthy and CF neutrophils. (A) Generation of 15-epi LXA_4 by healthy neutrophil donors in the presence of 14,15-EET (blue symbols) is reduced in the presence of recombinant Cif-WT (red symbols) but not by the catalytic mutant Cif-D129S (grey symbols). Freshly isolated human neutrophils from healthy donors (10^6) were incubated with 14,15-EET (1 µM) in either the presence of Cif-WT or Cif-D129S (1 µM). The reactions were stopped with cold MeOH, lipid extracted and analyzed for 15-epi LXA_4 concentrations by ELISA. * P <0.05, ** P <0.01; one-way ANOVA with Tukey's post hoc test; N≥3; Mean. (B) the production of 15-epi LXA_4 by CF neutrophil donors in the presence of 14,15-EET (blue symbols) is reduced in the presence of recombinant Cif-WT (red symbols) but not by the catalytic mutant Cif-D129S (grey symbols). * P <0.05, ** P <0.01; Paired one-way ANOVA with Tukey's post hoc test.

In a neutrophil-CFBE co-culture model, CFBE cells were treated with TNFα to induce the production of 14,15-EET and then incubated with neutrophils. Apical supernatants from the neutrophil-CFBE co-culture showed robust production of 15-epi LXA_4 following

TNFα stimulation. The addition of Cif to the neutrophil-CFBE co-culture substantially reduced transcellular generation of 15-epi LXA_4 to near baseline levels (**Figure 8A**). Since neutrophils have the capacity to produce both 15-epi LXA_4 and LXA_4 epimers, we examined whether Cif could reduce the biosynthesis of both pro-resolving lipid mediators. TNFα induced 14,15-EET did not stimulate transcelluar LXA_4 generation in the neutrophil-CFBE co-cultures. Therefore, Cif mediated hydrolysis of 14,15-EET had no effect on LXA generation in co-culture (**Figure 8B**).

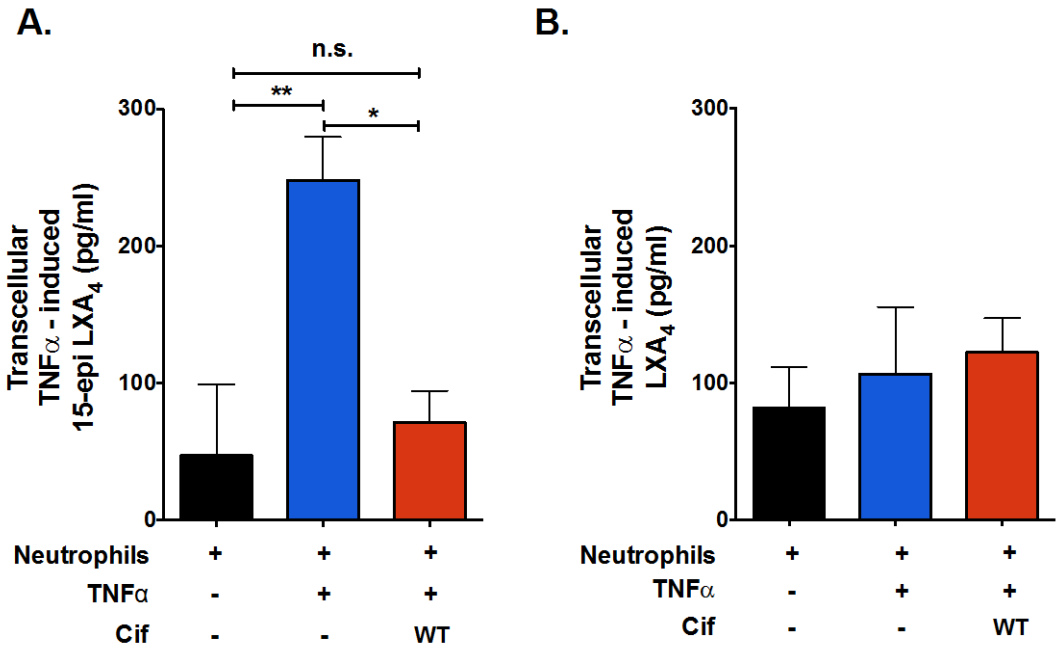


Figure 8. Cif mediated 14,15-EET hydrolysis reduced 15-epi LXA_4 production but did not have an effect on LXA_4 generation. (A) Cif hydrolysis of 14,15-EET suppresses the production of 15-epi LXA_4 in CFBE-neutrophil co-culture. Polarized CFBEs stimulated with TNFα (1 ng/ml) for 24 hours (blue bar) were treated apically with Cif-WT (1 µM) for 45 minutes (red bar), prior to the apical addition of $1.5x10^6$ of freshly isolated neutrophils for an additional 15 minutes. The apical supernatants from the co-cultures were collected and analyzed for 15-epi LXA_4 by ELISA. Data are normalized to CFBEs treated with TNFα. * P <0.05, ** P <0.01; one-way ANOVA with Tukey's post hoc test; N≥3; Mean ±SD. (B) Cif hydrolysis of 14,15-EET does not impact the production of LXA_4 in CFBE-neutrophil co-culture. The apical supernatants from the co-cultures as described above were collected and analyzed for LXA_4 by ELISA. Data are normalized to CFBEs treated with TNFα.

15-epi LXA_4 is a potent inhibitor of neutrophil transepithelial migration [187], so we next modified our co-culture system to examine whether Cif affects neutrophil transepithelial migration. Polarized CFBE cell monolayers were grown on the bottom of 3μM pore Transwell permeable membrane supports to physiologically model neutrophil transepithelial migration in the basolateral-to-apical direction. The neutrophil chemoattractant IL-8 was added in the apical compartment to drive neutrophil migration [204]. Exposure of neutrophils to 14,15-EET (100 nM, 45 minutes) or to 15-epi LXA_4 (100 nM, 15 minutes) significantly decreased transepithelial migration by 74% and 84% respectively **(Figure 9)**. Cif-WT, but not Cif-D129S significantly abrogated the inhibition of neutrophil transepithelial migration mediated by the addition of 14,15-EET. In addition, Cif-WT had no direct effect on migration when added in the presence of 15-epi LXA_4. These data demonstrate that Cif-mediated hydrolysis of 14,15-EET inhibits the generation of 15-epi LXA_4, disrupting its regulation of neutrophil transepithelial migration in the presence of IL-8.

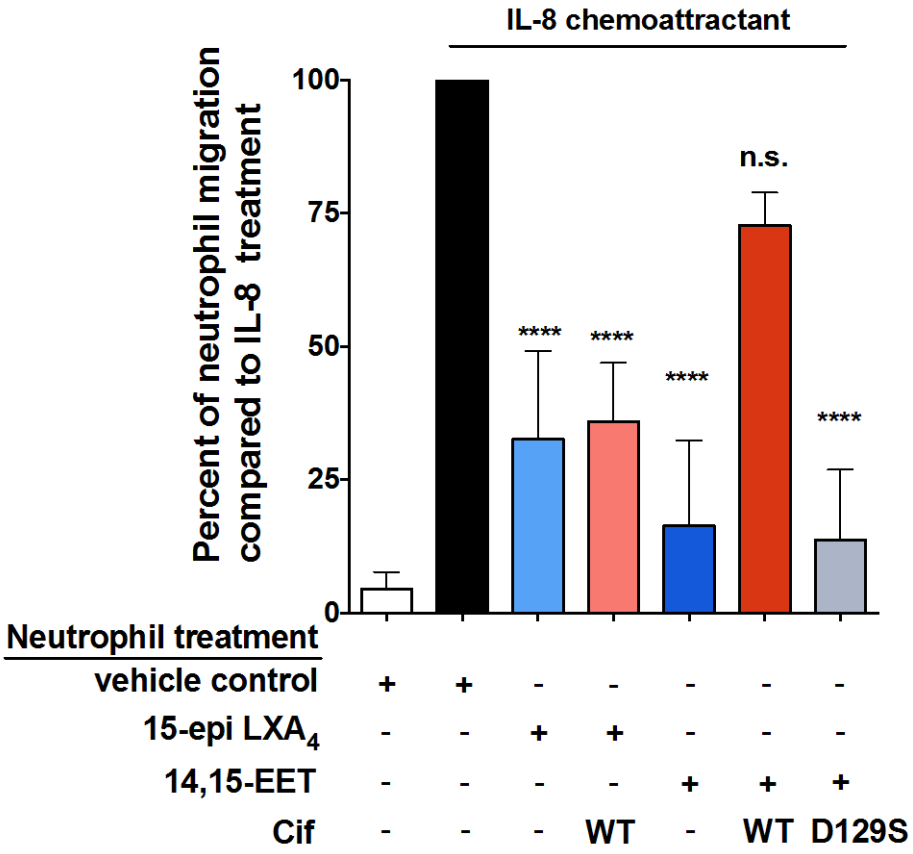


Figure 9. Cif hydrolysis of 14,15-EET abrogates the reduction of neutrophil transepithelial migration mediated by 15-epi LXA_4. Freshly isolated human neutrophils (5×10^5) were calcein-AM loaded and treated with either 15-epi LXA_4 (light blue bar) in the presence of Cif-WT (light red bar) or 14,15-EET (blue bar) in the presence of Cif-WT (red bar) or Cif-D129S (grey bar) for 15-45 minutes, as noted in the methods. The treated neutrophils were applied to the basolateral side of polarized CFBEs following the apical addition of the chemoattractant IL-8 (black bar, IL-8 positive control; white bar, no IL-8 negative control). After 2 hours, calcein-AM fluorescence was measured in the apical compartment to assess neutrophil transepithelial migration. Each experimental condition is compared to the migration measured in the positive control condition. **** P <0.0001; one-way ANOVA with Tukey's post-hoc test; N≥4; Mean ±SD.

3.3.5 *Pseudomonas aeruginosa* infection in an acute murine pneumonia model reduces 15-epi lipoxin A_4 in bronchioalveolar lavage fluid

To determine if Cif impacts 15-epi LXA_4 generation *in vivo*, we utilized an acute *P. aeruginosa* mouse pneumonia model [205]. *P. aeruginosa* strain PA14 or a PA14:*cif*-D129S

knock-in mutant were administered by oropharyngeal aspiration, and bronchoalveolar lavage fluid (BALF) was harvested 24 hours post-infection. Mice exposed to PA14 had BALF levels of 15-epi LXA_4 that were significantly lower than the PA14::Cif-D129S group and much closer to the mock-infected mice **(Figure 10)**. Thus, during an acute infection *in vivo*, the presence of a catalytically active Cif enables PA14 *P. aeruginosa* to disrupt production of 15-epi LXA_4.

