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Translational studies of neutrophils, macrophages, and T cells in the human lung: implications for cystic fibrosis and other chronic respiratory pathologies

By

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Doctor of Philosophy

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Advisor: Rabindra Tirouvanziam, Ph.D.

An abstract of
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Abstract

Translational studies of neutrophils, macrophages, and T cells in the human lung: implications for cystic fibrosis and other chronic respiratory pathologies

By Vincent D. Giacalone

Neutrophils comprise the largest subset of circulating leukocytes in humans, and maintain a significant presence in the lungs. In-depth investigation of their effector and regulatory functions has revealed new relationships with other leukocyte subsets such as macrophages and T cells. In cystic fibrosis (CF), sterile neutrophilic inflammation begins in the airways at a young age and drastically impacts the functions of other cell types while contributing to bronchiectasis. Clinical studies have traditionally relied on samples collected by invasive techniques such as bronchoalveolar lavage, but these samples are increasingly difficult to obtain.

The research presented in this dissertation focuses on four main areas of CF research with the common element of neutrophilic inflammation. First, we investigated the role of the PD-1 pathway in exhaustion of airway macrophages and how this coincides with the early stages of neutrophil recruitment. Second, we identified signatures of T-cell activation during acute pulmonary exacerbations in young children, which contrasts with the functional impairment of T cells later in CF lung disease. However, concomitant increases in neutrophil frequency in circulation and cytokine concentrations in the airways and plasma are suggestive of future waves of neutrophil recruitment to the airways. Third, we demonstrated that minimally invasive collection of induced sputum from young children with CF, already employed in routine care, can yield data on cellular and soluble markers of inflammation that are comparable to those from more invasive bronchoalveolar lavage. Finally, we used an in vitro transmigration model to generate new insights into neutrophil and monocyte function after their recruitment to the lung which would be difficult or impossible to obtain with primary samples, such as the dynamics of acid sphingomyelinase, IL-29, and nitric oxide production. By adapting this model to study pulmonary *Yersinia pestis* infection, we obtained data suggesting that *Y. pestis* virulence factors block calcium flux in neutrophils.

This dissertation advances the knowledge of CF lung disease by providing novel insights about coordinated immune responses in the lung, with implications for other acute and chronic pulmonary diseases.

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Acknowledgements

The work presented in this dissertation is the result of a collective research effort by multiple esteemed investigators and dedicated trainees. The members of our consortium from the United States of America, the Kingdom of the Netherlands, and the Commonwealth of Australia are devoted to furthering our knowledge of CF lung disease with the goal of improving life for all people with CF. Throughout my graduate training I grew as a scientist and as a professional through hard work and dedication, but I owe gratitude to many individuals who have supported and mentored me along the way, both prior to and during my time as a graduate student.

As an undergraduate student at the University of Massachusetts Amherst I was fortunate in having the opportunity to build a strong foundation as an aspiring biomedical researcher. I would like to thank Dr. John Stoffolano for giving me my first opportunity to work in a research laboratory, where I began as freshman and continued until my graduation. Working in an entomology lab taught me the critical skills of observation and pattern recognition, which have helped me innumerous times in everything I have done since, from cell culture to analyzing flow cytometry plots. I would also like to thank Dr. Wilmore Webley, in whose lab I conducted my honors thesis on infectious asthma and where I decided to pursue a career in immunology. Dr. Webley was the first mentor to instill in me a passion for this field, and I thank him for giving me the confidence and training to qualify for a competitive PhD program. During the summers I trained as an intern with Dr. Hyun-Hee Lee at Merck Research Labs and Yana Ostrovsky at Genocea Biosciences. These experiences in private industry also had an integral role in my early training as a scientist by teaching me how to pursue a clear scientific goal with focus, which I believe has made me a strong asset to all teams I have since worked with.

I have been fortunate to work with a truly outstanding team of researchers in the Tirouvanziam Lab these past 5 years. As a new student I learned a great deal from Osric Forrest, Bijean Ford, and Camilla Margaroli who shared with me their knowledge as senior students and each have been essential as peer mentors. I thank fellow student Brian Dobosh who, despite occasional acts of sabotage, has been a great source of support as we shared the burdens of last-minute clinical samples and readjusting workflow during the COVID-19 pandemic. Countless times I have depended on our research specialists Milton Brown and Mila Aldeco, who brought tremendous experience as scientists and as leaders and never failed to keep our operations running smoothly no matter what obstacles we faced.

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I am thankful for the guidance and feedback from my thesis committee, including Dr. Byron Au-Yeung, Dr. Eliver Ghosn, Dr. Periasamy Selvaraj, and Dr. Ioanna Skountzou in addition to my advisor Dr. Rabindra Tirouvanziam. My ability to present and communicate

scientific data has been greatly enhanced by their mentorship, and I thank them for helping me reach this point. Each of their individual expertise have helped me to refine my projects and prepare a well-structured dissertation. I would also like to thank our center director Dr. Nael McCarty for his unwavering support of me and all other graduate students in our division. In addition, the resources offered by the Pediatrics/Winship Flow Cytometry Core have been instrumental to my graduate work, and I thank Aaron Rae for his superb efforts in running this essential core facility.

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Finally, the work that I and other researchers are fortunate to conduct is generously supported by the Cystic Fibrosis Foundation, the Center for Cystic Fibrosis and Airways Disease Research (CF-AIR), and CF@LANTA. This support is crucial for maintaining our team of research coordinators, clinicians, and scientists. We are thankful for the willingness of patients and their families to participate in these research studies which enable us all to come together with the goal of one day achieving a cure for cystic fibrosis.

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Table of abbreviations

Acronym	Expansion
A1AT	Alpha-1 antitrypsin
ABPA	Allergic bronchopulmonary aspergillosis
APE	acute pulmonary exacerbation
ARDS	acute respiratory distress syndrome
Arg1	arginase-1
ASO	Antisense oligonucleotide
BAL	bronchoalveolar lavage fluid
BCL6	B cell lymphoma-6
CF	cystic fibrosis
CFASN	cystic fibrosis airway supernatant
CFTR	Cystic fibrosis transmembrane conductance regulator
cGAS	Cyclic-GMP-AMP synthase
COPD	chronic obstructive pulmonary disease
CXCL	C-X-C motif chemokine ligand
DNase	Deoxyribonuclease
ECP	Eosinophil cationic protein
EV	Extracellular vesicles
FEV1	Forced expiratory volume in 1 s
GRIM	Granule releasing, immunomodulatory, and metabolically active
IAV	Influenza A virus
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible nitric oxide synthase
KC	keratinocyte chemoattractant
LAIR-1	leukocyte-associated Ig-like receptor 1
LTB4	Leukotriene B4
MAVS	mitochondrial antiviral-signaling protein
miRNA	micro RNA
MMP-9	matrix metalloproteinase-9
MPO	myeloperoxidase
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
MyD88	myeloid differentiation primary response 88
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NET	neutrophil extracellular traps
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NO	nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOX	nicotinamide adenine dinucleotide phosphate oxidase

OAS	Oligoadenylate synthetase
oxCAMKII	oxidized calmodulin-dependent protein kinase II
PAD4	peptidyl arginine deiminase 4
PD-1	Programmed cell death protein 1
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I
RNase	Ribonuclease
ROS	reactive oxygen species
RSV	respiratory syncytial virus
SARS-CoV	severe acute respiratory syndrome coronaviruses
SPADE	spanning-tree progression analysis of density-normalized events
STING	Stimulator of interferon genes
TGF-β	Transforming growth factor-β
Th	T helper cells
TLR	Toll-like receptor
TM	transmigration
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRPM8	transient receptor potential melastatin-8

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Chapter 1

Introduction

1.1 Neutrophil Adaptations upon Recruitment to the Lung:

New Concepts and Implications for Homeostasis and Disease

1.2: Immunomodulation in cystic fibrosis: why and how?

Sections of this chapter have been published in the International Journal of Molecular Sciences

1.1: Neutrophil Adaptations upon Recruitment to the Lung: New Concepts and Implications for Homeostasis and Disease

1.1.1. Introduction

Neutrophils comprise the largest proportion of circulating leukocytes in the human body and maintain a major presence in organs such as the lung. Consequently, despite being considered as terminally differentiated and endowed with a short lifespan after leaving the bone marrow, they are a major player in innate immunity. Their hallmark function is clearance of debris and pathogens through phagocytosis but they exhibit a diverse array of other immune functions. In addition to the direct phagocytosis of bacteria [1] and fungi [2], they limit the spread of microbes by releasing neutrophil extracellular traps (NETs) made of DNA through a process known as NETosis [3]. Although neutrophils are professional killers, they also have significant capacity to modulate the function of other immune cells. For example, through secretion of arginase-1 (Arg1) they suppress T-cell proliferation in the airways of cystic fibrosis (CF) patients [4] and limit T-cell function in the tumor microenvironment [5]. Similarly, the release of the protease neutrophil elastase (NE) by activated neutrophils has been linked to the alteration of macrophage function by the cleavage of Toll-like receptors (TLRs) and cytokines [6], T-cell function through the cleavage of surface co-receptors [7], and the modulation of secreted antibodies [8]. Diverse effector functions of secreted proteins, some of which are summarized in **Table 1.1.1.1**, are key for neutrophil adaptability and their far-reaching effects on immune responses.

Protein	Role	Function
Arg1	pro/anti-inflammatory	Suppresses T-cell proliferation [4]
BCL6	pro/anti-inflammatory	Suppresses neutrophil apoptosis [9]
CD200R	anti-inflammatory	Attenuates oxidant production by neutrophils [10]
CXCR2	pro-inflammatory	promotes chemotaxis as receptor for CXCL1 [11]
CXCR4	homeostatic	promotes retention in bone marrow/lung as receptor to CXCL12 [12]
Dectin-1	pro-inflammatory	promotes phagocytosis of fungi [13]
iNOS	pro-inflammatory	supports the generation of nitric oxide [14]
LAIR-1	anti-inflammatory	suppresses neutrophil recruitment [15, 16]
MPO	pro-inflammatory	supports generation of hypochlorous acid [17]
MMP-9	pro-inflammatory	degrades the extracellular matrix [18]
NOX	pro-inflammatory	supports the generation of superoxide [1]
NE	pro-inflammatory	degrades phagocytosed microbes [1] and extracellular matrix [19]
oxCAMKII	pro-inflammatory	Activates STAT1 and generation of inflammatory mediators [20]

Table 1.1.1.1. Diverse roles of neutrophil signaling and effector proteins. Neutrophil effector proteins, such as proteases and phagocytic receptors, and receptors involved in chemotaxis, contribute to the plasticity of neutrophils in driving inflammation, promoting resolution, or maintaining homeostasis. Abbreviations: Arg1, arginase-1; iNOS, inducible nitric oxide synthase; LAIR-1, leukocyte-associated Ig-like receptor 1; MPO, myeloperoxidase; MMP-9, matrix metalloproteinase-9; NOX, nicotinamide adenine dinucleotide phosphate oxidase; NE, neutrophil elastase; oxCAMKII, oxidized calmodulin-dependent protein kinase II.

Not only do neutrophils exhibit greater functional diversity than once thought [21], but they demonstrate the ability to reprogram and adapt to local microenvironments upon recruitment to tissues. This is in contrast with the conventional view holding these cells as terminally differentiated once released from the bone marrow. For example, a recent report details neutrophil reprogramming in a model of atherosclerosis via oxidized calmodulin-

dependent protein kinase II driving a pro-inflammatory phenotype alongside the suppression of homeostatic transcription factors [20]. Neutrophilic inflammation is also a key component of progressive lung damage in patients with CF [22, 23], which is one of the most common fatal hereditary diseases [24]. Consequently, neutrophil adaptation in the context of CF lung disease has been reviewed extensively [25, 26] and has incited further investigation into neutrophil reprogramming in other diseases. Neutrophil adaptation upon recruitment to the lung is of particular interest, due to the role this organ plays as a major neutrophil reservoir [27, 28]. There is a rapidly increasing prevalence of chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) in the global population, in part due to increased exposure to air pollution [29]. In addition, aging populations are faced with increased risk for bacterial pneumonia, which results in sustained neutrophil recruitment to the lung but reduced efficacy in clearing infections [30]. A greater understanding of neutrophil responses to endogenous and exogenous stimuli when recruited from circulation into the lungs (**Figure 1.1.1.1**) is critical to improving treatment options for both acute and chronic inflammatory diseases.

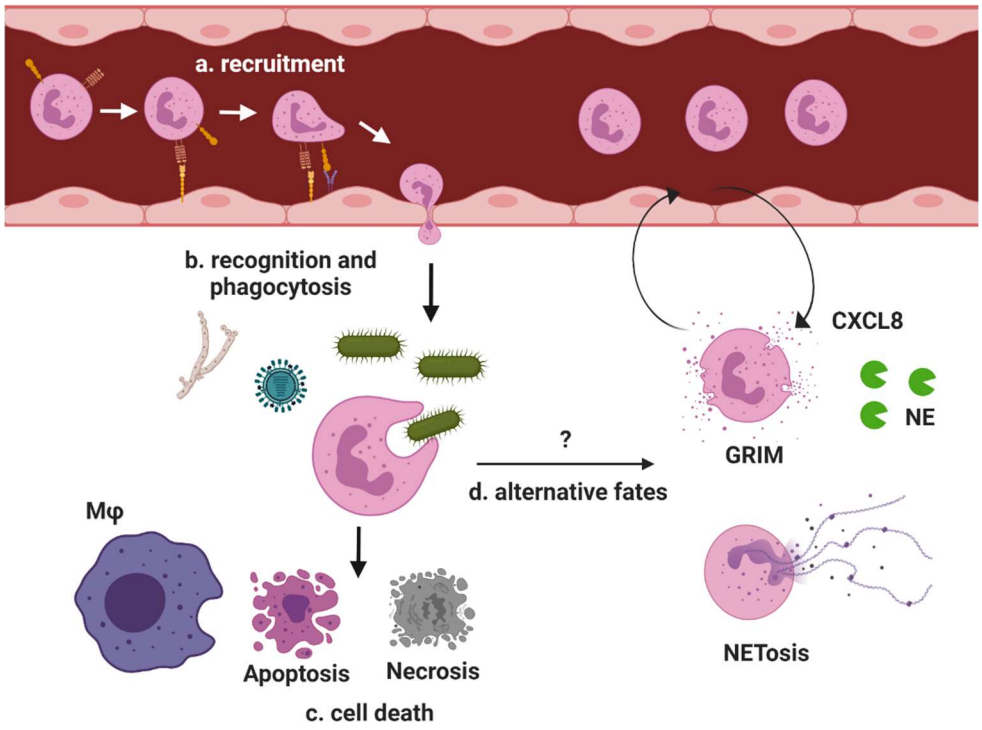

Figure 1.1.1.1. Overview of neutrophil recruitment and responses in the lung. As a major neutrophil reservoir, the lungs are an important environment in the study of neutrophil biology, both at homeostasis and in responding to inflammatory stimuli. **(a)** Endothelial cells upregulate P-selectin to bind P-selectin glycoprotein ligand-1 on circulating naive neutrophils. Upon slowing down, neutrophil β2 integrin binds with higher affinity to ICAM-1 on the endothelial cell surface followed by extravasation into the tissue. **(b)** Recruited neutrophils recognize pathogen-associated molecular patterns from all types of pathogens by surface pattern recognition receptors. Phagocytosed pathogens are degraded internally by fusion of the granules with the phagosome. **(c)** Neutrophils quickly apoptose and are cleared by tissue macrophages. **(d)** Neutrophil recruitment can also lead to alternate fates. Dysregulated neutrophil responses in diseases such as CF and COPD include the GRIM (granule releasing, immunomodulatory, and metabolically active) phenotype which exhibits active degranulation but impaired pathogen clearance. The release of NE damages host tissue while sustained CXCL8 production drives further neutrophil recruitment. Neutrophils can also expel their DNA through NETosis, but may survive and retain phagocytic capability.

1.1.2 Stress responses

Unlike some of the longer-lived leukocytes featured in both arms of the immune system, such as mast cells in the innate system and memory T cells and long-lived plasma cells in the adaptive system, neutrophils do not maintain long-term tissue residence. However, they are invaluable for their ability to respond rapidly and in massive manner to almost any type of stress inflicted upon peripheral tissues. The hallmark function of neutrophils is microbial clearance, especially of bacteria, by phagocytosis. It is now well understood how bacteria are captured and digested internally [1, 31], but neutrophils are also well equipped to clear viruses [32, 33] and fungi [2], as well as contribute to defense against larger parasites [34].

1.1.2.1 Bacterial infections

Responding to bacterial infections is the most well-characterized function of neutrophils. With the increased focus on innate immunity in recent years, we are now learning more about the complexity of pathogen identification and clearance in specialized areas such as the lung. Although neutrophils are characterized by pre-programmed functions, their ability to carry out these functions is highly dependent on the specific conditions of their microenvironment. For example, a recent report by Lei et al. details the differences in neutrophil response to Group A *Streptococcus* in the lungs compared to the skin. While this bacterial species is efficiently cleared from the lungs of mice by a nicotinamide adenine dinucleotide phosphate oxidase (NOX)-dependent mechanism, clearance is impaired in the skin [35]. This discrepancy may be due to creation of an anoxic environment in the skin, which favors the growth of this organism but hampers oxidative burst by neutrophils. Indeed, NOX-dependent generation of reactive oxygen species (ROS) is a crucial component of a neutrophilic response [1]. The CD200 receptor

has been shown to play a role in driving lung pathology during influenza infection, as blocking this receptor attenuated macrophage-associated inflammation [36]. However, blocking this receptor on neutrophils during pulmonary *Francisella tularensis* infection in mice augmented infection by reducing ROS production [21]. Although there are scenarios in which it would be beneficial to counteract excessive ROS production, for bacterial and fungal infections [37] as well as viral infections [38], this finding demonstrates the potential benefit of boosting ROS production in certain cases. For example, boosting innate immune responses following influenza infection in mice by overexpression of granulocyte-macrophage colony-stimulating factor in the lungs was found to protect against *Staphylococcus aureus*-induced pneumonia by enhancing ROS production in alveolar macrophages but not neutrophils. Neutrophils were, however, essential for protection and the mice did not experience excessive inflammation resulting from elevated ROS production [39]. In another example, ROS production was enhanced by treatment with an angiotensin-converting enzyme inhibitor, which promoted the killing of methicillin-resistant *S. aureus* (MRSA) [40]. This mechanism may not only apply to enhancing the antimicrobial response. For example, it has also been implicated in the stimulation of wound healing through enhancing the differentiation of pro-resolution macrophages in the liver [41].

Of equal importance to ROS in the destruction of bacteria are reactive nitrogen species (RNS) such as nitric oxide (NO) [42] which can be produced by neutrophils to a high degree in diseased airways [43]. Inducible nitric oxide synthase (iNOS) is the enzyme complex responsible for generating NO using arginine as a substrate [14] and has long been known to be highly activated in neutrophils in response to bacterial infection [44]. However, neutrophils are not the sole source of NO produced in tissues, as it is also produced by endothelial cells [45] and macrophages. NO production by all cells can be inhibited by Arg1, which competes with iNOS

for arginine as a substrate [46], and is actively secreted by neutrophils in chronic diseases such as CF [4] and cancer [47]. While RNS are important microbicidal mediators, they can have detrimental effects when released from activated neutrophils and other cells. In a study by Kumar et al. looking at septic patients with confirmed bacterial infections, neutrophils were found to have increased iNOS activity. Nitrite, a metabolite of NO, was measured in the plasma and found to inversely correlate with lung function [48]. While lung function was likely impacted by other aspects in this severe pathological condition, these findings emphasize the potency of a neutrophilic response in impacting the function of organs such as the lungs. In an in vitro model of sepsis, Shelton et al. found that neutrophil iNOS activity contributed to leakage across an endothelial barrier with evidence that peroxynitrite, produced by NO reacting with O_2^-, mediates this effect [49]. While this model did not directly use bacterial challenge, it employed mixtures of cytokines important for sepsis in humans, which is defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection" [50], in which neutrophilic inflammation is an important component [51].

Another antimicrobial mechanism involves the release of histone-bound DNA complexed with primary granule proteins, such as NE and myeloperoxidase (MPO), in the form of NETs [52]. Formation of NETs is regulated by a complex pathway requiring histone citrullination by peptidyl arginine deiminase 4 (PAD4) followed by decondensation of the chromatin [53], which has more recently been shown to be promoted by histone acetylation using broadly acting inhibitors of histone deacetylase [54]. NETosis independent of PAD4 has also been described [55, 56]. NETosis has been classically viewed as a cell death pathway but neutrophils have been shown to maintain viability and anti-bacterial functionality after NET release, at least for a few hours [57, 58]. Although the exact role of NETosis in disease pathogenesis is still under debate

[59, 60], this process has demonstrated importance in the neutrophilic response to some bacterial lung infections, including bacterial pneumonia. In a study of patients with ventilator-associated pneumonia, the presence of NETs was assessed by measuring complexes of DNA and MPO. NET presence was found to be elevated in acute respiratory distress syndrome (ARDS) patients with ventilator-associated pneumonia compared to ARDS alone, and correlated with both bacterial burden and CXCL8 [61]. Considering that MPO has been found to be elevated in the airways of ARDS patients [62] and that this enzyme is associated with lung damage in CF [63, 64], NET-associated MPO may be a contributing factor in progression of disease in ARDS. Studies of NETosis are also contributing to a better understanding of neutrophil plasticity. Although typically thought of as having minimal transcriptional activity, NETosis has been shown to be dependent on transcription. Khan and Palaniyar demonstrated that inhibition of transcription using actinomycin D attenuated NETosis in response to the bacterial stimulants lipopolysaccharide and ionomycin [65]. This finding implicates transcription as a potential target of inhibition for treating NET-related pathologies. Another therapeutic target related to NETs formation is type I interferon (IFN). A recent finding suggests that type I IFN-driven NETosis may promote respiratory *P. aeruginosa* infections in mice by providing a scaffold to support biofilm production [66]. Interruption of IFN signaling may, therefore, be useful for suppressing infection by biofilm-capable organisms, but care would need to be taken not to increase susceptibility to viral infection. Finally, when considering the cost-benefit of NETosis, with regards to its anti-microbial properties and its association with disease pathology, one should consider the balance between the number of neutrophils present at the site of inflammation vs. those undergoing NETosis. Indeed, NETosis occurs in 1-5% of a neutrophil population in vitro. Also, complexation of neutrophil cationic proteins (NE, MPO) with extracellular DNA can occur

outside of neutrophils as a result of exocytosis combined with necrosis and electrostatic interaction between molecules. Therefore, NETosis is often overestimated by simple measures of NE/MPO/DNA complexes. While the accurate quantification of NETosis may be challenging to achieve, especially in vivo, it may clarify its impact on the fitness of the host in specific inflammatory settings.

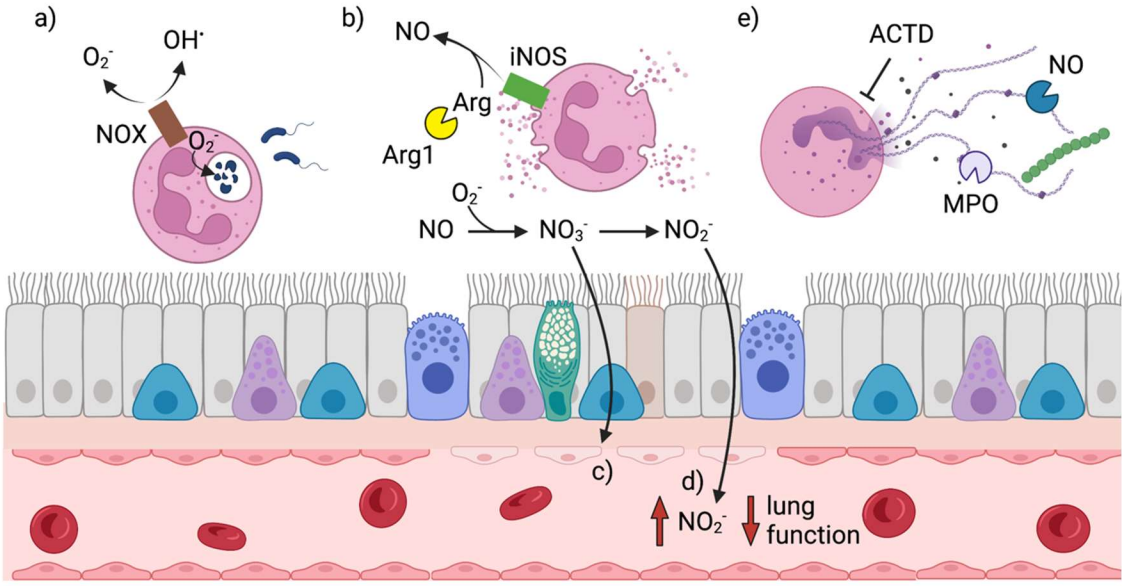

Figure 1.1.2.1. Neutrophil responses to bacterial infection. Neutrophils have multiple mechanisms for clearing bacteria, which can also have detrimental effects on host tissues. **(a)** Production of ROS is an important effector mechanism, generating superoxide (O_2^-) and its derivatives such as hydroxyl radical ($OH^\bullet$) via NADPH oxidase (NOX). ROS contribute to destruction of pathogens captured in phagosomes. **(b)** Reactive nitrogen species produced by iNOS are also important effector molecules, but can injure host tissue. NO is produced by iNOS using arginine (Arg) as a substrate, which competes with the enzyme arginase-1 (Arg1) secreted by neutrophils via granule exocytosis in lung diseases such as CF. Peroxynitrite is a product of NO reacting with O_2^- which can promote endothelial leakage **(c)** while nitrite, a further metabolite of NO, can inversely correlate with lung function in a model of sepsis **(d)**. **(e)** Release of DNA complexed with NE and MPO via NETosis can trap bacteria and is associated with bacterial pneumonia. A role for transcription in NETosis has been demonstrated by blocking NET release with the RNA polymerase inhibitor actinomycin D (ACTD).

1.1.2.2 Viral infection

Although neutrophils are most thoroughly studied in the context of antibacterial responses, a growing body of literature demonstrates their importance in responding to viral infection, as well. The adaptations they undergo in this role, especially when recruited to the airways, have major implications for disease outcomes and eventual resolution of inflammation since neutrophil-driven innate immune mechanisms can mount a rapid antiviral response even in the absence of memory B- and T-cell responses [67, 68]. The fine balance of neutrophilic inflammation in response to respiratory tract viral infections is exemplified by a variety of findings that may appear contradictory. For example, a recent study demonstrated that activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome improves survival during influenza A virus (IAV) infection in mice by recruiting neutrophils via interleukin-1β [69]. However, neutrophils do not provide a universal protective effect during influenza infection. Studies using aged mice have demonstrated overall higher neutrophil presence in the lung during IAV infection, but impaired migration towards the chemoattractant CXCL1 and reduced expression of the corresponding receptor CXCR2 on bone marrow neutrophils. Depleting neutrophils after infection was also shown to promote survival without impairing clearance [11]. Since overall neutrophil recruitment was not shown to be impacted while migration towards specific chemoattractant gradients was dysregulated, this demonstrates the need for more targeted immunomodulatory therapies focused on neutrophilic responses. A new potential target described in a recent study using a murine model of IAV infection is BCL6. This transcriptional regulator was shown to suppress neutrophil apoptosis specifically in airway neutrophils near the site of infection, while cells in the bone marrow and in circulation were unaffected. Mice with a myeloid cell deficiency in BCL6 exhibited improved survival and

reduced inflammation when infected with IAV [9]. Modulating neutrophilic inflammation may therefore provide clinical benefit when chronic lung disease results from influenza infection [70]. However, careful consideration will likely be needed to determine which patients would benefit from an enhanced neutrophil response during viral infections, and which would benefit more from suppressing neutrophil activity. While neutrophil adaptations in response to influenza infection are some of the best studied, important findings with other viruses are continuing to direct much-needed focus toward the innate immune response to viral infections (**Figure 1.1.2.2**). It was recently demonstrated in a mouse model of respiratory syncytial virus (RSV) infection that while signaling through myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) is essential for neutrophil recruitment to the lung, signaling through mitochondrial antiviral-signaling protein (MAVS) is required for neutrophil activation and the production of key mediators including matrix metalloproteinase 9 (MMP-9), MPO, and NE [71]. RSV infection is associated with severe neutrophilic inflammation, sometimes contributing to mortality [72]. Resolving neutrophilic inflammation is therefore as important as suppressing the infection, and a recent report suggests that leukocyte-associated Ig-like receptor 1 (LAIR-1) fills this role during RSV infection. This receptor is not expressed on circulating neutrophils, but may be upregulated upon migration into tissues and subsequent activation [15]. RSV-infected mice lacking functional LAIR-1 exhibited greater neutrophil influx into the airways but had no indication of enhanced viral clearance [16].

In addition to bacterial infection, NETosis has been described in the context of neutrophil responses to viral infection in previous reviews [73, 74]. Studies of NETosis in response to respiratory viruses further establish the important implications of neutrophils in responding to viral infections, for both enhancing clearance and contributing to pathology. Murano et al.

demonstrated that RSV is capable of inducing NETosis by the classical PAD4-mediated pathway. They also observed possible virion trapping demonstrated by co-localization of extracellular DNA lattices and primary granule proteins, including NE and MPO, with RSV F protein [75]. NETs have also demonstrated efficacy in the neutrophil response against HIV. Saitoh et al. demonstrated that the initiation of NETosis by signaling through TLR-7 and TLR-8 promotes trapping and inactivation of HIV through activity of the effector proteins MPO and α-defensin [76]. However, viral-induced NETosis can also have a detrimental impact on the host. In a mouse model of influenza infection, neutrophils from the bronchoalveolar lavage (BAL) fluid were highly NETotic when co-incubated with infected epithelial cells and contributed substantially to lung injury [77]. In addition, blood neutrophils from influenza-infected patients have demonstrated a high propensity for NETosis when stimulated ex vivo, and NETosis was found to increase vascular permeability using an in vitro model [78]. Moreover, extracellular host DNA released in accordance with rhinovirus infection has been observed to correlate with a shift towards a type 2 immune response in humans, typically associated with allergic disease. Using a murine model of rhinovirus infection, Toussaint et al. then demonstrated that infection promotes NET formation and inhibition of NETosis reduced type 2 immune pathology [79]. While sometimes effective in limiting viral infection, further research is needed to determine if the risks of anti-viral NETosis outweigh the potential reward as a therapeutic target.

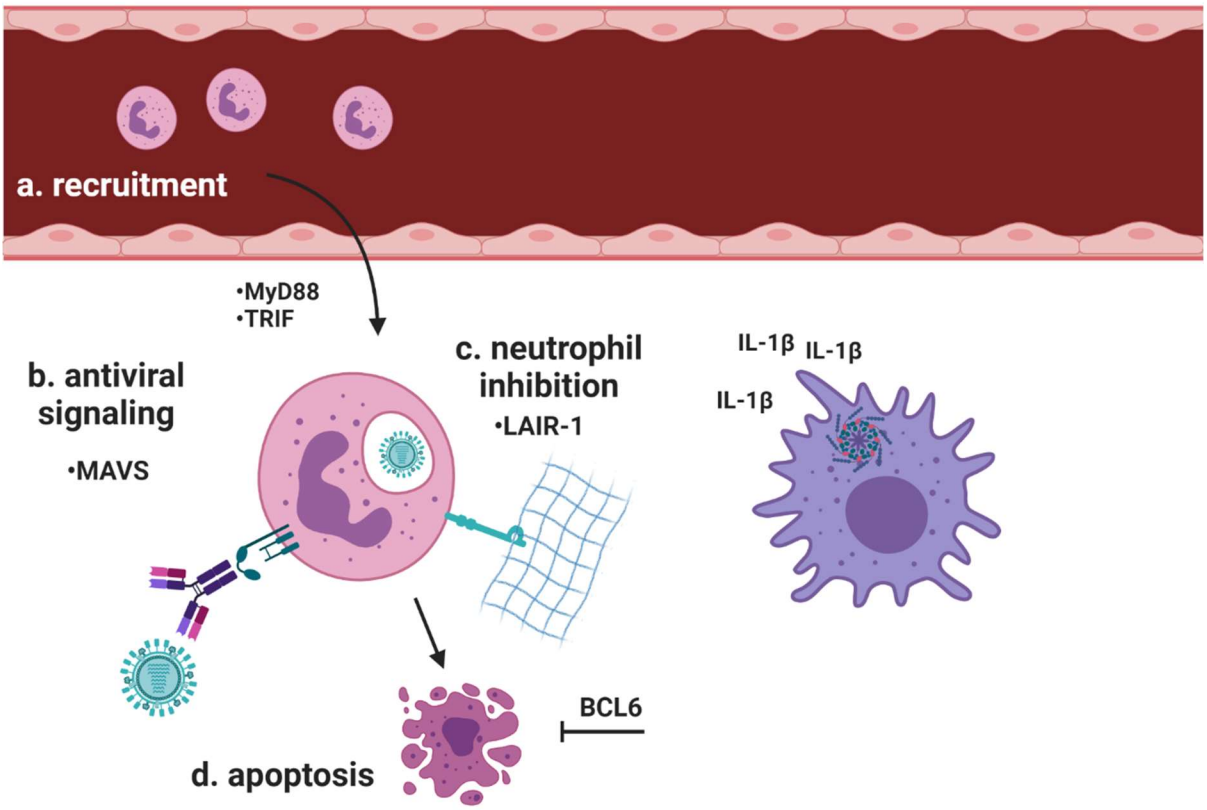

Figure 1.1.2.2. Neutrophil responses to viral infection. Neutrophils have an important role in antiviral immunity. **(a)** Neutrophils are recruited to sites of viral infection in the lung via signaling through MyD88 and TRIF. Interleukin-1β production by the NLRP3 inflammasome in resident antigen presenting cells drives recruitment. **(b)** once in the tissue, MAVS signaling initiates neutrophil activation and production of inflammatory mediators. Neutrophils engulf antibody-bound virions via surface Fc receptors. **(c)** The inhibitory receptor LAIR-1 binds collagen and suppresses neutrophil activity. **(d)** Transcriptional regulators such as BCL6 suppress apoptosis and represent a potential target for enhancing neutrophil-mediated antiviral immunity.

1.1.2.3 Fungal infections

In addition to bacteria and viruses, neutrophils can mount a powerful response to fungi, as well. One of the major culprits behind respiratory fungal infections is *Aspergillus fumigatus*, which promotes potent neutrophilic inflammation [80] induced in part by the regulation of the Von Willebrand factor via cleavage by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 [81]. In addition to their role in clearance of spores and hyphae [82], neutrophils play a key role in regulating the antifungal adaptive immune response. In neutropenic mice infected with *A. fumigatus*, dendritic cells were found to accumulate in the lungs, but had impaired homing to the mediastinal lymph nodes. Dendritic cells were also found to lack expression of surface costimulatory molecules, but this defect was rescued by coincubation with neutrophils following *Aspergillus* exposure [83]. Considering that neutrophils are essential for guarding the lungs against fungal pathogens it is surprising that CF patients, who experience widespread neutrophilic inflammation in the airways, are highly susceptible to respiratory fungal infections [84]. The cause may be neutrophils themselves. Neutrophils acquire a dysfunctional phenotype in the CF airways which hampers their ability to clear bacteria, despite attaining a high state of activation where they exocytose their primary granules [85]. Exocytosis of primary granules releases various effectors including NE into the extracellular environment, and this protease has been found to cleave the pattern recognition receptors dectin-1 and 2 [13], which are important phagocytic receptors for fungal pathogen-associated molecular patterns such as β-glucan [86, 87]. Cleavage of these receptors by NE inhibited the antifungal response in infected mice [13], and the airway fluid from CF patients has been found to have a high prevalence of extracellular NE [88]. NE drives multiple aspects of CF lung disease, including increased mucus production and impairment of mucociliary clearance

[89]. Both outcomes promote colonization of the airways by opportunistic fungal pathogens such as *Aspergillus*, which has a complex array of interactions with airway mucins [90]. Considering that this protease is being actively secreted by neutrophils undergoing pathological conditioning in the CF airways [85], with similar dysfunctions observed in other respiratory diseases [91], NE inhibition may offer a therapeutic option for treating pulmonary fungal infections. Indeed, pyrimidine derivatives in some NE inhibitors have antifungal properties [92].

1.1.3 Neutrophils in chronic respiratory pathologies

We are now building a greater understanding of how neutrophil responses to infectious challenge, especially in the lung, are far more complex than simply locating and clearing microbes. Their responsiveness to non-infectious challenge, for example with allergen and smoke exposure, is also a potent factor in innate immune responses. The ability of neutrophils to quickly resolve these challenges or contribute to pathology has major implications for both organ-specific and systemic health.

In the absence of infection, neutrophilic inflammation can be initiated and result in severe inflammatory pathologies. When neutrophils are recruited to the lungs in the absence of infection, in genetic disorders such as CF [26], or in diseases linked to environmental conditions such as COPD [93], they can cause extensive damage through release of their destructive granule contents such as NE and MPO [26, 93]. They also exhibit potent immunomodulatory capabilities whereby they can substantially alter the immune balance of various environments [4, 94]. In addition to ongoing tissue damage and altered adaptive immune responses, chronic neutrophilic pathologies are also characterized by neutrophil dysfunction where these cells are not able to effectively conduct their normal duties of debris and microbe clearance. A better understanding

of how neutrophilic pathologies are initiated and how they might be corrected is essential for treating patients with rare diseases like CF that currently have few anti-inflammatory treatment options, as well as widespread diseases like asthma and COPD which are becoming ever-larger public health burdens each year.

1.1.3.1 Cystic fibrosis

CF is a severe monogenic multiorgan disease that affects multiple epithelial organs, with the majority of morbidity and mortality due to airway disease [95]. Neutrophils have a central role in the development and persistence of airway disease and their role in pathology has been studied extensively [25, 26]. Importantly, they have been shown to develop a unique inflammatory phenotype after recruitment into the CF airway lumen, where they maintain viability and exocytose their primary granules but have reduced ability to phagocytose bacteria in a distinct fate termed GRIM (granule releasing, immunomodulatory, and metabolically active) neutrophils [85, 96, 97]. Exocytosis of the primary granules, which are usually sequestered in the cytoplasm, results in release of effectors including NE, Cathepsin G, MPO and Arg1, which has been found to correlate with disease progression in mice with CF-like lung disease and young children and older patients with CF [98-100]. This pro-inflammatory phenotype with reduced ability to clear pathogens poses an intriguing paradox and is becoming a focal point in addressing CF lung disease. This failure of a major defense mechanism in the lungs is likely a contributing factor to the high susceptibility of CF patients to common environmental bacteria [101]. Directly causing lung damage through protease and oxidase release further implicates neutrophil dysfunction in being a major problem in CF lung disease that warrants additional research [89]. Prior studies of metabolic reprogramming in CF airway neutrophils may offer

some explanation to how this dysfunctional phenotype is acquired. CF airway neutrophils activate the mechanistic target of rapamycin (mTOR) pathway [102] and increase expression of the Glut1 glucose transporter [103], which is controlled by mTOR [104]. Activation of the mTOR pathway and expression of Glut1 promote utilization of glucose in CF airways [105]. Another aspect of reprogramming in CF airway neutrophils is increased production of the regulatory protein resistin [106], which is closely tied to insulin resistance [107]. While resistance to insulin impairs the uptake of glucose by cells, anabolic reprogramming of neutrophils in the CF airways enables them to effectively take in and utilize glucose to fuel pro-survival pathways [85, 102]. The downside of this adaptation is that resistin decreases the ability of neutrophils to kill bacteria by inhibiting ROS production and actin polymerization, as noted for CF-associated pathogens *P. aeruginosa* and *S. aureus* [108].