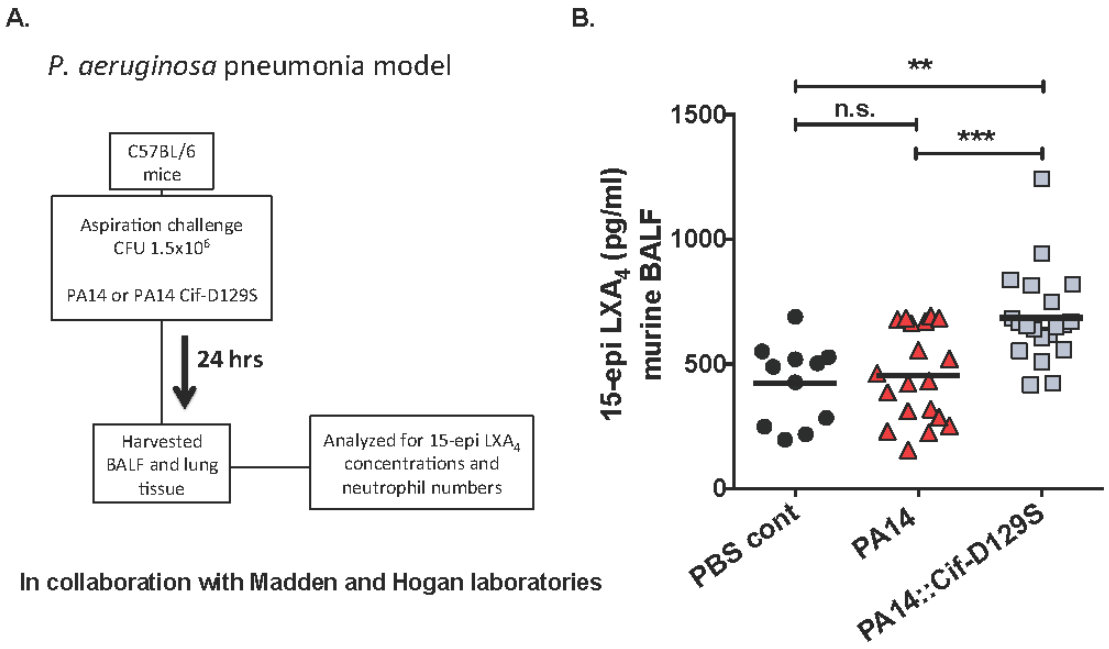


Figure 10. Oropharyngeal aspiration of a Cif producing *P. aeruginosa* strain reduces 15-epi LXA_4 in bronchial alveolar lavage fluid in a murine pneumonia model. (A) Experimental design for murine pneumonia model. (B) In a acute pneumonia model, 15-epi LXA_4 concentrations are significantly reduced in murine BALF when infected with PA14 compared to the Cif mutant strain PA14::Cif-D129S. Adult male C57Bl/6J mice were inoculated with 40 µl (1×10^6 CFU) of *P. aeruginosa* PA14 or isogenic PA14:Cif-D129S via oropharyngeal aspiration. BALF was collected in PBS 24hpi and immediately methanol trapped. The samples were solid-phase lipid extracted and analyzed for 15-epi LXA_4. ** $P \leq 0.001$, *** $P \leq 0.0001$; one-way ANOVA.

3.3.6 Cif expression in CF patients is widespread and correlates with elevated IL-8, loss of 15-epi LXA_4 and reduced pulmonary function

Based on our *in vitro* preclinical data and *in vivo* studies in the mouse pneumonia model, we hypothesized that Cif levels in patients with CF would be positively correlated with lung inflammation and inversely correlated with BALF levels of 15-epi LXA_4 and with several measures of lung function. To test these hypotheses, we obtained BALF from a random cross-sectional cohort of pediatric CF subjects who had undergone bronchoscopy (**Table 1**). Using newly developed Cif-specific antisera (**Figure 11**) we quantified Cif abundance in BALF samples that tested positive for the presence of *P. aeruginosa* lipopolysaccharide.

Table 1. Demographics of the patient cohort from which CF bronchoalveolar lavage fluid was obtained

Patient	Age	Gender	Binned Cif group
1	13	F	High
2	16	M	Low
3	18	F	Low
4	17	F	Low
5	4	M	High
6	13	F	Low
7	11	M	High
8	17	F	Low
9	13	F	High
10	7	F	High
11	13	M	Low
12	12	F	Low
13	16	F	Low
14	14	M	High
15	16	F	Low
16	11	F	High
17	17	F	Low

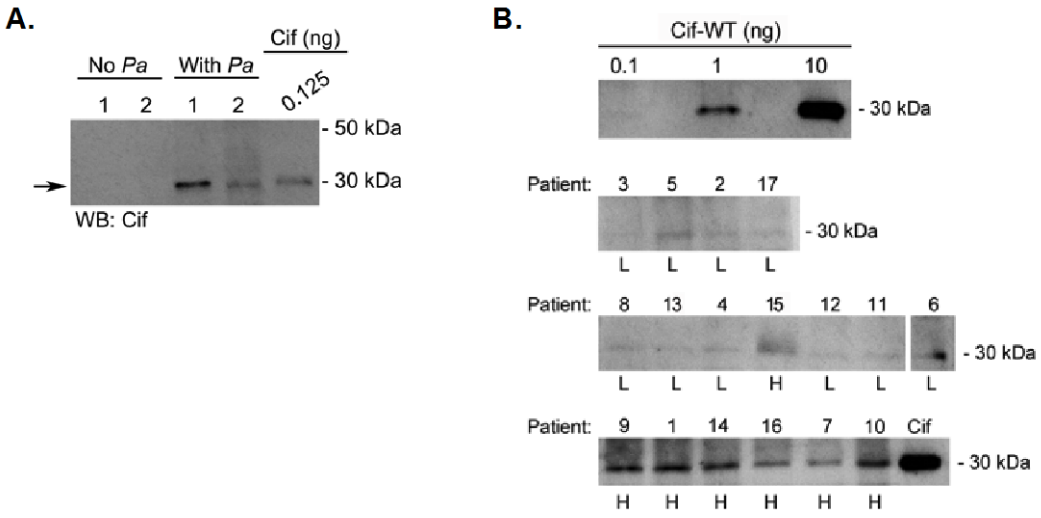


Figure 11. Cif is detected in bronchoalveolar lavage fluid from CF patients. (A) Cif antisera is specific for Cif protein in CF patient BALF. CF patient BALF (6µg/lane) was separated by SDS-PAGE and anti-Cif polyclonal rabbit serum was used for detection by Western blot. Samples were tested for *P. aeruginosa* polysaccharide and were categorized as *P. aeruginosa* positive (With Pa) or negative (No Pa). Recombinant Cif-WT protein is shown as a positive control. Arrow denotes the molecular weight of Cif. (B) Cif protein is detected in CF patient BALF. Bronchial alveolar lavage fluid was obtained from CF pediatric patients who received bronchoscopy. N=17. A standard curve was generated using recombinant Cif-WT protein (top panel). BALF for each study subject (6µg/lane) was separated by SDS-PAGE and Cif protein was detected by Western blot. Cif was quantified in each BALF sample by densitometry utilizing a standard curve of known amounts of recombinant Cif protein. Cif protein concentrations are reported in Figure 13. Patient ID number and the associated classification as low Cif (L) or high Cif (H) are shown above and below each lane, respectively.

We also quantified the levels of IL-8 and 15-epi-LXA_4 in these samples by ELISA. A Pearson cross-correlation matrix of available data demonstrated patterns clearly consistent with our predictions **(Figure 12).** Pulmonary function tests (solid outline) showed strong positive correlations with each other, consistent with CF airway disease. 15-epi LXA_4 levels positively correlated with lung function (dashed outline), consistent with the idea that pro-resolution signals are protective against inflammatory lung damage in CF. As predicted, Cif inversely correlated with both 15-epi LXA_4 and pulmonary function measures (dotted outline), and IL-8 inversely correlated with 15-epi LXA4 and positively with Cif (red line).

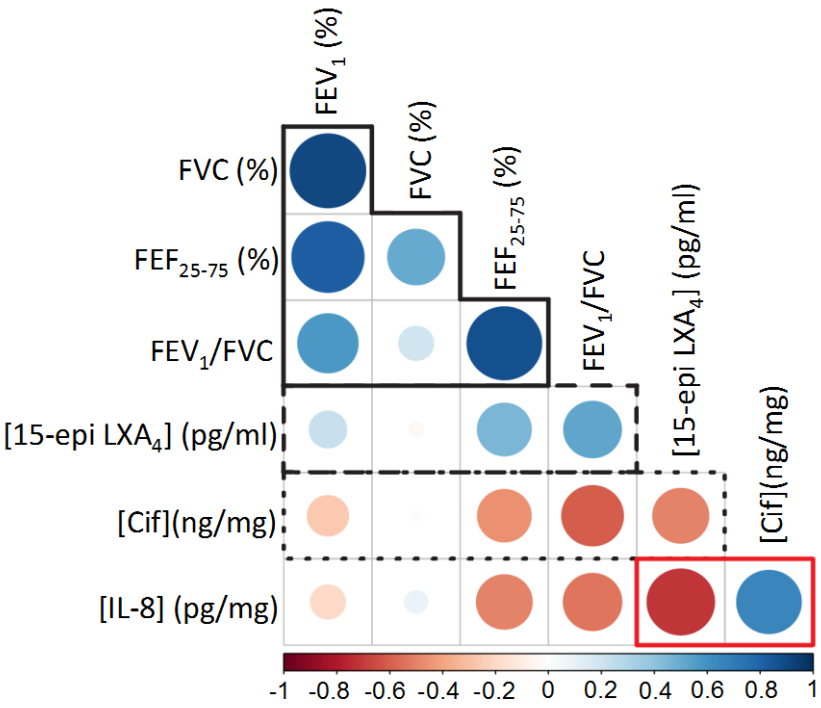


Figure 12. Cif levels in CF patient BALF positively correlated with lung inflammation and inversely correlated with 15-epi LXA_4 and with several measures of lung function. Elevated levels of Cif in CF patient bronchial lavage fluid result in lower 15-epi LXA_4 and higher IL-8 concentrations and worsening pulmonary function. A visualization of a Pearson correlation matrix depicts positive correlations in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Below the correlogram, the scale shows the correlation coefficients and the corresponding colors.

To investigate these patterns in more detail, we divided patients into "Cif low" and "Cif high" groups (**Figure 13A**), using 10 ng Cif per mg of total protein to delineate groups. Individual analyses confirmed significantly reduced levels of 15-epi LXA_4 and poor lung function and pulmonary obstruction as measured by FEV_1/FVC (**Figure 13B, and E**). Furthermore, IL-8 an important inflammatory chemokine was elevated in patients with higher levels of Cif, demonstrating that Cif abundance correlated with worse pulmonary inflammation **(Figure 13D**). Lastly, in the patient cohort, IL-8 and 15-epi LXA_4 are strongly inversely correlated (**Fig. 13C**). Thus, among subjects with *P. aeruginosa* infections, Cif levels are associated with worse obstructive lung disease.

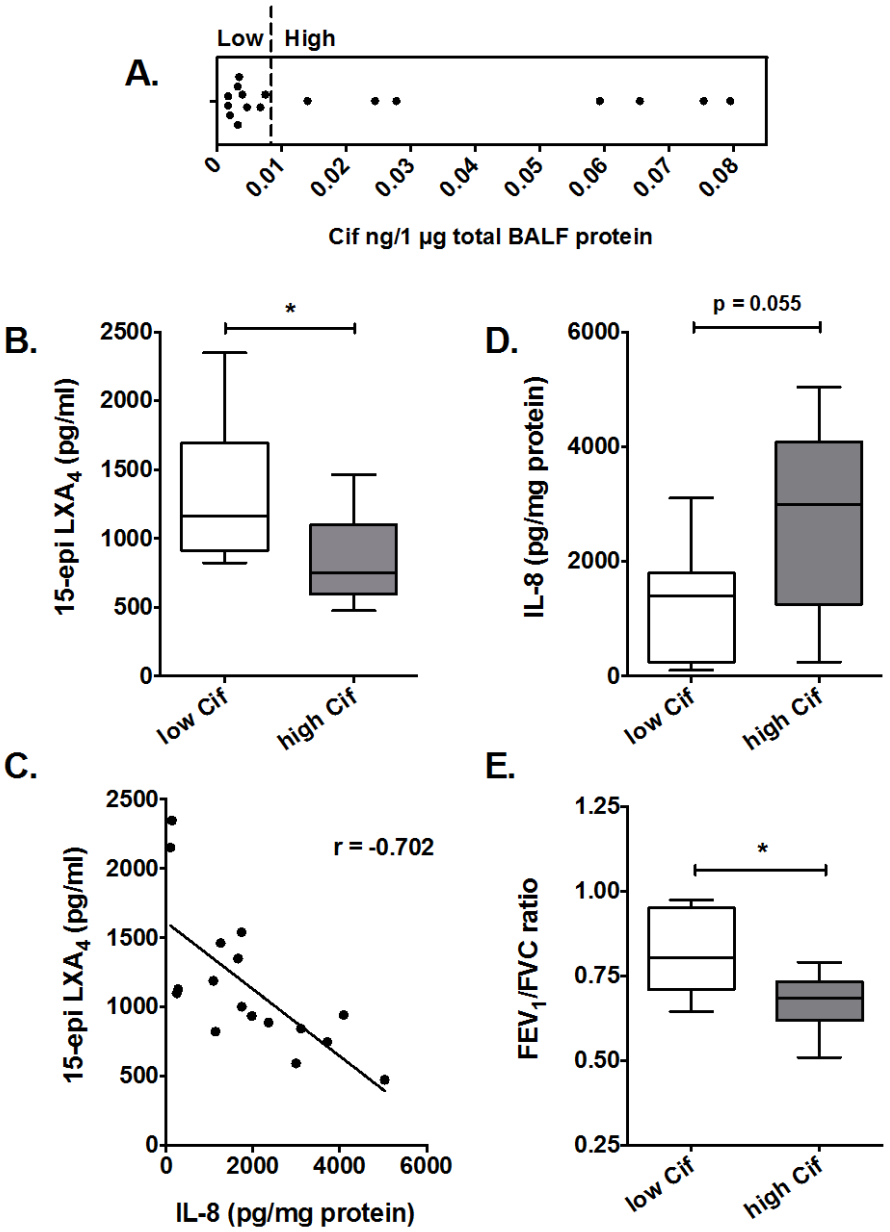


Figure 13. Elevated levels of Cif in CF patient BALF resulted in less 15-epi LXA_4 levels, higher IL-8 concentrations and more pulmonary obstruction. (A) Cif expression in CF patient BALF was determined by Western blot analysis, and binned into "low" and "high" categories. (B) 15-epi LXA_4 concentrations are significantly reduced in CF patients with elevated levels of Cif. CF BALF samples were lipid extracted and analyzed for 15-epi LXA_4 concentrations by ELISA. * $P < 0.05$ Wilcoxon Rank-Sum Test. (C) Increased IL-8 concentrations strongly correlate with lower 15-epi LXA_4 concentrations in CF patient BALF. ** $P = 0.0016$, Pearson correlation. (D) CF patients with elevated Cif have increased IL-8 concentrations in the airways. IL-8 concentrations in CF BALF were determined by sandwich ELISA and normalized to total protein in each sample. $P = 0.055$ Wilcoxon Rank-Sum Test. (E) CF patients with high Cif have worse lung obstruction. Patient FEV_1/FVC ratio was measured at time of bronchoscopy, stratified by high or low Cif groups. * $P < 0.05$, Wilcoxon Rank-Sum Test.

3.4 DISSCUSSION

A major hallmark of the disease cystic fibrosis is vigorous and persistent pulmonary inflammation, which damages host lung tissue, eventually causing respiratory failure and death. Chronic *P. aeruginosa* infections often accompany robust airway inflammatory processes, provoking continuous immune cell infiltration into the lung. In the current study, we identified the epithelial-derived eicosanoid 14,15-EET as an endogenous substrate for the *P. aeruginosa* virulence factor Cif. We also showed that the Cif-mediated reduction of 14,15-EET disrupted paracrine signaling to neutrophils to produce 15-epi LXA_4, blocking its pro-resolving functions.

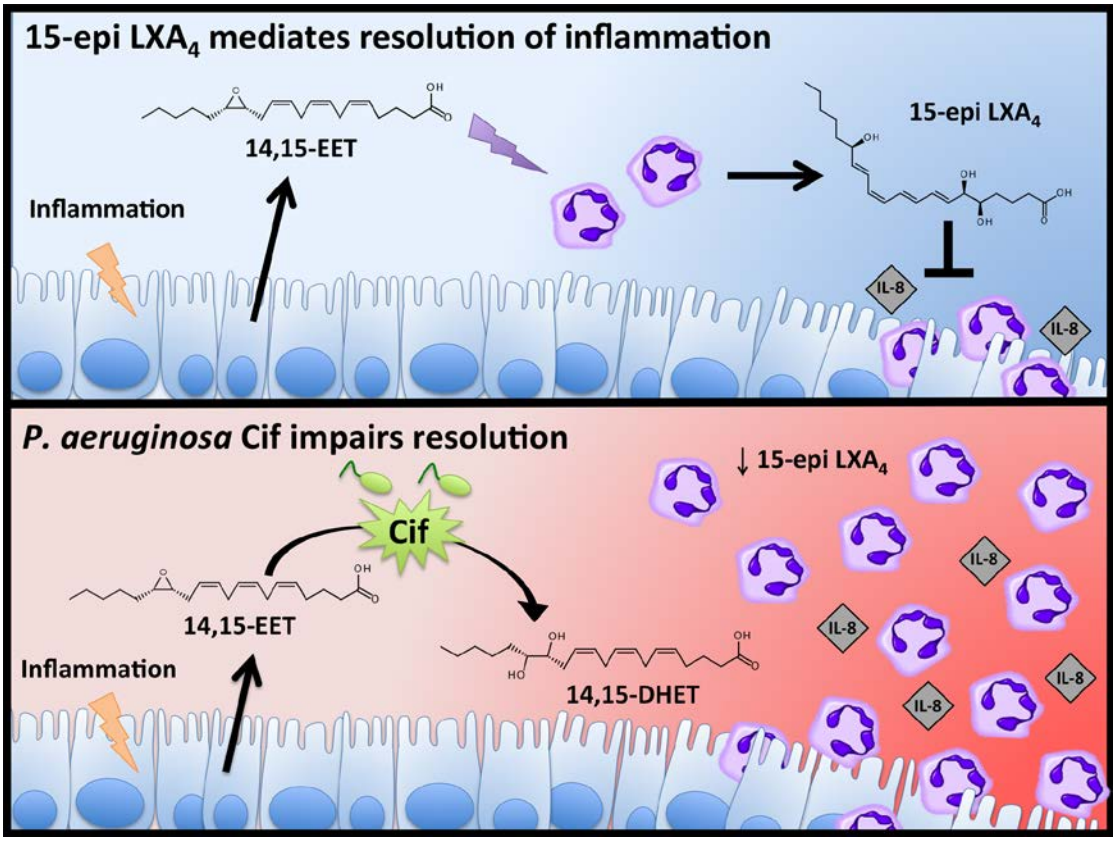


Figure 14. Summary Figure

Consistent with the Cif-mediated effect of blocking the generation of pro-resolving mediators, we demonstrated that higher Cif levels in the bronchoalveolar lavage fluid of CF patients correlated with reduced 15-epi LXA_4, increased inflammatory marker IL-8 and reduced pulmonary function. Taken together, our results provide evidence for a novel role of the bacterial epoxide hydrolase Cif in obstructing normal resolution pathways in the airway and promoting pulmonary inflammation in CF patients colonized with *P. aeruginosa*.

In contrast to the pro-inflammatory effects of Cif, several pathogens manipulate lipid mediators to suppress the immune response and evade detection. *Mycobacterium tuberculosis* and *Toxoplasma gondii* both enhance the generation of lipoxins [209, 210], while the fungal pathogen *Candida albicans* synthesizes resolvin E1 [184]. *Mycobacterium tuberculosis* can also inhibit the formation of the pro-inflammatory lipid LTB_4, further shifting the host lipid environment towards an anti-inflammatory state [211]. *P. aeruginosa* produces the 15-lipoxygenase LoxA [176, 212], as well as the phospholipase ExoU [170], both of which can potentially generate anti-inflammatory signals. However, unlike many airway pathogens, *P. aeruginosa* survives in a hyper-inflammatory environment, particularly in the context of chronic lung disease [8]. As a result, *P. aeruginosa* may be uniquely poised to exploit both pro- and anti-inflammatory strategies to thwart host defense mechanisms.

14,15-EET is the first host substrate identified for *P. aeruginosa* Cif. Previous crystallization studies identified Cif as a member of the α/β-hydrolase family of proteins, with epoxide hydrolase activity focused on small, monosubstituted substrates [191]. Thus, it was surprising to discover that Cif could accommodate the extended carbon chain of an eicosanoid such as 14,15-EET. Our crystal structure revealed an unexpected rearrangement of specific residues that define the boundaries of the active site. Small-molecule inhibitors

were recently reported to drive conformational changes in an epoxide hydrolase active site [213], but our study represents the first example of a substrate-induced fit in this class of enzymes. Furthermore, while our work shows that Cif cannot hydrolyze the other EET regioisomers, its active-site flexibility may enable it to hydrolyze other members of the broad network of regulatory lipids [214].

Among its multiple anti-inflammatory functions [215, 216], 14,15-EET plays a particularly critical role as a paracrine stimulus for the production of the pro-resolving lipid 15-epi LXA_4 by neutrophils [151]. In addition to enzymatically targeting 14,15-EET, our study also demonstrated that Cif indirectly reduced levels of the pro-resolving lipid mediator 15-epi LXA_4. Our data provide the first evidence that the capacity to generate 15-epi LXA_4 is fundamentally intact in the context of CF, yet susceptible to Cif-mediated reductions when *P. aeruginosa* is present in the CF airway. Specifically, the production of 14,15-EET and the associated modulation of neutrophil behavior were both observed with primary cells derived from CF patients. Consistent with this proposal, elevated levels of Cif in CF patient BALF correlated with reduced concentrations of 15-epi LXA_4, increased levels of the inflammatory cytokine IL-8, and worsened pulmonary function. Our study suggests that by inhibiting normal host resolution programs, the secreted *P. aeruginosa* virulence factor Cif contributes to the characteristic hyper-inflammatory environment of the CF airway.

These results have important implications for our understanding of CF disease pathogenesis. The targeting of the pro-resolution 14,15-EET/15-epi LXA_4 axis represents an unexpected bacterial virulence strategy. As is demonstrated by decades-long chronic infections in the inflamed lungs of CF patients, *P. aeruginosa* thrives in a hyper-inflammatory environment [217]. Here we show that a majority of pulmonary clinical

isolates from CF patient express Cif, implying this conserved virulence mechanism may be vital during chronic pulmonary infection. Since our data demonstrates that Cif enables *P. aeruginosa* to manipulate the host inflammatory environment, it may play an important role in defining the nature of these on-going infections, which ultimately lead to lung tissue damage and respiratory failure. Functional Cif homologs have been identified in other airway pathogens, including *Acinetobacter nosocomialis* and *Burkholderia cepacia* [97, 101]. As a result, these bacterial epoxide hydrolases may represent a new class of therapeutic targets in CF and other airway diseases in which hyper-inflammatory responses accelerate tissue damage.