1.1.3.2 Asthma

Asthma is among the most common chronic diseases in children and adults, and typically viewed as a Th2-mediated allergic disease featuring profound eosinophilic inflammation [109]. However, there is a growing focus on neutrophilic inflammation in non-atopic asthma [110, 111]. One of the contributing factors to neutrophilic asthma is respiratory infections. For example, Patel et al. demonstrated that young asthmatics who tested positive for *Chlamydia pneumoniae* exhibited elevated neutrophil counts and CXCL8, a powerful neutrophil chemoattractant, in BAL fluid [112]. Following on this discovery, Patel and Webley then used a mouse model of respiratory *Chlamydia* infection to demonstrate that airway neutrophils produce large amounts of the inflammatory mediators hepoxilin A3 and histamine [113]. However, the propensity of neutrophils to exacerbate inflammatory conditions in asthma is neither restricted to

bacterial pathogens nor to the airways. Neutrophils isolated from the peripheral blood of asthmatics were found to have enhanced secretion of CXCL8 in response to the viral surrogate and TLR agonist R848 compared to non-asthmatics [114], which may provide an explanation for why patients with viral respiratory tract infections are more likely to experience treatment failures [115]. While most therapies are directed at treating eosinophil-mediated allergic asthma, options for developing neutrophil-directed asthma treatments have been investigated. The macrolide antibiotic clarithromycin has previously shown promise in suppression of neutrophilic inflammation in patients with refractory asthma as shown by a reduction in sputum CXCL8 concentration and neutrophil presence [116]. While a variety of therapies targeting neutrophilic asthma have proven ineffective, as reviewed recently by Seys et al. [117], an increased understanding of neutrophil dysregulation in diseased airways will hopefully enable much-needed therapeutic breakthroughs.

1.1.3.3 Chronic Obstructive Pulmonary Disease

COPD has emerged as one of the most common causes of morbidity and mortality worldwide. Caused mostly by exposure to environmental factors such as tobacco smoke and pollution [118], COPD also depends on underlying genetic predispositions [119]. As with CF, lung disease in COPD is characterized by a heavy neutrophil component [120], with extracellular NE being associated with severity of disease and exacerbations [121-123]. In addition, it was observed by Chrysanthopoulou that exposure to cigarette smoke induces NET formation which contributes to lung fibrosis [124]. Recently, Genschmer et al. highlighted a new mechanism of NE-induced lung damage, showing that NE in the airways of COPD patients is localized on the surface of exosomes [125] forming an active NE pool resistant to inhibition by alpha-1

antitrypsin (A1AT). While this study provides strong evidence for the damage phenotype, Garratt et al. showed that NE inhibition by A1AT mitigated epithelial repair [126], suggesting a potential physiological role for NE-associated exosomes in mediating epithelial repair. Indeed, NE acts on epithelial surfaces by triggering pro-reparative epidermal growth factor signaling [127, 128].

Exposure to tobacco smoke and pollution have been well studied, but a new trend showing intriguing effects on innate immunity is the use of electronic cigarettes. Exposure to vaporized nicotine and e-liquid base from electronic cigarettes was found several years ago to impair bacterial killing by neutrophils [129], as with a more recent finding using neutrophils exposed to total particulate matter from conventional cigarette smoke [130]. While neutrophil bactericidal capacity was found to be impaired, the activation and production of inflammatory markers and mediators were augmented upon exposure to e-cigarette vapor extract, as demonstrated by Higham et al. Neutrophils showed increased expression of surface CD11b and CD66b and secreted more MMP-9 and CXCL8, while NE activity was found to be increased in culture conditions [131]. In addition, blood neutrophils isolated from e-cigarette users showed elevated susceptibility to the induction of NETosis, which was supported by an increased presence of NET-related proteins in the sputum of these subjects compared to non-smokers [132]. These findings of neutrophil activation in response to e-cigarette components closely mirror what is observed in COPD due to smoke and air pollution exposure and provide solid evidence for similarly detrimental impact on lung health due to induction of neutrophilic inflammation.

1.1.4 Conclusion

Recent advances in understanding neutrophil biology in health and disease emphasize the plasticity of these cells. Despite comprising the largest proportion of circulating leukocytes, studying the molecular mechanisms of neutrophil function is still a relatively minor endeavor in the field of immunology. While the identification and clearance of microbes by neutrophils are well understood, theses represent only a small part of their functional capabilities. These functional capabilities depend on an array of signaling and effector proteins (**Table 1.1.1.1**).

There is now a growing interest in the contribution of neutrophils to chronic diseases, especially those relating to the lungs. First, while neutrophilic inflammation has long been known to have a role in CF and COPD, new mechanisms are uncovered by which they may contribute to lung damage. Second, new studies of asthma are shedding light on pathological mechanisms that are driven by a neutrophilic response despite typically being thought of as a type 2-dominated eosinophilic airway disease. Third, considering the monogenic disease CF where the focus has historically been on the mutated CF transmembrane conductance regulator ion channel (expressed primarily in epithelial cells), recent studies have indicated that early and sustained neutrophil recruitment to the airways and activation in the mucostatic environment of the CF lung is a major factor in the initiation and progression of lung disease [23, 133, 134]. With our improved understanding of neutrophil contribution to chronic diseases and new data demonstrating metabolic and transcriptional adaptions of neutrophils in these circumstances, neutrophil-directed therapies may soon become an option for the innovative treatment of diseases characterized by chronic neutrophilic inflammation, such as CF, COPD, and asthma.

1.2: Immunomodulation in cystic fibrosis: why and how?

1.2.1 Introduction

The study of CF lung disease has generated a wealth of knowledge on epithelial cell dysfunction and potential options for targeted therapeutics [135-137]. While these findings have provided key information needed for the development of modulator therapies directed at the cystic fibrosis transmembrane conductance regulator (CFTR), growing interest in the role of immune cells in CF, especially in the lungs, is advancing our understanding of previously unknown disease mechanisms. CFTR mutations have been associated with impaired pathogen clearance by myeloid cells [138-142], altered B-cell activation [143], and cytokine secretion by T cells [144].

However, the exact role that CFTR plays in modulating key immune cell functions remains unclear, as recent studies have challenged early findings that CFTR deficiency impairs pathogen clearance by myeloid cells [145, 146]. As such, the adaptability of immune cells, particularly neutrophils [147], to diseased microenvironments may be of greater importance than loss of CFTR regarding their role in CF. In addition, there is a great variety of CFTR mutations, with over 2,000 identified mutations spread across seven classes. Each of these classes represents different mechanisms causing CFTR deficiency or dysfunction, and thus many opportunities for precision medicine [148]. However, no therapy to date has been able to address the onset of chronic inflammation in the CF lung [149].

New therapies directly addressing cellular mechanisms of inflammation, especially regarding neutrophils, are urgently needed. Neutrophils are the most abundant leukocyte subset in the human body in terms of new cells produced per day (approximately 109 per kg) and an

essential component of innate immunity for their role as highly efficient phagocytes and regulators of immune responses [31]. In CF patients, neutrophils are massively recruited to the airways and are major drivers of lung inflammation [25, 26]. Among resident immune cells, tissue-resident macrophages are important for maintaining lung homeostasis [150], and another focal point of immune imbalance in CF airways [151].

Immunological dysfunction or reprogramming of innate cells is gaining significant attention as a contributing factor to the inability to control infections, either due to intrinsic or acquired defects. These infections include common CF pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [152], but also emerging and possibly more dangerous pathogens such as *Mycobacterium abscessus* [153]. Although innate immune cells have captured much of the growing interest in the immunology of CF, important discoveries have been made in adaptive immune cells, as well. T cells are heavily suppressed by neutrophil activity in CF lungs [4]. While this suppression may avoid autoimmune responses to self-antigens present in this chronically inflamed environment, it also be problematic by excluding regulatory subsets of T cells. Fewer studies have been conducted on the role of B cells in CF, but there is preliminary evidence for CFTR deficiency contributing to heightened B-cell activation and development of lymphoid follicles [143]. These observations form a foundation from which to investigate in more depth the interplay between immune subsets in the CF lung, and identify key mechanisms of immunomodulation for therapeutic targeting.

1.2.2 Targeting immune cells in the CF lung

1.2.2.1 Neutrophils

The progression of events in early CF lung disease is still under debate. Traditionally, bacterial infections established after mucus obstruction were viewed as the driving force for reactive inflammation, but recent reports proposed that inflammation may be initiated by early mucus plugging in the absence of infection [154], as reviewed in [134]. Although the exact sequence of events has not yet been defined, murine models have been used to demonstrate that hypoxia-induced necrosis of airway epithelia can initiate neutrophil influx via the interleukin (IL)-1 receptor signaling in the absence of infection [154]. Neutrophilic inflammation quickly becomes a major factor in the pathogenesis of CF lung disease due to acquisition of a pathological phenotype. CF airway neutrophils exhibit exocytosis of primary and secondary granules, loss of phagocytic receptors, and metabolic reprogramming that contributes to delayed apoptosis [85, 102]. These phenotypic shifts create a critical role for neutrophils in the CF lung environment, which has been reviewed extensively [25, 26, 155, 156]. Although mechanisms explaining how neutrophils acquire this phenotype are still being investigated, it is readily apparent how much it contributes to disease progression.

Exocytosis of the primary granules releases the serine protease NE into the extracellular environment. NE causes degradation of connective tissue in the lung [157], promotes mucus production in the airways [158], and is capable of degrading CFTR [159]. Furthermore, activity of soluble NE in the BAL fluid from infants with CF can serve as a reliable predictor of future bronchiectasis [23]. NE can be captured on the cell surface following release or become associated with exosomes, enabling it to resist inhibition by tissue anti-proteases and maintain

catalytic activity [125, 160]. These attributes demonstrate the therapeutic potential of targeting NE as a way to reduce inflammation and lung damage, which has indeed been a topic of discussion [92, 161]. Importantly, inhibition of NE has been shown to promote wound healing in primary airway cells from CF patients [126]. It is important to note that NE is not the only protease implicated in the progression of CF lung disease. The MMP family has also been studied for its role in tissue degradation and as potential therapeutic target [162].

In addition to proteases, other neutrophil granule proteins play important roles in CF lung disease. MPO, also contained in neutrophil primary granules, generates potent oxidants including hypochlorous acid [17]. The presence of MPO in the BAL fluid of patients with CF has been found to strongly correlate with development of bronchiectasis [64], as does methionine sulfoxide, a by-product of MPO activity [63]. Neutrophil degranulation has also been identified as a possible source of elevated resistin in CF patient sputum. As a potent inducer of neutrophil recruitment via ligation of TLR-4, resistin may serve as a mechanism by which neutrophils exponentially increase their own recruitment to CF lungs as they degranulate [106].

The clear relationship between release of inflammatory mediators and progression of CF lung disease creates substantial need to modulate neutrophil exocytosis. Several compounds have been identified that can inhibit neutrophil granule exocytosis without impeding normal antimicrobial function by specifically targeting granule docking proteins [163, 164] (**Figure 1.2.2.1a**). However, because CF patients are at high risk for contracting respiratory infections, it is essential that any suppression of neutrophil activity not leave patients vulnerable to common pathogens.

1.2.2.2 Eosinophils

Eosinophils are a class of granulocytes primarily involved in defense against parasites, but they can fulfill diverse roles including responding to fungal and viral infections, as well as interfacing with the adaptive immune system [165]. Recent studies have reaffirmed that eosinophils, which have a more prominent inflammatory role in asthma [166], typically are not a driving force in CF lung pathology [167]. However, research into eosinophil activation [168] and their contribution to comorbidities such as allergic bronchopulmonary aspergillosis (ABPA) [169], eosinophilic esophagitis [170], and nasal polyps [171] in CF patients suggest they do have clinical relevance.

As demonstrated by Koller et al., eosinophils may be more activated in CF airways even if they are not proliferating. In one of the earliest reports of activated eosinophils having a potential role in CF lung pathology, increased serum levels of eosinophil cationic protein (ECP), an eosinophil activation marker, were detected in CF patients compared to healthy control subjects [167]. Interestingly, this increase in sputum ECP levels was not linked to a change in peripheral blood eosinophil counts but correlated strongly with sputum ECP levels. This finding was confirmed by stimulating granulocytes ex vivo and demonstrating higher release of ECP from eosinophils of CF patients compared to those of healthy controls and bronchial asthma patients [172]. Since eosinophil activity was also shown to impact clinical variables in that study, it suggests that even though this rare cell population may not have increased prevalence in CF patients, their heightened activity may have clinical relevance. Such finer details are important to consider when planning anti-inflammatory treatments for patients, especially considering other factors such as sex. Indeed, significantly higher populations of both eosinophils and mast cells

have been detected in female CF patients compared to their male counterparts and may therefore require different dosages of anti-inflammatory drugs [173].

The kinase inhibitor (R)-Roscovitine has recently been suggested as having potential efficacy in treating CF by reducing release of peroxidase from activated eosinophils [174] (**Figure 1.2.2.1b**). Eosinophil peroxidase has structural similarities to MPO [175], which is implicated in CF lung damage [25], but has both pro- and anti-inflammatory properties [175]. (R)-Roscovitine has the additional benefit of inducing eosinophil apoptosis. Without proper efferocytosis, this may however lead to secondary necrosis [176] and result in acute inflammation, so care must be taken to avoid driving the inflammatory response in an attempt to reduce it.

Another therapeutic that may have efficacy in counteracting eosinophil activation in CF patients is benralizumab. This monoclonal antibody blocks the IL-5 receptor and induces eosinophil apoptosis, showing efficacy in treating eosinophilic asthma [177]. A study of benralizumab in asthmatics by Pham et al. showed reduced serum eosinophil-derived neurotoxin and ECP following eosinophil depletion [178]. While CF patients were excluded from this study, it may have efficacy in CF patients who display markers of eosinophil activation [168, 172].

Additionally, it has been reported that the eotaxin receptor CCR3 on eosinophils from CF patients with ABPA (CF-ABPA) is upregulated compared to patients without this comorbidity [179]. This finding complements an earlier report that methylprednisolone, which has been shown to reduce eosinophil chemotaxis [180] and degranulation [181], improves clinical outcomes in CF-ABPA patients [182]. Another therapy evaluated in CF-ABPA patients is the anti-immunoglobulin E (IgE) monoclonal antibody omalizumab. Early case reports using this antibody therapeutically demonstrated reduced exacerbations and improved lung function in CF-

ABPA patients with eosinophilia, elevated total serum IgE, and elevated *Aspergillus*-specific IgE [183-186], but larger retrospective studies did not find such efficacy [187, 188]. Due to the difficulty in treating established *Aspergillus* infections in CF patients, identifying infection early is crucial. In a study by Keown et al., peripheral eosinophilia and exhaled NO were significantly higher for CF patients with confirmed ABPA compared to those who were only sensitized, while serum ECP levels showed a positive trend [189].

1.2.2.3 Basophils

In addition to neutrophils and eosinophils, other innate immune cells have been studied in CF. Basophils are a rare granulocyte subset in the bloodstream that have a potent role in allergic inflammation [190]. Their potential role in CF was observed as early as 1980, when CF patients with *P. aeruginosa* infections were shown to have greater histamine release by basophils compared to those without such infections [191]. The potency of this vasodilating agent in driving the inflammatory response, especially for CF patients, is demonstrated by its use in assessing bronchial hyperreactivity [192]. Similar to the observation that eosinophils in CF patients may be more activated but not more abundant [168, 172], basophils in CF patients have been shown to be more active in releasing histamine but not more abundant compared to control subjects [193, 194].

Another similarity to eosinophils is the role of basophils in CF-ABPA. Gernez et al. analyzed basophils from CF-ABPA patients and identified increased expression of the activation marker CD203c compared to non-sensitized CF patients and those without *Aspergillus* infection [179]. Katelari et al. were later able to identify an increase in expression of both CD203c and CD63 using the basophil activation test in CF-ABPA patients vs non-ABPA CF patients,

highlighting the potential diagnostic value of this method [195]. Given the severe pathology seen in CF patients with ABPA, and the heightened activation of histamine-producing basophils, antihistamine treatments may warrant increased attention. Antihistamines including loratadine, cetirizine, and fexofenadine are common medications for CF patients with allergies and are more selective for histamine receptors than muscarinic receptors [196]. Still, it is desirable to minimize the tendency of antihistamines to cause drying or thickening of mucus in the airways, as has been demonstrated for cyproheptadine hydrochloride [197]. Continued investigation into the role of basophils in CF-ABPA patients could improve treatments by accurately distinguishing allergic exacerbations in CF patients from those caused by bacterial infections, which drive a neutrophilic response [198].

1.2.2.4 Mast cells

Mast cells, which are active in allergy and immunomodulation, harbor numerous homeostatic roles such as wound healing and angiogenesis, are involved in early initiation of inflammation through secretion of vasodilators like histamine [199]. Given their wide-ranging abilities to modulate immune responses, a greater understanding of mast cell function in complex inflammatory diseases like CF could yield new strategies for improving treatment options. In a study of mast cells in lung disease, Andersson et al. observed a reduction in total mast cell density in the small airways of CF patients. This was attributed to decreased density of the mucosal mast cell subpopulation associated with healthy lung function while the connective tissue mast cells were unchanged. However, production of IL-6 by mast cells was increased in CF patients [200]. IL-6 has been shown to block the production of regulatory T cells (Tregs) normally induced by tumor growth factor-β (TGF-β) [201], and due to its role in maintaining the

balance between Tregs and T helper 17 (Th17) cells [202], could be important for Th17-related, neutrophil-dominated pathology in CF. Since the proportion of connective tissue mast cells in small airways correlated negatively with lung function, mast cell-derived IL-6 could be a target for inhibition in CF patients, especially considering successful use of IL-6 trans-signaling blockade in a mouse model of pulmonary fibrosis [203]. Another cytokine target relevant to mast cell activity is IL-9. In a recent study by Moretti et al., IL-9 was shown to promote production of IL-2 by mast cells, leading to expansion of type 2 innate lymphoid cells and subsequent activation of inflammatory Th9 cells. Imatinib, a tyrosine kinase inhibitor that reduces IL-9-mediated lung mastocytosis, reduced mastocytosis and production of inflammatory cytokines in a CF mouse model [204] (**Figure 1.2.2.1c**).

Modulation of mast cell activity may be of special benefit to CF patients with nasal polyps. Polyps from CF patients have been shown to have elevated mast cell numbers and greater degranulation compared to those from non-CF individuals [205]. It may also be beneficial to promote autophagy in mast cells, as inhibition of this process attenuated bacterial killing by mast cells while induction by rapamycin promotes killing of *P. aeruginosa* [206]. Mast cell modulation may also be beneficial in *Aspergillus* infections. Production of the mast cell protease tryptase was decreased in patients with *Aspergillus*-specific IgE compared to IgE- patients [207]. As recently reviewed by Piliponsky et al., mast cells can effectively respond to *Candida* infections at mucosal surfaces [208]. Modulating mast cell activity in CF patients with *Aspergillus* infections may aid in controlling this opportunistic pathogen.

1.2.2.5 Macrophages

In addition to neutrophils, macrophages comprise a large proportion of CF airway leukocytes. Their major functions include clearance of debris and pathogens, promoting tissue remodeling, and regulating immune response [150]. Because of these crucial roles, the role of macrophages in CF lung disease has been studied extensively [150, 151, 209]. An important aspect of lung macrophages in CF is that like neutrophils [210], they exhibit defects in pathogen clearance despite acquiring highly pro-inflammatory phenotypes [151] (**Figure 1.2.2.1d**). The effect of CFTR deficiency on neutrophils has been studied [139, 140], but has been described more in depth for macrophages in recent years [138, 141, 142]. These reports demonstrate clear evidence for deficiencies in CF macrophage function and suggest the opportunity for therapeutic intervention, including possible direct benefit to macrophage function from CFTR modulatory therapy. Although one of the primary roles of airway macrophages is to maintain a homeostatic environment, they have been shown to greatly increase production of pro-inflammatory cytokines in CF patients compared to those of healthy subjects [211]. A recent study by Lara-Reyna et al. suggests this may be due to metabolic reprogramming broadly affecting innate immune cells in CF. This study demonstrates that activation of the inositol-requiring enzyme 1 α pathway due to endoplasmic reticulum stress in macrophages increases glycolysis and drives production of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) and IL-6 [212].

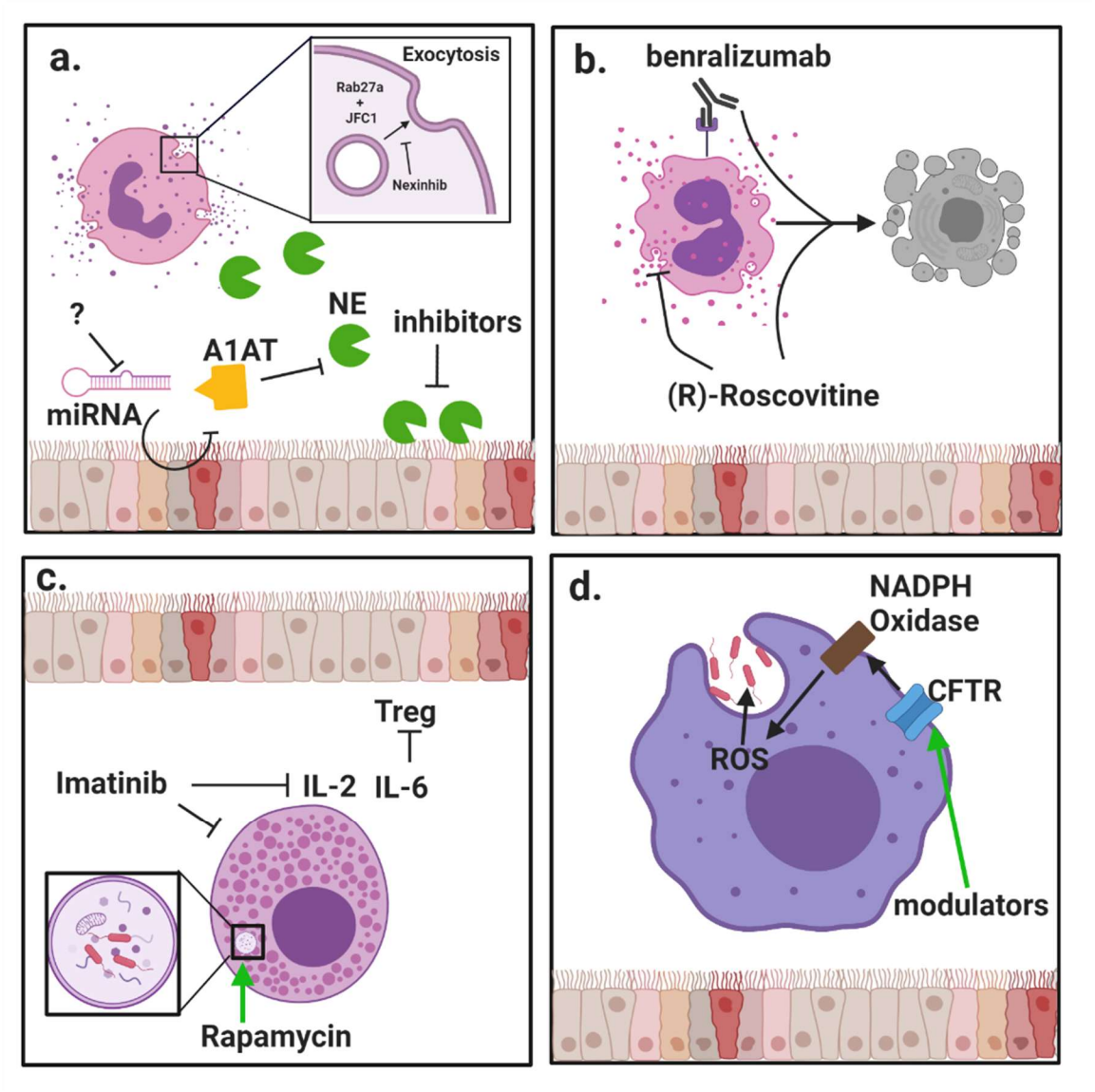

Figure 1.2.2.1. Modulation of innate immune cell function by protein-directed therapies. Innate immune cells, including granulocytes and macrophages, have important roles in the pathophysiology of cystic fibrosis (CF) lung disease. **(a)** Neutrophils are massively recruited to the airways in CF and fail to clear pathogens despite their highly inflammatory activity. Neutrophils rapidly exocytosis their granules, releasing destructive enzyme such as neutrophil elastase (NE) into the extracellular space. Alpha-1 antitrypsin (A1AT) is a crucial anti-protease in the lung but is overwhelmed by the burden of NE in

advanced stages of disease. New potential mechanisms to counter NE-driven inflammation include inhibition of microRNA (miRNA) against A1AT and novel NE inhibitors with increased potency. Specific inhibition of granule exocytosis without affecting other functions can be achieved by neutrophil exocytosis inhibitors (nexinhibs) via inhibition of the interaction between the two docking proteins Rab27a and JFC1. **(b)** Eosinophils are much rarer than neutrophils but they can have a potent role in comorbidities with CF such as allergic bronchopulmonary aspergillosis (APBA). (R)-Roscovitine may be effective in suppressing eosinophilic inflammation by blocking degranulation and inducing apoptosis. Benralizumab has also shown promise in promoting apoptosis in studies of asthma. **(c)** Mast cells are another rare granulocyte that present the opportunity for new therapies. IL-6 blockade may relieve mast cell-mediated suppression of regulatory T cells (Tregs), and this may be achieved through use of imatinib which has been shown to suppress mast cell infiltration and secretion of inflammatory cytokines. Induction of autophagy by rapamycin has also improved bacteria killing by mast cells. **(d)** Besides neutrophils, macrophages are the other major phagocyte in the lungs. CF macrophages have reduced ability to kill bacteria but the direct role of CFTR deficiency is still debated. Use of CFTR modulators has shown some ability to restore bacteria killing, which could be due to restored activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increased generation ROS.

1.2.2.6 T cells

While the airway lumen of CF patients experiences substantial influx of neutrophils, lymphocytes are actively recruited into the bronchial mucosa, and accumulate under the basement membrane [213]. Similar to neutrophils, T cells in CF patients demonstrate distinct pathological behavior. An important modulator of T-cell function is Arg1, which was first evaluated in CF patients in 2005 by Grasemann et al., identifying an overabundance of Arg1 in CF sputum samples, which negatively correlated with lung function [214]. This enzyme is involved in the impairment of T-cell signaling via downregulation of the CD3ζ chain [215].

Ingersoll et al. demonstrated the importance of Arg1 in negative regulation of T-cell function in CF patients by identifying the link between Arg1 from CF airway fluid and suppression of T-cell proliferation. Furthermore, they attributed much of this regulatory activity to neutrophils by demonstrating a positive correlation between expression of Arg1 on airway neutrophils and Arg1 activity in airway fluid [4]. The suppression of T-cell activity may be contributing to the observation that CF patients have a significant increase in reported respiratory illness, even if they do not experience higher frequency of actual infection [216]. These findings suggest that inhibition of Arg1 activity, which has already shown promise in animal models of inflammatory disease [217, 218], could have efficacy in treatment of CF. The importance of T cells in respiratory health is emphasized by a case report detailing a CF patient with mucosal-associated invariant T cell deficiency. Lung function was too high for considering a lung transplant, but the patient had exceptional risk for bacterial infections [219]. While this deficiency was likely attributed to an inherited defect independent from CF, it highlights the susceptibility of CF patients to immunological impairments.

Tregs are a crucial subset of T cells that regulate the immune response by maintaining self-tolerance and limiting inflammation [220]. Unsurprisingly, Treg dysfunction is implicated in CF. A reduction of $CD4^{+}CD25^{high}FoxP3^{+}$ Tregs in CF patients was first identified in 2014, and the prevalence of this cell population was found to positively correlate with lung function as measured by forced expiratory volume in 1 second (FEV1) [221]. Chronic *P. aeruginosa* infections have been linked to reduced Treg counts in CF patients, especially those over the age of 16 [222]. As life expectancy continues to improve for CF patients, this problem may require therapeutic attention. One potential treatment relative to Tregs centers on indoleamine 2,3-dioxygenase (IDO) (**Figure 1.2.2.2**). This enzyme is involved in tryptophan degradation and

when dysfunctional it has been attributed to an imbalance between Tregs and Th17 cells [223-225]. After demonstrating that IDO deficiency is associated with imbalanced Th17 and Treg populations in a murine model of CF, Ianitti et al. successfully corrected helper T cell populations and resolved inflammation by IDO restoration therapy. Although T cells are largely excluded from the airway lumen, Th17 T cells have been identified in the airway submucosa of CF patients in both early and established airway disease [226], and the Th17 pathway has been proposed to exert a major role in CF [224]. These findings suggest that correction of T cell imbalance in the CF lungs, especially Tregs for their anti-inflammatory role, could improve the course of airway inflammation.

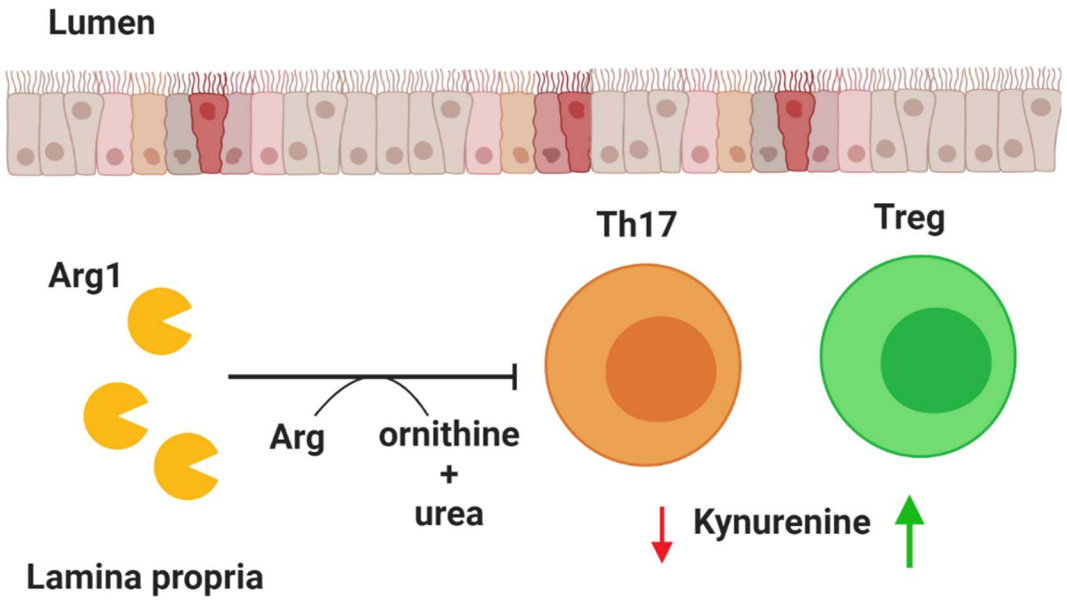

Figure 1.2.2.2. Modulation of T cell-activity. Arginase-1 (Arg1) is secreted by neutrophils upon granule exocytosis and has a potent inhibitory effect on T cells in the lung by cleaving the essential amino acid arginine (Arg) to produce ornithine and urea. A deficiency of regulatory T cells (Tregs) in CF patients may be explained by reduction of indoleamine 2,3-dioxygenase (IDO). Mouse models have demonstrated that correction of IDO deficiency by administration of kynurenines can rectify T cell imbalance by promoting Treg populations. Decreased availability of kynurenine (red arrow) reduces T helper type 17 (Th17) populations, while increased availability (green arrow) promotes Treg phenotype.

1.2.2.7 B cells

B lymphocytes form the other arm of the adaptive immune system and are also altered in CF. An earlier study by Sorensen et al. identified a higher frequency of plaque-forming cells, signifying differentiation into antibody-secreting cells, in CF patients who did not yet have severe pulmonary disease [227]. They noted normal plasma cell presence in patients with advanced disease and identified impaired B-cell differentiation in cells activated in vitro.

Similarly, Hubeau et al. found larger B cell-containing lymphoid aggregates in airways of CF compared to non-CF subjects, and similar presence of plasma cells in CF and non-CF tissue samples. They speculated that B cells rather than plasma cells are contributing to inflammation in CF airways, possibly due to their role as antigen-presenting cells [228]. It may be worth investigating if the influx of B cells with apparent lack of differentiation to plasma cells is a CF-related defect or possible control mechanism to prevent antibody-mediated inflammation. The latter could be explained by the resistance of CF B cells to treatment with dexamethasone. Indeed, while IgG production was found to be similar in CF and control patients, dexamethasone was demonstrated to enhance antibody production in stimulated control cells but had no effect on CF cells [229]. While CF and normal B cells were found to present staphylococcal superantigen to T cells in similar capacity, presentation by CF B cells was not inhibited by dexamethasone treatment [230]. This finding may indicate that a potential inflammatory role of CF B cells proposed by Hubeau et al. may be due to a resistance to acute inhibition of antigen presentation rather than an intrinsically heightened activity.

Consistently, an increased frequency of lymphocyte-containing tertiary lymphoid structures has been identified in the lungs of CF patients, and chronic bacterial infection has been shown to induce their formation in mice [231]. When the B cell-depleting anti-CD20 antibody rituximab was administered to two CF patients prior to receiving lung transplants, lymphoid aggregates were not disrupted and their role in CF lung pathogenesis could not be determined [232]. Although lymphoid follicle formation can be induced by bacterial lung infection [231] and their presence has been identified in end-stage CF [173], further investigation into the role of lymphoid neogenesis in CF is needed to determine if they should be targeted therapeutically [233]. Possible therapeutics to disrupt lymphocyte aggregation in the airways could target the

chemokines responsible for B-cell recruitment. An abundance of C-X-C motif chemokine ligand 12 (CXCL12) and 13 (CXCL13) has been identified in lymphoid aggregates from the lungs of individuals with bronchiectasis and CF compared to the airway epithelium of control nonsmokers [231]. In the same study, IL-17A was found to be increased in the epithelium of bronchiectasis and CF subjects compared to controls [231]. These cytokines are important for the recruitment of B cells [234, 235] and are potential targets for cytokine blockade therapy. Cytokine blockade is currently being evaluated for efficacy in other inflammatory diseases, for example through targeting of the pro-inflammatory mediators IL-17 and TNF-α in rheumatoid arthritis [236].

1.2.3 Protein-directed therapies

No cure has yet been developed for CF, but an improved understanding of CFTR biogenesis and regulation has opened new opportunities for therapeutic development [237]. Extensive work has been done to identify and develop CFTR modulators that can correct and potentiate the function of the mutated protein. Two major groups of these drugs include CFTR potentiators, such as ivacaftor, and correctors, such as lumacaftor and tezacaftor. These drugs used as single or combination therapies have shown efficacy in restoring lung function and other core clinical features of CF, and have been reviewed extensively [238-241]. However, drug development is complicated by a recent observation that CFTR correctors differentially affect expression of the N- and C-halves of the protein [242]. New approaches acting independently of CFTR will likely be needed. A recent study by Gianotti et al. investigated the use of small molecule anionophores to facilitate exchange of chloride and bicarbonate as a way to circumvent

CFTR deficiency [243]. Another recent study has suggested the use on amphotericin to generate small unselective ion channels can modulate CF-related host defense defects [244].

The need for alternative therapies is reinforced by observations that managing long-term inflammation still remains a challenge despite implementation of CFTR modulator therapies [245]. Continued development of innovative protein-directed therapies could yield new breakthroughs in treating CF lung disease. For example, Reihill et al. demonstrated that inhibition of proteases that activate signaling through the epithelial sodium channel in primary CF airway epithelial cells reduces channel activation and fluid absorption, which would possibly stimulate improved airway hydration and mucociliary clearance if used therapeutically as a CFTR-independent treatment [246]. These channel-activating serine proteases are secreted in great quantity by neutrophils in the CF airways where they acquire a pathological phenotype [102, 103].

Neutrophil-directed protein-targeting therapies are a major area of unmet need given their broad roles in CF [25, 26]. A recent report shows the potential breakthroughs of neutrophil-directed therapies by demonstrating that inhibition of the nucleotide-binding oligomerization domain-like receptors (NOD)-like receptor protein 3 inflammasome in a murine model of CF promotes clearance of *P. aeruginosa* and resolution of airway inflammation via reduced IL-1β production [247]. Targeting mechanisms of neutrophil exocytosis, as well as the activity of proteases, could offer new anti-inflammatory treatment options. However, the effect of CFTR modulators on macrophage function is also an area of interest and intriguing reports have been published on opportunities for targeting macrophages in CF, as detailed below.

1.2.3.1. Neutrophil Exocytosis

The enormous impact of neutrophils on progression of lung disease in CF makes them an obvious target for new therapies. Given the role of neutrophil exocytosis in driving disease progression [98], due to the release of factors that promote bronchiectasis [22, 23], inhibition of exocytosis specifically in neutrophils offers the potential to improve patient outcomes without adversely impacting normal immune functions. The antiprotease secretory leukoprotease inhibitor was identified as having efficacy in reducing CF neutrophil exocytosis by interrupting calcium flux via reduced inositol 1,4,5-triphosphate production [248]. However, the effect on other essential neutrophil functions such as phagocytosis was not assessed. A potential breakthrough was published by Johnson et al. several years ago with the identification of neutrophil exocytosis inhibitors (nexinhibs). The group described several small molecules that inhibit the interaction between Rab27a and JFC1, which are key regulators of exocytosis. These compounds were shown to potently inhibit exocytosis of the primary (azurophilic) granules without reducing the ability to phagocytose and kill microbes. Additional functions including NETosis were also unaffected [164]. Interestingly, blood neutrophils from CF patients have demonstrated reduced propensity for exocytosis compared to those of healthy controls [249], although more recent studies have demonstrated high exocytic activity of neutrophils in an in vitro CF airway model [96] and those from the airways of CF patients [98]. In this study it was found that treatment with the CFTR modulator ivacaftor increased neutrophil exocytosis to levels comparable to that of healthy control cells through activation of Rab27a [249]. These findings are important to consider as implementation CFTR modulatory therapies has failed to reverse chronic airway inflammation despite some recovery of lung function [149].