Our observations may also suggest novel therapeutic approaches, as increased Cif levels correlated with more severe obstructive lung disease, as measured by FEV_1/FVC ratios, in CF patients. As increasing FEV_1/FVC ratio remains a key therapeutic objective of clinical therapy, one strategy may be direct replacement of the absent lipoxin. Multiple studies have demonstrated that exogenous administration of specialized pro-resolving mediators improve morbidity and mortality outcomes following infection [167]. In particular, the administration of a LXA_4 analog in mice challenged intratracheally with *P. aeruginosa* led to reduced neutrophil infiltration, weight loss, and bacterial burden, resulting in overall lessening of disease severity [54]. An alternative strategy may be to inhibit Cif activity with a targeted small-molecule approach [96, 218]. Our observations a murine acute pneumonia model indicate that eliminating Cif epoxide hydrolase activity would in fact elevate 15-epi LXA_4 levels in the airway during *P. aeruginosa* infection. In addition to blocking the enzymatic degradation of lipid mediators, inhibitors would also block the ability of Cif to subvert rescue of CFTR by recently approved clinical correctors [219]. Finally, since Cif

represents a key link between chronic infections and the damaging, hyper-inflammatory environment present in the CF airway, it may serve as a valuable biomarker of airway disease and treatment options in CF.

4.0 15-EPI LIPOXIN A_4 TREATMENT REDUCES RSV BURDEN AND TYPE III INTERFERON MEDIATED *P. AERUGINOSA* BIOFILM GROWTH

Portions of this chapter is adapted from:

Respiratory syncytial virus infection enhances *Pseudomonas aeruginosa* biofilm growth through dysregulation of nutritional immunity

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4.1 INTRODUCTION

Respiratory viral infections are associated with the increased acquisition of secondary bacterial superinfections resulting in greater immunopathology [220-222]. This association is of great research interest, especially in the area of CF, where respiratory viral infections are linked to pulmonary exacerbation and enhanced bacterial acquisition. Respiratory viral infections are associated with disease progression in CF, promoting early respiratory tract morbidity and poor lung function [125, 223, 224]. Clinical studies in CF patients also link RSV infections to the development of *P. aeruginosa* chronic infections [116, 117, 225]. This clinical observation is supported in scientific studies as mice co-infected with RSV and *P. aeruginosa* have significantly higher colony forming units of *P. aeruginosa* in the lung homogenates compared to bacterial infection alone [226, 227]. Although numerous reports demonstrate an epidemiological link between viral infections, increased bacterial load and pulmonary exacerbation in CF patients, the molecular mechanisms that govern these complicated biologic interactions is unclear and need further examination.

The airway epithelium is a critical component of the innate immune system, providing the first line of defense against respiratory pathogens [7, 8]. AECs respond to viral infections by producing interferon (IFN), which signals in an autocrine and paracrine manner to induce antiviral cellular activities [228]. Both type I (IFN α/β) and type III (IFN-λ) interferon's can be secreted by the epithelium, however, IFN-λ is expressed in greater

abundance [229]. Moreover, the receptor for IFN-λ receptor is restricted to mucosal sites and primarily expressed by epithelial cells, dendritic cells and neutrophils [230-233], whereas IFN α/β receptors are universally expressed on all nucleated cells [228]. IFN-λ is structurally related to the IL-10 cytokine family and signals through a heterodimer composed of the unique IL-28αR chain and the shared IL-10R chain [234, 235]. IFN-λ signaling results in a variety of antiviral activities included the degradation of RNA, interference with vial assembly and release as well as up-regulation of MHC class I antigen expression [228]. Multiple studies have linked IFN α/β to promoting secondary bacterial infections [236-238]. However, few studies have determined whether IFN-λ, whose receptor mainly is concentrated at mucosal surfaces, contributes to detrimental secondary bacterial infections. As it is well known that *P. aeruginosa* thrives in biofilm communities in CF airway, we investigated if the appropriate production and signaling of IFN-λ following RSV infection of AECs enhanced bacterial biofilm growth.

The inflammation generated by the host is a significant factor in RSV disease pathology. Robust immune cell infiltration, epithelial cell sloughing and increased mucus secretion is characteristic of RSV respiratory infection, resulting in bronchiolitis and airway obstruction [239]. With few available effective treatment options and failure to develop natural immunity, perhaps enhancing endogenous pathways that resolve inflammation could be modern strategy to curb damaging RSV pathology.

Specialized pro-resolving lipid mediators (SPM) are key signaling molecules that down-regulate inflammatory pathways and promote the return to tissue homeostasis [186]. Although well recognized for their role in sterile and allergic inflammation, the contributions of SPMs in restraining inflammation from infectious insults is recently becoming more

appreciated. One study demonstrated the importance of both 5-lipoxygenase and 15-lipoxygenase activities in macrophage, for promoting the resolution of RSV induced inflammation [240]. This study focused on RSV induced pathology and macrophage responses to RSV did not show viral burden in their murine model. AECs are the primary cell type infected by RSV. Therefore, in this study we assessed SPM treatment on viral burden and the host response to the infection in AECs.

4.2 MATERIALS AND METHODS

Epithelial cell culture. Primary human CF bronchial epithelial cells (CF HBE) were obtained from explanted CF patient lungs and cultured by the University of Pittsburgh Airway Cell and Tissue Core under an Institutional Review Board approved protocol at the University of Pittsburgh (IRB# 11070367). CF HBE were seeded on 6.5 mm transwell inserts with a 0.4 mm pore size (Corning Life Sciences) at 2.5×10^5 cells per filter for 4-6 weeks at air–liquid interface at 37°C with 5% CO_2 until they were polarized and differentiated [241]. CFBE41o- cells, an immortalized human bronchial epithelial cell line derived from a ΔF508 homozygous cystic fibrosis patient, and the WT CFTR complemented CFBE41o- cell line (CFBE41o- wt) were generously provided by Dr. J.P. Clancy. The cells were cultured in minimum essential media (MEM, Gibco) supplemented with 2 mM L-Glutamine (Corning), 5 U/mL Penicillin and 5 μg/mL Streptomycin (Sigma), 0.5 μg/mL Plasmocin prophylactic (InvivoGen), and 10% Fetal Bovine Serum (FBS, Gemini Bio-Products). CFBE41o- or CFBE41o- wt cells were seeded onto collagen-coated 6.5 mm transwell inserts at 7×10^4 cells per filter and grown at an air-liquid interface at 37°C and 5% CO_2 for 8 – 10 days to allow for cell polarization.

RSV and *P. aeruginosa* infections. Respiratory Syncytial Virus (RSV) line A2 strain was propagated in NY3.2 cells, immortalized murine fibroblast cells from a $STAT1^{-/-}$ mouse [242]. For virus infection, primary CF HBEs or the cell lines CBFE41o- or CFBE41o- wt were washed with MEM supplemented with 2 mM L-Glutamine and the basolateral media was replaced with fresh media without antibiotics. The cells were apically inoculated with RSV diluted in MEM supplemented with 2 mM L-Glutamine to an MOI of 0.1 or 1. The

cells were incubated for 7 hours at 37˚C before the apical media was removed to allow the airway cells to retain their polarity. RSV infections then were allowed to proceed for 72 hours prior to inoculation with *P. aeruginosa.*

mRNA Expression. Prior to RNA isolation, the cells were washed apically and basolaterally twice with PBS (Gibco) supplemented with 0.1 mM Calcium Chloride (Fisher Scientific) and 1 mM Magnesium Chloride (Fisher Scientific). Total RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad). Quantitative Real-Time PCR by iQ SYBR Green Supermix (Bio-Rad) was performed using a StepOne System (ThermoFisher Scientific) to evaluate viral infection and interferon signaling gene expression. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fold change of target mRNA expression was determined by the $2^{-\Delta\Delta CT}$ method with control cells as calibrators. The following primers were used

GAPDH	5'-CGACCACTTTGTCAAGCTCA-3’(F),
	5'-AGGGGAGATTCAGTGTGGTG-3’(R)
RSV	5’-GCTCTTAGCAAAGTCAAGTTGAATGA-3’ (F)
	5’-TGCTCCGTTGGATGGTGTATT-3’ (R)
IFNL1	5'-CGCCTTGGAAGAGTCACTCA-3’ (F)
	5'-GAAGCCTCAGGTCCCAATTC-3’ (R)
IL-8	5'- CTGGCCGTGGCTCTCTTG-3’ (F)
	5'- CCTTGGCAAAACTGCACCTT-3’ (R)

CCL20	5'- TATATTGTGCGTCTCCTCAG-3' (F)
	5'- GCTATGTCCAATTCCATTCC-3' (R)
ALX/FPR2	5'- AGTCTGCTGGCTACACTGTTC-3' (F)
	5'- TGGTAATGTGGCCGTGAAGA-3' (R)

Biotic Biofilm Assay. Following 72 hours of RSV infection, AECs were inoculated apically with *P. aeruginosa* strain PAO1. Overnight cultures of PAO1 grown at 37˚C in LB were washed and diluted in MEM supplemented with 2mM glutamine. The AECs were inoculated with ~$7x10^6$ bacteria, corresponding to a multiplicity of infection of 25. After 1 hour of bacterial attachment, unattached bacteria were removed and the apical media was replaced with MEM containing 2mM glutamine and 0.4% L-Arginine (Sigma-Aldrich). After an additional 5 hour incubation, apical media was removed and cells were washed with MEM apically and basolaterally to remove unattached bacteria. The attached biofilms were then disrupted with MEM containing 0.1% Triton X-100 (Bio-Rad). Bacteria were serially diluted and plated on LB agar to determine colony-forming units (CFU).

IFN-λ ELISA. To measure the apical secretion of IFN-λ following 72 hours of RSV infection, MEM supplemented with 2mM glutamine was added during the final 18 hours to the apical compartment of the CFBE cells. The apical secretions were collected and frozen at -80°C until analysis. IFN-λ (IL-29/IL-28B) concentrations was measured with the human IL-29/IL-28B DuoSet ELISA (R&D Systems) as per manufacturer's instructions.

IFN-λ signaling and biofilm growth. Following RSV for 72 hours and CFBE cells were treated with 10 μg/mL IL-10Rβ neutralizing antibody (IL-10Rβ Ab; R&D systems) for the final 12 hr of infection. The CFBE cells were inoculated with *P. aeruginosa* and biotic biofilm growth was measured by CFU/ml. For siRNA experiments CFBE were transfected with scrambled siNeg or siRNA targeting IL-28Rα (siIL28Rα) prior to IFN-λ treatment and biotic biofilm assay. Abiotic biofilm growth in the presence of IFN-λ (200 ng/mL) was measured following 24 hour growth in 96-well microtiter plate. Biofilm growth was measured as absorbance at A_{550} following crystal violet staining as previously described [243].

15-epi lipoxin A_4 treatment of RSV infected AECs. 15-epi LXA_4 (Calbiochem) was diluted in MEM supplemented with 2 mM L-Glutamine to a working concentration of 2μM as determined by OD_{302}. The 15-epi LXA_4 was further diluted to a final concentration of 100nM by direct addition to the apical and basolateral media previously added to the CFBE cells. 15-epi LXA_4 was added either at the same time as RSV infection (T=0), 24 or 48 hours post RSV infection.

Plaque assay. NY3.2 cells were seeded at 4×10^5 cells/well in 24-well plates (Corning) that were coated with polyethylenimine (Sigma). To measure infectious RSV particles released from CFBE cells, MEM supplemented with 2mM glutamine was added during the final 18 hours to the apical compartment of the CFBE cells. The apical secretions were collected and serially diluted in DH125 media. NY3.2 cells were inoculated with the diluted apical secretions for 2 hours at 37˚C prior to the addition of a methyl cellulose (Sigma-

Aldrich)/polyethylene glycol overlay (Fisher Scientific). After 48 hours incubation, the NY3.2 cells were fixed in 10% Buffered Formalin Phosphate (Fisher Scientific). Anti-RSV antibody (Meridian Life Science, Inc.), anti-Goat-AP conjugated antibody (Santa Cruz Biotechnology) and SIGMAFAST BCIP/NBT (Sigma) were added in sequence to detect plaque-forming units (PFU).

Statistics. Experimental differences for in vitro assays were evaluated for statistical significance using GraphPad 6.0 Software. All in vitro data are shown as Mean ± SD. $P < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Respiratory syncytial virus infection enhances *P. aeruginosa* biofilm growth on CF airway epithelial cells

To model *P. aeruginosa* biotic biofilm growth in the CF airway, we utilized an *in-vitro* co-culture system with well-differentiated polarized human AECs grown at an air-liquid interface cells [244]. To determine if we could model the clinical observation that viral infection enhances *P. aeruginosa* colonization, we first tested if viral infection in primary CF bronchial epithelial cells (CF HBE) altered biofilm growth using a static biotic biofilm co-culture system (**Figure 15A**). Preceding infection of RSV (MOI=1) for 72 hours in two CF HBE codes resulted a significant increase in *P. aeruginosa* biofilm growth compared to no viral infection control (**Figure 15B**).

In order to further examine this observation and explore the potential mechanism mediating this increase in biofilm formation, we utilized the genetically tractable immortalized CF bronchial epithelial cell line (CBFE), which we infected with RSV for 72 hours prior to inoculation with *P. aeruginosa* (**Figure 15C**). We found that RSV infection with a MOI=1 significantly increased biofilm formation as seen previously with the primary CF HBE codes. Previous studies reported that *P. aeruginosa* biofilm growth is increased on airway epithelial cells with the CF genotype, as compared to non-CF cells [102], therefore we tested if CFTR activity was facilitating the virus mediated biofilm growth. We infected a CFBE cells complemented with wt CFTR and found that the presence of a functional CFTR

did not abrogate virus induced biofilm production (**Figure 15D**). These results demonstrate that preceding RSV infection increases the production of *P. aeruginosa* biofilm on CF AECs and this effect is independent of CFTR function.

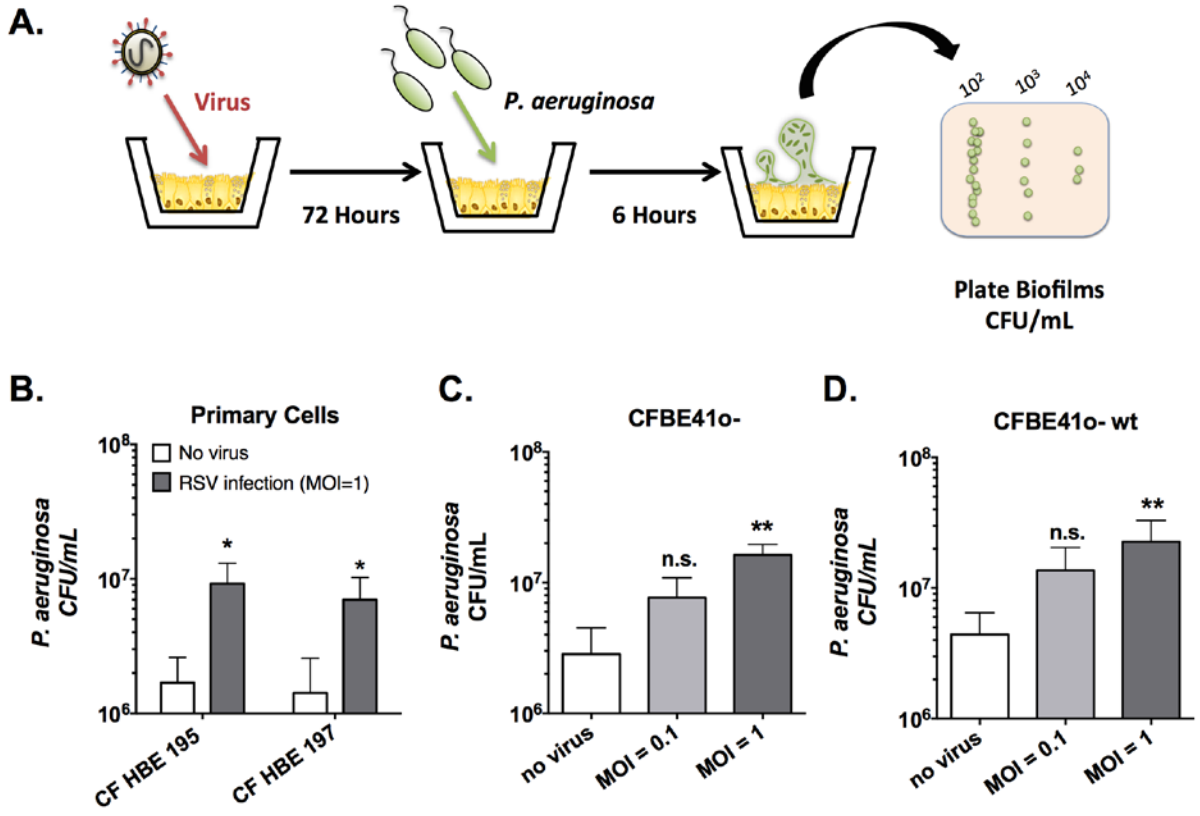

Figure 15. RSV co-infection induces robust *P. aeruginosa* biofilm growth on CF AECs. (A) Experimental design for assessing biotic *Paeruginosa* biofilm grown on AECs. (B) RSV infection promotes *P. aeruginosa* biofilms grown on primary CF HBE cells from two individuals with CF. CF HBE cells were infected with RSV (MOI=1) for 72 hours prior to apical inoculation with PA01 (MOI=25). Following 6 hour incubation at 37˚C biofilms disrupted with 0.01% Triton and quantified by CFU/ml. * $P \leq 0.05$; two-way ANOVA with Sidak's post-hoc test; N=3 for each code; Mean ±SD (C) CFBE or (D) CFBE + wt CFTR were infected with RSV at a MOI = 0.1 or 1 for 72 hours prior to apical inoculation with PA01 (MOI=25). Following 6 hour incubation at 37˚C biofilm were disrupted with 0.01% Triton and quantified by CFU/ml * $P < 0.05$, ** $P < 0.01$; one-way ANOVA with Tukey's post-hoc test compared to no virus control; N=3; Mean ±SD.

4.3.2. Antiviral IFN-λ enhances *P. aeruginosa* biotic biofilm growth

AECs orchestrate innate host defense against respiratory viral infections by secreting antiviral interferons (IFN). Therefore, we sought to determine if the increase in bacterial biofilm following RSV infection was due the secretion of IFN-λ. We therefore measured the production of IFN-λ in CFBE cells upon RSV infection over time to determine if there was a temporal relationship between IFN-λ secretion and *P. aeruginosa* biofilm production. We observed in our model that the greatest *P. aeruginosa* biofilm production occurred 72 hours post RSV infection **(Figure 16A)**. Interestingly this time point corresponded with peak apical secretion and mRNA expression of IFN-λ1 (IL-29) during the course of RSV infection (**Figure 16B and 16C**). We also observed increased IFN-λ1 expression levels in primary CF HBE cells at 72 hours post RSV infection (**Figure 16D**). These results indicate a temporal association between CFBE apical IFN-λ secretion and *P. aeruginosa* biofilm induction.

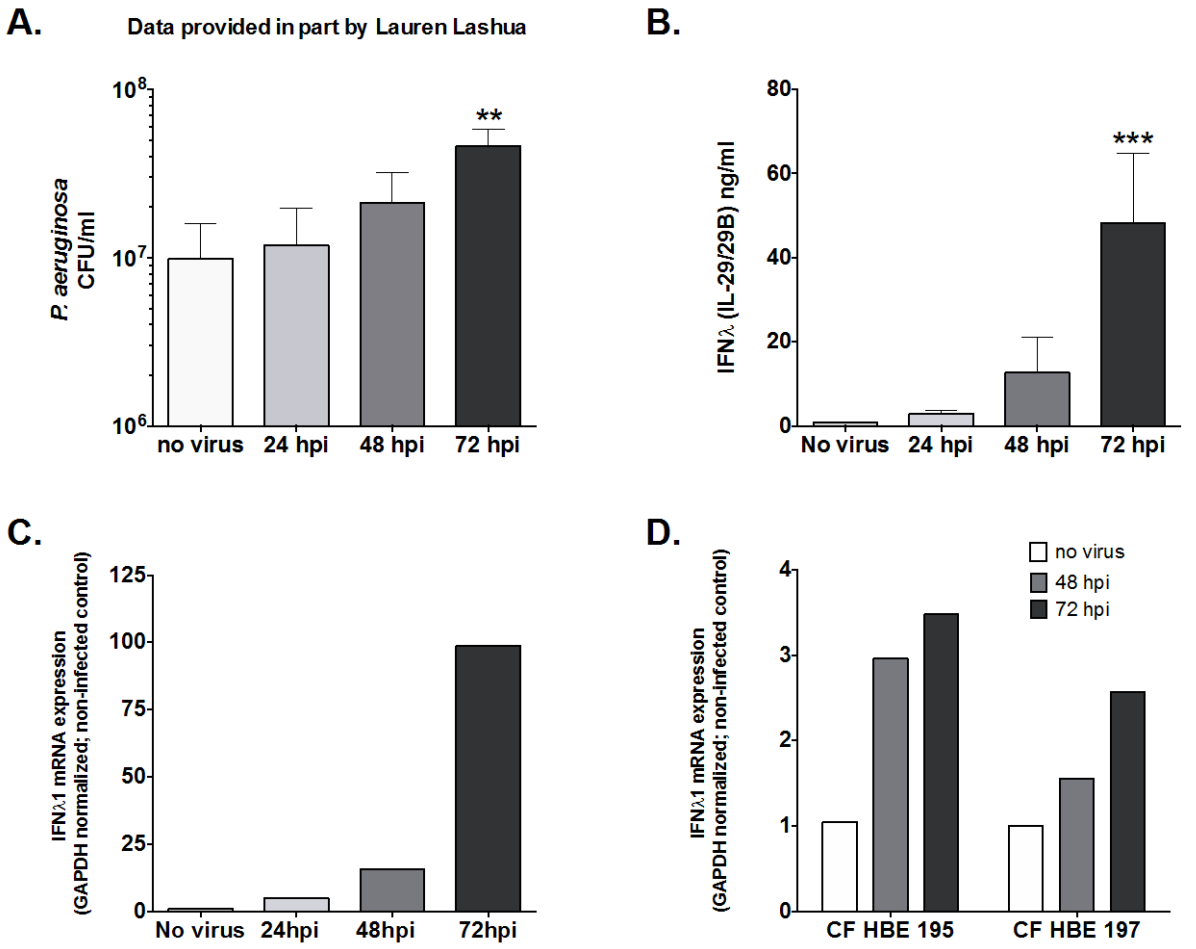

Figure 16. The temporal production of IFN-λ following RSV infection correlates with enhanced *P. aeruginosa* biofilm growth. (A) CFBE cells were infected with RSV at a MO=1 for the time points indicated, prior to apical inoculation with PA01 and biofilm production was quantified by CFU/ml ** $P < 0.01$; one-way ANOVA with Tukey's post-hoc test; N=3; Mean ±SD B) Apical secretion of IFN-λ1/3 (IL-29/28B) by ELISA from CFBE cells infected with RSV (MOI=1) for the indicated number of hours (hpi). *** $P < 0.001$; one-way ANOVA with Tukey's post-hoc test; N=3; Mean ±SD (C) IFN-λ1 gene expression in CFBE cells infected with RSV (MOI=1) measured by qPCR over the indicated time points, normalized for GAPDH and presented as fold change from the uninfected control. N=1 (D) IFN-λ1 gene expression in two primary CF HBE codes infected with RSV for either 48 hours or 72 hours (MOI=1) measured by qPCR, normalized for GAPDH and presented as fold change from the uninfected control.