1.2.3.2. Neutrophil Elastase

Exocytosis of the neutrophil granules releases their destructive cargo into the extracellular environment where they can damage host tissue. A major example of this is NE, which has attracted significant attention as therapeutic target over the past decade [92, 250, 251]. The interest in NE as a target has been reinforced by recent findings demonstrating that surface-bound NE on neutrophils is associated with disease severity [98, 252]. In addition, common treatments for CF patients such as deoxyribonuclease (DNase) [253] may increase NE-related lung pathology by promoting enzyme activity [254], further demonstrating the need for developing therapies directed toward inflammatory mediators. NE activity in CF sputum promotes an aggregation phenotype in *P. aeruginosa* demonstrated by antibiotic resistance and reduced invasiveness independent of biofilm-promoting mechanisms [255]. These findings demonstrate that rather than exerting bactericidal functions as NE normally does intracellularly, extracellular NE in CF can actually aid in establishing infection.

Several recent trials have demonstrated the efficacy of therapeutically targeting NE in CF lung disease. The small molecule NE inhibitor KRP-109, which was previously shown to potently suppress neutrophilic inflammation in a mouse model of pneumococcal pneumonia [256], demonstrated efficacy in reducing the degradation of mucins in sputum from CF patients and thus may be effective at improving mucociliary clearance [257]. Oral administration of the new NE inhibitor POL6014 was shown to effectively target the airways and inhibit NE activity in the sputum, with only low concentrations detected in plasma [258]. Further research is needed to identify the best compounds for selectively inhibiting NE with minimal off-target effects. Using sulfur fluoride exchange, Zheng et al. identified a novel inhibitor of NE, benzene-1,2-disulfonyl fluoride, and a derivative of this compound with even greater potency, 2-

(fluorosulfonyl)phenyl fluorosulfate. Importantly, this inhibitor was not cross-reactive with the homologous protease cathepsin G [259]. This technique will facilitate discovery of new compounds that can selectively target proteins with pathological activity and minimize the risk for interrupting normal immune functions. Indirect targeting of NE may also prove efficacious. As discussed recently by Hunt et al., inhibition of microRNAs that target the antiprotease A1AT could also help counter the massive NE burden in the lung by promoting the function of one of its endogenous inhibitors [260]. microRNAs that target the mRNA transcripts of A1AT suppress production of this key antiprotease. Inhibition of these non-coding negative regulators could allow for increased production of A1AT to counteract the destructive effect of excessive NE release.

1.2.3.3. Matrix Metalloproteinases

MMPs are another class of proteases implicated in CF lung disease, produced not only by neutrophils but also by macrophages and epithelial cells [162]. MMP-9 is especially interesting in CF lung disease, since NE has been shown to cleave and activate pro-MMP-9, as well as degrade the tissue inhibitor of metalloprotease-1 [261]. MMPs have a potent ability to degrade extracellular matrix and MMP-9 is capable of inducing the release of matrikines such as proline-glycine-proline [262] and drive further neutrophilic inflammation in vivo (reviewed in [263]). MMP-9 has also been shown to inhibit wound healing of epithelial cells when exposed to bacterial infection [264]. The importance of this particular protease was reinforced in a report by Garratt et al. demonstrating that in early disease, measurement of MMP-9 in BAL fluid is an effective predictor of bronchiectasis, while MMP-1 and MMP-7 were minimally present and MMP-2 was possibly degraded by NE [22].

Although much attention is given to neutrophil-derived MMP-9, Averna et al. demonstrated that peripheral blood mononuclear cells from CF patients are also predisposed to secrete MMP-9 compared to healthy donor cells. This was attributed to enhanced signaling through calpain and protein kinase Cα promoted by increased intracellular calcium due to loss of CFTR [265]. Although no trial has yet been conducted to specifically target MMPs in CF, Xu et al. treated exacerbating CF patients with doxycycline, an antimicrobial with anti-MMP activity, and demonstrated reduction of MMP-9 activity and notable clinical outcomes in a single-center clinical study [266]. In addition, Hentschel et al. conducted a study of CF patients who were administered intravenous antibiotics by assessing the presence of inflammatory mediators before and after treatment. They found that within a short time after treatment (median time of 6 days) there was a significant reduction in MMP-9 and several proinflammatory cytokines in nasal lavage fluid, but this decrease was not observed for NE [267].

These findings demonstrate that, as expected, not all inflammatory processes respond to treatments with the same kinetics and as such offer opportunities for personalized treatment approaches. In a rat model for liver ischemia-reperfusion injury, which exhibits protease-induced tissue damage, Wang et al. demonstrated that cleavage of vascular endothelial growth factor by MMP-9 exacerbated injury, and inhibition of MMP-9 rescued this defect [268]. However, vascular endothelial growth factor is implicated in progression of CF lung disease [269], so careful consideration must be given for therapies aiming to modulate protease function.

1.2.3.4. Effect of CFTR Modulators on Macrophages

Since the identification of mutated CFTR protein as the cause of CF in 1989, several classes of drugs have been developed to promote expression of functional protein [270]. These

CFTR modulators were designed to primarily correct epithelial cell function in the airways, but recent studies have demonstrated the benefits of these therapies in immune cells, too. Macrophages are the dominant leukocyte subset in healthy airways and are essential for clearing pathogens and debris. As such, altered macrophage function in CF is detrimental to lung health [150, 151]. A pro-inflammatory poise of macrophages in the CF lung was suggested by Tarique et al. to be directly linked to CFTR deficiency [141]. In that study, blood monocytes from CF patients were less able to differentiate to the anti-inflammatory M2 macrophage phenotype compared to healthy donor cells. Furthermore, inhibition of CFTR in healthy donor cells recapitulated the pro-inflammatory phenotype observed with CF monocyte-derived macrophages. A growing body of literature now suggests that CFTR offers opportunities for therapeutic targeting. Zhang et al. demonstrated that blood monocyte-derived macrophages from CF patients have decreased phagocytic capability and are more prone to apoptosis than cells from non-CF subjects. Interestingly, while cells obtained from CF patients on ivacaftor therapy exhibited restored phagocytosis of *P. aeruginosa* compared to untreated patients, lumacaftor/ivacaftor combination therapy worsened the phagocytic response in cells from both CF and non-CF subjects treated in vitro [142]. Conversely, a separate study showed that lumacaftor improved killing of *P. aeruginosa* while addition of ivacaftor abrogated this effect in a dose-dependent manner [138]. Further research in needed to understand the possible beneficial effects of single therapies on pathogen clearance but abrogation of this effect by combination treatments.

One possible explanation for the lack of bactericidal activity in CF macrophages is impaired calcium influx due to reduced signaling through transient receptor potential vanilloid 2. Expression of this channel was found to be reduced on CF macrophages and inhibition of CFTR

function on non-CF macrophages impaired calcium influx [271]. Impaired production of ROS has been postulated as another mechanism for the inability of CF macrophages to kill bacteria. Impaired activation of the NADPH oxidase complex necessary for ROS production has been demonstrated in CF macrophages [272], and this defect in ROS production has been linked to an inability to kill intracellular bacteria [273]. These findings demonstrate that in addition to restoring ion flux in airway epithelial cells, targeting expression of CFTR in macrophages can rescue macrophage function by acting through a variety of pathways. However, more recent studies have challenged these findings. Using surface-enhanced Raman spectroscopy-based nanosensors, Law et al. demonstrated that CF monocyte-derived macrophages do not have impaired acidification of phagolysosomes compared to those of healthy controls [145]. Furthermore, Leuer et al. investigated the phagocytic capacity of neutrophils and monocytes from venous blood of CF and healthy subjects and found no significant difference for either cell type [146]. Further research is needed to ascertain the effect of CFTR loss on immune cell function.

1.2.4 Conclusion

As exemplified by the development of CFTR-directed therapies, including approval of Trikafta® in 2019 [238], the majority of recent clinical trials for CF have focused on incorporating these novel small molecule therapeutics into the drug regimen. Indeed, there are currently 56 interventional clinical trials (active or recruiting) in the US for CF (clinicaltrials.gov), many of which are aimed at modulating CFTR. A few trials are focused on inflammatory and infectious components of the disease (reviewed in [274]), three of which are in phase II. Of note, a clinical trial exploring the safety and tolerability of full length CFTR mRNA

delivery to the lungs by inhalation is also ongoing (CFF.org/trials), possibly a sign of things to come with regards to upcoming CF gene therapy trials.

Unmet Need for Immunotherapies

There is a plethora of candidate protein or nucleic acid-based therapies being developed that show promising results for either personalized medicine-based or one-size-fits-all approaches to curing CF disease. Although some have moved forward to the clinical testing phase most of these candidate therapies have not been tested in patients yet. A critical bottleneck in drug development is that existing CF animal models do not include all of the relevant barriers to entry nor fully recapitulate the peculiar immunological landscape in the lung of CF patients. Improved in vitro and animal models are therefore needed to further optimize not only candidate treatments, but also delivery methods to target specific airway cells. Further research into the complex interplay resulting in CF lung inflammation will provide important direction toward development of better treatments, either based on direct immunomodulation, or as adjunct therapies for nucleic acid delivery approaches. Moreover, particular attention should be directed to the mechanisms underlying the architecture of lung tissue, as chronic inflammation triggers tissue remodeling and repair mechanisms. To this end, the investigation of small molecules targeting both inflammation and tissue remodeling is of interest. However, while novel therapies have reduced toxicity, using drugs affecting a large variety of cell types may lead to increased side effects, particularly in CF, where patients are subjected to an already heavy therapeutic regimen. It will also be critical to assess use of these new therapies in combination with CFTR modulators, which have become the “new normal” in CF clinical management.

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Chapter 2

Macrophage PD-1 associates with neutrophilia and reduced bacterial killing in early cystic fibrosis airway disease

Sections of this chapter have been published in the Journal of Cystic Fibrosis

2.1 At-a-glance commentary

Current scientific knowledge on the subject: Macrophages are the main immune cell residing in the airways and are responsible for maintaining homeostasis. Normally macrophages prevent mass infiltration of neutrophils into tissues, but are not only unable to fulfill this critical regulatory role in CF lung disease but also demonstrate impaired ability to clear bacteria. Lack of CFTR expression in macrophages from people with CF may play a role in this dysregulated phenotype, but CFTR modulators do not fully resolve lung disease. It is unknown what other factors influence macrophage dysfunction in the airways of people with CF.

What this study adds to the field: We demonstrate that expression of the programmed cell death protein 1 (PD-1) receptor on airway macrophages correlates with measures of lung disease and neutrophilia. Signaling through this receptor has been shown to impair macrophage effector functions in several chronic diseases, but we show for the first time that this pathway contributes to macrophage exhaustion in CF. Exposure to soluble factors secreted by CF airway-like neutrophils promoted PD-1 expression on healthy control monocytes, and blocking PD-1 on primary airway leukocytes augmented their bacteria killing ability. However, modulation of CFTR activity to promote or block expression on CF-donor or healthy-donor monocytes, respectively, did not directly influence PD-1 expression. These data demonstrate a novel mechanism by which CF airway macrophages fail to exert key effector functions and how this relates to the incomplete resolution of lung disease by CFTR modulator treatment.

2.2 Abstract

Background: Macrophages are the major resident immune cells in human airways coordinating responses to infection and injury. In CF, neutrophils are recruited to the airways shortly after birth, and actively exocytose damaging enzymes prior to chronic infection, suggesting a potential defect in macrophage immunomodulatory function. Signaling through the PD-1 receptor controls macrophage function in cancer, sepsis, and airway infection. Therefore, we sought to identify potential associations between macrophage PD-1 and markers of airway disease in children with CF.

Methods: Blood and BAL fluid were collected from 45 children with CF aged 3 to 62 months and structural lung damage was quantified by computed tomography. The phenotype of airway leukocytes was assessed by flow cytometry, while the release of enzymes and immunomodulatory mediators by molecular assays.

Results: Airway macrophage PD-1 expression correlated positively with structural lung damage, neutrophilic inflammation, and infection. Interestingly, even in the absence of detectable infection, macrophage PD-1 expression was elevated and correlated with neutrophilic inflammation. In an in vitro model mimicking leukocyte recruitment into CF airways, soluble mediators derived from recruited neutrophils directly induced PD-1 expression on recruited monocytes/macrophages, suggesting a causal link between neutrophilic inflammation and macrophage PD-1 expression in CF. Finally, blockade of PD-1 in a short-term culture of CF BAL fluid leukocytes resulted in improved pathogen clearance.

Conclusion: Taken together, these findings suggest that in early CF lung disease, PD-1 upregulation associates with airway macrophage exhaustion, neutrophil takeover, infection, and structural damage.

2.3 Introduction

Resident macrophages play a key role in tissue homeostasis and responses to infection and injury [1]. In the airways, macrophages quickly adapt to variations in oxygen pressure, inflammation, and the presence of pathogens [2] . Exposure of airway macrophages at birth and thereafter to host- and microbe-derived factors shapes long-term responses and the likelihood to mount an aberrant response to later insults [3]. In CF, airway epithelial cells and macrophages display intrinsic defects due to mutations in the gene encoding the CFTR protein. In particular, mucin hyperconcentration and dehydration can alter airway macrophages [4], which may contribute to abnormal immune regulation, and subsequent recruitment and activation of neutrophils, with ensuing lung damage [5-7]. In itself, CFTR dysfunction in CF airway macrophages may result in impaired phagocytosis and pathogen clearance [8-10]. While the impact of intrinsic CFTR-linked defects on CF airway macrophage responses has been well studied, little is known about their regulation by extrinsic immunomodulatory pathways.

PD-1 is an immune checkpoint protein that regulates cell behavior during immune responses, with best known functions related to T-cell control in cancer and viral infection [11]. Comparatively, the role of PD-1 in macrophages is less well described, however there is increasing evidence of its role in tumor-infiltrating myeloid cells [12]. In blood monocytes during sepsis and in tumor-associated macrophages, PD-1 expression is associated with reduced uptake of bacteria and cell debris, respectively, suggesting a role for this pathway in shaping macrophage function [13-15]. Recently, PD-1 signaling was also found to mediate extrinsic control of resident macrophages by immunosuppressive leukocytes recruited in a model of airway infection [16]. We previously showed that neutrophils recruited to CF airways undergo reprogramming, leading to complex functional changes [17-21]. Among those is the acquisition

of immunosuppressive functions [22], including expression of the PD-1 ligand, PD-L1, observed in adult patients with CF [23].

The early and massive recruitment of neutrophils to the CF airways [20] suggests a change in the poise of resident airway macrophages, which have an essential role in repairing tissue damage and preventing untimely neutrophil recruitment to sites of injury [24]. Inhibitory signaling in airway macrophages may reduce their capacity for blocking neutrophil recruitment in the early stages of inflammation in CF airways [25], while simultaneously inhibiting their ability to clear bacteria. Since PD-1 signaling has been shown to downregulate macrophage poise in multiple inflammatory diseases [13, 15, 16], we hypothesized that expression of PD-1 on airway macrophages in young children with CF would associate with progressive airway disease, and that blocking this signaling cascade could enhance bacteria killing. Therefore, we investigated the presence of the PD-1 pathway in airway macrophages and its relationship with intrinsic and extrinsic inflammatory factors critical for the early stages of CF lung disease.

2.4 Methods

Study visits

Prospective study visits were conducted on 45 CF children aged 3 to 62 months of age enrolled in AREST-CF (Perth, Australia), I-BALL (Rotterdam, The Netherlands) and IMPEDE-CF (Atlanta, GA, USA) early disease surveillance programs, and 10 age-matched non-CF controls undergoing bronchoscopy for clinical indications at the Aerodigestive Clinic at Children's Healthcare of Atlanta (Atlanta, GA, USA). The study was approved by relevant Institutional Ethical Review Boards at each site (1762/EP for AREST-CF; NL49725.078.14 for I-BALL; IRB#00097352 for IMPEDE-CF).

H441 cell line

H441 cells (ATCC, Cat# HTB-174) were cultured in DMEM/F-12 media supplemented with 10% FBS (Corning), 2 mM glutamine (Sigma), and 100 U/mL-0.1mg/mL penicillin / streptomycin (Sigma). 2.5 ×10 5 H441 were harvested at passages 2-3 and cultured at air-liquid interphase on the Alvetex scaffold (Reprocell) coated with rat tail collagen (Sigma) as previously described [26]. The basolateral medium DMEM/F-12 media supplemented with 2% Ultroser G (Crescent Chemical Co), 2 mM glutamine (Sigma) and 100 U/mL-0.1 mg/mL penicillin / streptomycin (Sigma) was replaced every two days. After 14 days the membranes were inversed to allow neutrophil loading on the basolateral side and apical migration as described below.

Human sample collection and processing

Blood and BAL fluid were collected from CF children (N = 42) and non-CF disease controls (N = 10). Demographic information for CF and non-CF disease control subjects is provided in **Table 2.4.1**. Blood was collected in K2-EDTA tubes by venipuncture, cells and plasma were separated by centrifugation and the cellular fraction was used for flow cytometry. BAL fluid was collected under general anesthesia using sterile saline as per the standard clinical procedure previously described [20]. BAL fluid was then mechanically dissociated on ice in presence of 2.5 mM EDTA, cells were recovered after an 800 x g, 10-minute centrifugation at 4 °C, then washed and used for downstream assays. BAL fluid supernatant was further spun at 3,000 x g for 10 minutes at 4 °C to remove debris and bacteria, and stored at -80 °C until use. Differential cell counts and Oil Red O staining with lipid laden macrophage index [27] were performed by the clinical pathology laboratories at the Erasmus MC/Sophia Children's Hospital and Emory University / Children's Healthcare of Atlanta. Infection status was determined by clinical microbiology using standard aerobic culture techniques at each respective institution, and classified for presence of the pro-inflammatory pathogens *P. aeruginosa*, *S. aureu*s, *H. influenzae*, and *Aspergillus* spp. [28]. Due to the nature of the sample collection process, the age of the patients, and cell yield, it was not possible to perform all assays on all collected samples. A detailed tally describing the assays performed on each sample is provided in **Table 2.4.2**.

	CF pediatric cohort	Non-CF disease control pediatric cohort
	(N = 42)	(N = 10)
Age in months, mean (SD)	30.4 (22.3)	21.4 (7.5)
Sex		
Males	20	5
Females	22	5
Genotype		
F508Del homozygous	18	N/A
F508Del heterozygous	19	N/A
Other	5	N/A
Infection status (pathogens detected)		
None	32	6
One, or more	10	4

Table 2.4.1. Summary of patient demographics.

Subject	Age (months)	Sex	Site	CFTR mutation 1	CFTR mutation 2	Infection	Pancreatic sufficiency	Race	Assays	PRAGMA CF CT
1	22	F	P	G85E	unknown	*M. catarrhalis*	Yes	White	FC, E, C	Yes
2	23	M	P	F508	F508	*Aspergillus spp, H. influenzae, P. aeruginosa*	data unavailable	White	FC, E, C	Yes
3	23	F	P	F508	F508	None	data unavailable	White	FC, E, C	Yes
4	24	M	P	F508	F508	*E. coli, P. lilacinum*	No	White	FC, E, C	Yes
5	23	F	P	F508	R117H	None	data unavailable	White	FC, E, C	Yes
6	24	M	P	F508	1898+1G->A	None	No	data unavailable	FC, E, C	Yes
7	11	M	P	F508	F508	None	No	data unavailable	FC, E, C	Yes
8	12	M	P	I507	621+1G->T	*H. influenzae*	No	data unavailable	FC, E, C	Yes
9	10	M	P	F508	F508	None	No	data unavailable	FC, E, C	Yes
10	11	F	P	F508	I502T	None	No	data unavailable	FC, E, C	Yes
11	11	F	P	F508	F508	None	No	White	FC, E, C	Yes
12	4	F	P	F508	1717-1G->A	None	data unavailable	data unavailable	FC, E, C	Yes
13	9	M	P	F508	F508	*Cytomegalovirus*	No	data unavailable	FC, E, C	Yes
14	12	F	P	F508	R1158X	None	data unavailable	data unavailable	FC, E, C	Yes
15	3	M	P	F508	F508	None	No	data unavailable	FC, E, C	Yes
16	3	M	P	F508	F508	None	No	data unavailable	FC, E, C	Yes
17	7	F	P	F508	c.3718-2477C>T	*Rhinovirus*	Yes	White	FC, E, C	Yes
18	3	F	P	F508	F508	*A. niger, E. coli, C. albicans*	No	White	FC	Yes
19	39	M	R	F508	G542X	*P. aeruginosa*	No	White	FC, E, C	Yes
20	60	M	R	F508	A455E	*H. influenzae*	No	White	FC, E, C	Yes
21	62	M	R	F508	A455E	*H. influenzae, S. aureus*	Yes	White	FC, E, C	Yes
22	61	M	R	F508	R117H-7T	None	Yes	White	FC, E, C	Yes
23	61	F	R	F508	F508	*P. aeruginosa*	No	White	FC	Yes
24	61	M	R	E60X	4015delATTT	*S. aureus*	No	White	E, C	Yes
25	61	M	R	F508	F508	*A. fumigatus*	No	White/Asian	FC, E, C	Yes
26	60	F	R	F508	F508	None	No	White	FC, E, C	Yes
27	60	M	R	F508	R117H	None	Yes	White	E, C	Yes
28	62	F	R	F508	R117H	None	Yes	White	FC, E, C	Yes
29	61	F	R	F508	F508	*H. influenzae, S. aureus*	No	White	FC, E, C	Yes
30	61	M	R	F508	Dele2,3(21KB)	None	No	White	FC, E, C	Yes
31	12	F	R	F508	F508	None	No	White	FC, E, C	Yes
32	13	F	R	Y275X	A559T	None	No	Black	FC, E, C	No
33	36	F	R	F508	N1303K	None	No	White	FC, E, C	No
34	62	M	R	F508	N1303K	None	No	White	FC, E, C	No
35	12	M	R	F508	E60X	None	No	White	FC, E, C	Yes
36	12	F	R	F508	A455E	None	Yes	White	E, C	No
37	60	F	R	F508	NK1303K	None	No	White	FC	No
38	61	F	R	F508	F508	None	No	White	FC	No
39	36	F	R	F508	F508	None	No	White	FC, C	Yes
40	12	F	R	F508	F508	None	No	White	FC, C	Yes
41	13	M	R	R1162X	R1162X	None	No	White	FC, E, C	No
42	12	F	R	F508	1682dup	None	No	White	FC, C	Yes
43	25	F	A	NA	NA	None	NA	Hispanic	FC, E, C	No
44	13	M	A	NA	NA	None	NA	White	FC, E, C	No
45	18	F	A	NA	NA	*H. influenzae, P. aeruginosa*	NA	African American	FC, E, C	No
46	17	F	A	NA	NA	None	NA	African American	FC, E, C	No
47	37	M	A	NA	NA	*H. influenzae*	NA	White	FC, E, C	No
48	17	M	A	NA	NA	None	NA	data unavailable	FC, E, C	No
49	12	M	A	NA	NA	None	NA	data unavailable	FC, E, C	No
50	24	F	A	NA	NA	MRSA, *S. aureus*	NA	Hispanic	FC, E, C	No
51	26	M	A	NA	NA	None	NA	African American	FC, E, C	No
52	25	F	A	NA	NA	*M. catarrhalis*	NA	Hispanic	FC, E, C	No

Table 2.4.2. Detailed list of assay implementation by patient (1-42: CF; 43-52: non-CF disease control). A: Atlanta control cohort; BK: bacterial killing assay; C: multiplexed cytokine assay; E: enzyme activity assays; F: female; FC: flow cytometry; M: male; MRSA: methicillin-resistant *S. aureus*; P: Perth cohort; R: Rotterdam cohort. No subjects were prescribed CFTR modulators.

Chest-computed tomography (CT) scans

Patients underwent a chest CT-scan without anesthesia using a Siemens SOMATOM®Force ultra-fast scanner at the Erasmus MC/Sophia Children's Hospital or under general anesthesia at the Perth Children's Hospital. The CT-images were scored for structural lung damage using the Perth-Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis (PRAGMA-CF) scoring method [29]. Repeatability of PRAGMA-CF score has been previously described. Disease score (%Dis) was the total lung volume proportion of bronchiectasis, mucus plugging, and bronchial wall thickening.

Flow cytometry and SPADE analysis

Multiparametric flow cytometry analysis of whole blood and BAL fluid cells was standardized across study sites by integrating two stringent calibration steps prior and post sample acquisition using a fluorescent bead-based method (Biolegend), as previously illustrated [20] which provide constant and robust output from the flow cytometers, and used premixes of the antibodies listed above to stain blood and BAL cells. Samples were pre-stained for 10 minutes on ice in the dark with the Human TruStain FcX Fc blocking solution and the Zombie Aqua reagent (Biolegend). In addition, we also check for specificity of antibody staining with fluorescence-minus one controls, as previously explained [30]. The following staining was

subsequently added on ice in the dark for 30 minutes: anti-human CD3 (pan-T cells), CD45 (pan-leukocytes), CD63, PD-1, PD-L1, PD-L2, CD66b, CD33, CD41a (all from Biolegend), CD66a (Novus Biologicals) and Siglec-8 (R&D Systems). Cells were washed, fixed in Lyse/Fix Phosflow (BD Biosciences) and acquired on a FACS Fortessa or LSRII (BD Biosciences). Analysis and compensation were performed in FlowJo V9.9.5 (FlowJo, LLC). Macrophages, neutrophils, and T cells were gated from blood and airway samples as detailed in **Figure 2.4.1**. While gating of blood leukocyte populations is straightforward, gating of airway leukocytes is more challenging. Our gating strategy was devised to identify all monocyte/macrophage-lineage cells but was not sufficient to identify subsets. CD45 is a pan-leukocyte marker and was used to exclude epithelial cells and debris. CD3 was used to separate T cells from myeloid cells, which were separated into monocyte/macrophage and neutrophil populations using CD33 [31]. Clustering analysis and group comparisons were performed using the panning-tree progression analysis of density-normalized events (SPADE) V4.0 algorithm [32] using the following parameters: arcsinh transformation cofactor (150), neighborhood size (5), local density approximation factor (1.5), maximum allowable cells in pooled downsampled data (50,000), target density with fixed number of cells remain (20,000), K-means algorithm with 100 clusters.

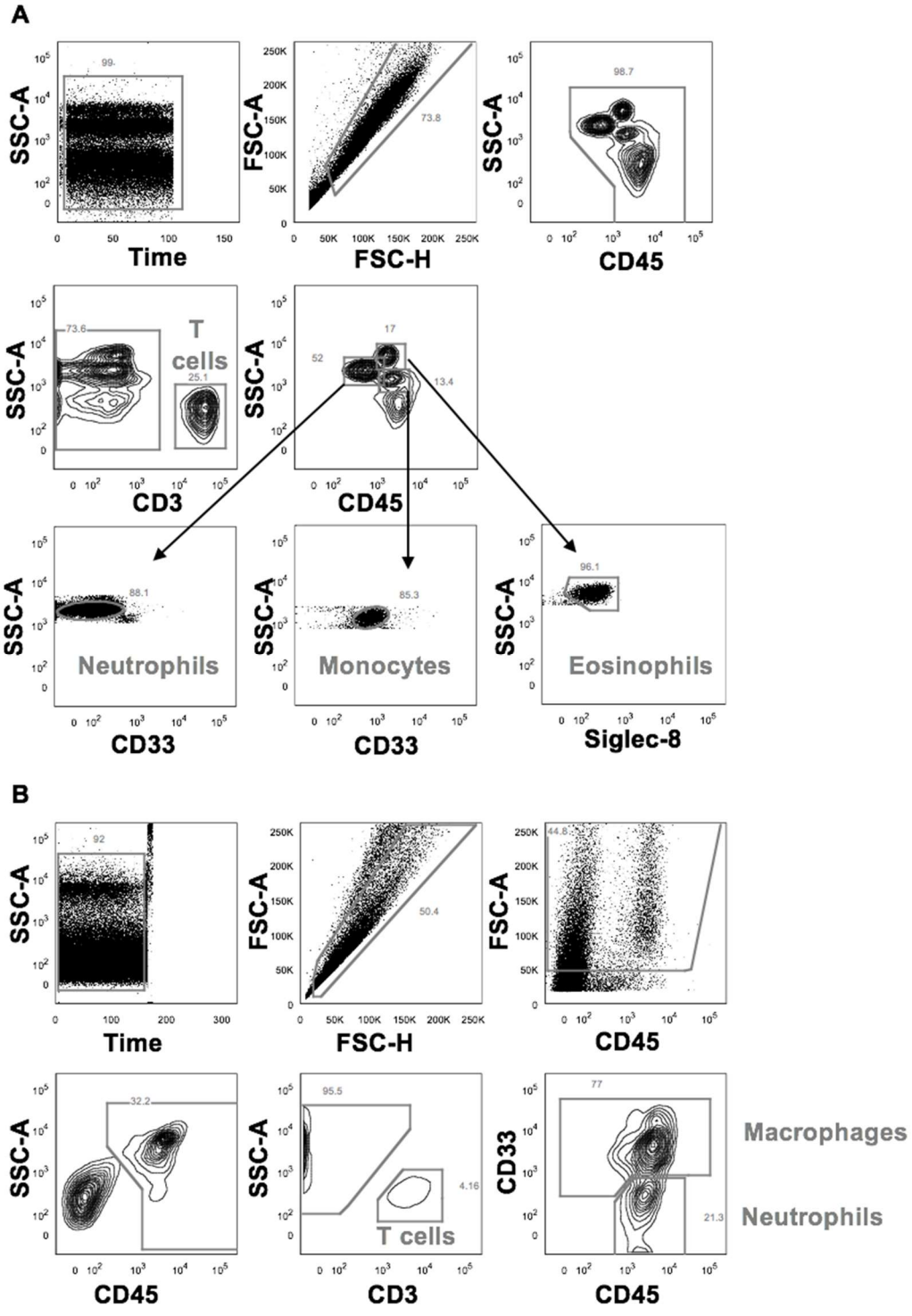

Figure 2.4.1. Gating strategy for blood and BAL. Blood **(A)** and BAL cells **(B)** were analyzed by flow cytometry following the illustrated gating strategy.

Assays for soluble mediators in BAL fluid

BAL fluid cytokines were quantified using a U-PLEX multiplex chemiluminescent ELISA assay (Meso Scale Diagnostics), per manufacturer's protocol. In vitro enzymatic activity assays for NE and MMP12 were performed using Förster resonance energy transfer probes NEmo-1 and LaRee-5 respectively (Sirius Fine Chemicals SiChem GmbH), as described [33].

In vitro transmigration (TM) model

Blood neutrophils and monocytes were isolated from healthy donors using Polymorphprep (Accurate Chemical) and RosetteSep (STEMCELL technologies) with Ficoll (GE Healthcare) kits, respectively, per manufacturers' protocols. Isolated blood neutrophils were resuspended in RPMI (Corning), loaded on the wells and transmigrated towards the chemoattractant control leukotriene B4 (LTB4, 100 nM, Sigma) or CF airway supernatant (CFASN, devoid of cells and bacteria), as we have detailed previously [18]. Neutrophils transmigrated through the epithelium to either LTB4 (TM control) or CFASN (CF airway-like condition) were collected after 4 hours, washed three times, and cultured in fresh RPMI for 8 hours at 37 °C to generate conditioned media. These conditioned media were further purified by sequential centrifugations at 800 x g for 10 minutes, followed by 3,000 x g for 10 minutes. Isolated blood monocytes were then transmigrated in turn through the epithelium into the neutrophil-derived conditioned media in new TM chambers for 5 hours and analyzed by flow cytometry as detailed above, with the replacement of anti-human CD33 by anti-human CD115 (Biolegend). All transmigrations were performed in an incubator with 5% CO_2 at 37°C. Monocytes from CF donors were isolated using the same procedure, with subject demographics summarized in **Table 2.4.3**.

Subject	Age (years)	Sex	Site	CFTR mutation 1	CFTR mutation 2	Infection	Pancreatic sufficiency	Race	CFTR modulators	Samples used
1	6	F	P	F508del	F508del	unavailable	No	White	lumacaftor/ ivacaftor	BALF cells, BK
2	5.1	F	P	F508del	I502T	none	data unavailable	data unavailable	None	BALF cells, BK
3	2.2	M	P	F508del	1078delT	none	No	White	None	BALF cells, BK
4	2.3	F	P	F508del	F508del	Parainfluenza virus type 3	data unavailable	White	None	BALF cells, BK
5	1.71	F	P	G551D	S549R	Mixed oral flora	data unavailable	White	None	BALF cells, BK
6	2.9	F	A	F508del	F508del	normal flora	No	White	None	BALF cells, BK
7	2.9	M	A	F508del	R553X	multiple Gram+ and Gram-	No	White	None	BALF cells, BK
8	1.4	M	A	F508del	F508del	MRSA	No	White	None	BALF cells, BK
9	39	F	A	F508del	F508del	*P. aeruginosa*, *P. putida*	No	White	ETI	Blood monocytes, TM
10	67	M	A	F508del	R117H	*P. aeruginosa*	No	White	ETI	Blood monocytes, TM
11	31	M	A	F508del	R560T	None	No	White	ETI	Blood monocytes, TM

Table 2.4.3. Demographics of subjects used for in vitro assays. A: Atlanta CF cohort; BALF: Bronchoalveolar lavage fluid; BK: bacteria killing; ETI: elexacaftor/tezacaftor/ivacaftor; F: female; M: male; MRSA: methicillin-resistant *S. aureus;* P: Perth CF cohort; TM: transmigration.

Ex vivo bacterial killing assay

Overnight cultures of the pro-inflammatory bacteria *Pseudomonas aeruginosa* (strain PAO1) were subaliquoted and grown to reach the exponential growth phase. The bacteria were then incubated in RPMI, supplemented with 10% FBS (Corning), on an end-over-end rotating wheel for 30 minutes at 37°C. Fresh BAL fluid leukocytes were resuspended in RPMI, 10% FBS and incubated at 37°C, 5% CO_2 for 15 minutes. Co-incubation of bacteria and immune cells, in presence or absence of PD-1 blockade, was performed at a multiplicity of infection of 1, in

RPMI, 10% FBS, on an end-over-end rotating wheel for 1 hour at 37°C, 5% CO_2. PD-1 blockade was accomplished using 1 μg/mL of Ultra-LEAF purified anti-human PD-1 blocking antibody (Biolegend) combined with 80 nM of SHP099 (Cayman Chemical), a small molecule SHP2 inhibitor [34]. Total bacterial killing was assessed by gently lysing the cells in 0.1% Triton-X and plating of bacteria on an agar plate overnight at 37°C. Bacterial killing capacity was calculated using colony forming units (CFU), with the bacteria, RPMI, 10% FBS condition set as 100% survival. Treatment efficacy was calculated by subtracting the effect of PD-1 blockade on bacteria alone and the killing capacity of immune cells without treatment from the condition where bacteria, immune cells, and PD-1 blockade were all present. Demographic information for CF subjects used for the bacteria killing assays is summarized in **Table 2.4.3**.

Statistical analysis

Data were compiled in Excel (Microsoft) and transferred to JMP13 (SAS Institute) and Prism v7 (GraphPad) for statistical analysis and graphing, respectively. The effect of study site on measured variables was corrected, while adjusting for age using a non-parametric empirical Bayesian method as previously described [20]. Predictor screening analysis was performed using bootstrap forest partitioning in JMP13, while simple linear fitting modelling was performed in R. Correlations were calculated using Spearman's rank-order correlation, a non-parametric rank-based calculation that is relatively insensitive to outlier effect.

2.5 Results

CF airway macrophages express high levels of surface PD-1

To determine the presence of the PD-1 signaling pathway in early CF airway inflammation, immune cells from blood and BAL fluid from 45 children with CF, aged 3 to 62 months, were analyzed by flow cytometry (**Figure 2.4.1**). The cohort included equal numbers of male and female subjects, with a mean age of 30 months, and 11/45 subjects were diagnosed with proinflammatory pathogen infections. Twenty subjects were homozygous for F508del, twenty had compound heterozygous mutations with F508del, and five had other mutations (**Table 2.4.1**). These subjects were primarily White with pancreatic insufficiency, and none were prescribed CFTR modulators at the time of sample collection. For Perth cohort subjects, demographic data on genotype, sex and birth date were obtained from clinical records. Data on ethnicity, pancreatic sufficiency status and current treatment were recorded by parent questionnaires, with "data unavailable" indicating this was not answered on the questionnaire (**Table 2.4.2**).

PD-1 expression on airway macrophages was higher than on airway neutrophils and T cells, by 10- and 17-fold respectively (**Figure 2.5.1A**). Clustering analysis of CF airway macrophages with the SPADE algorithm [32] revealed a distinct pattern of PD-1 expression in different airway macrophage clusters, highlighting the intrinsic diversity of this immune cell population with regards to the PD-1 pathway (**Figure 2.5.1B**). Airway macrophages and T cells showed significant expression of the PD-1 ligands, PD-L1 and PD-L2 (**Figure 2.5.1C, D**), suggesting potential for activation of PD-1 signaling. While we observed differences in expression of PD-L1 and PD-L2 between neutrophils, macrophages and T cells, these may be

partially caused by outliers. Indeed, there were 5-6 outlier points for expression of PD-L1 and PD-L2 on macrophages and T cells, and there were four subjects common to these groups of outliers. However, these subjects did not differ from the rest of the cohort based on demographic or inflammatory outcomes.

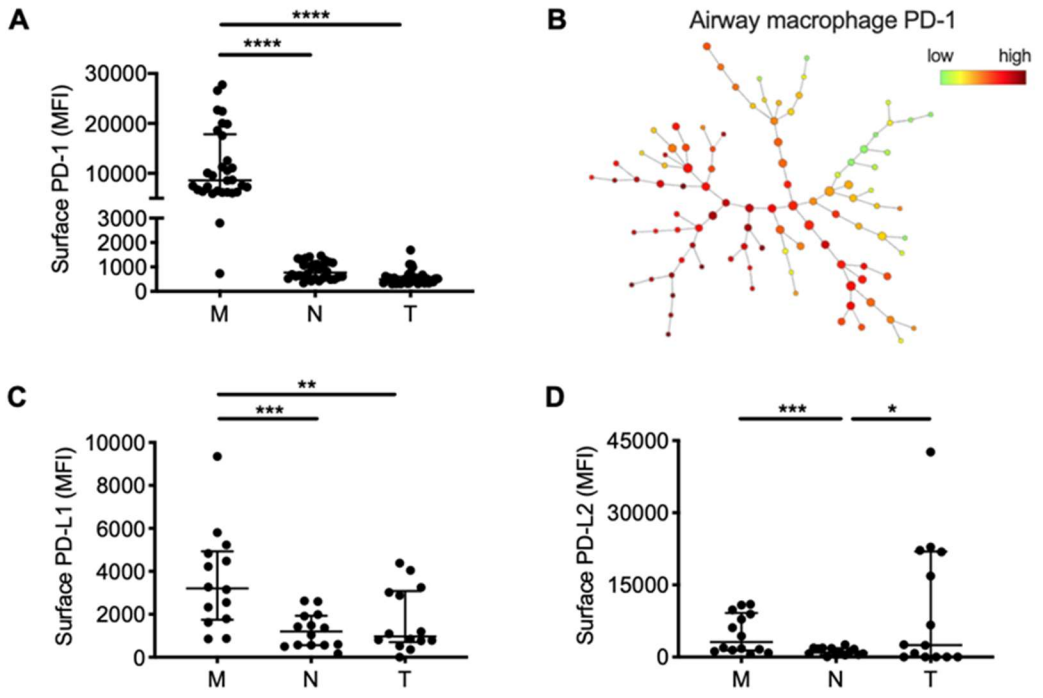

Figure 2.5.1. PD-1 and its ligands, PD-L1 and PD-L2, are expressed in CF airway leukocytes. Flow cytometry analysis of BAL fluid leukocytes from CF children shows higher PD-1 expression on airway macrophages (M) compared to neutrophils (N), and T cells (T) **(A)**. SPADE analysis of airway macrophage PD-1 expression in different clusters **(B)**. PD-L1 **(C)** and PD-L2 **(D)** expression in airway macrophages (M), neutrophils (N), and T cells (T) measured by flow cytometry shown as median fluorescence intensity (MFI). Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

Given the pattern of expression of PD-1 and its ligands on airway immune cells, we investigated their relationship with clinical variables of early CF lung disease. No significant relationships were observed between the clinical outcomes and the levels of expression of PD-L1 or PD-L2. However, in a subset of 24 children with CF, for whom flow cytometry data and date-matched CT scans were acquired, PD-1 expression on airway macrophages correlated with both the total score of structural lung damage (%Dis), ranging between 0 and 5% (Rho = 0.51, p = 0.01, **Figure 2.5.2A**), and the score for bronchiectasis (%Bx), ranging from 0 and 2.1% (Rho = 0.47, p = 0.01, **Figure 2.5.2B**). Furthermore, while PD-1 expression on airway macrophages did not correlate with absolute macrophage count (Rho = -0.03, p = n.s., **Figure 2.5.2C**), it correlated negatively with airway macrophage percentage (Rho = -0.57, p = 0.003, **Figure 2.5.2D**), and positively with airway neutrophil absolute count (Rho = 0.50, p = 0.01) and percentage (Rho = 0.51, p = 0.009). These findings suggest a potential link between neutrophilic inflammation, airway macrophage PD-1 expression, and early structural lung damage in CF.

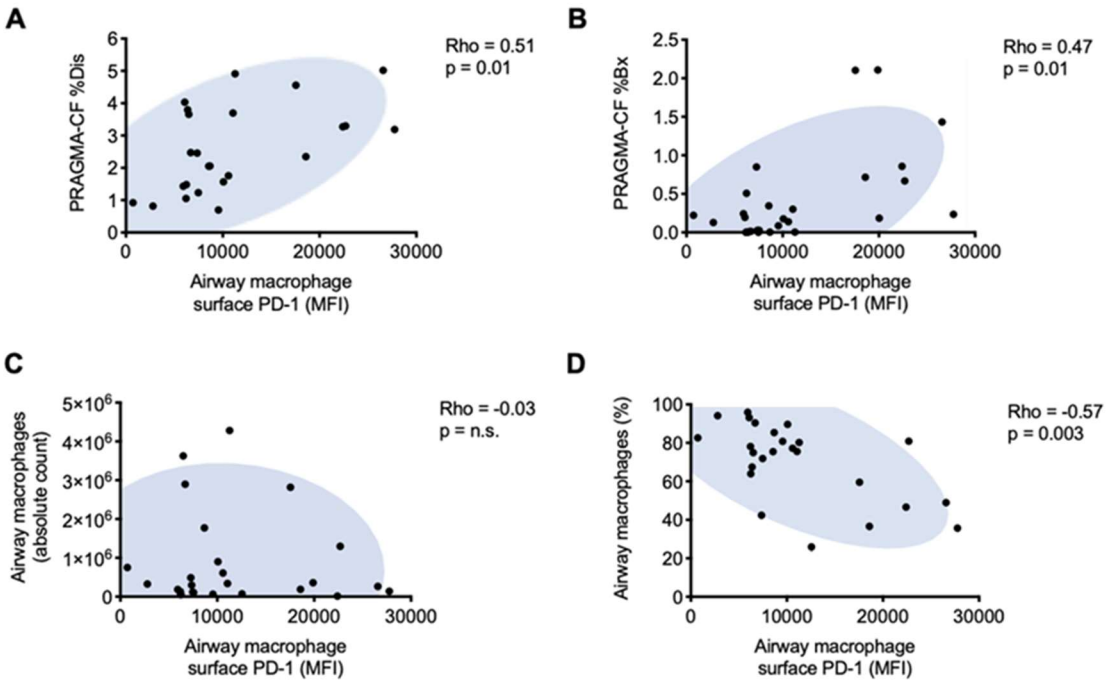

Figure 2.5.2. PD-1 expression on airway macrophages correlates with lung disease in children with CF. PD-1 expression on CF airway macrophages is associated with PRAGMA-CF total disease score measured by CT (%Dis) **(A)** and with the bronchiectasis score (%Bx) **(B)**. PD-1 expression on airway macrophages did not correlate with airway macrophage absolute count **(C)**, but correlated negatively with airway macrophage percentage **(D)**. Correlations were performed using the non-parametric Spearman's rank order correlation test and are shown as coefficient strength of Spearman Rho (left panel) and significance levels of p-values.

CFTR genotype and sex do not influence PD-1 expression in CF airway macrophages

Next, we investigated which intrinsic or extrinsic factors could contribute to PD-1 expression in airway CF macrophages. No significant difference was observed in the level of PD-1 expression based on sex (**Figure 2.5.3A**) or between patients bearing F508Del homozygous vs compound heterozygous genotypes (**Figure 2.5.3B**). Likewise, while uptake of

oxidized lipids and the subsequent inability to efficiently metabolize them (lipidation index) correlates with a dysfunctional state in macrophages [35], as described in atherosclerosis [36] and several lung conditions including CF [37, 38], in the subset of 22 children with CF for which Oil Red O staining was performed, no relationship was found between airway macrophage PD-1 expression and the lipidation index [27] (**Figure 2.5.3C**).

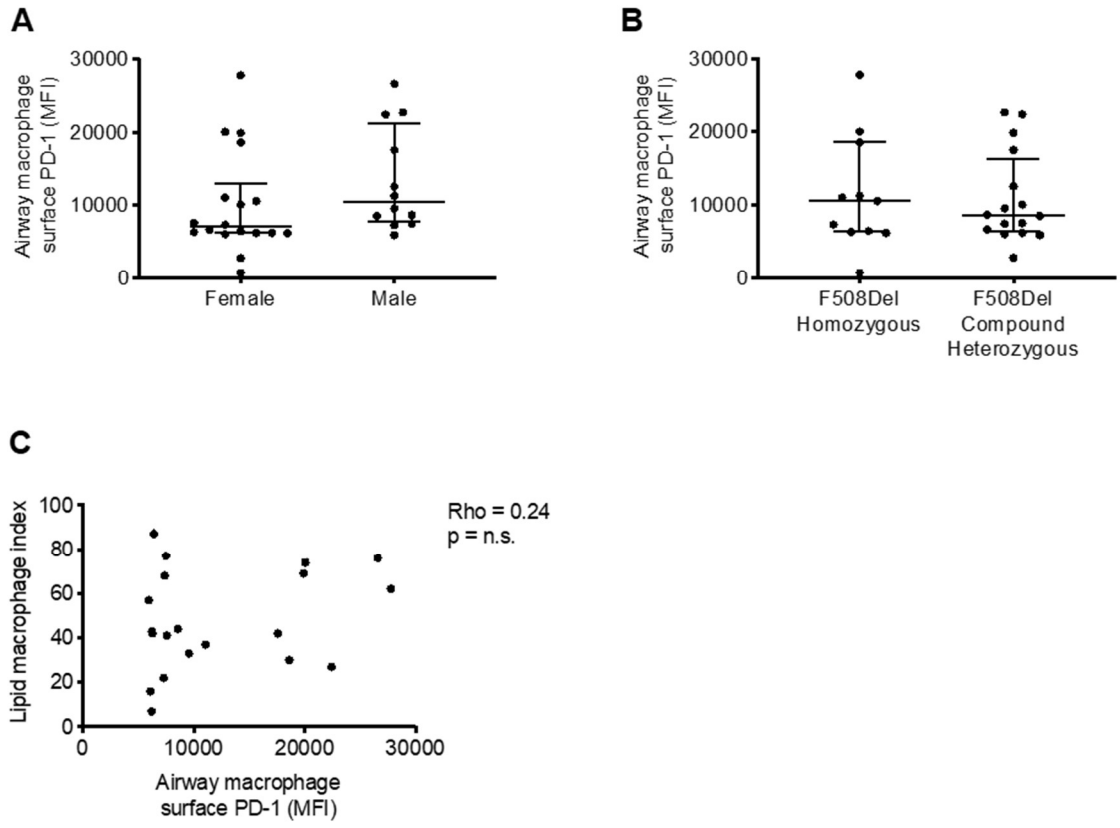

Figure 2.5.3. Neither sex nor CFTR genotype influence PD-1 expression. PD-1 expression on CF airway macrophages did not differ upon sex **(A)** or genotype of CFTR mutations **(B)**, and did not correlate with the airway macrophage lipidation index **(C)**, as measured by Oil Red O stain on cytospin slides. Statistical analysis was performed by the Wilcoxon rank sum test and the Spearman's Rho test for correlations. Data in **(A)** and **(B)** are shown as median and interquartile range.

Since lipidation, sex, and CFTR genotypes were not related to PD-1 expression on CF airway macrophages, we measured soluble and cell-bound inflammatory mediators in BAL fluid and combined them with clinical variables in a bootstrap forest partitioning analysis (**Table 2.5.1**, N = 42 patients included). The main predictors for PD-1 expression on airway macrophages were patient age, neutrophil mediators, and infection with pro-inflammatory pathogens, defined by the presence of at least one of four pro-inflammatory pathogens, namely *P. aeruginosa*, *S. aureus*, *H. influenzae*, and *Aspergillus* spp. [28], at the time of BAL fluid collection. While these factors may be related to each other, we recently showed that the presence of neutrophils in the airways of children with CF and their active exocytosis of NE-rich granules precedes colonization by pro-inflammatory pathogens, and that the extent of NE exocytosis did not differ between infected and non-infected groups at the single cell level [20]. However, LPS is an inducer of PD-1 expression on macrophages [14] and upon infection with pro-inflammatory pathogens, neutrophils are increasingly recruited to the airways, resulting in the extracellular accumulation of neutrophil-derived mediators. Therefore, to untangle the contribution of neutrophil and bacterial mediators in inducing PD-1 expression, we performed subsequent analyses discriminating between patients that presented with pro-inflammatory pathogens and those that were classified as not infected.

Term	# of Splits	Portion
Infection	13	0.1144
G-CSF	16	0.099
% Macrophages	20	0.0826
M-CSF	18	0.0723
IL-8	18	0.0661
Age	11	0.0648
% Neutrophils	12	0.0644

Table 2.5.1. Predictors of PD-1 expression on airway monocytes/macrophages. The Bootstrap Forest method for predictive modeling was applied to soluble mediators, cell surface markers, and clinical variables to determine predictors of PD-1 expression on airway monocytes/macrophages. Clinical variables included age range (up to 1 year old, 1-3 years old, and 3-5 years old), sex, CFTR mutation (F508 homozygous, F508 heterozygous, and other mutations), PRAGMA scores (%Dis and %Bx) and infection status (uninfected, or infection with pro-inflammatory pathogens). Differential cell counts, cell surface marker expression, inflammatory cytokine concentration, and activity of MMP12 and NE were also included. The number of splits and portion for top results are summarized.

Neutrophil activation modulates PD-1 expression by CF airway macrophages

Considerable overlap was observed between infected and non-infected groups in inflammatory mediator levels, suggesting that a high state of immune activation is not exclusive to patients with airway infections (**Figure 2.5.4**).

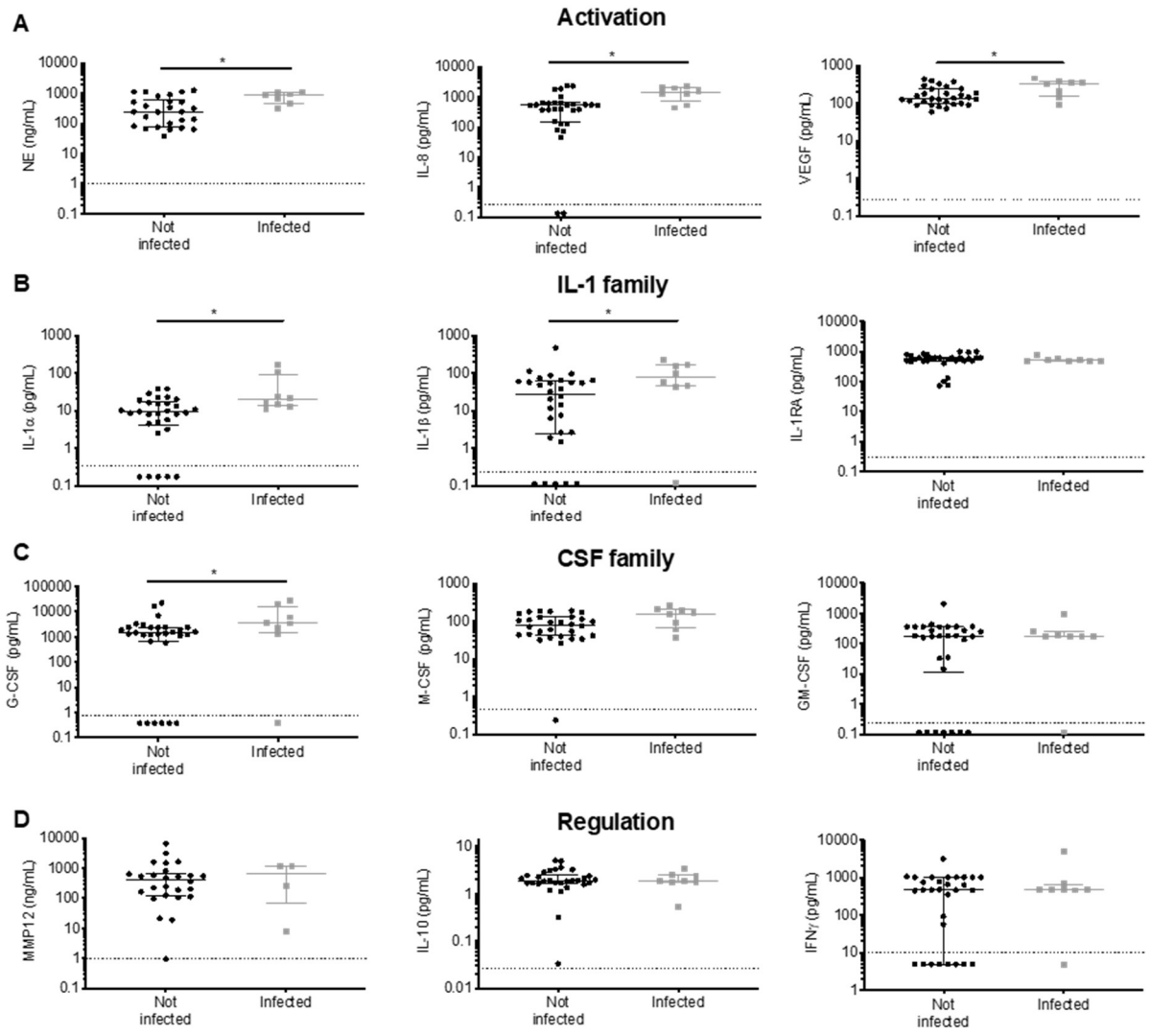

Figure 2.5.4. BAL fluid mediators are modulated upon infection by pro-inflammatory pathogens. BAL fluid mediators are grouped into functional categories and compared between groups of CF children relative to the infection status by pro-inflammatory pathogens, as determined by clinical microbiology. Significant modulation is observed in mediators belonging to the neutrophil activation **(A)**, IL-1 **(B)** and CSF **(C)**, groups, but not in the regulatory group **(D)**. Data are shown as median and interquartile range. Statistical analysis was performed with the Wilcoxon rank sum test and corrected for multiple testing using false discovery rate and q-value calculation (*$q<0.05$).

We then assessed factors correlated with PD-1 expression on airway macrophages in the absence of infection (**Figure 2.5.5A**). Significant correlations were observed for extracellular NE (Rho = 0.62, p = 0.02) and IL-8 (Rho = 0.48, p = 0.04), suggesting a role for neutrophil-derived soluble factors in the upregulation of PD-1 in airway macrophages.

To determine whether neutrophil-released mediators could directly modulate airway monocyte/macrophage surface PD-1 expression, we exploited an in vitro TM model previously used to recreate the CF airway neutrophil phenotype observed in vivo [18], which includes increased lifespan, active release of pro-inflammatory cytokines and exocytosis of granule enzymes such as NE and MPO. Upon in vitro generation of CF airway neutrophils, media was conditioned with these cells and then subsequently applied to a blood monocyte TM model [39] (**Figure 2.5.6**). Interestingly, monocytes that transmigrated into the conditioned media from CF airway-like neutrophils showed increased expression of surface PD-1 compared to both their blood counterpart and also to those monocytes that transmigrated into the TM control supernatant (conditioned media from neutrophils transmigrated towards LTB4) (**Figure 2.5.5B**). Our experimental model suggests soluble mediators from neutrophils recruited to CF airways could be directly modulating airway monocyte/macrophage PD-1 expression.

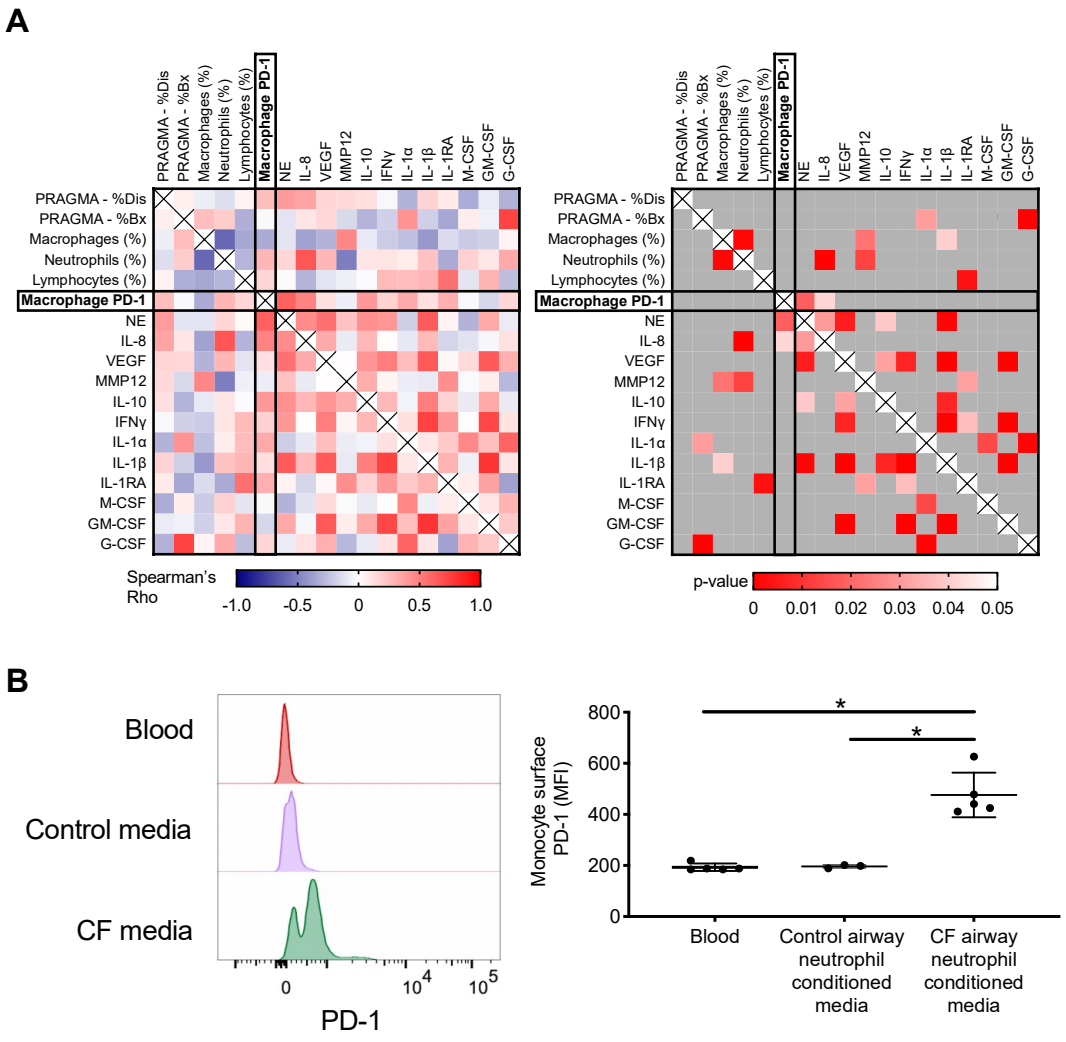

Figure 2.5.5. PD-1 expression on CF airway macrophages is associated with neutrophilic inflammation. PD-1 expression on CF airway macrophages correlates with neutrophil-derived mediators in young children with CF with negative airway cultures. Correlations are shown as coefficient strength of Spearman Rho (left panel) and corresponding significance levels of p-values (right panel, with grey indicating $p > 0.05$) **(A)**. *In vitro* TM of monocytes into neutrophil conditioned media (illustrated in **Figure S4**) show modulation of PD-1 expression in monocytes exposed to the CF-like neutrophil conditioned media **(B)**. Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as * for $p < 0.05$.

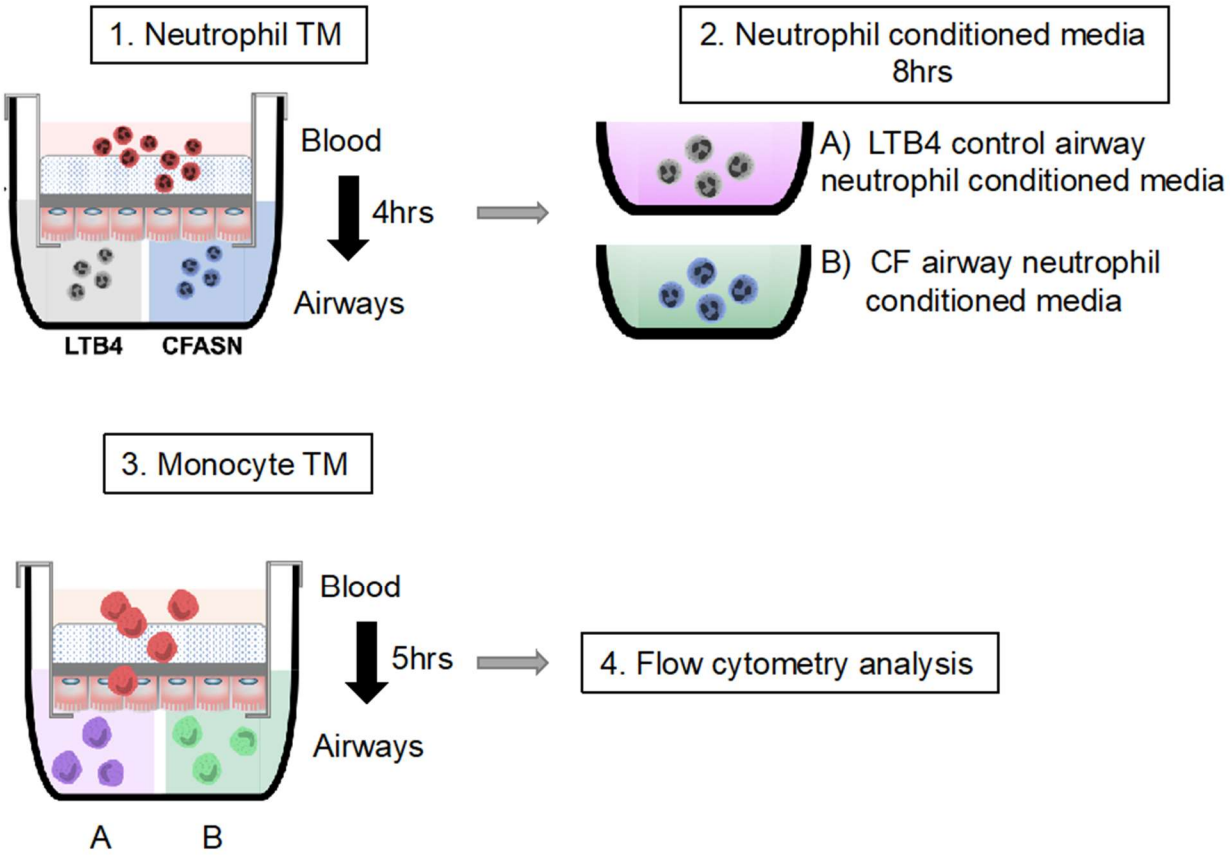

Figure 2.5.6. Schematic of in vitro TM experiments. (1) Blood neutrophils were transmigrated (TM) in an *in vitro* TM chamber through an epithelial monolayer towards a chemoattractant control (LTB4) or towards the CF airway supernatant as previously published [17]. **(2)** Airway neutrophils were washed and incubated to condition their own media. **(3)** This supernatant was subsequently used as airway fluid for monocyte TM. **(4)** Flow cytometry analysis was conducted on blood and airway monocytes for surface PD-1 expression.

PD-1 increases uniformly on CF airway macrophages upon infection

Since the likelihood of infection increases with age and correlates with enhanced neutrophil recruitment to CF airways [40, 41], we next fitted a logistic regression model for infection given age, and then a multivariate linear regression model for PD-1 expression on

airway macrophages adjusted for age and infection. Unsurprisingly, we found that older children were more likely to harbor infections ($p = 0.038$) and that age was also a significant predictor of PD-1 expression ($p < 0.001$). However, even after adjusting for age, the effect of infection on PD-1 expression remained significant ($p < 0.001$). These findings support the notion that while age increases the risk of infection in CF, both age and infection can independently impact PD-1 expression on airway macrophages in children with CF.

Next, we investigated whether the increase of PD-1 expression on airway macrophages upon infection was confined to CF or if it could be applicable to age-matched controls. Therefore, we collected blood and BAL from 10 age-matched non-CF disease control children with aerodigestive diagnosis, prone to aspiration-induced inflammation, and analyzed the cells by flow cytometry. Similar to children with CF, airway macrophages showed higher PD-1 expression compared to airway neutrophils and T cells by 10- and 18-fold, respectively (**Figure 2.5.7A**). Furthermore, we found that the two cohorts did not differ in their lipidation index (**Figure 2.5.7B**), neutrophil percentage (**Figure 2.5.7C**), or macrophage percentage (**Figure 2.5.7D**). However, macrophage count was elevated in CF compared to non-CF disease control BAL fluiud, consistent with previous studies [42]. CF patients with the highest absolute macrophage counts tended to be younger (approximately 0.25-2 years) (**Figure 2.5.7E**).

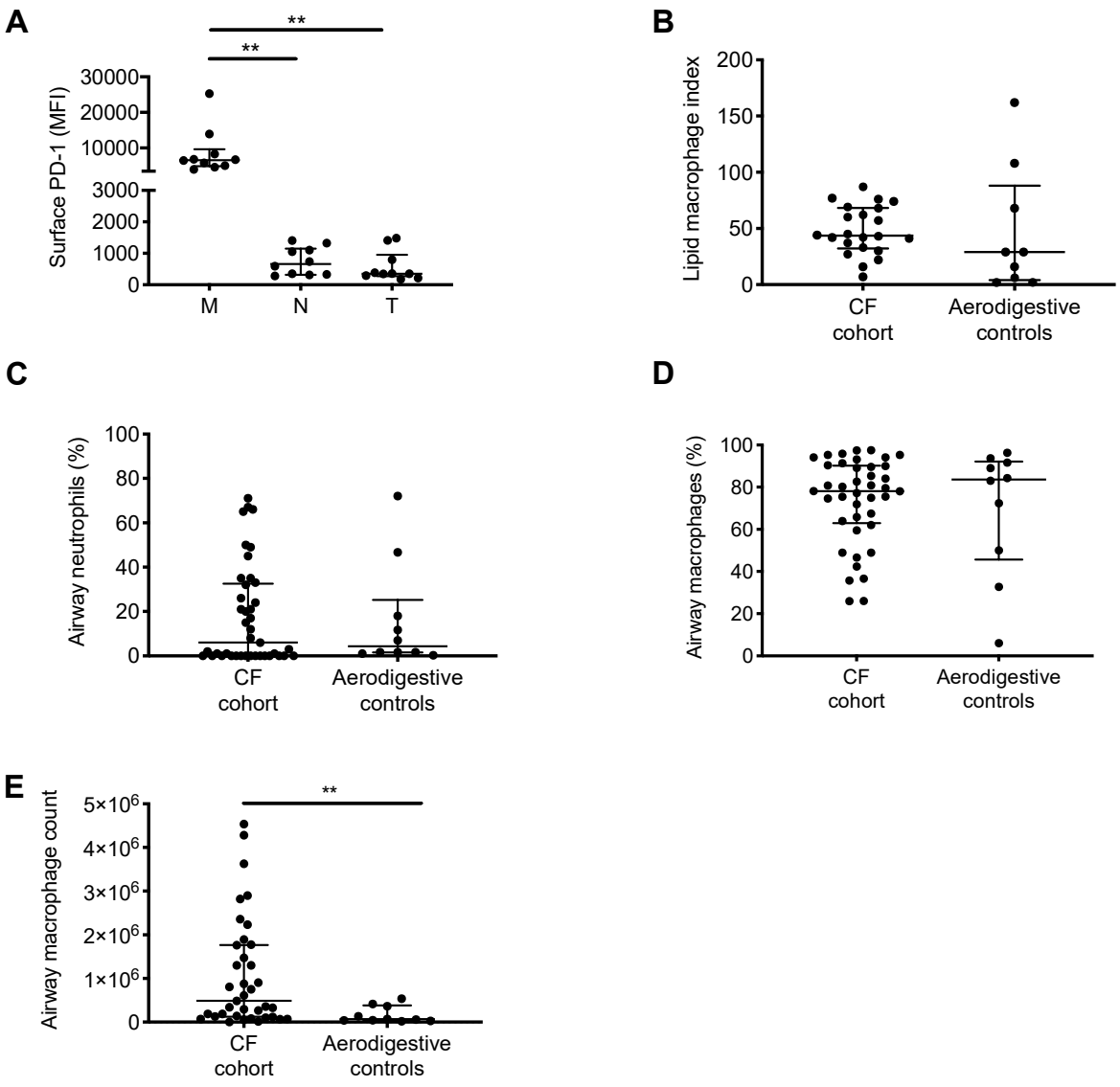

Figure 2.5.7. CF and age-matched disease controls show similar airway immune cell profiles. PD-1 expression on age-matched disease control airway macrophages (M), neutrophils (N), and T cells (T) **(A)**. The two cohorts did not differ in their lipidation index **(B)**, neutrophil percentage **(C)**, or macrophage percentage **(D)**, but CF patients showed higher macrophage counts than non-CF disease control **(E)**. Data are represented as median and interquartile range. Statistical analyses were performed by the Wilcoxon rank sum test, ** $p<0.01$.

The significant increase in PD-1 expression on CF airway macrophages in the presence of infection compared to non-CF aerodigestive controls (**Figure 2.5.8A**) suggests differential regulation of this pathway in CF airway disease. Clustering analysis showed that PD-1 expression was upregulated uniformly when comparing infected and non-infected subgroups (**Figure 2.5.8B**), suggesting that infection with pro-inflammatory pathogens does not correlate with the modulation of PD-1 expression on any particular SPADE-defined subset of airway macrophages. Interestingly, among all surface markers included in our cytometry panel, PD-1 emerged as the most significant feature in the airway macrophage population discriminating between infected and non-infected children with CF ($p < 0.0001$, paired sample t-test). None of the other surface markers listed as significant discriminators between the two groups reproduced the uniform pattern observed with PD-1. Furthermore, differences in airway macrophage cell frequencies between the infected and not infected groups were observed (**Figure 2.5.8C**). Out of the 100 clusters in the SPADE tree, 52 clusters, matching the lower PD-1 expression originally shown in **Figure 2.5.1B**, were more prevalent in the non-infected group. Taken together, these results show that while certain airway macrophage subsets become more prevalent during infection, all airway macrophages upregulate surface PD-1 expression upon presence of pro-inflammatory pathogens.

PD-1 blockade in CF airway cells improves bacterial clearance

Next, we investigated whether presence of pro-inflammatory bacteria could modulate PD-1 expression on airway macrophages. To this end, we designed a short-term culture assay in which fresh BAL fluid samples from CF children were collected (demographics in **Table 2.4.3**), minimally processed to remove endogenous bacteria and incubated with the pro-inflammatory

pathogen *P. aeruginosa*. Co-incubation with bacteria did not change overall level of PD-1 expression on macrophages or the frequency of cells positive for PD-1 compared to cells not exposed to bacteria in vitro (**Figure 2.5.8D-E**). To determine whether surface PD-1 on airway immune cells was active and influencing cell behavior we leveraged the same short-term culture assay and assessed bacterial killing in the presence or absence of PD-1 signaling blockade using a combination of a PD-1 blocking antibody and treatment with SHP099 (inhibitor of the PD-1-associated phosphatase SHP2). In a subset of 8 primary BAL fluid samples from children with CF with sufficient leukocyte yield to conduct this assay, bacterial killing significantly improved upon PD1 blockade (**Figure 2.5.8F**), suggesting that surface PD-1 is indeed active in airway leukocytes in CF and that its activity reduces bacterial clearance.

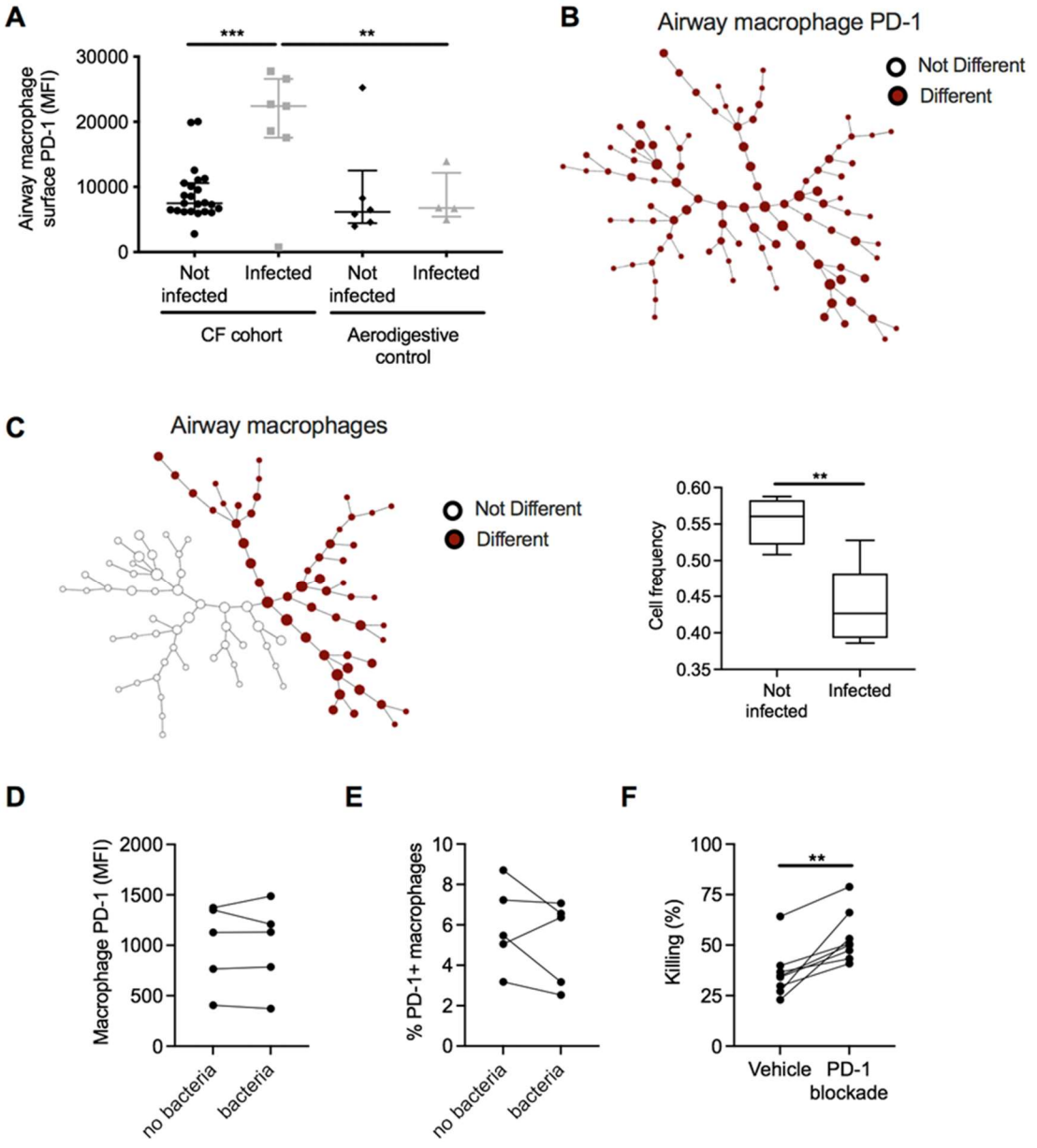

Figure 2.5.8. PD-1 expression and function in CF airway macrophages associates with airway infection. Children with CF showed higher PD-1 expression on airway macrophages upon infection compared to non-CF disease control children (N = 22 and N = 10, respectively). Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as ** for p< 0.01, *** for p<0.001 **(A)**. SPADE analysis of CF airway macrophages shows that the difference in PD-1 expression levels measured between infected vs. non-infected CF children is due to

uniform PD-1 modulation across all macrophage subsets (dark red) **(B)**, and changes in frequency in defined cell clusters **(C)**. Subset size reflects the number of macrophages with similar levels of marker expression. BAL fluid leukocytes from eight young children with CF were treated *ex vivo* with anti-PD1 (antibody) combined with a SHP2 inhibitor (SHP099) while co-incubated with *P. aeruginosa* for 1 hour **(D-F)**. Expression of PD-1 on macrophages and frequency of PD-1% macrophages was compared in samples with and without coincubation. Subject-matched data points are connected and analyzed by the Wilcoxon rank sum test (N = 5, no differences). Killing was determined by CFU counts (N = 8). Treatment effectiveness was calculated by subtracting the effect of therapy on bacteria alone and killing capacity of the cells without treatment. Subject-matched data points are connected, and analyzed by the Wilcoxon rank sum test with significance levels indicated as ** for $p< 0.01$.