We next sought to determine if IFN-λ signaling was required for virus stimulated *P. aeruginosa* biofilm growth. We treated CFBE cells with neutralizing IFN-λR antibody prior to *P. aeruginosa* inoculation and observed a decrease in the virus enhanced *P. aeruginosa* biofilm growth **(Figure 17A).** These results indicated that IFN-λR signaling was important

for mediating viral enhanced bacterial biofilm growth. We then tested if direct addition of IFN-λ1 to CFBE cells was sufficient to induce *P. aeruginosa* biotic biofilm growth. IFN-λ1 was added apically to CFBE cells for 14 hours prior to inoculation with *P. aeruginosa*. (**Figure 17B**). IFN-λ1 treatment was sufficient to promote *P. aeruginosa* biofilm growth on CFBE cells in a dose-dependent manner. Furthermore, when we inhibited IFN signaling through using RNAi-mediated IL-28Rα knockdown prior to IFN-λ treatment we also saw a decrease in biofilm growth **(Figure 17C)**. Together, these results show that host anti-viral IFN-λ responses promote *P. aeruginosa* biofilm growth on AECs.

Lastly we tested if IFN-λ interacted directly with *P. aeruginosa* to enhance biofilm growth. Bacteria were grown in a 96-well microtiter plate in the presence of IFN-λ (200ng/ml) and biofilm were stained with crystal violet and measured by OD_{550} **(Figure 17D)**. The presence of IFN-λ did not alter biofilm growth, which indicates that the bacterium does not directly utilize IFN-λ for biofilm growth.

Data provided in part by Lauren Lashua and Matt Hendricks

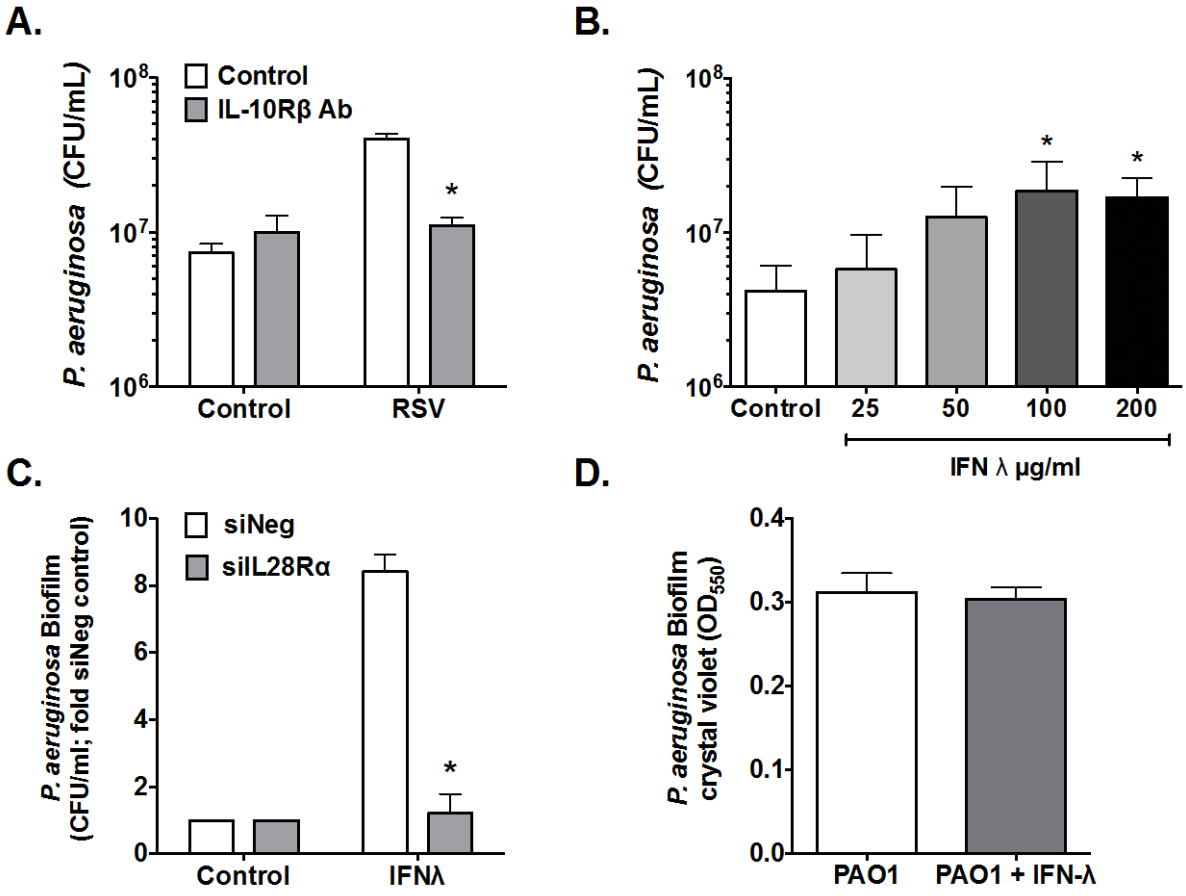

Figure 17. IFN-λ signaling is required and sufficient to mediate virus-stimulated *P. aeruginosa* biofilm growth. (A) CFBE cells were infected with RSV 72 hours and treated with IL-10Rβ–neutralizing antibody (gray bars) or were left untreated (white bars) * $P < 0.05$; $N \geq 3$; Mean ± SD; (B) CFBE cells were treated IFN-λ1 with the indicated concentrations for 14 hours prior to apical inoculation with *P. aeruginosa*. Biofilms were quantified by CFU/ml. * $P \leq 0.05$; one-way ANOVA with Tukey's post-hoc test; N=3; Mean ±SD. (C) AECs were transfected with scrambled siRNA (siNeg white bars) or siRNA targeting IL-28Rα (siIL28Rα grey bars) prior to IFN-λ1 treatment and biofilm assay * $P < 0.05$; $N \geq 3$; Mean ± SD. (D) *P. aeruginosa* biofilms were grown in a 96-well microtiter biofilm assay in the presence or absence of IFN-λ (200 ng/mL) diluted in MEM for 24 hours. Biofilm growth was measured as absorbance at OD_{550} following crystal violet staining. N = 3; Mean ± SD.

4.3.3 15-epi LXA_4 treatment reduces RSV burden

Recent studies demonstrate that SPM treatment can reduce viral replication and pathology associated with infection [107, 245]. Other reports have demonstrated that AECs express the lipoxin receptor FPR2/ALX, [246, 247]. To confirm that our CFBE cells express FPR2/ALX we measured receptor expression by qPCR. We found that CFBE cells express elevated FRP2/ALX transcripts compared to A549 cell expression, which have been shown to not express the receptor (**Figure 18A**). Since CFBE cells expressed FPR/ALX, we decided to investigate if 15-epi LXA_4 treatment during RSV infection could alleviate viral burden in infected epithelium. CFBE cells were infected with RSV (MOI=1) and treated with 15-epi LXA_4 at various time points over the course of infection (**Figure 18B**). Following 72 hours of RSV infection, CFBE cells were harvested and viral transcripts were measured by qPCR and infectious viral particles released in the apical secretions were quantified by plaque assay. 15-epi LXA_4 treatment done in parallel at the start of RSV infection (T=0) had no significant effect on either viral transcript production or plaque forming units (PFU) at 72 hpi. However, treating CFBE cells with 15-epi LXA_4 either 24 or 48 hours into viral infection resulted in significantly less viral transcript and PFU (**Figure 18C and 18D).** Interestingly, we observed that CFBE cells begin to ramp up inflammatory processes 48 hours following RSV infection, as seen by the increase in IL-8 and CCL20 by qPCR (**Figure 18E and F**). These results suggest that treatment of 15-epi LXA_4 can reduce viral burden but only if administered to AECs after the inflammatory processes induced by the viral infection have commenced.

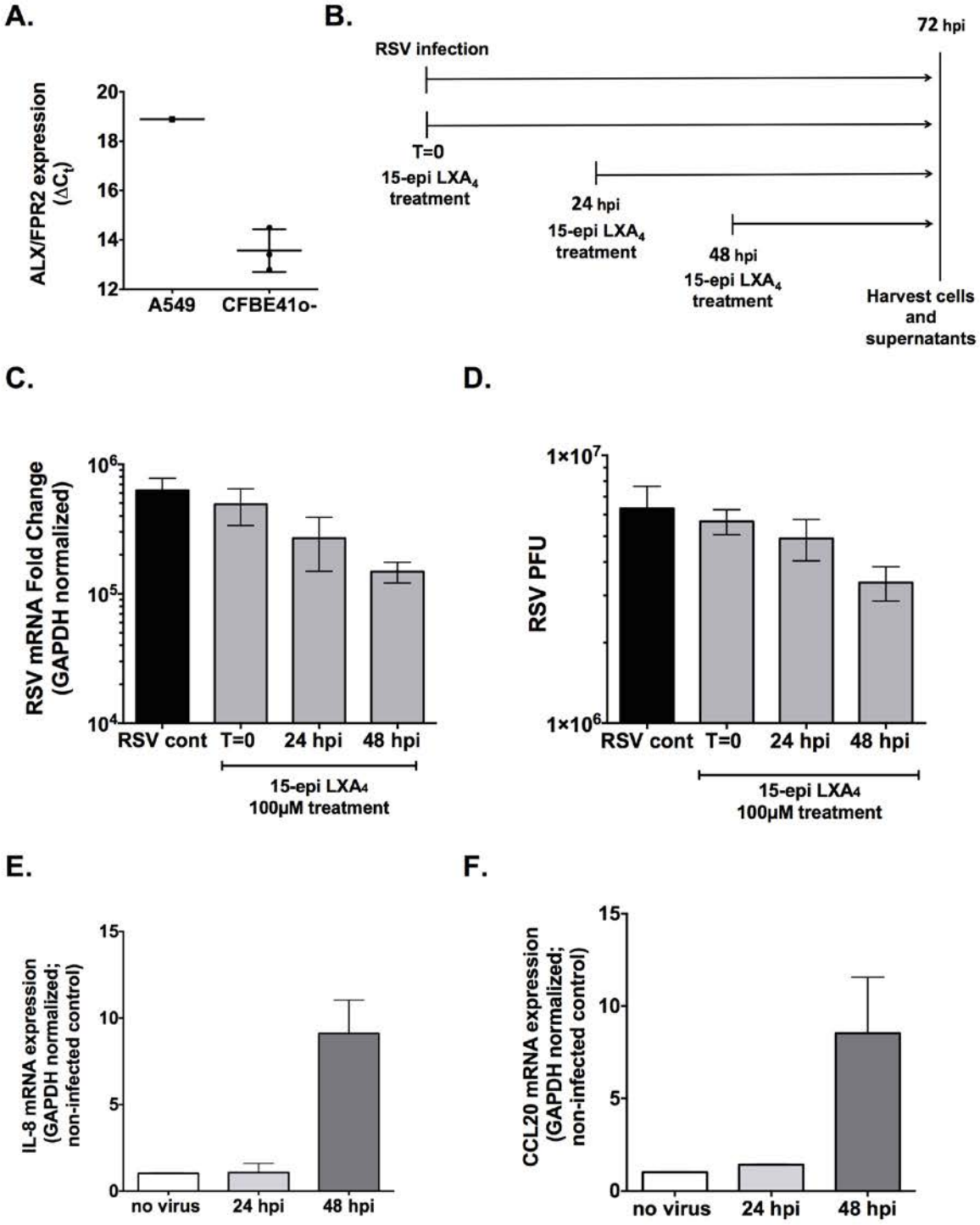

Figure 18. Viral burden is reduced upon 15-epi LXA_4 treatment in CFBE cells. (A) ALX/FPR2 expression measured by qPCR in untreated CFBE and A549 cells. (B) Experimental design for 15-epi LXA_4 treatment of RSV (MOI=1) infected CFBE cells. (C) RSV gene expression measured by qPCR in CFBE cells infected with RSV control (black bar) or with 100nM treatment of 15-epi LXA_4 over the indicated time points during infection (grey bars). Data normalized for GAPDH and presented as fold change from the

uninfected control; N=2; Mean ±SD. (D) The apical release of infectious RSV particles quantitated by plaque assay. RSV control (black hashed bar) or with 100nM treatment of 15-epi LXA_4 over the indicated time points during infection (grey bars); N=2; Mean ±SD. (E) IL-8 and (F) CCL20 gene expression measured by qPCR in CFBE cells infected with RSV control at the time points indicated. N=3; Mean ±SD.