Finally, we investigated if PD-1 expression was dependent on CFTR function using our in vitro monocyte TM model (**Figure 2.5.6).** Monocytes from healthy control (CFTR-competent) donors were incubated with CFTR inhibitor 172 and monocytes from CF (CFTR-defective) donors were incubated with elexacaftor/tezacaftor/ivacaftor, then transmigrated to LTB4 or CFASN with the same drugs present. Neither treatment significantly impacted expression of PD-1 or PD-L1 in either TM condition, though some donor variability was observed (**Figure 2.5.9**). Overall, our findings are consistent with the notion that the CF airway microenvironment, rather than intrinsic differences in CFTR function in monocytes/macrophages, leads to a change in poise in part linked to PD-1 signaling. This change is concomitant with early neutrophilic inflammation and a decrease in bacterial killing capacity, opening the path for chronic CF airway disease (**Figure 2.5.10**).

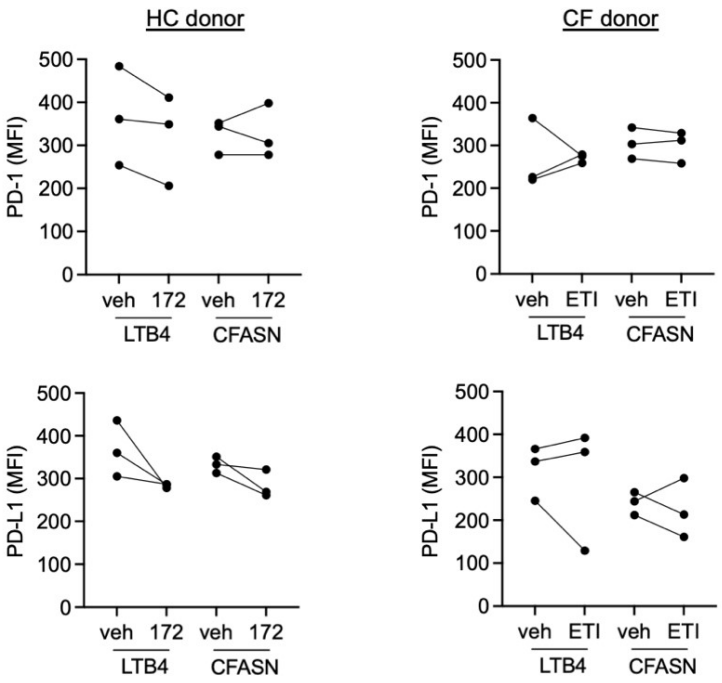

Figure 2.5.9. Pharmacological modulation of CFTR expression in healthy donor or CF donor monocytes does not directly impact expression of PD-1 or PD-L1. Monocytes were isolated from the blood of healthy control (HC, n = 3) or CF donors (n = 3) and transmigrated to either LTB4 control chemoattractant or CFASN. HC monocytes were pre-treated with either vehicle control (veh) or CFTR-inhibitor 172 (172, 10 μM), and CF monocytes with vehicle or elexacaftor/tezacaftor/ivacaftor (ETI, each at 5 μM) for 1 hour prior to TM. The same drugs were added to the TM wells for respective donors. Transmigrated monocytes were harvested and stained for flow cytometry.

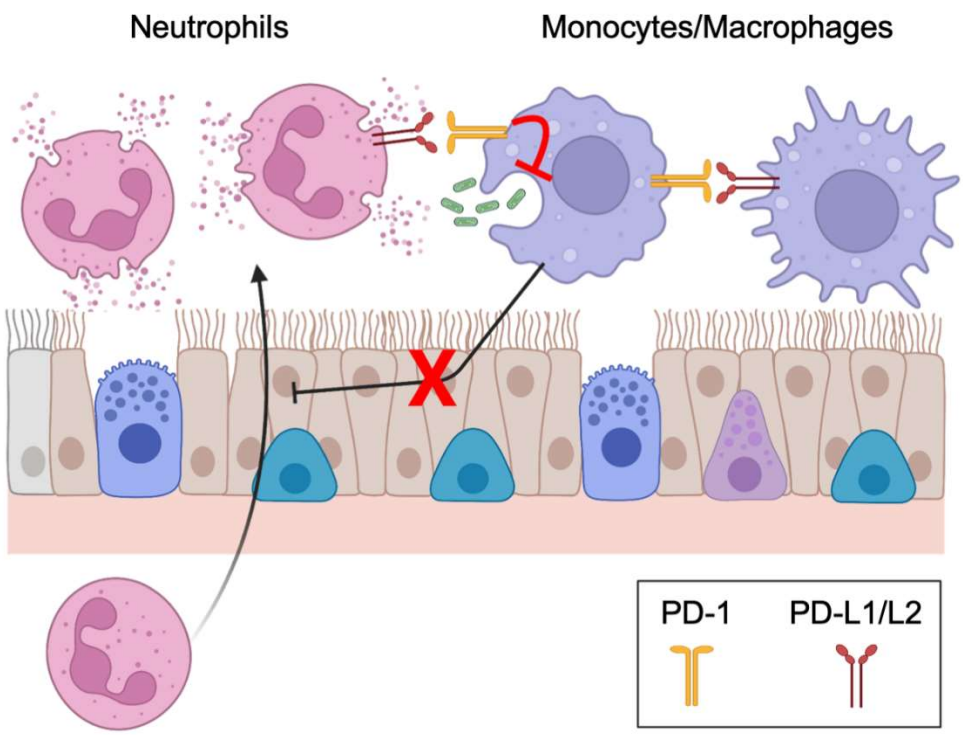

Figure 2.5.10. Pathological model of PD-1 signaling in CF airway monocytes/macrophages. Increased PD-1 expression on CF monocytes/macrophages increases with age, and associates with chronic neutrophilic inflammation and hyperexocytosis and bacterial infection in the airways.

2.6 Discussion

Immune responses in CF airways depend on close interactions between the CF epithelium, which shows enhanced pro-inflammatory signaling [43, 44], resident macrophages and recruited neutrophils, mucus, and colonizing pathogens, which evolve over the course of the disease [45, 46]. Macrophages and neutrophils in particular collaborate and modulate each other during responses [47, 48], by dynamically sharing released factors such as NE [49], implementing bacterial clearance [50], and orchestrating gene expression [51]. Taken together, data presented here are consistent with a model in which airway macrophages in children with CF undergo apparent exhaustion, marked by increased PD-1 expression, which associates with neutrophilic inflammation and infection with pro-inflammatory pathogens, resulting in significant lung damage. However, while at later stages of disease this phenomenon may be more widespread to the whole lung, in early CF lung disease, upregulation of PD-1 on airway macrophages likely occurs only in discrete diseased areas.

We recently showed that presence of a pathogenic neutrophil subset in CF airways, which actively exocytoses NE-rich granules, is detectable prior to the sustained presence of pro-inflammatory pathogens, and whose prevalence is not influenced by a patient's infection status [20]. Here, we showed that macrophage PD-1 expression was not directly modulated by presence of pro-inflammatory bacteria in vitro, but expression was elevated in children with CF who had detectable airway infection. Further, the differential expression of PD-1 observed between patients with CF and the control cohort could be due to epigenetic reprogramming induced by the local microenvironment [52]. Macrophages have previously been proposed as potential therapeutic targets for CF airway disease [53]. However, studies addressing the impact of new CFTR modulators and correctors have yet to fully address their influence on intrinsic defects of

CF airway macrophages [54]. In that context, there is a clear need for the development of new therapeutic agents aiming at the direct modulation of extrinsic regulatory pathways in macrophages, which may reinvigorate pathogen clearance and delay neutrophil takeover seen in the early phase of pro-inflammatory pathogen infection and lung damage in children with CF. Here, we showed preliminary evidence that PD-1 blockade in airway leukocytes improved bacterial clearance. Because PD-1 is known to play a key role in controlling macrophage responsiveness in several conditions, modulation of PD-1 signaling may be of interest in airway diseases other than CF which also feature chronic bacterial infections [13, 15, 16].

Further studies are needed to address downstream effects of PD-1 signaling in CF airway macrophages, although prior evidence linked PD-1 signaling to changes in autophagy [55], which controls pathogen clearance by macrophages [56]. Moreover, while *P. aeruginosa* did not change PD-1 levels on macrophages, it may modulate macrophage responsiveness by altering expression of the PD-1 ligand PD-L1, which would increase the likelihood of PD-1 inhibitory signaling in these cells [57]. While we did not observe a direct link between CFTR function and PD-1 expression on airway-conditioned monocytes in vitro, further studies are needed to determine if altered PD-1 signaling and/or intrinsic effects of CFTR deficiency in airway macrophages cause an inability to clear pathogens in CF children. While it has been previously shown that CFTR mutations can affect in vitro responses of monocyte-derived macrophages [58], less is known about CFTR function in resident human airway macrophages.

Limitations of study

In this study we consider the transition from macrophage dominance to neutrophil dominance in the lung, but we do not define specific subsets of macrophage-lineage cells, such

as inflammatory monocytes, monocyte-derived macrophages, and alveolar macrophages in BAL samples. Future studies implementing new techniques such as CITE-Seq are needed to pursue deeper investigation of CF airway monocyte/macrophage subsets revealed in our initial flow cytometry and SPADE analysis, with a particular focus on changes in PD-1 expression upon infection and neutrophil recruitment. Also, while this study shows a novel immunomodulatory pathway present in CF airway disease, it does not delineate whether the enhanced PD-1 expression on airway macrophages observed in patients with detectable infection is a cause or a consequence of the progressive neutrophil takeover. Indeed, a limitation in both this and prior [20] studies is that sensitive bacterial metagenomic analyses were not available, such that we cannot fully state that given patients were free of pro-inflammatory pathogens even if clinical microbiology cultures were negative. However, the in vitro modulation of PD-1 expression by neutrophil-derived factors did occur in the absence of pathogens or pathogen-derived molecules, suggesting a host-host interaction between CF airway neutrophils and monocytes/macrophages. Given limitations in BAL volume and cell number from pediatric subjects, we made a concerted effort to maximize use of each sample, but were not able to assess all possible targets of interest. One example is IFN-α which has been reported to induce PD-1 expression in macrophages and should be assessed in future studies. Even though our in vitro data suggest little acute impact of CFTR modulator therapy on lung-recruited monocytes, it will also be interesting to assess lung macrophage PD-1 expression and poise in BAL from infants with CF as CFTR modulator therapy becomes available to toddlers and infants.

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Chapter 3

Acute pulmonary exacerbations in early cystic fibrosis lung disease are associated with CD3 and PD-1 modulation on lung T cells

3.1 At-a-glance commentary

Current scientific knowledge on the subject: Acute pulmonary exacerbations (APEs) are complex episodes of increased lung disease severity experienced by people with CF, often instigated by bacterial infections. Treatment with intravenous antibiotics usually resolve these events. However, each APE can lead to incremental loss of lung function and increases the risk for future APEs. While pro-inflammatory cytokines are associated with APEs, little is known about how immune cells in the airway or circulation respond during these events.

What this study adds to the field: In this study, we compared young children with CF who: 1) have no history of hospitalization for APEs; 2) have prior occurrence of APEs but are currently stable; and 3) are currently hospitalized for an APE. We identified signatures of T-cell activation during APEs that are in stark contrast to the later stages of CF lung disease where T cells are potently inhibited by infiltrating neutrophils. Furthermore, we identified an increased frequency of circulating neutrophils and elevated G-CSF concentration in plasma among patients with a current APE, providing insights into why APEs may accelerate lung function decline.

3.2 Abstract

Background: In chronic CF lung disease, neutrophilic inflammation and T-cell inhibition occur concomitantly, partly due to neutrophil-mediated release of the T-cell inhibitory enzyme Arg1. However, the onset of this tonic inhibition of T cells, and the impact of APEs on this process remains unknown.

Methods: Children with CF aged 0-5 years were enrolled in a longitudinal cohort study at Emory University. Blood (n = 38) and BAL fluid (n = 20) were collected at stable outpatient clinic visits or inpatient APE visits and analyzed by flow cytometry (for immune cell presence and phenotype) and 20-plex chemiluminescence assay (for immune mediators). Patients were sorted by APE history including none, prior to stable visit, or current APE.

Results: APEs were associated with increased concentration of both pro- and anti-inflammatory mediators in BAL and increased neutrophil frequency and G-CSF in circulation. APE BAL samples showed a trend for an increased frequency of hyperexocytic GRIM neutrophils previously identified in chronic CF. Expression of the T-cell receptor associated molecule CD3 and the inhibitory programmed death-1 (PD-1) receptor was significantly decreased and increased, respectively, on BAL vs blood T cells from total samples. There was no difference in CD3 or PD-1 expression on blood T cells according to APE status, but CD3 was decreased and PD-1 was increased on BAL T cells from APE samples.

Conclusions: These data suggest that airway T cells are engaged during early-life APEs prior to the onset of chronic neutrophilic inflammation. However, increased neutrophil frequency in circulation and trend towards increasing frequency of GRIM neutrophils in the airway offers new insights into the mechanisms of how childhood APEs may accelerate inflammation and lung function decline and increase the risk for future APEs.

3.3 Introduction

APEs are a frequent occurrence for people with CF, with up to 20,000 episodes recorded in the United States each year in a population of about 50,000 patients [1]. Exacerbations are often promoted by pulmonary infections and require treatment with intravenous antibiotics. These events are characterized by constriction of the airways, reduced lung function, increased coughing, and mucus obstruction [2]. APEs can be detrimental even for children with CF who do not yet present major symptoms of lung disease, as repeated exacerbations have been shown to accelerate lung function decline [3]. Pro-inflammatory cytokine secretion has been reported in numerous studies as a consequence of APEs [4-6], particularly the potent neutrophil chemoattractant CXCL8 [7, 8]. Osteoclast activity and total circulating leukocyte counts are elevated during APEs and have been shown to decrease following antibiotic treatment [6, 7], but little is known about phenotypic or functional changes that immune cells undergo in the airway during exacerbations. There are several limitations common to prior studies of inflammatory markers associated with APEs, including discrepancies in criteria used to define APEs in different countries, differences in sample collection and processing procedures, and the time at which samples are collected between the advent of APEs and the inception of high-impact treatment [9].

As CF lung disease progresses, infiltrating neutrophils become dominant in the airway lumen and suppress the functions of other immune cell populations including macrophages [10] and T cells [11]. Early life APEs may offer the opportunity to study non-neutrophil populations in the airway before neutrophils have begun to dominate this microenvironment and alter their activity. Furthermore, these events may prefigure impending neutrophilic inflammation, since a recent APE occurrence is the most important risk factor for occurrence of future APEs [12].

To gain further understanding of both soluble and cellular markers of inflammation related to APEs, we obtained blood and BAL samples from young children with CF at stable clinic visits and hospitalizations for treatment of an APE. Some of these subjects enrolled for collection of samples at stable clinic visits had prior hospitalizations for an APE, but all subjects in the study were in the early stages of CF lung disease. Through measurement of cytokines, NE, and leukocyte phenotypes we demonstrate that early life APEs are not characterized by elevated biomarkers of neutrophilic inflammation in the airway. Rather, early life APEs associate with changes in neutrophil poise in the circulation which may foreshadow future neutrophilic inflammation in the airways. Furthermore, we show evidence for T-cell activation during early-life APEs, which differs from later stages of CF lung disease when T cells in the airways are strongly inhibited by neutrophils [11].

3.4 Methods

Human subjects and samples

A total of 45 children with CF between the ages of 0-5 years old were enrolled for collection of blood and/or BAL samples as part of the IMPEDE-CF study at Emory University and Children's Healthcare of Atlanta. All aspects of subject enrollment and sample collection were approved by the Emory University Institutional Review Board (IRB00097352). Subject demographics are summarized in **Table 3.4.1**. Samples were collected from subjects coming to the clinic for a planned stable visit or from patients admitted to the hospital an APE. Patients were classified into three groups based on their history of APE as follows: 1) None- samples collected at a stable clinic visit, with no history of hospitalization for treatment of APE; 2) Prior- samples collected at a stable clinic visit but subject had previously been hospitalized for treatment of an APE; 3) Current- samples were collected during current hospital admission for an APE. As is common for pediatric studies, not all samples/outcomes could be obtained for all visits, due to limitations in material available to research. Sample collection totals are recorded in **Table 3.4.2**, with a summary of assays performed provided in **Table 3.4.3**.

	Sample collection				Sex		CFTR mutation			Modulators	
APE status	Subjects enrolled	Mean age (years)	Blood samples	BAL samples	Male	Female	F508 HO	F508 HZ	Other	IVA	LUM+ IVA
None	21	3.5	19	8	12	9	10	9	2	0	1
Prior	15	3.8	12	7	13	2	5	5	5	1	3
Current	9	3.9	7	5	7	2	6	3	0	0	0

Table 3.4.1. Demographics of enrolled subjects. A total of 38 blood samples and 20 BAL samples were collected at 45 clinic visits. Subjects were divided by APE history, including stable clinic visit with no history of exacerbation (None), stable clinic visit but previously hospitalized for APE (Prior), and currently hospitalized for treatment of APE (Current). HO: homozygous; HZ: heterozygous; IVA: ivacaftor; LUM: lumacaftor.

Sample collection and processing

Venous blood was collected using K2 EDTA tubes which were first centrifuged at 400 x g to separate plasma from blood cells. The plasma was then removed for further centrifugation at 3000 x g to remove platelets and remaining red blood cells. The debris-free plasma was frozen at -80°C and banked for later quantification of cytokines. The blood cell pellet was washed with 10 mL of sterile ice-cold phosphate-buffered saline with 2.5 mM EDTA added (PBS+EDTA). After centrifugation and aspiration of supernatant the blood cell pellet was resuspended to its original volume using PBS+EDTA. The washed blood pellet was used for flow cytometry analysis of blood leukocytes.

BAL was performed during clinically-indicated bronchoscopy procedures under general anesthesia at stable visits and hospitalizations for APEs. A 3.1 mm bronchoscope (BF-XP190, Olympus, Japan) with a 1.2 mm suction channel was used to retrieve BAL fluid. All BAL

samples were collected from the right middle lobe by instillation and aspiration of sterile 0.9% saline (1 mL/kg up to a maximum volume of 20 mL per aliquot). A total of 2 or 3 aliquots were instilled depending on the yield, with the first aliquot reserved for clinical microbiology. Microbiological cultures and cytology require 7 mL of BAL fluid, with additional volumes transported on ice to the research laboratory for immediate processing. EDTA was added to each BAL sample for a final concentration of 2.5 mM. Blood and BAL sample yields are summarized in **Table 3.4.2**. BAL samples were processed as described previously [13]. In brief, the sample was homogenized by passing through an 18-gauge needle for 12 cycles. The sample was centrifuged at 800 x g for 10 minutes at 4°C and the supernatant was removed for a further centrifugation at 3000 x g to yield debris-free supernatant. The BAL cell pellet was washed in PBS+EDTA and counted using fluorescent microscope with ethidium bromide + acridine orange staining. BAL supernatants were frozen at -80°C and banked for later quantification of cytokines and NE, and cells were stained for analysis by flow cytometry.

Subject	Blood		BAL	
	Whole blood (mL)	Plasma (mL)	Total volume (mL)	Live leukocytes
1	4.80	2.00	3.00	3.60E+05
2	3.50	1.40	8.36	1.90E+05
3	3.20	1.70	10.5	1.50E+06
4				
5	3.40	1.40	4.20	4.40E+05
6	3.50	1.80		
7	3.10	1.20	2.00	2.00E+06
8	4.50	2.30	1.40	2.70E+06
9	3.50	1.50		
10	2.80	1.10		
11	3.50	1.20	1.20	9.00E+04
12	2.50	0.90		
13	3.50	1.20	2.00	9.30E+04
14	2.50	1.00		
15	4.20	2.20		
16	3.60	2.00	1.35	1.08E+07
17	3.40	1.30		
18	3.50	1.70	2.70	2.00E+04
19				
20	2.05	1.00	4.45	5.00E+05
21	2.80	1.34	3.19	7.20E+05
22	3.10	1.40		
23	4.50	2.00	1.74	1.00E+06
24	2.43	0.95	2.40	4.50E+06
25	2.40	0.84		
26				
27	2.40	0.87		
28	2.90	1.22	3.80	2.40E+05
29	2.90	1.20	3.70	5.30E+05
30	2.60	1.00	0.35	4.20E+05
31				
32	2.80	1.90	3.20	7.30E+05
33	2.50	0.92		
34	7.60	3.40	3.53	4.00E+05
35	1.30	0.60		
36	2.40	1.20		
37	4.00	2.10	2.00	1.34E+06
38	2.45	1.00		
39	3.00	1.00		
40	3.00	1.00		
41				
42	3.00	1.10		
43	3.00	1.15		
44	3.00	1.50	3.50	1.60E+05
45				

Table 3.4.2. Sample collection totals. 45 clinic visits were conducted for collection of blood and/or BAL. Not all samples were able to obtained.

Subject	Clinical data	Soluble mediators			Flow cytometry				SPADE
		Plasma Cytokines	BAL cytokines	BAL NE	Blood P14	Blood P15	BAL P14	BAL P15	Mac P15
1	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X
4	X								
5	X	X	X	X	X	X	X	X	X
6	X	X			X	X			
7	X		X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X
9	X	X			X	X			
10	X	X			X	X			
11	X	X	X	X	X	X		X	
12	X	X			X	X			
13	X	X	X	X	X	X		X	X
14	X	X			X	X			
15	X	X							
16	X	X	X	X					
17	X	X			X	X			
18	X	X	X	X	X	X		X	X
19	X								
20	X	X	X	X	X	X		X	X
21	X	X	X	X				X	X
22	X	X				X			
23	X	X			X	X			X
24	X	X	X	X	X			X	X
25	X	X							
26	X								
27	X	X			X	X			
28	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X
31	X								
32	X	X	X	X	X	X	X	X	X
33	X	X				X			
34	X	X	X	X		X	X	X	X
35	X	X							
36	X	X			X	X			
37	X	X	X	X	X	X	X	X	X
38	X	X							
39	X	X							
40	X	X							
41	X								
42	X	X							
43	X	X							
44	X	X	X	X					
45	X								

Table 3.4.3. Total assays performed for blood and BAL samples. Soluble mediator assays, flow cytometry and SPADE analysis were conducted for blood and BAL samples where indicated by an "X.

Cytokines

A 20-plex panel of inflammatory response mediators was measured in plasma and BAL using a high-sensitivity chemiluminescence assay (U-PLEX, Meso Scale Diagnostics) according to the manufacturer's protocol. Analytes included cytokines related to neutrophil recruitment/activation (CXCL1, CXCL5, CXCL8, CXCL10, CXCL11, TNF-α), monocyte/macrophage recruitment/activation (CCL2, CCL4, IFN-γ, IL-6, VEGF-A), the IL-1 family (IL-1α, IL-1β, IL-18), the CSF family (G-CSF, M-CSF, GM-CSF), and anti-inflammatory mediators (IL-1RA, IL-10, TNFSF10). It is important to note that these are generalized groupings and individual cytokines may have different functions or classifications in specific contexts. For cytokine concentrations that fell below the lower limit of detection, a value of half the lower limit was assigned. These values are represented as open symbols in related figures. Statistical comparisons were not performed between groups if more than half of the data points from one group consisted of imputed values.

NE activity

Extracellular NE activity in BAL was measured by Förster resonance energy transfer (FRET) assay using the NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described [14-16].

Flow cytometry

Analysis of leukocytes in blood and BAL fluid by flow cytometry was performed in FlowJo as described previously [13]. A gating strategy was used to identify neutrophils, monocytes/macrophages, and T cells (**see Figure 4.4.2**).

Cells were pre-stained with Fc block to prevent non-specific binding of antibodies (Biolegend #422302) and calcein violet for viability for 10 minutes, followed by staining with antibodies for surface proteins for 20 minutes. Targets included CD3, CD16, CD36, CD41a, CD45, CD63, CD66b, CD115, CD304, EGFR, surface NE, PD-1, PD-L1, and Siglec-8. All incubations were performed on ice in the dark. Cells were washed with PBS+EDTA and fixed in BD Phosflow Lyse/Fix Buffer (BD #558049) by incubating overnight in the dark at 4ºC. The next day fixed cells were washed with PBS+EDTA and stored at 4ºC in the dark until acquisition. Samples were acquired in batches when possible but all were acquired within 2 weeks of staining. All samples were acquired on a BD LSRII or BD FACSymphony which were calibrated using 6-peak Rainbow Calibration Particles (Biolegend #422901), as previously described [17], to ensure consistent fluorescence output. Compensation was computed using single-stained UltraComp eBeads (Invitrogen #01-2222-42). All compensation, gating, and calculation of median fluorescence intensity (MFI) and cell frequencies was performed using FlowJo V9.9.5 (BD). A threshold of at least 50 events after all gating steps was established for populations to be considered reliable for analysis of fluorescence parameters.

Subset of flow cytometry analysis by SPADE

Following analysis of flow cytometry data in FlowJo, the FCS files for gated populations were extracted and analyzed with the SPADE clustering algorithm [18, 19]. Once the files were imported, all cells were combined and used to generate trees consisting of 100 nodes following similarity of surface marker expression. Only samples containing at least 1,000 cells in the population were included for SPADE analysis. Samples were identified by APE status (None,

Prior, Current) and SPADE was used to compare the frequency of cells from samples in each group within regions of the tree.

Statistical analysis

Data were analyzed in Prism (version 8; GraphPad Software) to conduct group comparisons, using nonparametric statistical tests due to the small sample size. Principal component analysis was conducted using MATLAB.

3.5 Results

Current APEs are associated with pulmonary infections and elevated BAL neutrophil frequency

We assigned each subject to one of three groups based on APE history as described in **3.4 Methods**, including None, Prior, and Current. We then compared airway colonization by pro-inflammatory pathogens and neutrophil frequency in BAL among these groups. Subjects in the None and Prior groups were evenly divided between infected and non-infected (10 uninfected vs. 11 infected, and 7 uninfected vs. 8 infected, respectively), while the majority of subjects currently experiencing an APE had detectable airway colonization (2 uninfected vs. 7 infected). We then compared airway neutrophilia in each group of subjects, using 10% as the threshold for determining elevated neutrophil frequency in BAL. Subjects with no history of APE were evenly divided (3 with <10% vs. 4 with >10%). However, the majority of subjects with prior and current APE (1 with <10% vs. 6 with >10% and 1 with <10% vs. 3 with >10%, respectively) had elevated neutrophil frequencies (**Table 3.5.1**).

	Infection status		Neutrophil frequency in BAL	
APE status	Uninfected	Infected	<10%	>10%
None	10	11	3	4
Prior	7	8	1	6
Current	2	7	1	3

Table 3.5.1. APEs are associated with airway infection and neutrophilia. Infections were defined as 2 out of 4 positive throat swab cultures with a pro-inflammatory pathogen. Neutrophil frequency in BAL was determined by flow cytometry. Subjects were divided by APE history, including stable clinic visit with no history of APE (None), stable clinic visit but previously hospitalized for APEs (Prior), and currently hospitalized for treatment of an APE (Current).

Pro- and anti-inflammatory cytokines are elevated in BAL and plasma during APEs

Previous studies have shown inflammatory response cytokines to be elevated in response to APEs and declining after treatment with antibiotics [4-8]. However, much of these data are from adolescent or adult subjects with less description of young children in the early stages of CF lung disease. We quantified 20 immune mediators in BAL and plasma and compared concentrations between subjects from None, Prior, and Current APE categories. Six neutrophil chemoattractants were significantly elevated in Current compared to None samples, including CXCL1, CXCL8, CXCL10, CXCL11, IL-6, and IL-18. In addition, CXCL8, IL-6, and IL-18 were significantly higher in Current vs Prior, but no difference between None and Prior were observed (**Figure 3.5.1**).

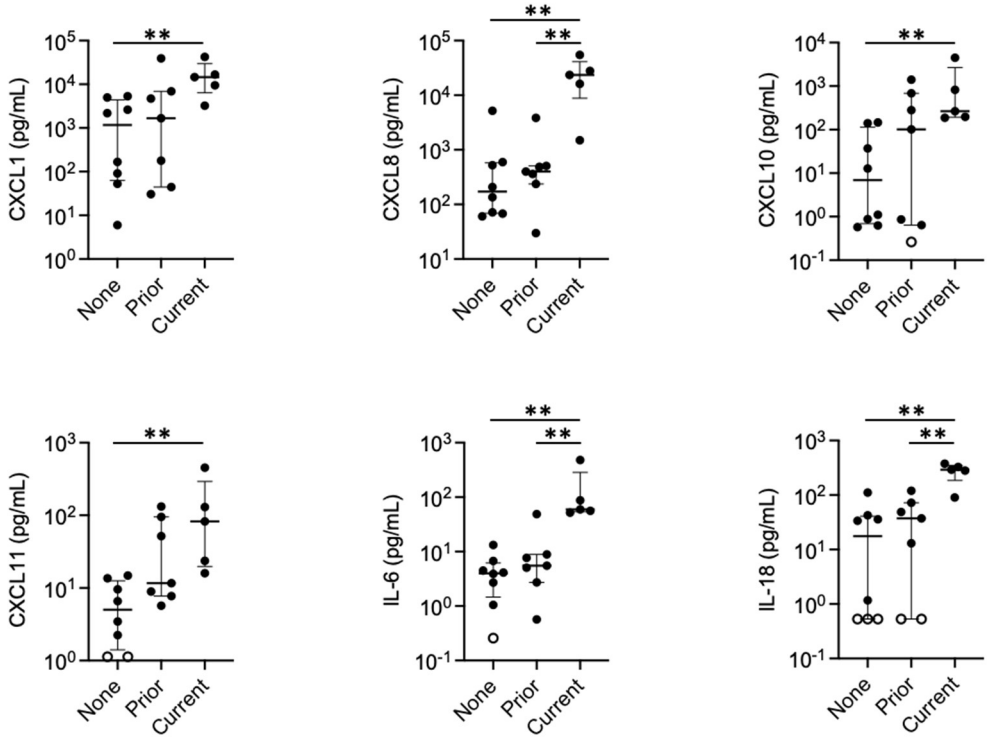

Figure 3.5.1. Neutrophil chemoattractants are elevated in BAL during APEs. A panel of 20 inflammatory cytokines was measured in BAL by multiplexed chemiluminescent assay and compared between subjects who had no history of APE (None, n = 8), a previous hospitalization for an APE (Prior, n = 7), or currently experiencing an APE (Current, n = 5) using the Mann-Whitney test. An imputed value of ½ the lower limit of detection was assigned for data points below the limit of detection and are represented by an open symbol. Significant differences are indicated as **$p \leq 0.01$ and ***$p \leq 0.001$. Non-significant comparisons are not shown.

We then conducted an unsupervised clustering by principal component analysis, excluding IFN-γ, IL-10, and CCL4 because many data points were below the limit of detection. We observed a separation of the Current APE subjects from the None and Prior, with the neutrophil chemoattractants CXCL1 and CXCL8 driving this difference (**Figure 3.5.2**).

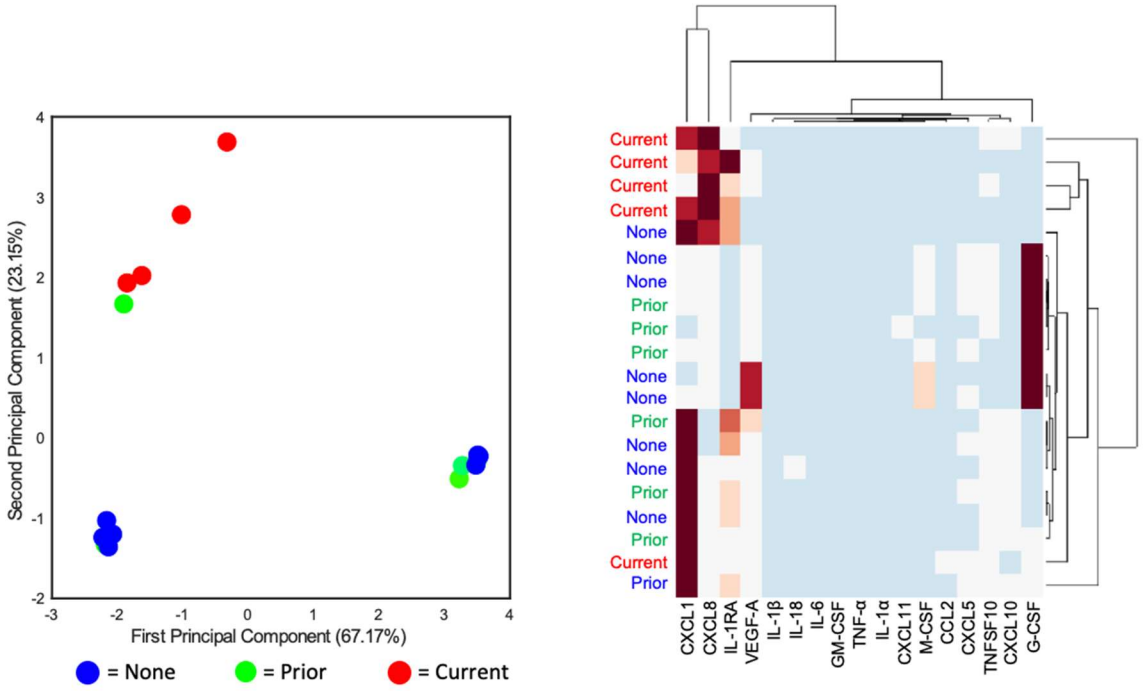

Figure 3.5.2. CXCL8 is a distinguishing signature of APE. Principal component analysis of BAL cytokines from subjects with no APE history (n = 8), a prior event (n = 7), or current APE (n = 5) demonstrates separation of the Current APE subjects, which is mainly driven by CXCL8.

We also observed increased concentrations in monocyte/macrophage-related and anti-inflammatory cytokines. CCL2, CCL4, and IFN-γ were elevated in Current compared to None and Prior groups (**Figure 3.5.3A**). Similarly, IL-1RA and TNFSF10 were higher in Current compared to the other groups while IL-10 was measurable in all samples from the Current group but largely undetectable in the Prior and None groups (**Figure 3.5.3B**).

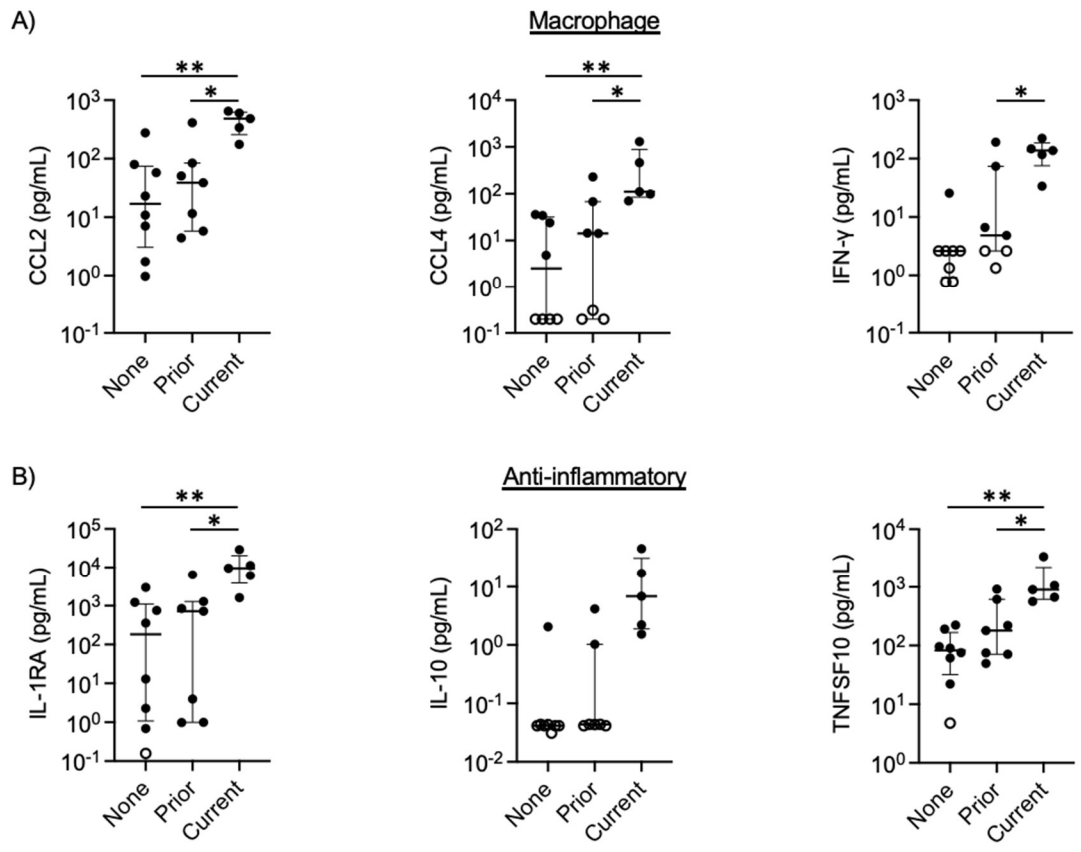

Figure 3.5.3. Macrophage-related (A) and anti-inflammatory (B) cytokines are elevated in BAL during APEs. A panel of 20 inflammatory cytokines was measured in BAL by multiplexed chemiluminescent assay and compared between subjects who had no history of APE (None, n = 8), a previous hospitalization for an APE (Prior, n = 7), or currently experiencing an APE (Current, n = 5) using the Mann-Whitney test. An imputed value of ½ the lower limit of detection was assigned for data points below the limit of detection and are represented by an open symbol. Significant differences are indicated as *$p \leq 0.05$ and **$p \leq 0.01$. Non-significant comparisons are not shown. Statistical comparison was not performed between groups for IFN-γ or IL-10 where the majority of points were below the limit of detection.

Next, we conducted the same comparisons in plasma samples. IL-1β was below the limit of detection in approximately half of the samples from the None and Prior groups, but was measurable in all but one sample from current APE. The concentration of IL-1β was comparable between the samples from each group that were within the limits of detection. Concentrations of IL-6, G-CSF, and IL-1RA were significantly higher in samples from the Current group compared to the None and Prior groups (**Figure 3.5.4**).

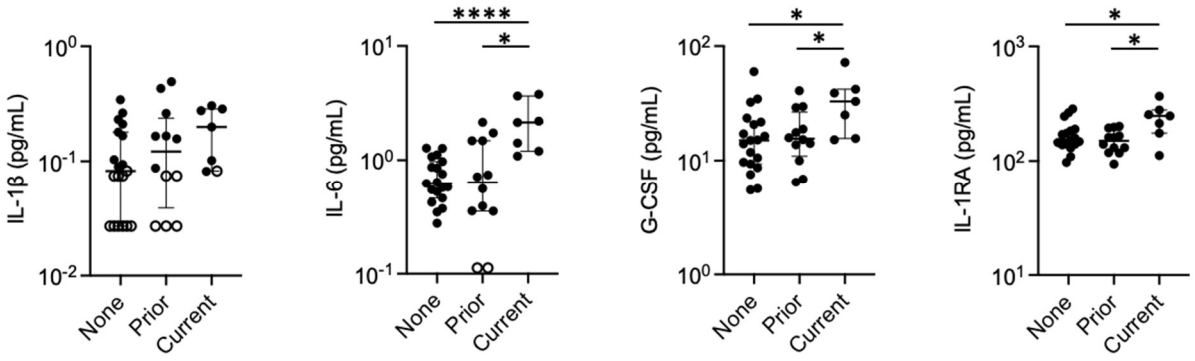

Figure 3.5.4. Neutrophil chemoattractant and anti-inflammatory cytokines are elevated in plasma during APE. A panel of 20 inflammatory cytokines was measured in plasma by multiplexed chemiluminescent assay and compared between subjects who had no history of APE (None, n = 19), a previous hospitalization for APE (Prior, n = 12), or currently experiencing APE (Current, n = 7) using the Mann-Whitney test. An imputed value of ½ the lower limit of detection was assigned for data points below the limit of detection and are represented by an open symbol. Significant differences are indicated as *$p \leq 0.05$ and ****$p \leq 0.0001$. Non-significant comparisons are not shown.

NE is not significantly secreted or scavenged during APEs

We quantified the activity of soluble NE in BAL and compared among the three groups of subjects and observed only a slight trend towards increased NE activity among the Current group compared to the None and Prior groups. We also measured surface-bound NE on BAL

neutrophils and monocytes/macrophages by flow cytometry but observed no significant differences between the groups (**Figure 3.5.5**).

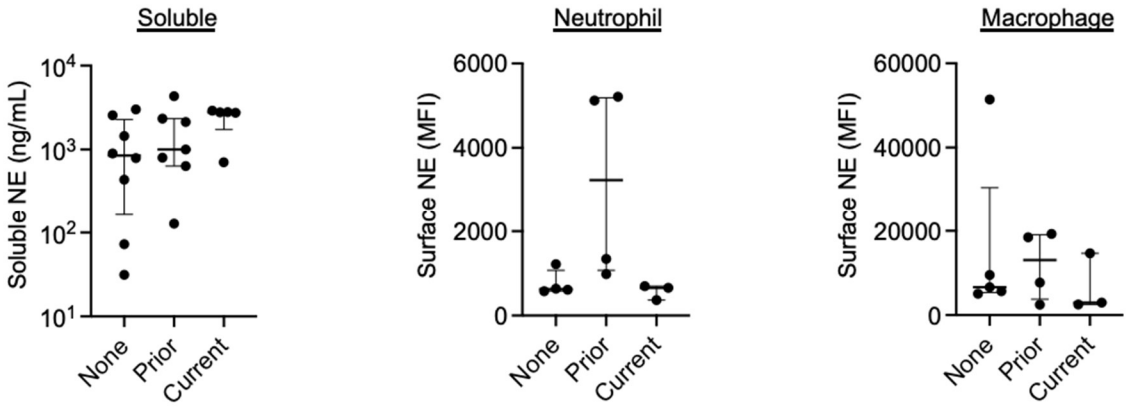

Figure 3.5.5. NE is not significantly secreted or scavenged during APE. Soluble NE activity was quantified in BAL using a FRET-based assay. Surface-bound NE on neutrophils and monocyte/macrophages from BAL was determined by flow cytometry. Comparisons between groups of subjects with no history of APE (None, n = 8 for soluble, n = 4 for neutrophil, n = 5 for monocyte/macrophage), a previous hospitalization for an APE (Prior, n = 7 for soluble, n = 4 for neutrophil, n = 4 for monocyte/macrophage), or currently experiencing an APE (Current, n = 5 for soluble, n = 3 for neutrophil, n = 3 for monocyte/macrophage) was conducted using the Mann-Whitney test (no significant differences).

Neutrophil frequency in circulation, but not the airway, increases during APE

We used flow cytometry to identify neutrophil, monocyte/macrophage, and T cell populations in blood and BAL and compared their frequencies out of total leukocytes in both fluids. We also calculated the proportion of neutrophils demonstrating the pathological GRIM phenotype defined as $CD63^{high}$ and $CD16^{low}$ [20], which have a major role in progression of lung disease [21]. The frequency of neutrophils in blood was significantly higher during APE

compared to subjects with no APE history, with a concomitant decrease in T-cell frequency but no differences for proportion of blood monocytes. There were no significant differences in airway leukocyte frequencies, although we noted a trend towards increased proportion of neutrophils endowed with the GRIM phenotype (**Figure 3.5.6**).

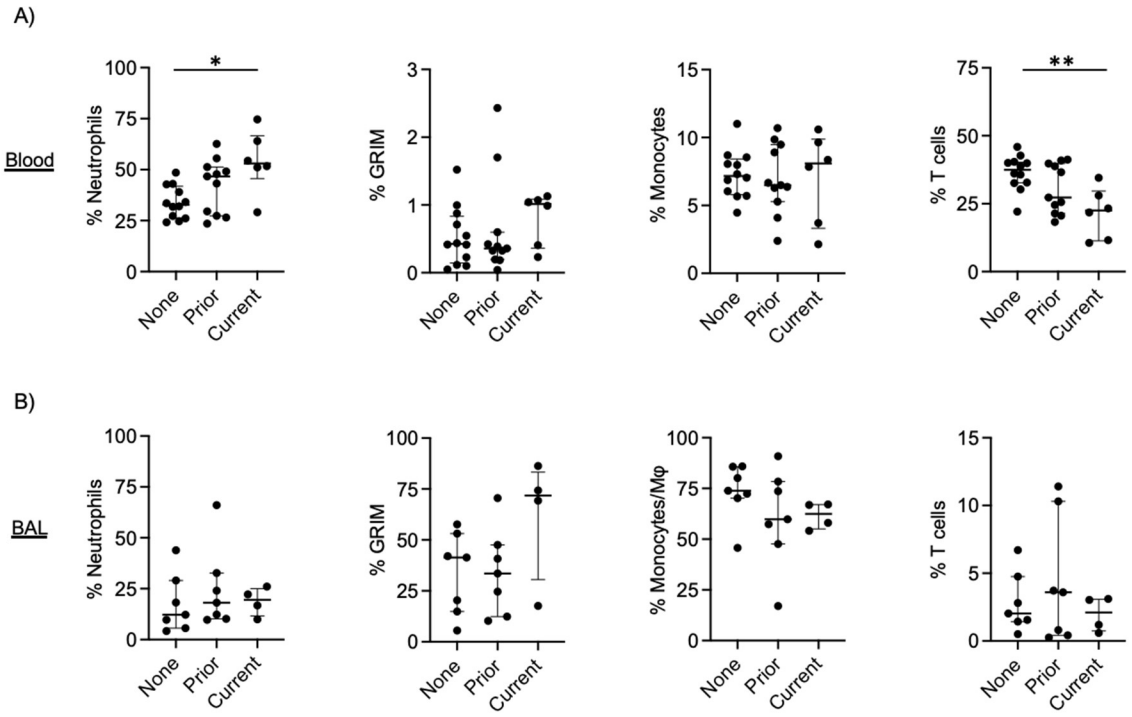

Figure 3.5.6. Neutrophil frequency in circulation, but not the airway, increases during APE. A flow cytometry gating strategy was developed to determine the frequency out of total leukocytes for neutrophils, monocytes/macrophages, and T cells in blood **(A)** and BAL **(B)**, as well as the proportion of neutrophils displaying the GRIM phenotype. Comparisons between groups of subjects with no history of APE (None, n = 12 for blood, n = 7 for BAL), a previous hospitalization for an APE (Prior, n = 11 for blood, n = 7 for BAL), or currently experiencing an APE (Current, n = 6 for blood, n = 4 for BAL) was conducted using the Mann-Whitney test with significant differences indicated as *$p \leq 0.05$ and **$p \leq 0.01$. Non-significant comparisons are not shown.

Clustering algorithm reveals phenotypic similarities among airway macrophages during APE

Next, we used the SPADE clustering algorithm [18, 19] to conduct a deeper investigation of BAL subsets. After using our gating strategy to identify airway monocytes/macrophages, we extracted these populations and assigned them to groups based on subject APE history. After constructing a tree using all cells included in the analysis, SPADE identified a region of the tree (51 out of 100 nodes) with a significantly higher frequency of cells from the Current vs. None groups (**Figure 3.5.7**).

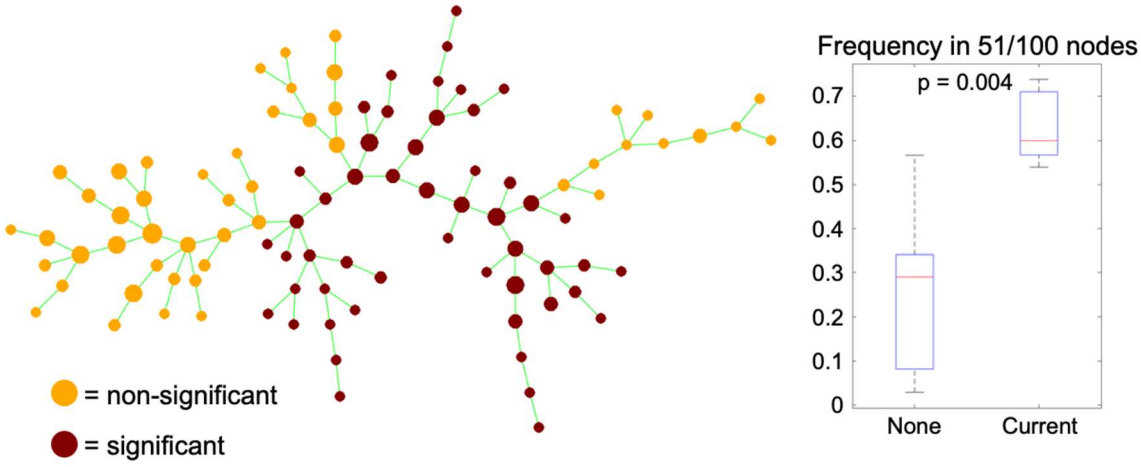

Figure 3.5.7. SPADE clustering identifies broad phenotypic similarity among airway monocytes/macrophage from APE BAL samples. Gated monocyte/macrophage populations (n = 18) were exported from FlowJo and analyzed using the SPADE clustering algorithm. A tree was generated based on similarity of marker expression across all cells, and individual samples were assigned to groups by APE status (None, n = 6; Prior, n = 7; Current, n = 5). Parameters included side scatter, CD45, CD63, CD66b, CD16, CD115, PD-1, PD-L1, and EGFR. SPADE compared all nodes of the tree to identify regions with significantly different frequencies of cells from the three groups of subjects.

T cells modulate activating and inhibitory receptors during APE

Previously we have shown that the process of neutrophilic inflammation in CF airways, particularly the release of Arg1 by infiltrating neutrophils, contributes to inhibition of T-cell activity and their eventual exclusion from the airway lumen [11]. Early in disease, however, T cells can still comprise 5-10% of total leukocytes obtained from BAL [13]. We used flow cytometry to conduct a basic phenotypic analysis of T cells from blood vs BAL and between the three groups of subjects reflecting APE history. When comparing T cells from blood vs those in BAL, we observed a significant decrease in CD3 expression and a significant increase in PD-1 expression in the latter. We then separated the subjects by APE history for both blood and BAL. There was no effect of APE history on expression of CD3 or PD-1 for blood T cells. However, CD3 expression was decreased and PD-1 expression was increased for airway T cells in Current compared to Prior groups (**Figure 3.5.8**).

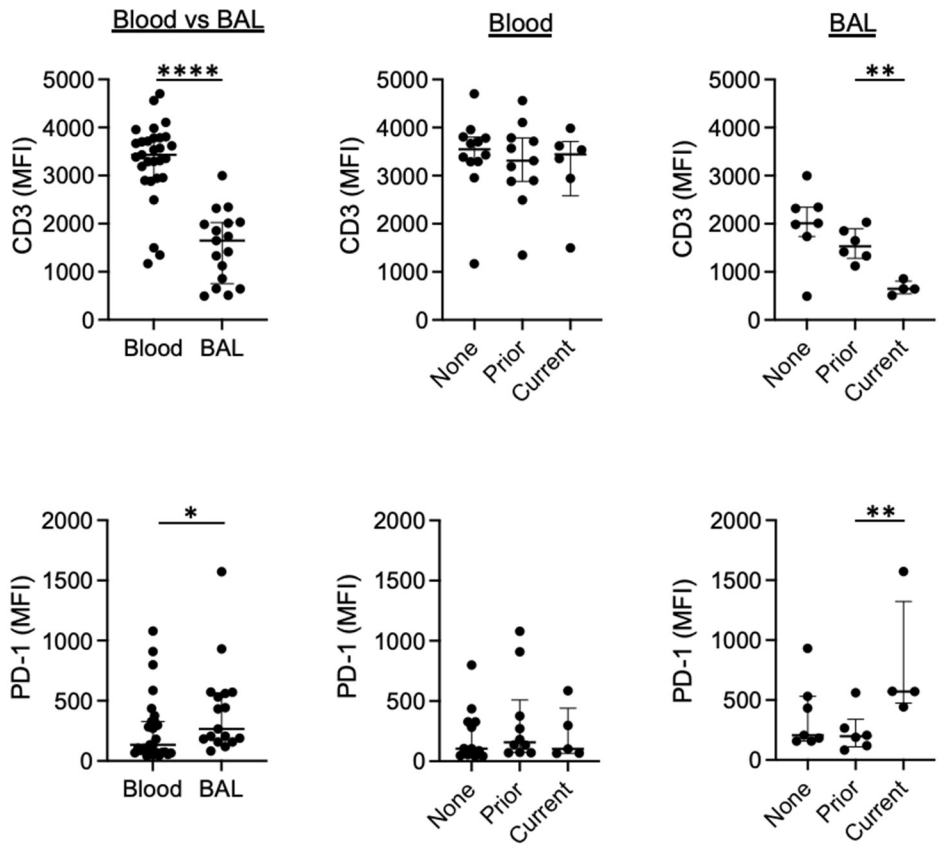

Figure 3.5.8. T cells downregulate CD3 and upregulate PD-1 during APE. Surface expression of CD3 and PD-1 on T cells from blood and BAL was measured by flow cytometry and reported as median fluorescence intensity (MFI). Comparisons between blood (n = 29 for CD3 and n = 27 for PD-1) and BAL (n = 17 for CD3 and PD-1) were performed by Mann-Whitney test. Groups of subjects with no history of APE (None), a previous hospitalization for APE (Prior), or current APE (Current) were compared by Mann-Whitney test. CD3: n = 12, 11, and 6 for None, Prior, and Current in blood and n = 7, 6, and 4 for None, Prior, and Current in BAL. PD-1: n = 12, 10, and 5 for None, Prior, and Current in blood and n = 7, 6, and 4 for None, Prior, and Current in BAL. Significant differences are indicated as *p ≤ 0.05, **p ≤ 0.01, and ****p ≤ 0.0001. Non-significant comparisons are not shown.

We also investigated expression patterns of CD45 and PD-L1. CD45 expression did not differ in blood vs. BAL samples, meanwhile, PD-L1 expression was significantly lower on airway vs. blood T cells. When grouping subjects by APE history, CD45 did not differ in blood but was significantly reduced on airway T cells from the Current group. PD-L1 expression was decreased on T cells from both blood and BAL in the Current group (**Figure 3.5.9**).

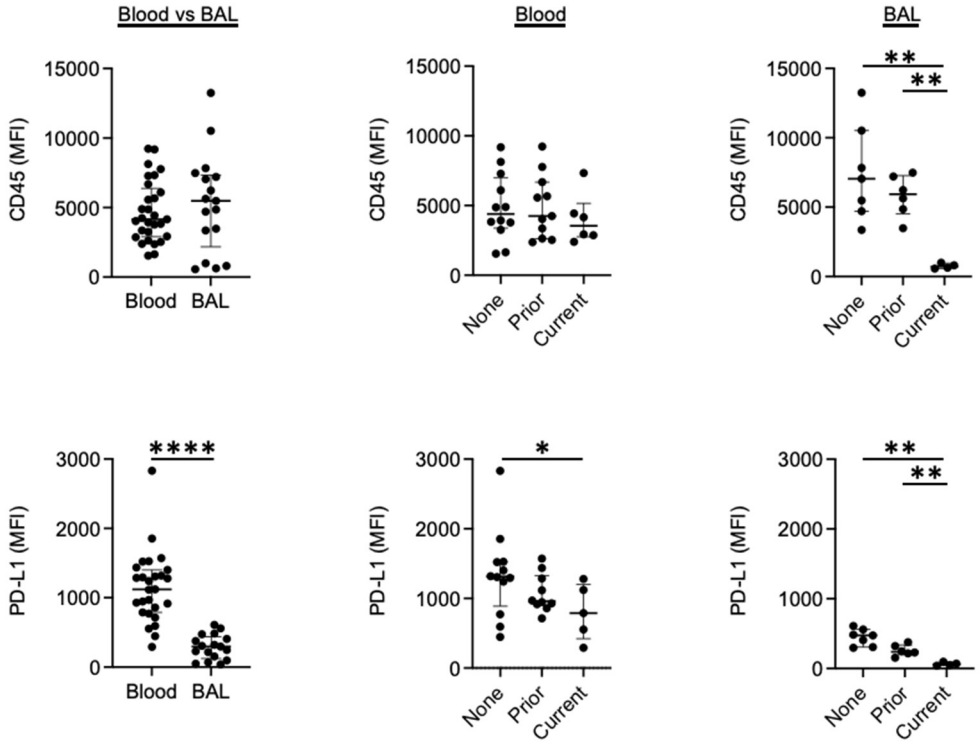

Figure 3.5.9. T cells downregulate CD45 and PD-L1 during APEs. Surface expression of CD45 and PD-1L on T cells from blood and BAL was measured by flow cytometry and reported as median fluorescence intensity (MFI). Comparisons between blood (n = 29 for CD45 and n = 27 for PD-L1) and BAL (n = 17 for CD45 and PD-L1) were performed by Mann-Whitney test. Groups of subjects with no history of APE (None), a previous hospitalization for APE (Prior), or current APE (Current) were compared by Mann-Whitney test. CD45: n = 12, 11, and 6 for None, Prior, and Current in blood and n = 7, 6, and 4 for None, Prior, and Current in BAL. PD-L1: n = 12, 10, and 5 for None, Prior, and Current in blood and n = 7, 6, and 4 for None, Prior, and Current in BAL. Significant differences are indicated as *p ≤ 0.05, **p ≤ 0.01, and ****p ≤ 0.0001. Non-significant comparisons are not shown.

3.6 Discussion

Through analysis of immune mediators and leukocyte subsets, this study provides new information on the coordination of immune responses in the airway during APEs in young children with CF. With expanding use of highly effective CFTR modulatory therapy in people with CF, including recent approval for elexacaftor/tezacaftor/ivacaftor at six years of age, management of CF lung disease is improving. However, this change in the disease landscape puts increasing focus on other aspects of living with CF such as nutritional balance and response to APEs. Considering that early-life APEs can accelerate lung function decline in children [3], an improved ability to predict and treat APEs will be beneficial for complementing the ability of modulatory therapy to improve lung function in the long term. An important aspect of this effort is to deepen our understanding of immune responses during APEs early in the course of lung disease, prior to the establishment of neutrophil dominance when the activities of other resident immune cells in the airways become altered [10, 11].

In this study we collected blood and BAL fluid from young children with CF, who had either no history of hospitalization for APE, a prior event but were currently stable, or who were currently hospitalized for treatment of an APE. Half of the subjects who provided samples at stable clinic visits tested positive for airway colonization with pro-inflammatory pathogens but, as expected, the majority of subjects currently experiencing an APE tested positive for infection. While only half of subjects with no APE history had neutrophil frequencies of >10% in BAL, the majority of subjects who had either prior or current hospitalizations had elevated BAL neutrophil frequencies. Although the overall comparison of neutrophil frequency in BAL was not different statistically between the three groups, this observation suggests that APEs promote a higher threshold for neutrophil presence in the lung which may foreshadow impending neutrophilic

inflammation in the airways that is characteristic of chronic CF lung disease. Indeed, experiencing APEs is the driving risk factor for occurrence of future events [12]. While neutrophils were not yet dominant in the airways during this early stage of disease, we did observe a trend towards increasing frequency of neutrophils bearing the GRIM phenotype out of total BAL neutrophils, suggesting that they are already beginning the process of reprogramming that directs their pathological activity [20, 22] even if they do not yet constitute the dominant cell population in the airways.

We measured a panel of 20 immune mediators, including a) neutrophil mediators, b) monocyte/macrophage mediators, and c) anti-inflammatory mediators. The Current group was associated with significant increases in concentrations of cytokines from each subcategory in BAL, including IFN-γ and IL-10 from subcategory c which were largely unmeasurable except in the Current group BAL samples. The significant increase in potent neutrophil chemoattractants such as CXCL1 and CXCL8 will likely contribute to rapidly increasing neutrophil recruitment to the airways, but the concurrent increases in mediators related to monocyte/macrophage recruitment, such as CCL2, and resolution of inflammation, such as IL-10, suggest that the system has not been overwhelmed and dominated by neutrophils as yet. However, the increased concentration of plasma G-CSF and concurrent increase in frequency of circulating neutrophils during APEs suggests that inflammatory signaling feedback from the lungs may promote ongoing neutrophil recruitment to the airways. This idea is supported by the modulation of osteoclast activity in response to APEs [6], and the decrease in total circulating leukocytes following intravenous antibiotic treatment [7]. These previous studies show that even though CF may not be synonymous with systemic inflammation, changes in immune responses in the lungs may directly influence circulating cytokines and leukocyte subset distribution. While prior

studies have documented an increase in inflammatory mediators in response to APEs and a decrease following treatment with antibiotics [4-8], the majority of these data are derived from adolescent and adult subjects with less data available from young children with CF who have yet to undergo persistent neutrophilic airway inflammation. Therapies aimed at blocking excessive neutrophil recruitment to the lung after resolution of an APE may therefore be successful in delaying airway inflammation in young children who experience these events frequently.

Airway neutrophils and macrophages did not demonstrate major phenotypic differences during APE when considering expression of individual surface proteins, but clustering analysis by SPADE revealed more subtle changes. After constructing a tree of all the cells included for analysis and assigning the individual samples to groups by APE history, SPADE identified a region with significantly higher frequency of cells from APE samples compared to no APE occurrence (significant region, **Figure 3.5.7**). A cluster of nodes within the tree contains cells that are similar to one another based on the total profile of surface markers, rather than comparing individual surface markers. This finding suggests that airway monocytes/macrophages have an overall phenotype that is slightly different from that of cells from subjects who did not yet experience an APE, and there may be other receptors not included in this study that would demonstrate significant differences. These may include scavenger receptors such CD14, CD36, and CD206 which may be used in follow-up studies to more specifically identify discrete subsets of lung macrophages in early CF airway fluid [23].

Airway T cells represent an important line of defense against bacterial and viral pathogens [24]. Although T-cell activity is inhibited by neutrophilic inflammation in the airways of people with CF [11], Th17 cells and rare T cell subsets are highly present in the submucosa [25]. The Th17 pathway is actively engaged during pulmonary infections associated with CF and

can promote neutrophilic inflammation [26], so understanding the level of T-cell engagement during APEs would be beneficial for improving immunomodulatory therapeutics which have shown promise but have yet to demonstrate definitive efficacy in treating CF lung disease [27].

In contrast to myeloid cells, T cells demonstrated phenotypic changes related to increased activation during APEs. CD3 is an important component of the T-cell receptor expressed at the cell surface with cytoplasmic tails that promote signal transduction. A decrease in CD3 is suggestive of activation, as TCR signaling has been shown to result both in degradation of CD3 at the cell surface [28] and reduced recycling [29]. Signaling through the PD-1 pathway is well characterized for its role in T-cell exhaustion during chronic viral infections and cancer [30], but expression of the receptor is induced on T and B lymphocytes upon cellular activation [31]. When comparing T cells from total blood and BAL samples, we observed decreased CD3 and increased PD-1 in BAL, suggesting that airway T cells exhibit an overall heightened state of activation compared to blood populations. It is important to note that these samples are collected from young children in the very early stages of CF lung disease, prior to established neutrophilic inflammation that represses airway T-cell activity [11]. However, the observed decrease in CD3 and increase in PD-1 was most pronounced on BAL T cells from the Current APE group, suggesting an active T-cell response to a bacterial infection which is likely instigating the exacerbation. $CD4^+$ T cells are important for orchestrating immune responses against bacterial infections, including in the respiratory tract [32]. However, the increase in PD-1 expression is unlikely to result in T cell exhaustion as we previously showed that T cell inhibition in the airways is primarily due to cleavage of arginine by neutrophil-derived Arg1 rather than the PD-1 pathway [11]. Moreover, as T cells increased PD-1 expression during APEs, PD-L1 was not increased on myeloid cells (data not shown) and was significantly reduced on T cells. Assessing

the level of airway T-cell activation during APE is complicated by the observation that CD45, which has roles for both positive and negative regulation of signaling in T cells [33], was dramatically reduced on T cells during current APE, but was not different in blood vs. BAL including all samples regardless of APE status. CD45 expression was also significantly reduced on airway neutrophils and monocytes/macrophages during APEs. Thus, more work is needed to determine how this receptor regulates different leukocyte populations during APEs compared to stable disease.

There are several limitations to consider for this study. This was a single center study, so validation of these findings with an independent cohort would be beneficial. The overall number of subjects was small, in particular for those with a current APE. The ability to follow up on these findings with future sample collection will be limited by the declining availability of BAL, but these samples may still be collected at hospital visits for treatment of APE. As such, these findings are particularly important for serving as a basis of future studies to make use of these valuable samples as BAL availability becomes increasingly limited. More frequent collection of airway samples will provide more data on the earliest inflammatory mechanisms and resolution of APEs and may be enabled by expanded use of induced sputum collection, which is discussed in **Chapter 4**. More in-depth phenotyping of T cells, including determination of subset frequency and analysis of additional activation markers and effector molecules, is also warranted. Studies with our local cohort were designed with a focus on myeloid cells in early CF lung disease and have recently been published [10, 13], but the findings presented here call for further investigation into adaptive immunity in the airways despite eventual dominance by neutrophils and exclusion of T cells as disease progresses.

In summary, we demonstrate that APEs in young children with CF are characterized by distinct inflammatory cytokine signatures but no significant phenotypic changes in airway neutrophils or monocytes/macrophages. However, we observe an increase in circulating neutrophil frequency and slightly higher frequency of GRIM neutrophils in BAL which may foreshadow the future mass-recruitment of neutrophils to the airway and their resulting contribution to lung disease. Furthermore, we identified a signature for increased T-cell activation during early CF APEs reflected by reduced CD3 and increased PD-1 expression. These data suggest that T cells may have a significant role in immune responses in the airway early in CF lung disease, particularly during APEs, prior to dominance of the airways by neutrophils. These findings may have important implications for unsolved questions regarding CF lung disease such as the effect on B- and T-cell repertoires in the lung later in life, and how this may relate to increased reporting of viral respiratory illness among people with CF, but not necessarily increased frequency of actual viral infection [34].

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Chapter 4

Inflammatory biomarkers in matched induced sputum and bronchoalveolar lavage of 2-year-olds with cystic fibrosis

Sections of this chapter have been published in the journal Pediatric Pulmonology

4.1 At-a-glance commentary

Current scientific knowledge on the subject: Monitoring of inflammation early in the course of CF lung disease can provide critical information on neutrophil recruitment to the airways and release of the destructive serine protease NE. BAL is the gold-standard technique for sampling the lower airways, but is an invasive technique with limited frequency of use. Induced sputum (IS) collection is minimally invasive but can also be used for measurement of inflammatory outcomes. It is unknown if induced sputum collected from young children with CF can yield data on the inflammatory poise of the airways that is comparable to BAL samples collected from specific lobes of the lung.

What this study adds to the field: We were able to collected IS, blood, CT scans, and two separate BAL samples all within a four-hour clinic visit (n = 11) with immediate processing of samples. Using flow cytometry to analyze leukocyte frequency and phenotype and assays to quantify soluble immune mediators and NE, we demonstrate that collection of minimally-invasive IS yields data comparable to that of invasive BAL collection from 2-year-old children with CF. We also demonstrate that inflammatory markers in BAL collected from the right middle lobe (gold-standard measurement) and the lingula (more diseased region in all subjects) were similar despite the differences in detectable disease. These findings justify the use of IS for more frequent but less invasive collection of samples to better track disease progression. Furthermore, we demonstrate that in early disease, leukocyte phenotype and immune mediator concentrations are similar in lobes with differing levels of observable disease.

4.2 Abstract

Background: In this pilot study, we investigated whether induced sputum (IS) could serve as a viable alternative to BAL and yield robust inflammatory biomarkers in toddlers with CF featuring minimal structural lung disease.

Methods: We collected IS, BAL (right middle lobe and lingula), and blood, and performed chest computed tomography (CT) scans from 2-year-olds with CF (n = 11), all within a single visit. Inflammatory biomarkers included 20 soluble immune mediators and NE, as well as frequency and phenotype of T cells, monocytes/macrophages, and neutrophils.

Results: At the molecular level, nine mediators showed similar levels in IS and BAL (CXCL1, CXCL8, IL-1α, IL-1RA, IL-6, CCL2, CXCL10, M-CSF, VEGF-A), four were higher in IS than in BAL (CXCL5, IL-1β, CXCL11, TNFSF10), and two were present in IS, but undetectable in BAL (IL-10, IFN-γ). Meanwhile, soluble NE had lower activity in IS than in BAL. At the cellular level, T-cell frequency was lower in IS than in BAL. Monocytes/macrophages were dominant in IS and BAL with similar frequencies, but differing expression of CD16 (lower in IS), CD115, and surface-associated NE (higher in IS). Neutrophil frequency and phenotype did not differ between IS and BAL. Finally, neutrophil frequency in IS correlated positively with air trapping.

Conclusions: IS collected from 2-year-olds with CF yields biomarkers of early airway inflammation with good agreement with BAL, notably with regard to molecular and cellular outcomes related to neutrophils and monocytes/macrophages.

4.3 Introduction

CF is an autosomal recessive disorder caused by one of more than 2000 variants in the gene encoding the CFTR anion channel [1]. Newborn screening is now being widely implemented to diagnose CF shortly after birth [2]. While multiple organs are affected in patients with CF, the majority of morbidity and mortality is due to airway disease [3]. Mutations in the CFTR protein disrupt ion flow, resulting in early and sustained airway inflammation and mucus abnormalities [4]. This environment is also highly susceptible to chronic infection by opportunistic pathogens [5].

However, the earliest stages of CF lung disease are characterized by sterile inflammation in the small airways. In a study of airway mucus composition, young children with CF already had elevated concentration of airway mucins, primarily MUC5AC and MUC5B, in BAL fluid compared to non-CF disease control subjects, despite being less than 5 years old and having stable disease status. Insoluble mucus flakes represented a large portion of total airway mucins and may contribute to hypoxia prior to widespread mucus obstruction [6]. Hypoxia is a major contributing factor to neutrophilic inflammation in the airways, often times prior to detectable infection [7]. Epithelial cells release IL-1α upon necrotic cell death induced by hypoxia, which potently activates neutrophils through the IL-1R signaling pathway [8]. Hypoxia further drives neutrophilic inflammation by promoting production of CXCL8 by macrophages as well as cellular adhesion and trans-epithelial migration of neutrophils [7]. Hypoxic pro-inflammatory signaling may be directly promoted by the lack of CFTR, which can interrupt the normal activation of HIF-1α [9] and NFκB [10] in response to hypoxia.

The early and sustained recruitment of neutrophils resulting from CFTR deficiency, despite the absence of infection, is observed in various models. This was first demonstrated by

implanting human fetal small airway grafts onto severe combined immunodeficient mice. While CF tissue caused sterile neutrophilic lung inflammation in the recipient mice, this was not observed for mice grafted with non-CF tissue [11]. Deletion of *cftr*, the gene encoding CFTR, from zebrafish results in massive neutrophil recruitment to wounds, causing further tissue damage [12]. In a ferret model of CF, which accurately reflects human lung disease, CXCL8 concentration is higher in BAL fluid from newborn CF animals compared to non-CF [13].

While recently approved CFTR-targeted modulator therapies are effective at restoring the function of mutant channels, the control of ongoing inflammation remains a challenge [14]. Therefore, understanding and tracking early events in CF airway inflammation is essential for improving patient outcomes. In prior studies, extracellular NE activity measured in BAL fluid at 3 months of age was found to be more effective than infection status and mucus obstruction at predicting lung damage at 1 and 3 years of age [15, 16]. Most of the extracellular NE activity in CF airway fluid stems from the active exocytosis of NE-rich granules by neutrophils acquiring the GRIM phenotype upon recruitment to CF airways [17, 18]. Live GRIM neutrophils are identifiable in BAL of young children with CF and correlate positively with structural lung damage [19], as quantified by chest computed tomography (CT) scans [20].

While BAL is the gold standard for assessing CF airway inflammation [21], it is an invasive procedure that requires sedation and bronchoscopy. In young children, performing a BAL requires general anesthesia, which makes it difficult to envision as a routine research tool and therefore often limits its use to clinically indicated procedures [22]. To better understand early events in CF airway inflammation, more frequent longitudinal sampling of young children using minimally invasive techniques would be desirable. One such option is induced sputum (IS), which has been used successfully to collect airway samples from children [23] and adults

[17, 24] with CF. An important difference is that BAL primarily samples material from distal airways, while IS samples material from both distal and proximal airways. Recently, a study assessing the ability of IS to yield a relevant picture of lower airway microbiology in young children and adolescents with CF found similarities to BAL, but also notable differences including higher sensitivity afforded by IS for detecting *Pseudomonas aeruginosa*, MRSA, *Burkholderia cepacia*, and *Burkholderia cenocepacia* in a subset of patients [25]. With regard to inflammation, IS in adults with CF has shown similarities to BAL based on immune mediator levels, total leukocyte count, and subset frequencies [24]. Critically, IS has not yet been used to study molecular and cellular biomarkers of inflammation in early CF lung disease.

To address this gap, we conducted a cross-sectional, single-center study of 2-year-olds with CF with sequential IS, BAL, and blood collections at the same visit. At this age, most patients have minimal structural lung damage, yet some have already developed signs of inflammation [26]. Through the analysis of soluble immune mediators and NE, leukocyte subsets and phenotype, and assessment of structural damage by chest CT scans, we demonstrate that IS can be used to detect early CF airway inflammation and lung damage, and document similarities and discrepancies with BAL fractions.

4.4 Methods

Human subjects and samples

Data were collected from eleven 2-year-old children at stable clinic visits. The study visits were conducted when the subjects had no recent acute respiratory illnesses or symptoms and had not required any oral or intravenous antibiotic therapy in the past 4 weeks. Subjects with CF were enrolled in the IMPEDE-CF study at Emory University and Children's Healthcare of Atlanta. All aspects of subject enrollment and sample collection were approved by the Emory University Institutional Review Board (IRB00097352). Consent for a sample collection from subjects was obtained from parents on the day of the clinic visit. Subject demographics are summarized in **Table 4.4.1**. Collection of IS, blood, chest CT scan, and BAL from the right middle lobe (RML) as a consistent measurement with a separate sample from the lingula (LIN) [27, 28] were completed in that order within the same 4-h visit. IS collection was done with a modified technique using hypertonic saline (7%) nebulization along with the use of a chest vest (high-frequency chest wall oscillation device) in all subjects. Samples were stored on ice following collection and delivered to the laboratory for immediate processing. Details of sample collection procedures are provided in **Figure 4.4.1**, with sample yields indicated in **Table 4.4.2**. The presence of aspiration was identified by clinical symptoms and positive results on a modified barium swallow test or the presence of lipid-laden macrophages in the BAL fluid.

Subject	Sex	CFTR allele 1	CFTR allele 2	Pulmonary exacerbations	Infection status	Aspiration	Pancreatic sufficiency	Wt for Length z-score
#1	Male	F508del	E585X	0	MSSA	No	No	0.19
#2	Male	F508del	F508del	0	MSSA	No	No	0.02
#3	Female	F508del	F508del	0	None	No	No	0.62
#4	Male	F508del	R553X	1	Multiple Gram+ and Gram- pathogens	No	No	1.57
#5	Female	F508del	5T with TG11	0	MRSA	No	Yes	1.41
#6	Male	3120+1 G>A	c.3468+2dupT	1	*E. coli*	Yes	No	1.21
#7	Male	F508del	F508del	2	MRSA	Yes	No	0.59
#8	Female	F508del	F508del	0	MSSA	No	No	-0.26
#9	Male	G542X	c.del_exon25_exon26	0	Negative	No	No	1.02
#10	Male	F508del	F508del	1	Negative	Yes	No	0.78
#11	Male	F508del	R553X	1	*Streptococcus* group A	Yes	No	1.57

Table 4.4.1. Demographics of enrolled subjects. Eleven patients at the age of two were enrolled to collect blood, induced sputum, and two BAL fractions. Infection status was identified by two positive throat swab cultures in the preceding year. MSSA: methicillin-susceptible *S. aureus*, MRSA: methicillin-resistant *S. aureus*.