4.3.4 15-epi LXA_4 treatment during RSV infection significantly reduces IFN-λ secretion by airway epithelial cells and lessens *P. aeruginosa* biofilm production

The observation that 15-epi LXA_4 treatment 48 hours post-infection reduced RSV viral burden suggested that SPM treatment was enhancing IFN-λ production, thereby accelerating epithelial control of the infection. Interestingly, we found the inverse; IFN-λ secretion was significantly decreased following 15-epi LXA_4 treatment at both 24 or 48 hours post infection (**Figure 19A**). This data indicates IFN-λ responses are attenuated in a manner that correlates with reduction in viral load, suggesting that 15-epi LXA_4 treatment is directly targeting virus replication rather than mediating the host response following RSV infection.

Given our previous observation that apical IFN-λ signaling enhances *P. aeruginosa* biofilm production, we decided to test if 15-epi LXA_4 treatment could reduce both viral and bacterial burden during co-infection (**Figure 19B**). 15-epi LXA_4 treatment either 24 or 48 hours after RSV infection resulted in a modest reduction in subsequent *P. aeruginosa* biofilm growth. These results are consistent with the conclusion that treating AECs with 15-epi LXA_4 during viral infection reduces IFN-λ secretion and lessens both viral and bacterial burden.

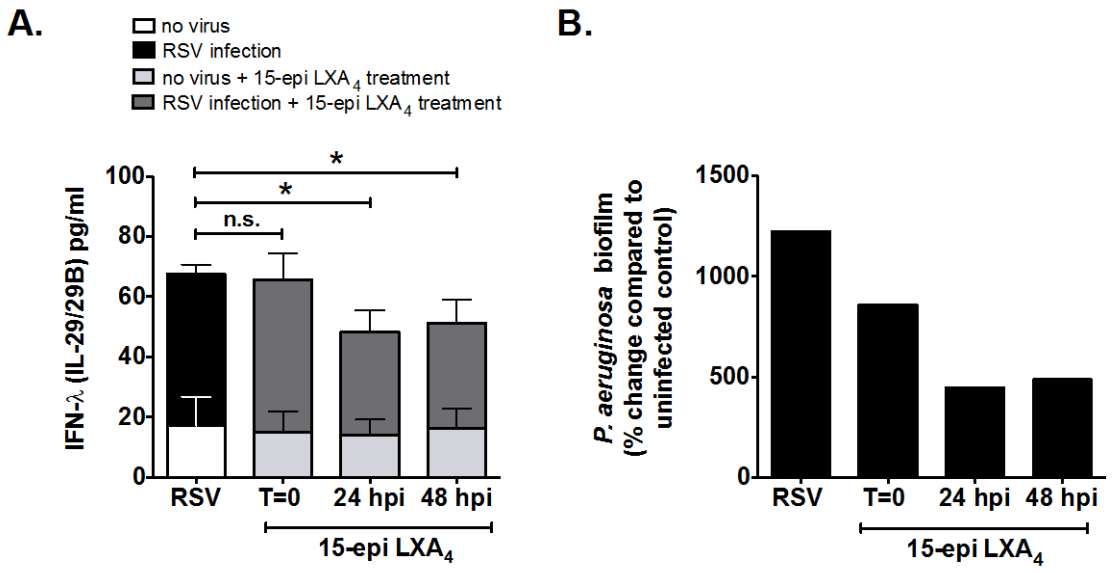

Figure 19. 15-epi LXA_4 treatment significantly reduces IFN-λ secretion and blunts virally enhanced *P. aeruginosa* biofim formation. (A) Secretion of IFN-λ (IL-29/28B) was measured by ELISA from untreated CFBE cells (white bar) or CFBE cells infected with RSV (black bar) in the presence of 15-epi LXA_4 over the indicated time points during infection (dark grey bars). Uninfected CFBE cells were treated with 15-epi LXA_4 in parallel (light grey bars). * $P < 0.05$; compared to RSV infection control, one-way ANOVA with Tukey's post-hoc test; N=3; Mean ±SD. (B) CFBE cells were infected with RSV for 72 hours and treated with 15-epi LXA_4 over the indicated time points during infection prior to apical inoculation with PA01 (MOI=25). Following a 6 hour incubation at 37˚C biofilms were disrupted with 0.01% Triton and quantified by CFU/ml. Data expressed as percent biofilm induction following RSV infection compared to uninfected control; N=1 done in replicate; Mean ±SD

4.4 DISCUSSION

Clinical observations have linked respiratory viral infection in CF with pulmonary exacerbations and enhanced *P. aeruginosa* chronic colonization. In this study, we demonstrate that RSV infection in primary CF HBE or immortalized CFBE cells promotes enhanced *P. aeruginosa* biofilm growth. We show this interaction is independent of functional CFTR activity and instead is due to host antiviral responses. We observed a temporal association between peak IFN-λ secretion and elevated *P. aeruginosa* biofilm growth. Interestingly, the direct addition of IFN-λ to uninfected cells recapitulated the increase in bacterial biofilm growth, demonstrating that *P. aeruginosa* is able to take advantage of the antiviral environment in the airway lumen. Thus, our study demonstrates that anti-viral IFN-λ responses to RSV infection in the airway promote secondary *P. aeruginosa* infection and biofilm growth.

Although the antiviral activities of IFN are well appreciated, recent viral-bacterial co-infection murine studies reveal that IFN responses have unfavorable consequences on secondary bacterial infections [248]. For example, in acute murine models of influenza or LCMV, IFN-α/β responses to viral infection impaired control of bacterial load in the airways [236-238]. More recently, IFN-λ signaling has also been shown to be detrimental to *P. aeruginosa* infections, as the bacterium was cleared more efficiently in mice that could not respond to IFN-λ [249]. Our data extend these findings and demonstrate that IFN-λ production by human AECs promotes *P. aeruginosa* biotic biofilm growth and chronic infections. Mucosal IFN-α/β responses have been shown to reduce both antimicrobial peptide release and IL-17 responses, both important for controlling extracellular bacterial infections

at the mucosa [238]. Others studies demonstrated that IFN target gene effector functions promote bacterial replication, suggesting that some pathogens have evolved to subvert and even benefit from the interferon response in host cells [250]. As IFN-λ receptor expression is restricted to mucosal surfaces, and *P. aeruginosa* thrives in the lumen of the airway, perhaps the bacteria have evolved mechanisms to take advantage of IFN-λ anti-viral host responses. Recently, Hendricks et al. demonstrated that viral infection of CFBE cells promotes the apical release of iron, which enhances *P. aeruginosa* biofilm growth [229]. Future studies, investigating how IFN-λ signaling in the CF the lung mucosa promotes an environment that is favorable to *P. aeruginosa* growth could potentially uncover new therapeutic targets that reduce bacterial burden.

While inflammation following a pathogenic insult is essential for host defense, excessive or inflammatory responses can cause tissue damage. A multitude of studies have demonstrated that endogenous SPMs orchestrate the resolution of inflammatory responses and promote the return to tissue homeostasis [152, 186]. However, utilizing SPMs to treat infectious disease is only recently becoming an area of active research interest for the treatment of pathogen-associated inflammation [173, 251, 252]. Given the limited treatment options for RSV, which causes robust and damaging pulmonary inflammation, in the current study we investigated if 15-epi LXA_4 could be used to treat this respiratory virus infection. We found that treatment with 15-epi LXA_4 during ongoing RSV infection significantly reduced IFN-λ secretion and attenuated viral burden. Interestingly, SPM treatment only had an effect if administered 24 or 48 hours post-infection. Thus, our data implies that 15-epi LXA_4 can only exert its pro-resolving signal when the AECs are responding to viral infection. Perhaps, AECs only initiate inflammatory programs that generate 15-epi LXA_4

signaling targets once a viral replication or PAMP accumulation has reached a threshold. Our study indicates that 15-epi LXA_4 treatment could be administered days into a viral infection, which would be a improvement over traditional anti-viral therapies which need to be employed at the early stages of infection to be effective.

Our study also demonstrated that 15-epi LXA_4 administration lessened IFN-λ associated *P. aeruginosa* biofilm growth. Although the effect on bacterial burden is small, our study suggests that mucosal SPM treatment could reduce IFN-λ responses that enhance secondary bacterial superinfection. The administration of SPMs may provide a novel way to target polymicrobial infections rather than using the current standard of cares that target one microorganism at a time. Additional work is needed to both titrate the optimal dose of 15-epi LXA_4, well as determine if other SPMs can be used to treat viral-bacterial co-infections. Furthermore, more studies are needed to identify the inflammatory pathways that 15-epi LXA_4 administration targets in AECs and other cells in the mucosa. Lastly, determining other intracellular pathogens that can be curbed by utilizing SPM treatment will be important area of future investigation.

In conclusion, our study supports use of SPM administration in the airway mucosa, which could provide a new avenue for therapeutic intervention that may alleviate both pathogen burden and detrimental host inflammatory responses.

5.0 OVERALL CONCLUSION AND FUTURE DIRECTIONS

Our studies demonstrate that not only does *P. aeruginosa* thrive in an inflammatory environment, but also actively promotes persistent inflammation in the CF lung. In our first study we determined that the epoxide hydrolase activity of the *P. aeruginosa* virulence factor Cif, mediated the hydrolysis of epithelial-derived 14,15-EET. The manipulation of AEC derived 14,15-EET reduced neutrophil generation of 15-epi LXA_4. We further showed that the presence of Cif in the BAL fluid of CF patients correlated with elevated inflammatory markers and pulmonary function. Cif is the first bacterial epoxide hydrolase that has been shown to promote inflammatory pathways. Interestingly, unlike other virulence factors expressed by *P. aeruginosa*, Cif expression is not reduced over time in the CF lung, indicating that its function is important for chronic infection. Other bacterial and fungal pathogens have been shown to also target host lipid pathways, however in the opposite manner [184, 209, 210]. These microorganisms mainly promote the production of pro-resolving lipid pathways, presumably to conceal themselves from the immune response. Our study reveals that *P. aeruginosa* takes a different approach in the CF lung, instead manipulating host lipid signals to promote continual inflammation. While our studies have focused on the CF airway, it is important to recognize that Cif may also skew inflammatory activities in other pulmonary diseases where patients are commonly infected with *P. aeruginosa* such as ventilator associated pneumonia and COPD. Furthermore, the *P.*

aeruginosa strain PA14, which highly expresses Cif was originally isolated from a burn patient. Thus, it is quite possible that *P. aeruginosa* utilizes this virulence mechanism to promote an inflammatory environment in other areas of the body, such as the skin. Future studies that determine Cif expression in *P. aeruginosa* isolates obtained from various host etiologies will be important for designing therapeutics and could also provide important clues for Cif expression regulation in the host.