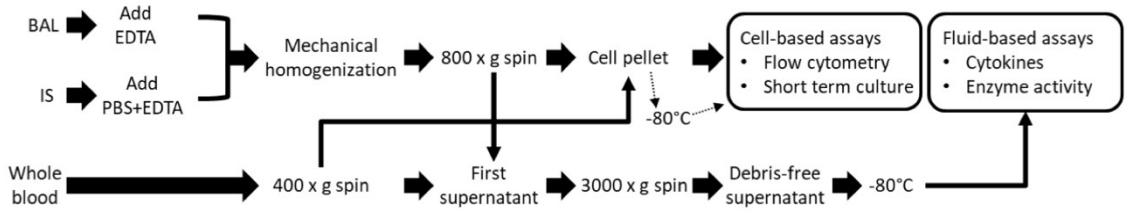


Figure 4.4.1. Sample processing workflow. EDTA was added to BAL for a final concentration of 2.5 mM. 3 mL of PBS+EDTA was added to each IS sample to facilitate processing and ensure adequate volume for analysis. BAL and IS were then mechanically homogenized by syringe and needle. Blood was spun to remove the plasma and resuspended to the original volume with PBS+EDTA. Isolated cells were then used for analysis by flow cytometry. Plasma, BAL, and IS supernatants were spun at 3000 x g to remove debris/bacteria, and debris-free supernatants were used for fluid-based assays.

Subject	IS Total Volume (mL)	IS (Live leukocytes)	RML BAL (mL)	RML BAL (Live Cells)	LIN BAL (mL)	LIN BAL (Live Cells)	Whole Blood (mL)	Plasma (mL)	Comments
#1	3.20	$1.57x10^5$	3.00	$3.60x10^5$	3.50	$3.60x10^5$	4.80	2.00	
#2	3.50	$8.00x10^3$	8.36	$1.90x10^5$	4.85	$3.20x10^4$	3.50	1.40	blood in RML
#3	n/a	n/a	10.50	$1.50x10^6$	2.10	$5.72x10^5$	3.20	1.70	
#4	5.30	$9.75x10^5$	4.20	$4.40x10^5$	3.03	$5.20x10^5$	3.40	1.40	
#5	4.45	$2.00x10^4$	1.20	$9.00x10^4$	2.25	$2.56x10^5$	3.50	1.20	
#6	5.00	$8.80x10^5$	2.00	$9.30x10^4$	4.00	$4.40x10^5$	3.50	1.20	
#7	2.80	$2.20x10^6$	2.70	$2.00x10^4$	1.20	$1.77x10^6$	3.50	1.70	blood in IS
#8	2.70	$3.21x10^5$	4.45	$5.00x10^5$	1.86	$4.65x10^5$	2.05	1.00	Dead leukocytes in IS
#9	3.50	0	3.80	$2.40x10^5$	7.50	$3.49x10^5$	2.90	1.22	IS contained $5x10^4$ epithelial cells but no leukocytes
#10	2.90	$1.25x10^5$	3.70	$5.30x10^5$	3.10	$4.90x10^5$	2.90	1.20	
#11	3.60	$3.13x10^6$	3.20	$7.30x10^5$	4.00	$1.00x10^6$	2.80	1.90	

Table 4.4.2. Sample collection totals. Eleven clinic visits were conducted, with blood and BAL obtained at all visits. IS collection was unsuccessful at one visit and another yielded a poor sample that was deemed unusable. Total volumes of each sample were recorded prior to aliquoting. Cell count was determined using fluorescent microscopy by staining with ethidium bromide + acridine orange. Epithelial cells comprised less than 1% of total nucleated cells in all IS samples except for one (excluded from analysis).

Sample collection and processing

Venous blood was collected into K2 EDTA tubes and spun at 400 x g to separate plasma from cells. Plasma was removed and centrifuged at 3000 x g to yield debris-free plasma by removing platelets and residual red blood cells. The cell pellet was washed with 10 mL of ice-cold 1x phosphate buffered saline + 2.5 mM EDTA (PBS+EDTA) and resuspended to its original volume. Washed blood was used to prepare aliquots for staining and fixation. All blood samples were analyzed by flow cytometry. In addition, immune mediators, but not NE, were quantified in all plasma samples.

Induced sputum (IS) samples were obtained by a modified sputum induction procedure using 7% hypertonic saline nebulization (4 mL) and use of a chest vest (high frequency chest

wall oscillation device). All subjects were in a fasting state, had not consumed any food or liquids in the preceding 6 hours and had dry mouths with no significant saliva. The caregivers performing IS collection procedure followed infection control guidelines with use of N95 mask, gown, gloves and eye-shields. The subject was seated in the parent's lap and pre-treated with 2 puffs of albuterol with an MDI attached to a spacer and mask. This was followed by inhaled 7% saline nebulization (4 mL) with a nebulizer and mask, along with their own chest vest using age-appropriate settings. The subject was encouraged to cough throughout the procedure and any spontaneous coughing was followed by suctioning of the pharynx, or after every 3 minutes of continued nebulization – whichever occurred sooner. The total duration of the sputum induction procedure was 15 minutes, which ensured that a minimum of 5 suctioning attempts (at every 3-minute interval or more if they started coughing) were made for each subject. A soft, flexible suction catheter (8 Fr) attached to a Lukens specimen container that was connected to a portable suction machine was used for pharyngeal suctioning throughout the procedure. To minimize salivary contamination, care was taken to not suction in the oral cavity at all and the suction machine was only turned on once the suction catheter had been advanced past the base of the tongue into the pharynx. The subject was allowed to watch their favorite movie/video on a phone/tablet device during the collection procedure to keep them distracted. The chest vest and nebulizer were paused every time pharyngeal suctioning was performed. After the completion of the hypertonic saline nebulization, any remnants of the sample in the suction tubing were collected into the specimen container by aspirating 3 mL of PBS+EDTA through the suction catheter into the specimen container. Final volumes of the collected samples after aspiration of the 3 mL are recorded in **Table 4.4.2**. Theoretically, every IS sample would therefore have a final volume of greater than 3 mL due to addition of true sample volume and the fixed volume of

3 mL PBS+EDTA. The final volumes for some samples were less than 3 mL, indicating some loss of volume in the tubing or specimen container. However, these samples still had adequate volume and cell yield to perform the various assays. After the completion of the procedure, the specimen container was detached from the suction tubing, sealed with a cap, and transported to the lab on ice for immediate processing. Use of IS samples in analytical assays is detailed in **Table 4.4.3**.

Subject	**Soluble mediators**		**FACS: Neutrophils**			**FACS: Monocytes/Macrophages**			**FACS: T cells**		
	NE	Cytokines	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16
#1	X	X				X	X	X			
#2	X	X				X	X	X			
#3											
#4	X	X	X	X	X	X	X	X		X	X
#5	X	X					X				
#6	X	X	X	X	X	X	X	X			
#7	X	X	X	X	X	X	X	X			X
#8	X	X		X			X				
#9											
#10	X	X	X			X					
#11	X	X	X	X	X	X	X	X			

Table 4.4.3. Total assays performed for IS samples. Soluble mediator and flow cytometry assays were conducted for each patient where indicated by an "X". Due to limitation of cell numbers, not all staining panels were applied to all samples.

BAL was performed under general anesthesia using a 3.1 mm bronchoscope (BF-XP190, Olympus, Japan) with a 1.2 mm suction channel. The selection of the site for BAL was based on initial assessment of Chest CT that was done prior to the bronchoscopy. If there was a significantly affected region of the lung, then it was prioritized in terms of the first BAL sample. Additional samples would be collected from the other lung (either from the right middle lobe (RML) or lingula (LIN)). In patients with no significant bronchiectasis noted on CT, the RML was the preferred site for the right lung and LIN was the preferred site for the left lung. This was based on the likelihood of getting the highest return (yield) during the BAL collection procedure. Two BAL samples were collected per bronchoscopy procedure, with the RML and LIN sampled for all study participants. BAL samples were collected using the standard method of wedging the bronchoscope in a distal bronchus, followed by the instillation and aspiration of sterile 0.9% saline (1 mL/kg up to a maximum volume of 20 mL per aliquot) at each site. A total of 2 or 3 aliquots were instilled per site depending on the BAL sample yield. The first aliquot was reserved for clinical microbiology using standard culture techniques. Clinical assessments including microbiological cultures and cytology required 7 mL. Any additional volume of BAL sample that was not used for the clinical lab was sent to the research lab on ice for immediate processing, with these BAL volumes recorded in **Table 4.4.2**. EDTA was added to BAL samples in the research lab for a final concentration of 2.5 mM. BAL cell counts with differential and BAL culture results were collected from the patient's electronic medical records. Use of RML and LIN BAL samples in analytical assays is detailed in **Table 4.4.4** and **Table 4.4.5**, respectively.

IS and BAL samples were then mechanically homogenized by passing through an 18-gauge needle and centrifuged at 800 x g for 10 minutes at 4°C. The cell pellet was washed in

PBS+EDTA and the supernatant was centrifuged at 3000 x g to removed bacteria and debris, yielding debris-free supernatant. Washed cells were used for staining and fixation, while debris-free supernatants were used for measurement of cytokine concentration and NE activity. Adequacy of IS and BAL samples was based on a minimum live leukocyte count of 5,000 cells.

Subject	**Soluble mediators**		**FACS: Neutrophils**			**FACS: Monocytes/Macrophages**			**FACS: T cells**		
	NE	Cytokines	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16
#1	X	X	X	X	X	X	X	X	X	X	X
#2	X	X	X	X	X	X	X	X	X	X	X
#3	X	X	X	X	X	X	X	X	X	X	X
#4	X	X	X	X	X	X	X	X			
#5	X	X		X			X			X	
#6	X	X		X			X			X	
#7	X	X		X			X			X	
#8	X	X		X			X			X	
#9	X	X		X		X	X		X	X	
#10	X	X	X	X	X	X	X	X	X	X	X
#11	X	X	X	X	X	X	X	X	X	X	X

Table 4.4.4. Total assays performed for RML BAL samples. Soluble mediator and flow cytometry assays were conducted for each patient where indicated by an “X”. Due to limitation of cell numbers, not all staining panels were applied to all samples.

Subject	Soluble mediators		FACS: Neutrophils			FACS: Monocytes/Macrophages			FACS: T cells		
	NE	Cytokines	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16	Panel 14	Panel 15	Panel 16
#1	X	X	X	X	X	X	X	X	X	X	X
#2	X	X	X	X	X	X	X	X			
#3	X	X	X	X	X	X	X	X	X	X	X
#4	X	X									
#5	X	X		X			X			X	
#6	X	X	X	X	X	X	X	X	X	X	X
#7	X	X									
#8	X	X	X	X		X	X		X	X	
#9	X	X	X	X		X	X		X	X	
#10	X	X	X	X		X	X		X	X	
#11	X	X	X	X	X	X	X	X	X	X	X

Table 4.4.5. Total assays performed for LIN BAL samples. Soluble mediator and flow cytometry assays were conducted for each patient where indicated by an “X”. Due to limitation of cell numbers, not all staining panels were applied to all samples.

Chest CT imaging and PRAGMA scoring

Chest CT scans were obtained under general anesthesia at Children's Healthcare of Atlanta [29] with full inspiratory and expiratory breath hold maneuvers and scored using the validated PRAGMA-CF method [30]. Scoring of mucus plugging (%MP), bronchiectasis (%Bx) and abnormal airways (%AA) after excluding areas of atelectasis were done with inspiratory scans, and scoring of trapped air (%TA) used expiratory scans. Total disease (%Dis) score was calculated by combining all abnormal areas as a percentage of total scored CT sections after excluding areas of atelectasis (**Table 4.4.6**).

Subject	**PRAGMA-CF scores**			
	% Total Disease (%Dis)	% Mucus Plugging (%MP)	% Abnormal Airways (%AA)	% Trapped Air (%TA)
#1	0.000	0.000	0.000	0.000
#2	2.107	0.335	1.389	0.739
#3	0.929	0.000	0.805	0.190
#4	0.320	0.000	0.320	0.211
#5	0.000	0.000	0.000	0.000
#6	2.348	0.000	2.047	1.576
#7	5.796	0.828	3.949	29.069
#8	5.908	0.995	4.291	3.534
#9	0.300	0.000	0.300	0.249
#10	3.946	0.185	2.836	2.154
#11	2.013	0.000	1.698	1.579

Table 4.4.6. PRAGMA-CF scores. Shown are scores of mucus plugging (%MP) and abnormal airways (%AA) using inspiratory scans, and of trapped air (%TA) using expiratory scans. Total Disease (%Dis) score was calculated by combining all abnormal areas as a percentage of total scored CT sections after excluding areas of atelectasis.

Total protein quantification

Total protein levels were quantified in IS and BAL using the Pierce copper sulfate/bicinchoninic acid (BCA) assay (Thermo Fisher Scientific), with bovine serum albumin used for calibration.

Soluble immune mediator quantification

A custom assortment of 20 soluble immune mediators was measured in IS and BAL using a highly sensitive chemiluminescent assay (U-PLEX; Meso Scale Diagnostics), according to the manufacturer's protocol. To enable comparison between IS and BAL samples, mediator concentrations were normalized to total protein concentration (measured using BCA assay), as illustrated in **Figures 4.5.1 and 4.5.3**. Normalization to total protein rather than to urea was performed as the latter has not been established for IS and its reliability was questioned for BAL [31].

Flow cytometry

Multicolor flow cytometry was performed on cells from blood, BAL, and IS using previously described methods [19]. A gating strategy was developed to identify all major leukocyte populations in blood and airway fluids, as detailed in **Figure 4.4.2**.

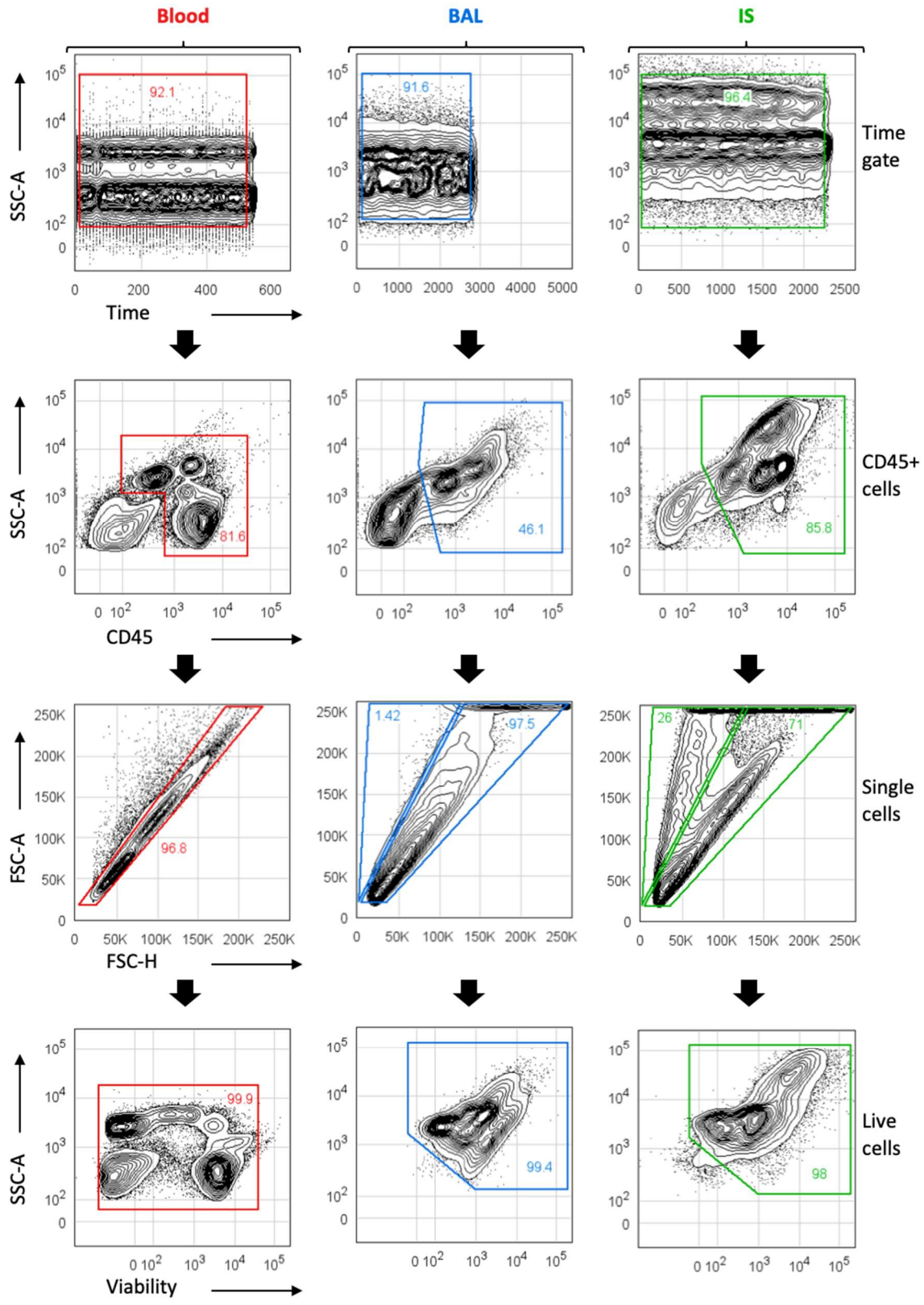
Blood and airway cells were pre-stained with Fc block to prevent non-specific uptake of antibodies (Biolegend #422302) and Calcein violet for 10 minutes in the dark on ice, followed by antibody staining for surface markers on ice in the dark for 20 minutes. A complete list of staining panels and the respective antibodies is found in **Table 4.4.7**. Cells were washed with PBS+EDTA and fixed overnight in the dark at 4ºC in BD Phosflow Lyse/Fix Buffer (BD

#558049). The next day cells were washed with PBS+EDTA to remove fixative reagent and stored at 4ºC in the dark and acquired within 2 weeks. All samples were acquired on a BD LSRII or BD FACSymphony. Cytometers were calibrated using 6 peak Rainbow Calibration Particles (Biolegend #422901) to ensure consistent fluorescence output was obtained over the course of this study [32]. Single-stained compensation controls were prepared using UltraComp eBeads (Invitrogen #01-2222-42). Analysis including compensation, gating, and calculation of fluorescence intensities and cell frequency was performed using FlowJo V9.9.5 (BD). A minimum required number of 50 events was chosen to include exported data in the analysis.

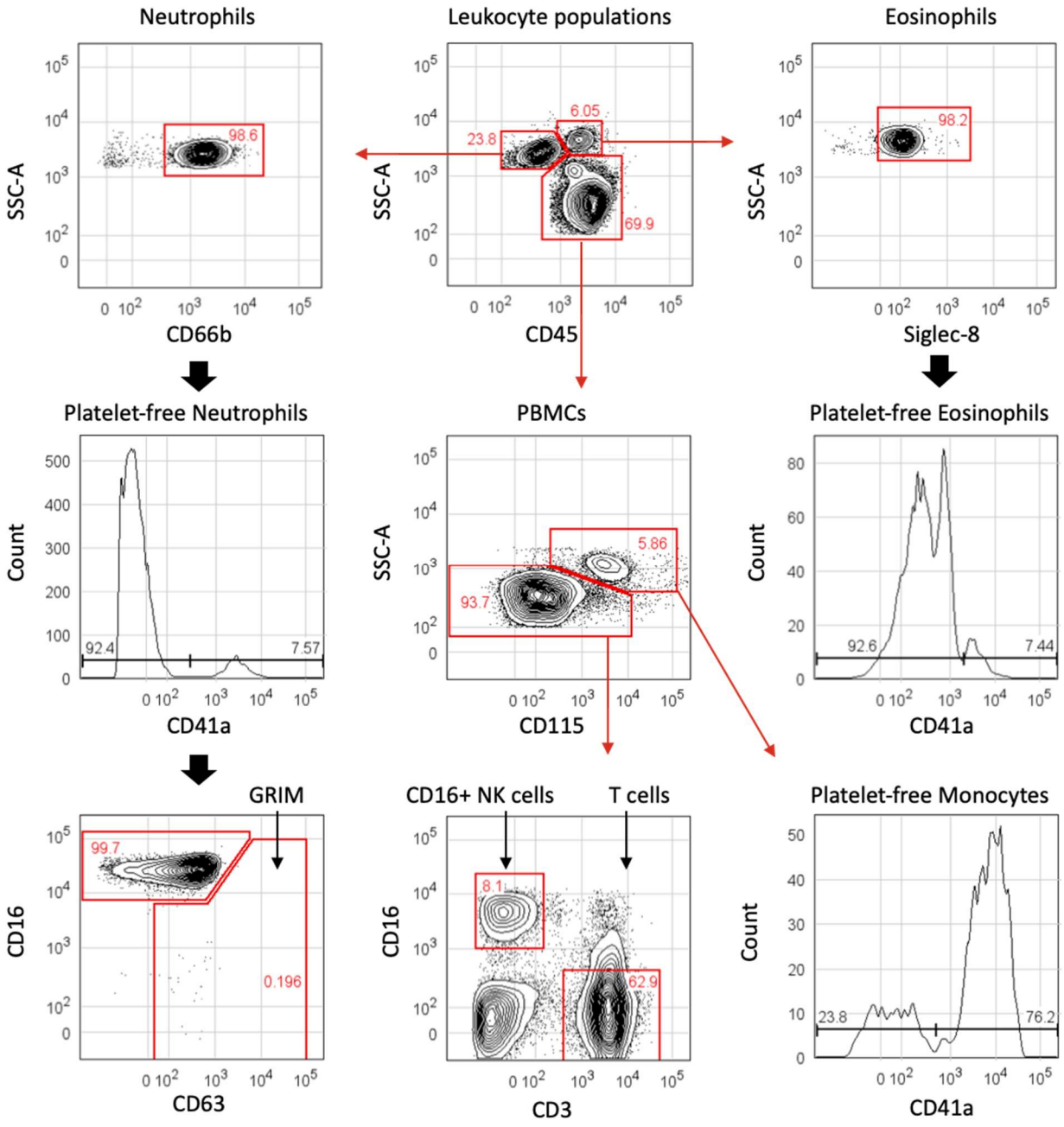
Color	Stain	Vendor	Catalog	Clone
Backbone				
PB	Calcein violet	Thermo Fisher	C34858	n/a
PB	CD41a	Biolegend	303714	HIP8
PB	CD3	Biolegend	300330	HIT3a
BV605	CD45	Biolegend	304042	HI30
BV650	CD63	Biolegend	353026	H5C6
PER CP CY 5.5	CD16	Biolegend	302028	3G8
PE	CD66b	Biolegend	305106	G10F5
PE CY7	CD115	Biolegend	347308	9-4D2-1E4
AF700	Siglec-8	R&D Systems	FAB7975N	837535
Panel 14				
FITC	NE	R&D Systems	IC91671G-100UG	950317
APC	CD36	Biolegend	336208	5-271
APC CY7	CD304	Biolegend	354524	12C2
Panel 15				
FITC	PD-1	Biolegend	329904	EH12.2H7
APC	PD-L1	Biolegend	329708	29E.2A3
APC CY7	EGFR (unconjugated)	Biolegend	352902	AY13
	APC Cy7 conjugation kit	Novus Biologicals	765-0010	n/a
Panel 16				
FITC	Exostain	SBI	EXOFLOW-800A-1	n/a
APC	CD163	Biolegend	333610	GHI/61
APC CY7	CD206	Biolegend	321120	15-2

Table 4.4.7. Flow cytometry staining panels. Three 10-color staining panels were designed and used over the course of this study. Each panel included a common set of backbone markers used in the gating strategy as well as three channels whose targets were panel-specific.

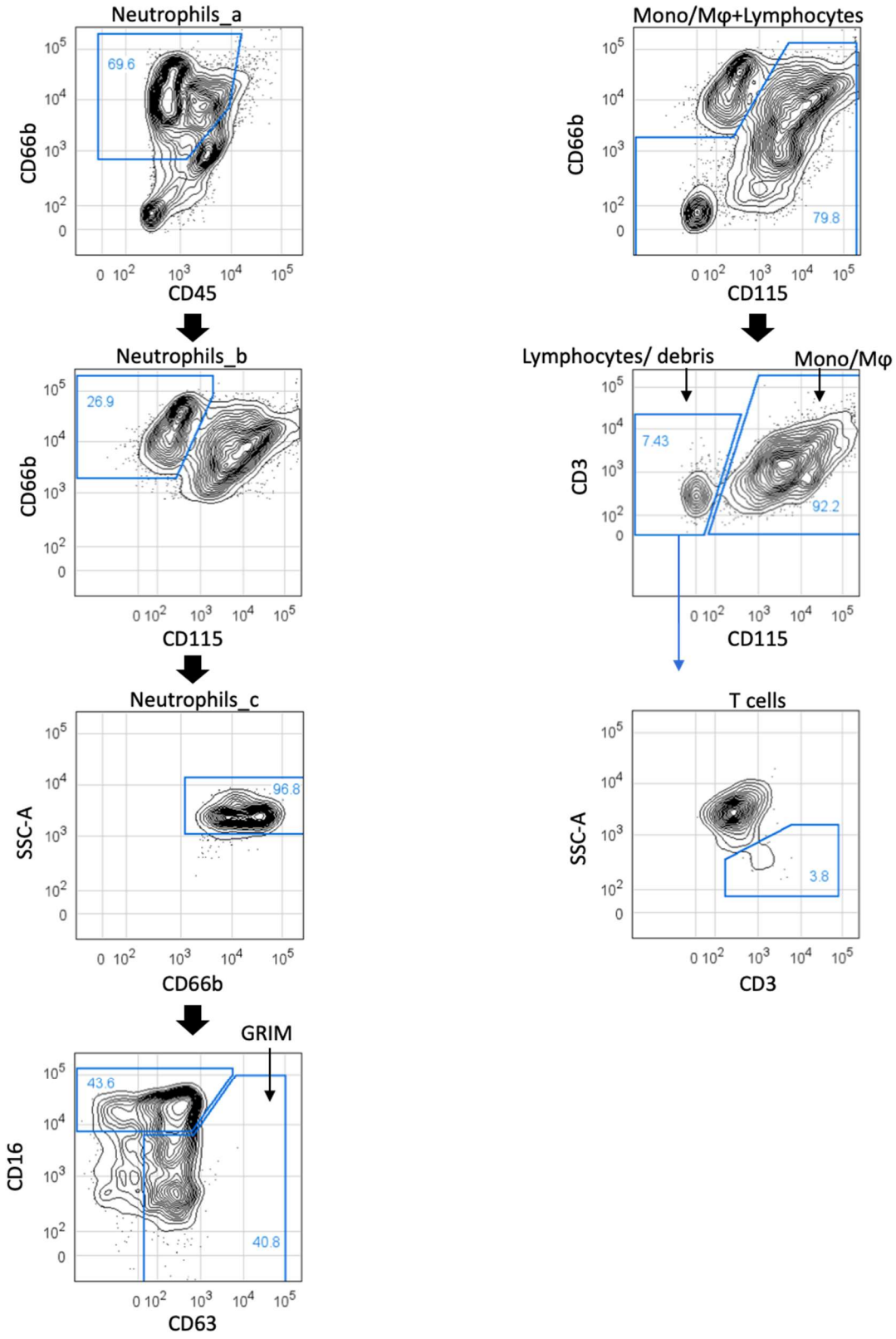
a)


Blood
BAL
IS
SSC-A
Time
92.1
91.6
96.4
Time gate
CD45
81.6
46.1
85.8
CD45+ cells
FSC-A
FSC-H
96.8
1.42
97.5
26
71
Single cells
Viability
99.9
99.4
98
Live cells

b)


Neutrophils
SSC-A
CD66b
98.6
Leukocyte populations
SSC-A
CD45
6.05
23.8
69.9
Eosinophils
SSC-A
Siglec-8
98.2
Platelet-free Neutrophils
Count
CD41a
92.4
7.57
PBMCs
SSC-A
CD115
5.86
93.7
Platelet-free Eosinophils
Count
CD41a
92.6
7.44
GRIM
CD16
CD63
99.7
0.196
CD16+ NK cells
T cells
CD16
CD3
8.1
62.9
Platelet-free Monocytes
Count
CD41a
23.8
76.2

c)


Neutrophils_a
69.6
CD66b
CD45
Neutrophils_b
26.9
CD66b
CD115
Neutrophils_c
96.8
SSC-A
CD66b
GRIM
43.6
40.8
CD16
CD63
Mono/Mφ+Lymphocytes
79.8
CD66b
CD115
Lymphocytes/ debris
Mono/Mφ
7.43
92.2
CD3
CD115
T cells
3.8
SSC-A
CD3

d)

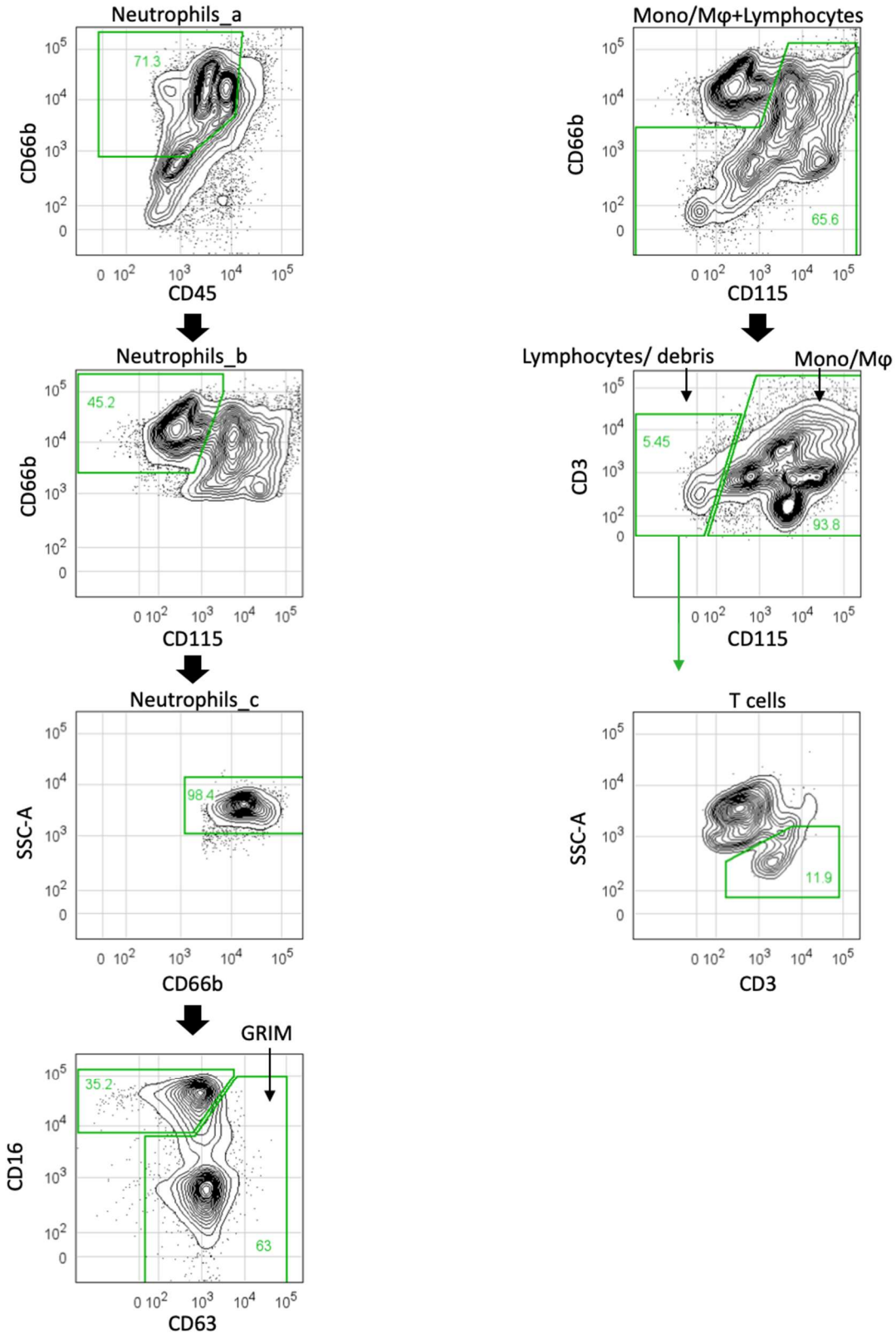

Neutrophils_a
71.3
CD66b
CD45
Neutrophils_b
45.2
CD66b
CD115
Neutrophils_c
98.4
SSC-A
CD66b
GRIM
35.2
63
CD16
CD63
Mono/Mφ+Lymphocytes
65.6
CD66b
CD115
Lymphocytes/ debris
Mono/Mφ
5.45
93.8
CD3
CD115
T cells
11.9
SSC-A
CD3

Figure 4.4.2. Detailed flow cytometry gating strategy. Cells from blood, BAL, and IS were stained for analysis by flow cytometry. All major leucocyte subsets in blood, BAL, and IS were identified. **(a)** The same upstream gates were applied to all samples beginning with a time gate to exclude air bubbles or other aberrant events if needed. A $CD45^+$ gate was used to exclude debris/red blood cells/epithelial cells. Single cells were identified using forward scatter-area and forward scatter-height. Live cells were then selected, from which individual cell populations were identified. **(b)** Eosinophil and neutrophil populations were identified from blood, from which platelet-free cells were selected by excluding $CD41a^+$ events. GRIM neutrophil subpopulations were identified using CD16 and CD63. Monocytes and lymphocytes were identified within PBMCs, and platelet-free monocytes were selected. Out of lymphocytes, T cells and $CD16^+$ NK cells were gated. **(c)** After identification of live cells, CD66b and CD45 were used to identify neutrophils, while CD66b and CD115 were used to select macrophages and lymphocytes for further gating. After the removal of the remaining macrophages, GRIM neutrophils were identified using the respective blood sample gates from individual subjects. T cells were then selected from $CD115^{low}$ events. **(d)** The same downstream gates were applied to IS as for BAL samples.

Extracellular NE activity

Extracellular NE activities in IS, RML BAL, and LIN BAL were measured with a Förster resonance energy transfer (FRET) assay using the NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described [33-35]. NE concentration was normalized to total protein concentration (measured using BCA assay) in the fluid.

Statistical analysis

Data were analyzed in Prism (version 8; GraphPad Software) using nonparametric statistics due to the limited number of samples, including Wilcoxon's matched-pairs signed-rank test and Spearman's test for correlation. Specifically for cytokines, a value of half the lower limit of detection or twice the upper limit of detection was assigned for data points that fell outside the limits of detection. Those values are clearly labeled as closed symbols in related figures. To avoid overrepresentation of imputed values, statistical comparisons of mediator concentration were not performed where more than half the data points of a sample group were imputed values. To conduct correlations, imputed values were removed, and correlations were conducted using R only for mediators with at least six non-imputed data points.

4.5 Results

Parallel collection of IS, BAL, and blood, and chest CT imaging in 2-year-olds with CF

The ability to collect IS from young children with CF was shown in prior studies, but focused on a limited set of cytokines [24] and on microbiology [25]. To assess the potential of IS to yield data on an extended set of inflammatory biomarkers, we collected it in parallel to BAL from the RML and LIN, blood, and chest CT, all within a single study visit. We attempted and achieved the collection of all samples and data on 11 subjects (demographics in **Table 4.4.1**), except for two subjects from whom we were unable to obtain IS, thus lowering our analyzable set of IS samples to 9 (**Table 4.4.2**). Chest CT scoring confirmed that this cohort was at an early stage of airway disease, with the %Dis score ranging from 0% to 5.9% (**Table 4.4.6**).

Similarities and differences in soluble immune mediators in IS and BAL

Prior studies of BAL have shown that the onset of neutrophilic inflammation is a key determinant of structural lung damage in young children with CF [26]. Thus, we selected 11 mediators impacting neutrophil recruitment and activation (**Figure 4.5.1**). Subject-matched samples were compared between each pair of sample groups using paired statistics, but data points are shown without connecting lines to improve readability. **Figure 4.5.2** includes graphs with connected points for select soluble mediators. Five mediators showed similar levels in IS and BAL, namely, CXCL1, CXCL8, IL-1α, IL-1RA, and IL-6 (**Figure 4.5.1a**). Six mediators showed differential levels between IS and BAL: CXCL5, G-CSF, IL-1β, and TNF-α were higher in IS than in one or both of the BAL fractions, while IL-18 was lower in IS than in LIN BAL.

Finally, IL-10 was below the limit of detection in most BAL samples but was measurable in most IS samples (**Figure 4.5.1b**).

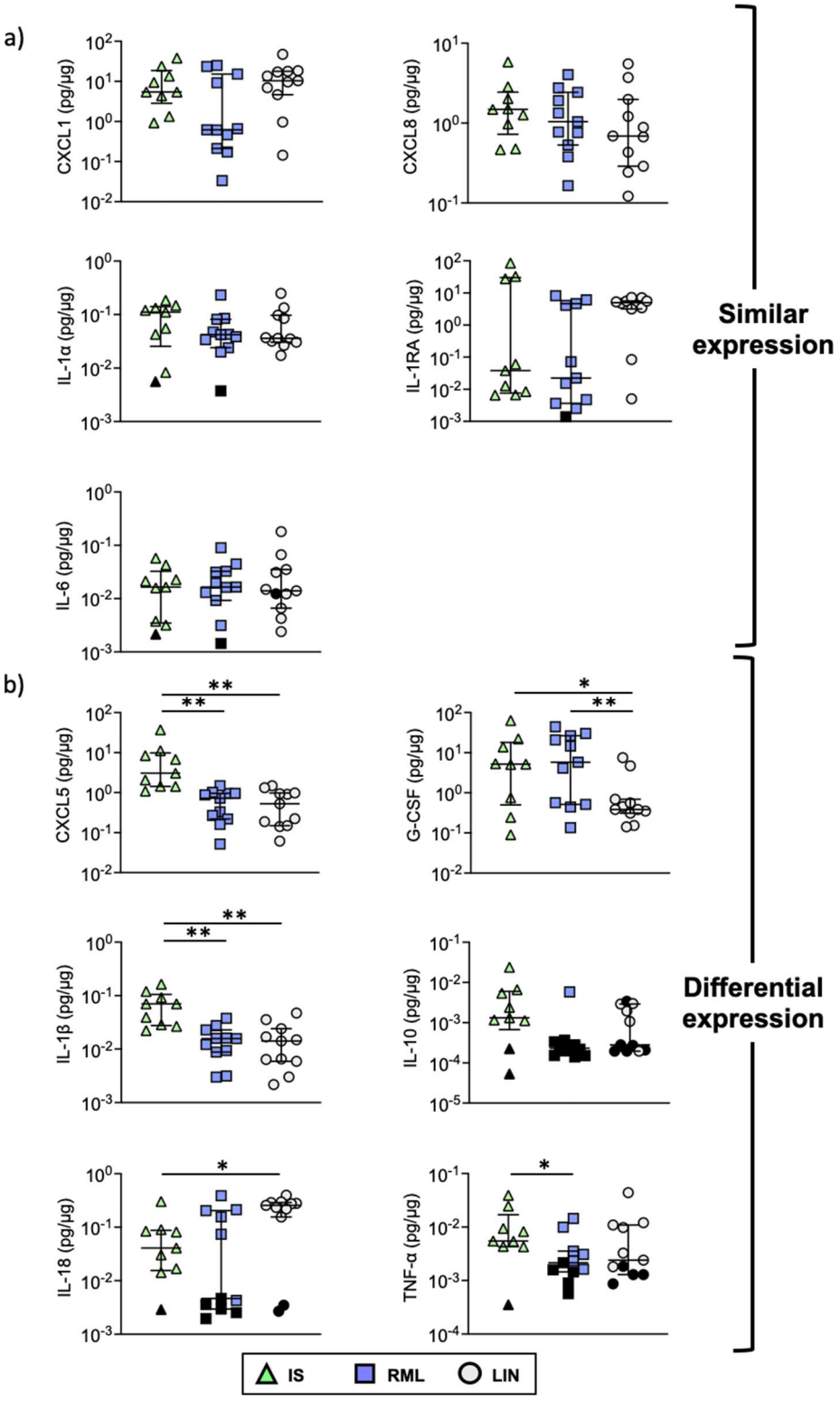


Figure 4.5.1. Neutrophil-related cytokines demonstrate similarities (A) and differences (B) between IS and BAL. Cytokine concentration was calculated using a 20-plex chemiluminescent assay and normalized to total protein concentration in each sample, then compared between subject-matched sample types using the Wilcoxon matched-pairs signed-rank test. Measurements below the lower limit of detection were assigned a value of ½ the lower limit of detection before normalization and represented by a black symbol. IS sample groups with more than 4/9 imputed values and BAL sample groups with more than 5/11 were not statistically compared to other groups. BAL, bronchoalveolar lavage; IS, induced sputum; RML, right middle lobe; LIN, lingula. Significant differences between groups are indicated as *$p \leq 0.05$ and **$p \leq 0.01$. Nonsignificant comparisons are not shown. IS vs. RML, n = 9; RML vs. LIN, n = 11; IS vs. LIN, n = 9.