Although it may seem counter productive for a bacterial pathogen to actively induce more inflammation, there are a few possible reasons that *P. aeruginosa* finds vigorous inflammation in the CF an accommodating niche. Perhaps, by inciting robust inflammation *P. aeruginosa* outcompetes other more susceptible microorganisms, thereby becoming the dominant microorganism in the lung [253]. Inflammation might also provide the bacteria with a steady supply of essential nutrients, as our studies demonstrate viral infection enhanced the secretion of apical iron by AECs [229]. It is also possible that by removing counter regulatory signaling and promoting continual inflammation, the accumulation of frustrated and necrotic neutrophils provides the bacteria with more host material to incorporate into bacterial biofilms [51, 53, 113]. Lastly, pulmonary inflammation promotes the secretion of mucus, which could provide the bacteria more surface area to grow biofilms [254, 255]. Although one can only postulate what the exact beneficial mechanism that continuous inflammation provides *P. aeruginosa*, it is clear that the bacteria can survive and adapt to the inflammatory CF lung. It is important to note that in immunocompetent hosts neutrophil responses are key in regulating *P. aeruginosa* infections, therefore in the CF lung, it is clear that neutrophil dysfunction along with poor mucocillary clearance are key elements that allow chronic colonization to occur.

During my studies I have gained an appreciation for the enormous contribution of epithelial cells in host defense. The production of antimicrobials, regulation of pH, and mucociliary clearance are all vital epithelial functions that maintain mucosal homeostasis. Although traditionally immunologists consider the epithelium to only afford barrier protection, I think of them to be specialized immune cell coordinators, as they orchestrate many immune responses at the mucosa. In our study, we show that 14,15-EET secreted from the AECs provide a key counter regulatory signal to neutrophils. Moreover, there is a large body of literature that show AECs secrete a variety of chemotactic proteins and lipids that help coordinate the traffic of granulocytes, lymphocytes and monocytes [7]. As epithelial cells are major constituent of many mucosal tissues, future therapies that target this cell type could potentially yield new anti-inflammatory therapies. However, much work remains to be done on cellular communication networks at the mucosa, particularly in terms of the resolution of inflammation.

There is substantial evidence that demonstrates SPMs can be useful for allergic or sterile inflammation. Recent studies are also beginning to show the utility SPM treatment for alleviating infectious inflammation [167, 245, 256]. Here we show that 15-epi LXA_4 administration to AECs reduces RSV burden and IFN-λ secretion. Although the effect we see in our study is small, changing the dose and timing of administration may increase the antimicrobial effect of 15-epi LXA_4. This investigation revealed that inflammatory pathways must be activated prior to 15-epi LXA_4 treatment to see an antimicrobial effect. This observation is exciting as SPMs treatment may be most effective when administered days into a viral infection which could be valuable given that traditional anti-viral therapies generally need to be dispensed during early stages of infection to be effective. Further

research on supplementing standard of care therapies with SPM treatment for antiviral therapy could be very beneficial, especially for chronic viral infections. Novel SPM analogues are in development, which have a longer half-life than their endogenous counterparts. Perhaps using a 15-epi LXA analogue on virally infected AECs in our model may enhance antimicrobial effects. In the future, studies that use SPM treatment may provide better patient outcomes during viral infections.

Future therapies that target the robust inflammatory response in the CF airway are on the horizon. Multiple drugs that enhance pro-resolving pathways including CTX-4430 and Resunab are currently undergoing clinical trials in CF patients. CTX-4430 is a selective inhibitor of leukotriene A4 hydrolase that blocks the production of leukotriene B4, a molecule that leads to inflammation and is known to be elevated in CF. Resunab is a first in class, synthetic oral endocannabinoid-mimetic drug that preferentially binds to the CB2 receptor expressed on activated immune cells [257]. CB2 activation triggers endogenous pathways that resolve inflammation and halt fibrosis. As the body of literature regarding SPM pro-resolving functions in the lung increases, it is likely that their use a therapeutic treatment for CF may be forthcoming in the near future.

APPENDIX A

THE *PSEUDOMONAS AERUGINOSA* LYPOXGENASE LOXA SUPPORTS BIOFILM GROWTH ON HUMAN AIRWAY EPITHELIAL CELLS

This appendix is adapted from:

Biochemical and Cellular Characterization and Inhibitor Discovery of *Pseudomonas aeruginosa* 15-Lipoxygenase

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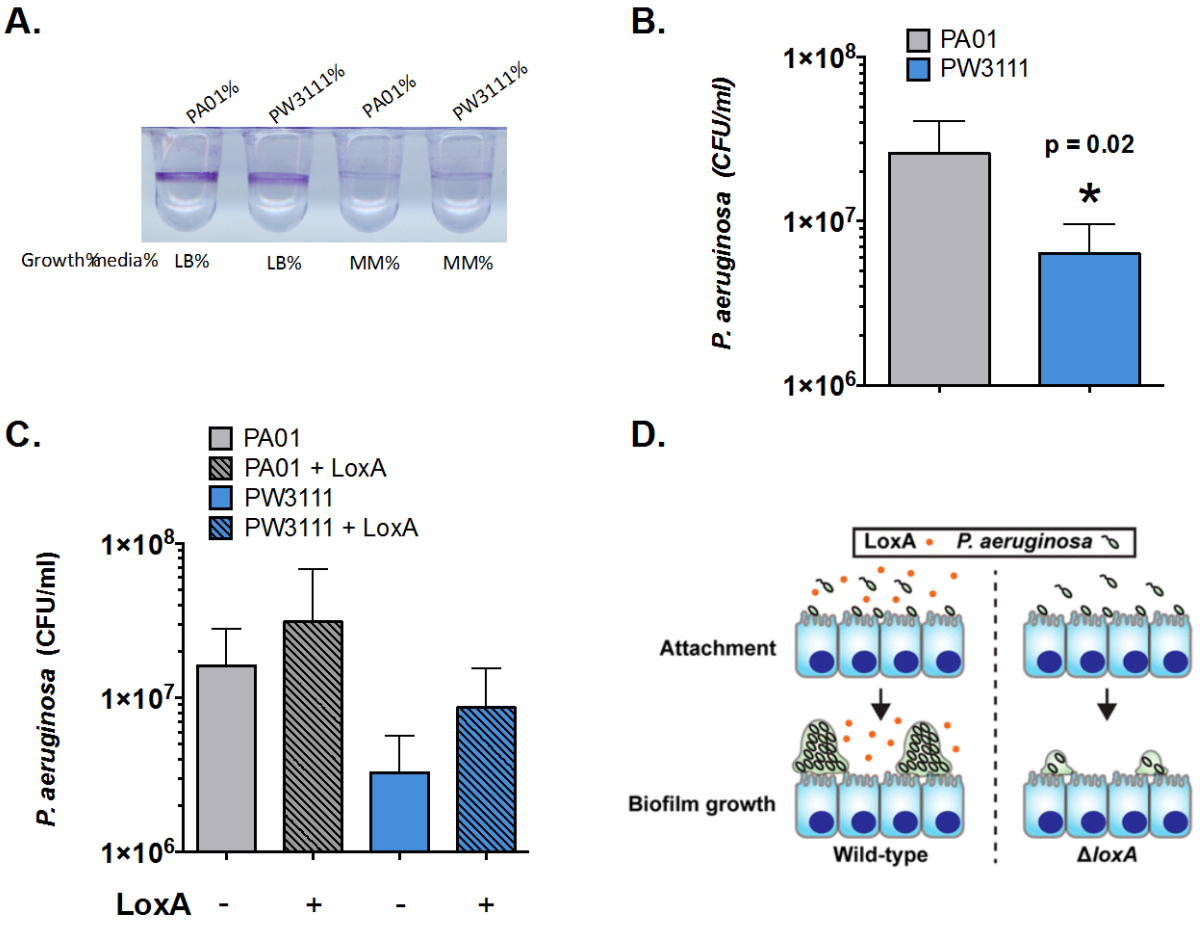
P. aeruginosa secretes the 15-lipoxygenase LoxA, that has the capacity to convert host arachidonic acid (AA) into 15-hydroxyeicosatetraenoic acid (15-HETE) [173, 251, 252]. LoxA has similar positional specificity for peroxidation of free fatty acids to that of human 15- lipoxygenase (15-LOX) [212]. It has been proposed that LoxA modulates host defense and by altering the host lipid environment, however, the biologic function *in vivo* is unknown. Here we tested the hypothesis that LoxA enhanced *P. aeruginosa* biofilm growth and thus facilitates chronic colonization in the host.

Biofilm growth of a LoxA transposon mutant PW3111 was tested in parallel with an isogenic laboratory strain PA01. Biofilm formation when grown on abiotic surfaces was similar between the two strains, indicating no functional consequence of LoxA on abiotic biofilm formation (**Appendix figure A**). However, when biofilms were grown on human AECs, PW3111 produced significantly less biofilm compared to PA01 (**Appendix figure B**). Furthermore, the direct addition of recombinant LoxA protein to PW311 or PA01 enhanced biofilm formation on AECs (**Appendix figure C**). These data suggest that LoxA positively correlates with *P. aeruginosa* biofilm production when associated with host epithelial cells.

AEC expression of 15-LOX is implicated in chronic lung inflammatory diseases such as asthma [176]. Type II inflammatory cytokines, IL-4 and IL-13 upregulate the expression of 15-LOX in epithelial cells [254, 255, 258]. Furthermore, the production of 15-HETE by 15-LOX in airway epithelial cells is associated with heightened mucus secretion [254, 255]. It is possible that the LoxA enhances epithelial cell mucus secretion allowing for more surface area for *P. aeruginosa* to attach and form biofilms. Future experiments examining the role of LoxA in pulmonary inflammation may provide further understanding of how *P. aeruginosa* manipulates the host inflammatory pathways to its own benefit.



Appendix Figure 1. (A) PA01 or the LoxA transposon mutant PW3111 we grown on a abiotic surface in Luria broth (LB) or minimal essential media (MM) for 24 hours and biofilm mass was quantified by crystal violet. (B) PA01 or PW3111 were grown on 16HBE airway epithelial cells for 6 hours and biofilm mass was quantified by CFU/ml. Paired T-test; N=4; Mean ±SD (C) PA01 or PW3111 biotic biofilms were quantified following the addition of 1µg of recombinant LoxA protein at the time of inoculation N=2; Mean ±SD. (D) Summary model figure.

LIST OF ABBREVIATIONS

14,15-EET	14,15 epoxyeicosatrienoic acid
15-epi LXA_4	15-epi lipoxin A_4
AA	Arachidonic acid
AEC	Airway epithelial cells
ASL	Airway surface liquid
BALF	Bronchoalveolar lavage fluid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFBE	CF bronchial epithelial cell line CFBE41o−
CF HBE	Primary human CF bronchial epithelial cells
Cif	CFTR inhibitory factor
Cif-WT	Cif protein
Cif-D129S	Cif-D129S mutant
COX	Cyclooxygenases
EETs	Epoxyeicosatrienoic acids
IFN α/β	Type I interferon
IFN-λ	Type III interferon
LXA_4	Lipoxin A_4

LOX	Lipoxygenases
SPM	Specialized pro-resolving lipid mediator
RSV	Respiratory syncytial virus

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