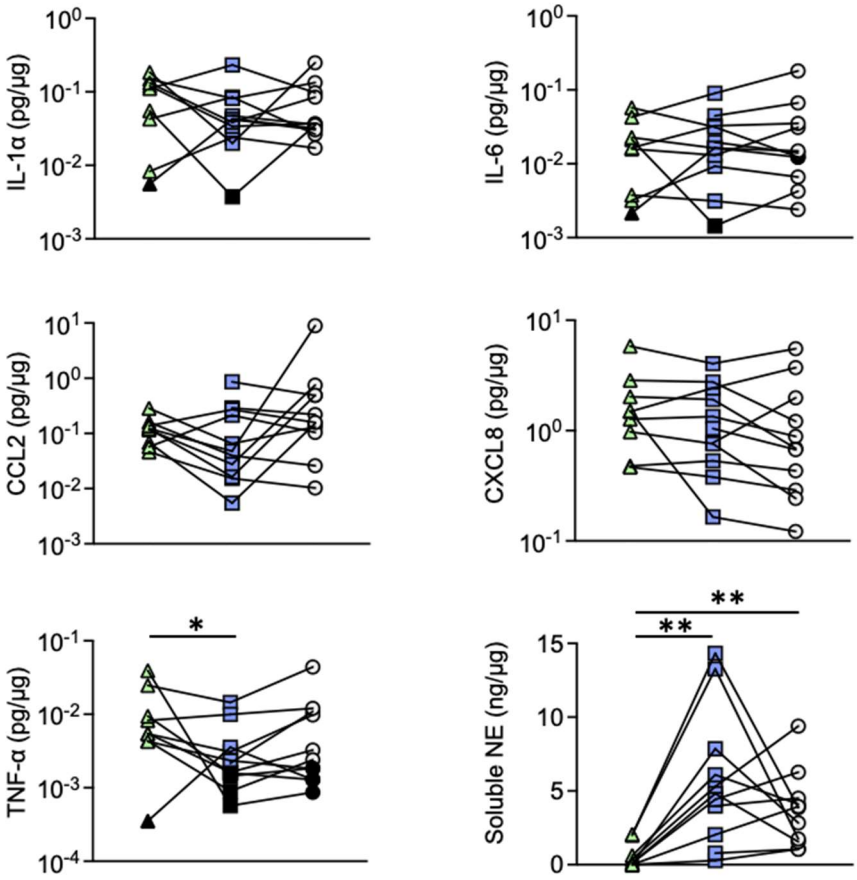


Figure 4.5.2. Connected points for soluble mediator concentrations in subject-matched samples. Graphs from **Figures 4.5.1**, **4.5.3**, and **4** are shown with lines connecting data points for subject-matched samples.

We also assessed nine mediators impacting the recruitment and activation of monocytes/macrophages and T cells (**Figure 4.5.3**). Four of those mediators showed similar levels in IS and BAL, namely, CCL2, CXCL10, M-CSF, and VEGFA (**Figure 4.5.3a**). Two of those mediators, CXCL11 and TNFSF10, showed higher levels in IS than in BAL. Finally, CCL4 and IFN-γ were below detection levels in most RML BAL samples, while GM-CSF was below the detection limit in most IS samples (**Figure 4.5.3b**).

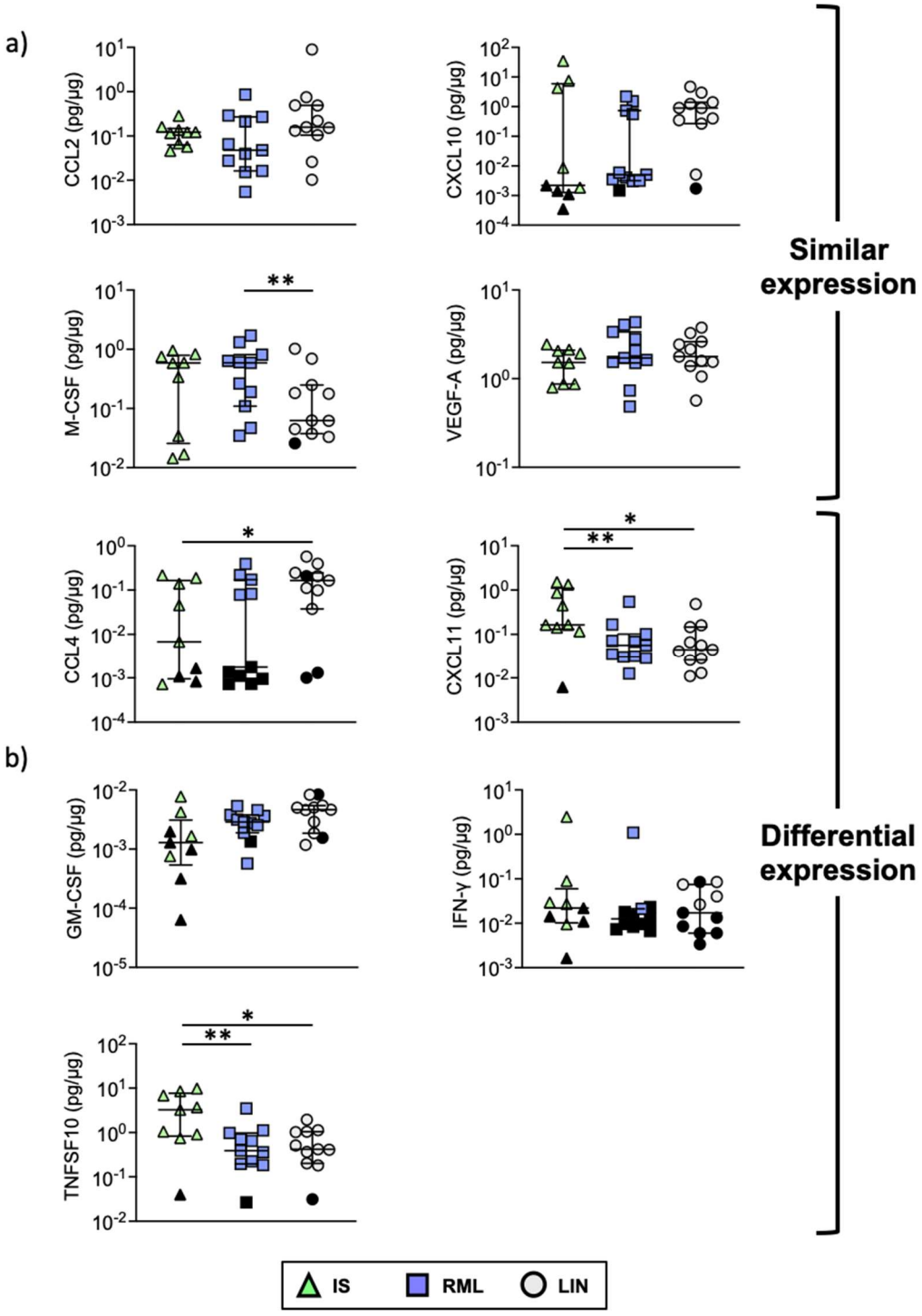

a)
CCL2 (pg/µg)
CXCL10 (pg/µg)
M-CSF (pg/µg)
**
VEGF-A (pg/µg)
Similar expression
CCL4 (pg/µg)
*
CXCL11 (pg/µg)
*
**
b)
GM-CSF (pg/µg)
IFN-γ (pg/µg)
TNFSF10 (pg/µg)
*
**
Differential expression
IS
RML
LIN

Figure 4.5.3. Monocyte/macrophage and T-cell-related cytokines demonstrate similarities (A) and differences (B) between IS and BAL. Cytokine concentration was calculated using a 20-plex chemiluminescent assay and normalized to total protein concentration in each sample, then compared between subject-matched sample types using Wilcoxon's matched-pairs signed-rank test. Measurements below the lower limit of detection were assigned a value of ½ the lower limit of detection before normalization and represented by a black symbol. IS sample groups with more than 4/9 imputed values and BAL sample groups with more than 5/11 were not statistically compared to other groups. BAL, bronchoalveolar lavage; IS, induced sputum; LIN, lingula; RML, right middle lobe. Significant differences between groups are indicated as *$p \leq 0.05$ and **$p \leq 0.01$. Nonsignificant comparisons are not shown. IS vs. RML, n = 9; RML vs. LIN, n = 11; IS vs. LIN, n = 9.

Correlations in soluble immune mediator levels among collected fractions

To explore potential relationships between the three airway fractions collected, we assessed correlations between IS and RML BAL, IS and LIN BAL, and RML and LIN BAL in the 20 immune mediators measured (**Figure 4.5.4**). Six neutrophil-associated mediators had significant positive correlations between IS and one or both of the BAL fractions, namely, CXCL8, G-CSF, IL-1α, IL-1RA, IL-6, and TNF-α. Two monocyte/macrophage-associated mediators had significant positive correlations between IS and one or both of the BAL fractions, namely, M-CSF and VEGF-A. Between RML and LIN BAL fractions, mediators with significant positive correlations included four neutrophil-associated mediators (CXCL5, CXCL8, IL-1β, and IL-6) and three monocyte/macrophage-associated mediators (CXCL11, GM-CSF, and VEGF-A).

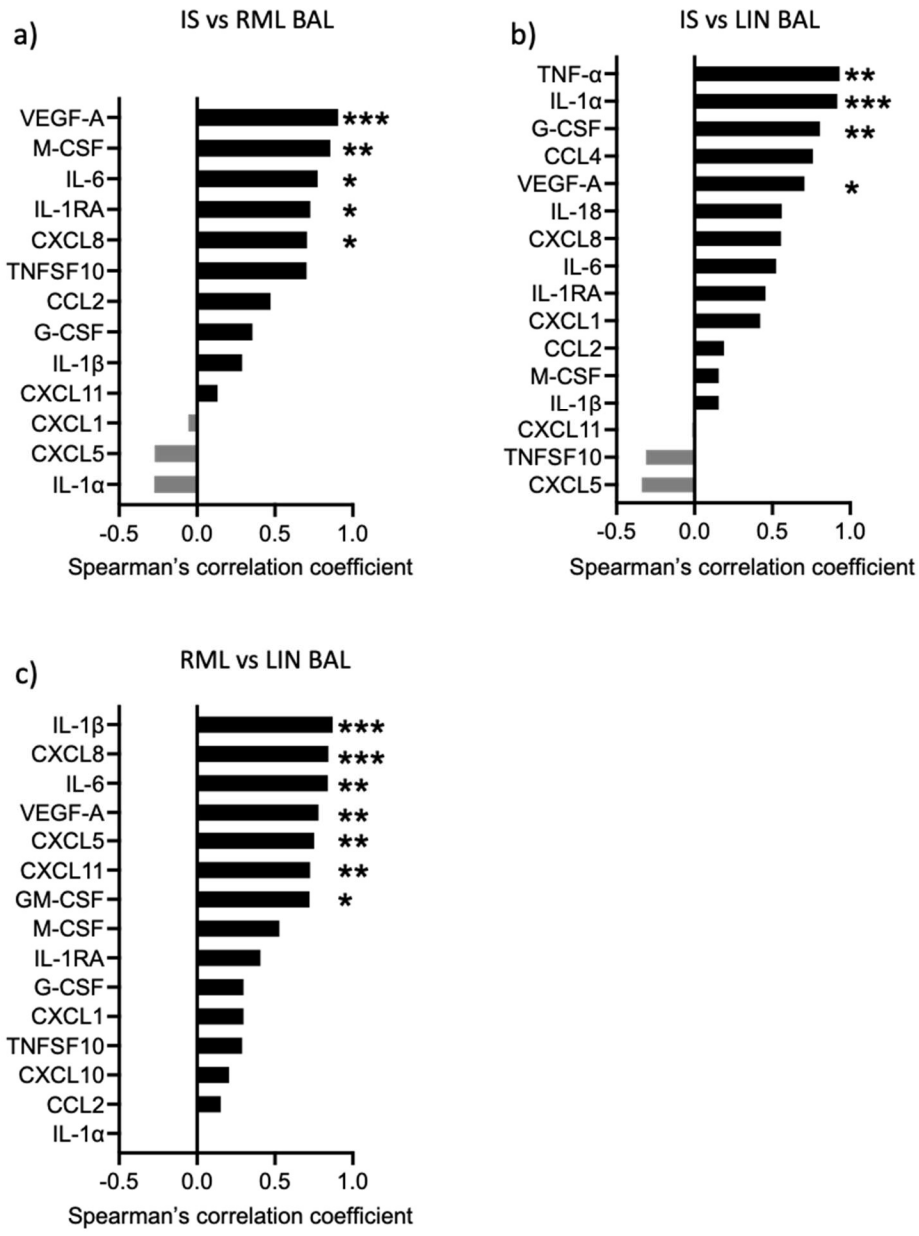


Figure 4.5.4. Concentrations of key mediators correlate between airway compartments. Twenty mediators were measured in IS and BAL using a chemiluminescent assay and normalized to total protein concentration in the fluid. Values below the lower limit of detection were excluded, and Spearman's correlation was conducted between subject-matched IS and RML BAL **(a)**, IS and LIN BAL **(b)**, and RML and LIN BAL **(c)** for mediators with at least 6 data points in each group. Significant correlations between groups are indicated as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Next, we assessed the cross-correlations of the three airway fluid fractions with plasma (**Figure 4.5.5**). In all three airway samples, we observed positive correlations between CXCL8 and IL-1β and CXCL11 with TNFSF10. In IS and RML BAL, TNF-α and TNFSF10 correlated positively and G-CSF and IL-1RA correlated negatively. In IS and LIN BAL, CCL2 and IL-6 correlated positively. In RML and LIN BAL, CXCL11 correlated positively with CCL2, CXCL10, IL-1β, and TNF-α and IL-1RA correlated positively with TNF-α. Plasma cross-correlations differed from those in airway samples, except for positive correlations between IL-10 and IL-18 observed in both plasma and IS; IL-1RA and TNF-α observed in plasma and both BAL fractions; and CXCL8 and IL-1α, as well as IL-1RA with IL-18, observed in both plasma and RML BAL.

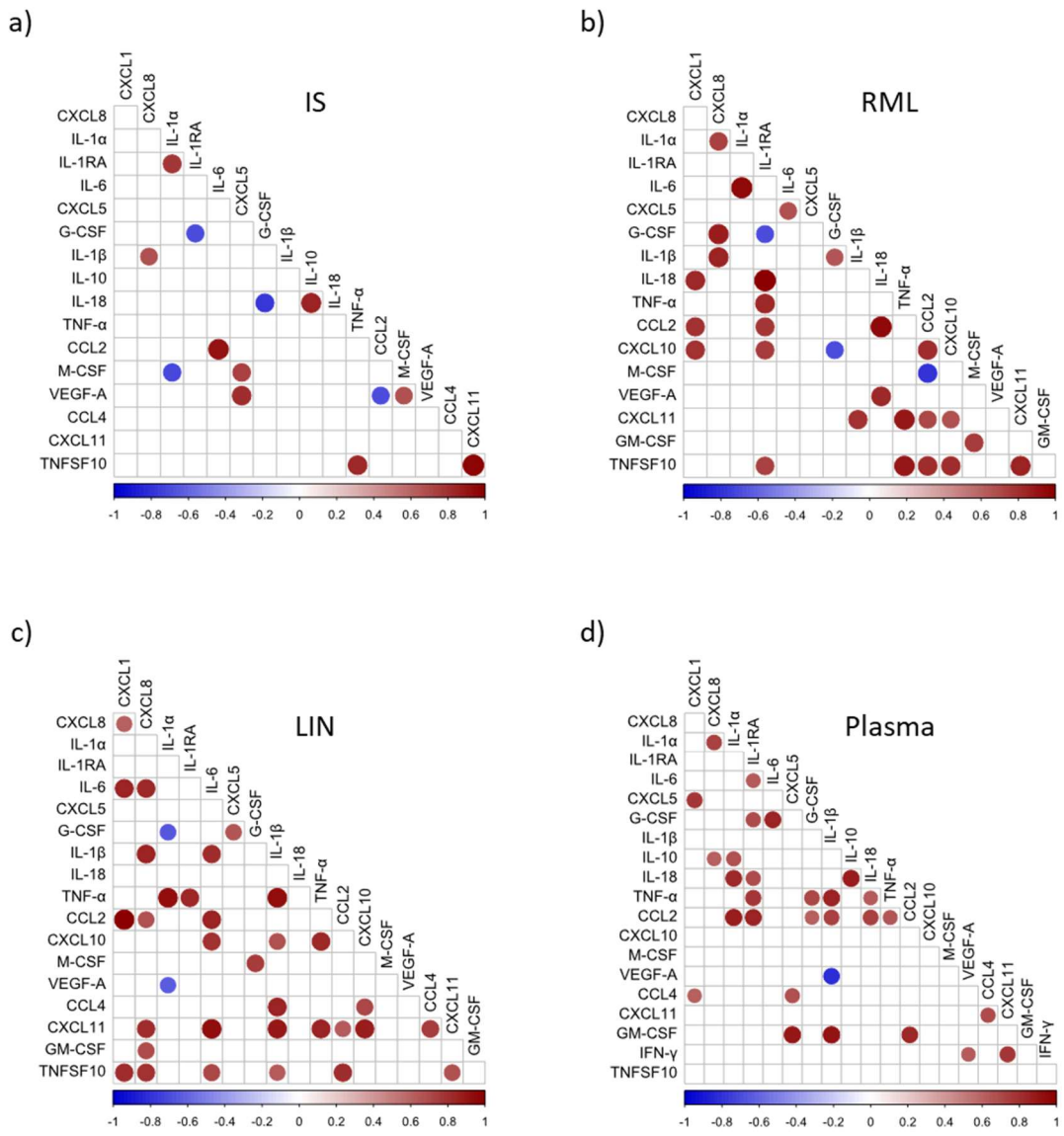


Figure 4.5.5. Cross-correlations of inflammatory response cytokines in each fluid. Spearman's correlations were calculated for IS **(a)**, RML BAL **(b)**, LIN BAL **(c)**, and plasma **(d)**. Only significant correlations are shown ($p<0.05$). Mediator concentrations in IS, RML, and LIN were normalized to protein concentration, while concentrations in plasma were not transformed. Values below the LLOD were excluded, and correlations were conducted only for mediators with at least 6 data points.

Cellular analysis of IS yields monocyte/macrophage, T cell, and neutrophil subsets, with phenotypes similar to BAL

By flow cytometry, we gated live monocytes/macrophages, T cells, and neutrophils in BAL as we showed before [19], and successfully applied the same gating strategy to IS **(Figure 4.4.2)**. Relative frequencies of neutrophils and monocytes/macrophages were similar in IS and BAL fractions, with a predominance of monocytes/macrophages in both. In contrast, the frequency of T cells was significantly lower in IS compared to BAL **(Figure 4.5.6a)**. Neutrophils were activated in IS compared to blood based on increased surface expression of CD66b (secondary granule exocytosis marker). However, surface CD63 (primary granule exocytosis marker) was not significantly increased in IS compared to blood, likely due to the very early stage of airway disease in this 2-year-old cohort. CD16 expression was reduced on BAL, but not IS, neutrophils compared to blood **(Figure 4.5.6b)**. Monocytes/macrophages in IS had significantly higher surface levels of CD115 (M-CSF receptor), while surface expression of CD163 (scavenger receptor) trended lower, as compared to BAL. Monocytes/macrophages in IS also showed decreased surface expression of CD16, which is sensitive to NE-mediated cleavage **(Figure 4.5.6c)**.

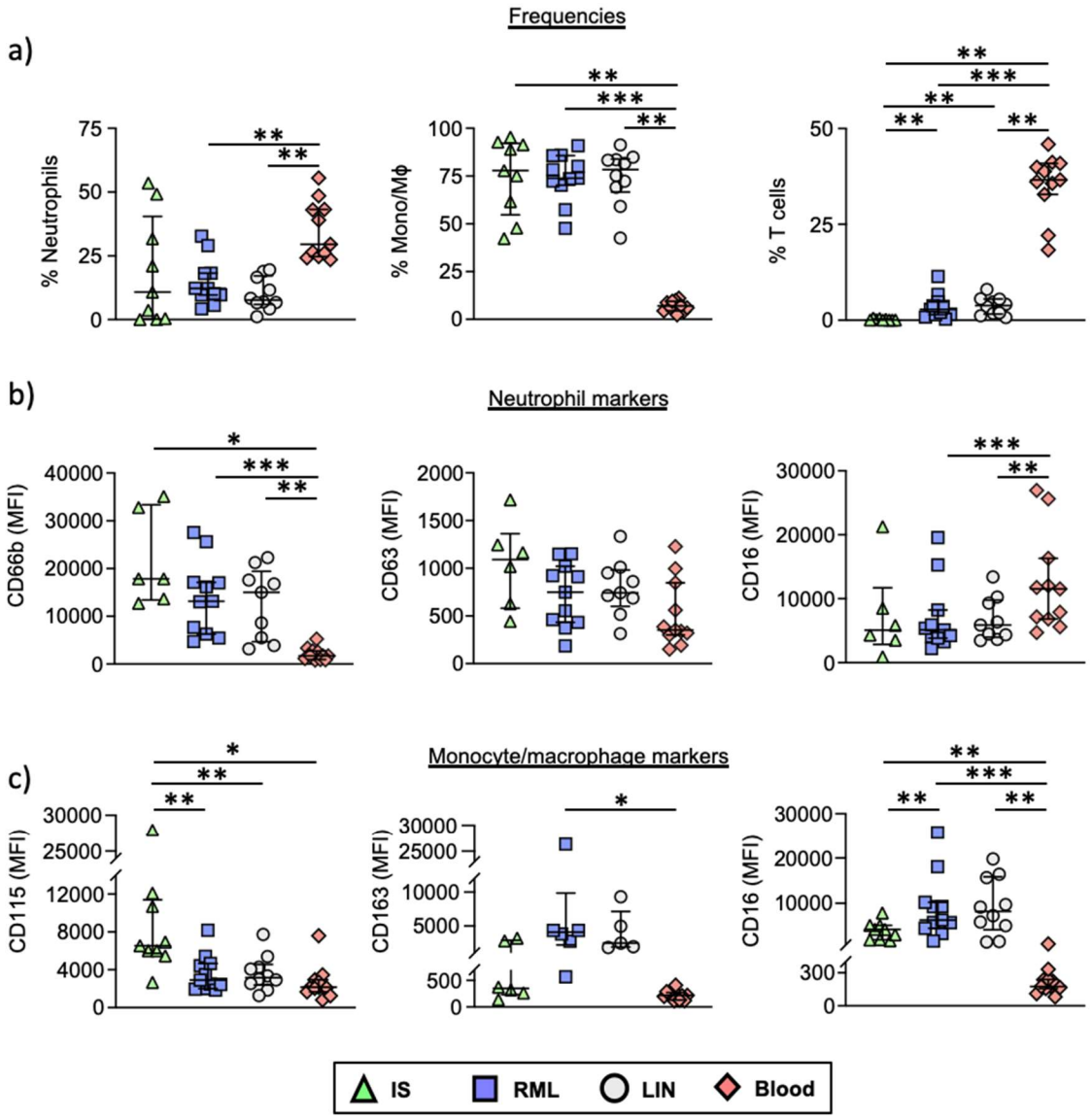


Figure 4.5.6. IS and BAL demonstrate similar leukocyte frequency and neutrophil surface marker expression but differences for macrophages. (a) Major leukocyte populations were identified by flow cytometry gating strategy. Surface protein expression of CD66b, CD63, and CD16 for neutrophils **(b)** and CD115, CD163, and CD16 for monocytes/macrophages **(c)** were reported as median fluorescence intensity (MFI). Statistical testing was performed on subject-matched sample types using Wilcoxon's matched-pairs signed-rank test. BAL, bronchoalveolar lavage; IS, induced sputum; LIN, lingula; RML,

right middle lobe. Significant differences between groups are indicated as *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

We further analyzed neutrophil populations by determining the proportion of neutrophils demonstrating the GRIM phenotype ($CD63^{high}$ $CD16^{low}$, as shown in **Figure 4.4.2**). The prevalence of these highly exocytic neutrophils was not significantly different between IS and BAL. Although there was a substantial dynamic range among airway samples, all blood samples had <1% GRIM neutrophils. Despite the high prevalence of GRIM neutrophils in some IS samples, soluble NE (following release from the primary granules of neutrophils) was lower in IS than in BAL **(Figure 4.5.7a)**. Extracellular NE can be scavenged by neutrophils and monocytes/macrophages, which we assessed by surface staining with flow cytometry. In contrast to the difference with soluble NE measurement, surface NE on neutrophils and monocytes/macrophages did not differ between IS and BAL, while blood cells had lower surface NE, as expected **(Figure 4.5.7b)**. Paired analysis was performed for comparisons of soluble mediators, cell surface markers, and NE activity for subject-matched samples and a selection of graphs with connected points linking samples from given individuals is provided in **Figure 4.5.2**.

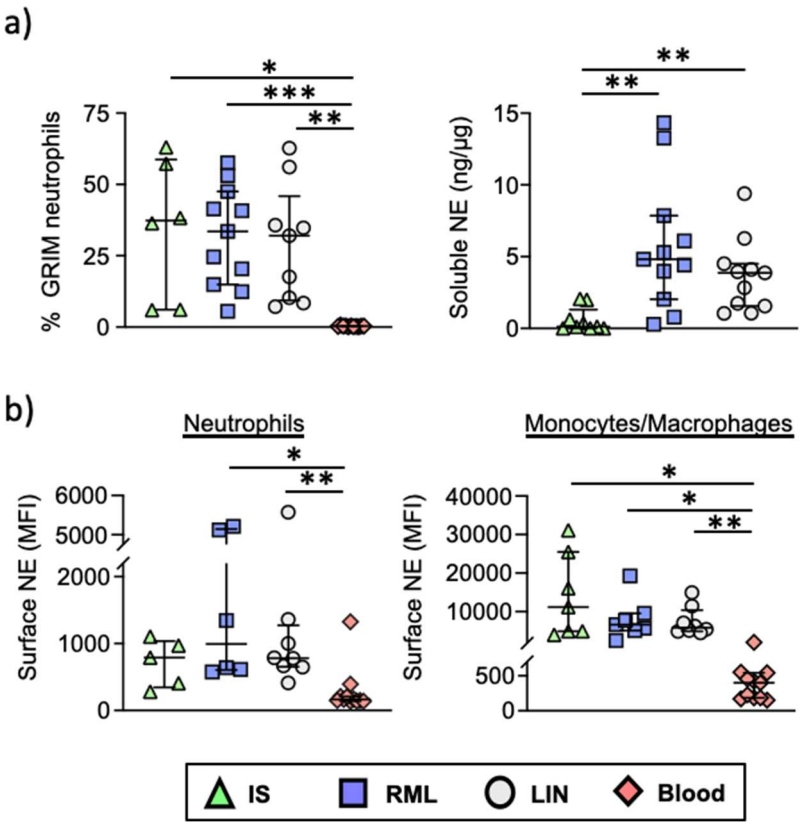


Figure 4.5.7. IS and RML display similar proportions of GRIM neutrophils, but soluble NE activity was lower in IS. (a) The proportion of neutrophils demonstrating the GRIM phenotype ($CD63^{high}$, $CD16^{low}$) was determined by flow cytometry, and soluble NE was quantified in IS and BAL using a FRET-based activity assay, then normalized to total protein concentration in the fluid. **(b)** Surface NE on neutrophils and monocytes/macrophages in blood, IS, and BAL was determined by flow cytometry. Subject-matched data points were compared using Wilcoxon's matched-pairs signed-rank test. BAL, bronchoalveolar lavage; GRIM, granule releasing, immunomodulatory, and metabolically active; IS, induced sputum; LIN, lingula; RML, right middle lobe. Significant differences between groups are indicated as *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$. Nonsignificant comparisons are not shown.

Neutrophil frequency in IS correlates with air trapping measured on CT scans

Collection of IS and BAL from the RML and LIN within an hour of chest CT scans allowed us to assess potential correlations between inflammatory biomarkers measured in these three sets of lung samples and structural lung damage. While this cohort of 2-year-olds displayed little to no bronchiectasis, we found strong internal correlations among the other measures of structural damage (**Table 4.5.1**), namely, total disease (%Dis), mucus plugging (%MP), abnormal airways (%AA), and air trapping (%TA) measured with the sensitive PRAGMA-CF scoring method. Air trapping was also correlated with neutrophil frequency in IS ($\rho = 0.7113$, $p = .0317$). Reflecting the early stage of disease in this cohort, none of the typical BAL predictors of structural lung disease observed in prior studies of older children with CF [15, 19, 26] including % neutrophils, % GRIM neutrophils, NE, and CXCL8, correlated with any of the PRAGMA-CF scores.

P values		PRAGMA			
		%Dis			
	%MP	0.0030	%MP		
PRAGMA	%AA	3.84E-09	0.0079	%AA	
	%TA	4.05E-05	0.0094	1.14E-05	%TA
	%N_IS	0.1447	0.4758	0.0805	0.0317
	%GRIM_IS	0.1562	0.1733	0.1562	0.1108
IS	%Mac_IS	0.5588	0.9628	0.3660	0.2318
	NE_IS	0.7268	0.3251	0.7268	0.6615
	CXCL8_IS	0.1684	0.2381	0.1524	0.2032
	%N_RML	0.8209	0.8778	0.8418	0.5637
	%GRIM_RML	0.9682	0.5343	0.9894	0.9682
BAL RML	%Mac_RML	0.7796	0.7345	0.7591	0.9258
	NE_RML	0.7899	0.6205	0.7490	0.7490
	CXCL8_RML	0.4160	0.4534	0.3843	0.3689
	%N_LIN	0.9867	0.9254	0.8282	0.6751
	%GRIM_LIN	0.7806	0.8994	0.7150	0.5292
BAL LIN	%Mac_LIN	0.9601	0.6927	0.8807	0.8282
	NE_LIN	0.7388	0.9510	0.8418	0.8627
	CXCL8_LIN	0.5005	0.5985	0.5363	0.8106

		IS				
	%N_IS	%N_IS				
	%GRIM_IS	0.6228	%GRIM_IS			
IS	%Mac_IS	0.0025	0.6228	%Mac_IS		
	NE_IS	0.9310	0.4993	0.5996	NE_IS	
	CXCL8_IS	0.1875	0.7040	0.1250	0.2772	CXCL8_IS
	%N_RML	0.2054	0.5441	0.2054	0.9310	0.3085
	%GRIM_RML	0.8647	0.5441	0.3807	0.0168	0.3558
BAL RML	%Mac_RML	0.5457	0.5441	0.3317	0.7611	0.8312
	NE_RML	0.8984	0.7872	0.4600	0.0102	0.3807
	CXCL8_RML	0.1116	0.5441	0.0358	0.3980	0.0016
	%N_LIN	0.6514	0.7471	0.1390	0.1702	0.9554
	%GRIM_LIN	0.4821	0.6000	0.7017	0.1294	0.8790
BAL LIN	%Mac_LIN	0.9108	0.8729	0.8225	0.3726	0.9554
	NE_LIN	0.4879	0.2657	0.7980	0.5091	0.5457
	CXCL8_LIN	0.3085	0.7872	0.1875	0.4802	0.0159

		BAL RML				
	%N_RML	%N_RML				
	%GRIM_RML	0.8525	%GRIM_RML			
BAL RML	%Mac_RML	0.2466	0.1601	%Mac_RML		
	NE_RML	1.0000	0.0008	0.1420	NE_RML	
	CXCL8_RML	0.2115	0.1097	0.4171	0.2006	CXCL8_RML
	%N_LIN	0.6761	0.0479	0.2443	0.0376	0.6761
	%GRIM_LIN	0.1875	0.0037	0.2440	0.0499	0.3558
BAL LIN	%Mac_LIN	0.8028	0.7770	0.1173	0.8287	0.7770
	NE_LIN	0.4334	0.6696	0.4500	0.4500	0.9366
	CXCL8_LIN	0.9155	0.4841	0.7293	0.5192	0.0053

		BAL LIN			
	%N_LIN	%N_LIN			
	%GRIM_LIN	0.2646	%GRIM_LIN		
BAL LIN	%Mac_LIN	0.9338	0.3085	%Mac_LIN	
	NE_LIN	0.9338	0.5165	0.0022	NE_LIN
	CXCL8_LIN	0.5796	0.2861	0.1173	0.2345

Rho coefficients		PRAGMA			
		%Dis			
	%MP	0.8020	%MP		
PRAGMA	%AA	0.9909	0.7493	%AA	
	%TA	0.9269	0.7387	0.9452	%TA
	%N_IS	0.5272	0.2739	0.6109	0.7113
	%GRIM_IS	-0.6571	-0.6375	-0.6571	-0.7143
IS	%Mac_IS	-0.2259	-0.0183	-0.3431	-0.4435
	NE_IS	0.1362	0.3714	0.1362	0.1702
	CXCL8_IS	0.5021	0.4382	0.5188	0.4686
	%N_RML	-0.0774	0.0526	-0.0683	0.1959
	%GRIM_RML	-0.0137	0.2106	-0.0046	0.0137
BAL RML	%Mac_RML	0.0957	0.1158	0.1048	-0.0319
	NE_RML	0.0911	0.1685	0.1093	0.1093
	CXCL8_RML	0.2733	0.2527	0.2916	0.3007
	%N_LIN	-0.0061	0.0341	-0.0790	-0.1520
	%GRIM_LIN	-0.1088	0.0495	-0.1423	-0.2427
BAL LIN	%Mac_LIN	-0.0182	-0.1434	0.0547	0.0790
	NE_LIN	-0.1139	-0.0211	-0.0683	0.0592
	CXCL8_LIN	0.2278	0.1790	0.2096	0.0820

		IS				
	%N_IS	%N_IS				
	%GRIM_IS	-0.2571	%GRIM_IS			
IS	%Mac_IS	-0.8667	0.2571	%Mac_IS		
	NE_IS	-0.0339	-0.3479	-0.2034	NE_IS	
	CXCL8_IS	0.4833	-0.2000	-0.5500	0.4068	CXCL8_IS
	%N_RML	0.4667	0.3143	-0.4667	0.0339	0.3833
	%GRIM_RML	-0.0667	-0.3143	-0.3333	0.7628	0.3500
BAL RML	%Mac_RML	-0.2333	-0.3143	0.3667	-0.1187	0.0833
	NE_RML	-0.0500	-0.1429	-0.2833	0.7967	0.3333
	CXCL8_RML	0.5667	-0.3143	-0.7000	0.3221	0.8833
	%N_LIN	-0.1905	-0.2000	0.5714	-0.5367	0.0238
	%GRIM_LIN	-0.3214	-0.4000	-0.1786	0.6301	0.0714
BAL LIN	%Mac_LIN	-0.0476	-0.1000	0.0952	-0.3660	0.0238
	NE_LIN	-0.2667	-0.5429	0.1000	0.2543	0.2333
	CXCL8_LIN	0.3833	-0.1429	-0.4833	0.2712	0.7667

		BAL RML				
	%N_RML	%N_RML				
	%GRIM_RML	0.0636	%GRIM_RML			
BAL RML	%Mac_RML	-0.3818	-0.4545	%Mac_RML		
	NE_RML	0.0000	0.8545	-0.4727	NE_RML	
	CXCL8_RML	0.4091	0.5091	-0.2727	0.4182	CXCL8_RML
	%N_LIN	-0.1515	-0.6364	0.4061	-0.6606	-0.1515
	%GRIM_LIN	-0.4833	0.8500	-0.4333	0.6667	0.3500
BAL LIN	%Mac_LIN	0.0909	-0.1030	0.5273	-0.0788	-0.1030
	NE_LIN	0.2636	0.1455	0.2545	0.2545	0.0273
	CXCL8_LIN	-0.0364	0.2364	-0.1182	0.2182	0.7727

		BAL LIN			
	%N_LIN	%N_LIN			
	%GRIM_LIN	-0.4167	%GRIM_LIN		
BAL LIN	%Mac_LIN	0.0303	-0.3833	%Mac_LIN	
	NE_LIN	-0.0303	-0.2500	0.8424	NE_LIN
	CXCL8_LIN	0.2000	0.4000	-0.5273	-0.3909

Table 4.5.1. Correlations between CT scores and inflammatory outcomes. Shown are Spearman P (top) and rho (bottom) for PRAGMA-CF scores (%Dis, %MP, %AA, %TA) and inflammatory outcomes (%neutrophils -%N-; %GRIM neutrophils -%GRIM-; %macrophages -%Mac-; NE and CXCL8 levels) in IS, BAL RML and BAL LIN samples. Highlighted are significant correlations.

4.6 Discussion

Based on the analysis of select soluble immune mediators as well as leukocyte subset frequency and phenotype, findings of this pilot study suggest that collection of IS in 2-year-olds with CF is achievable and yields inflammatory biomarkers that are broadly comparable to BAL. As highly effective CFTR modulator therapy is expected to be introduced earlier and earlier in the course of early CF airway disease in the coming years, ongoing changes are expected in the onset and rate of progression of structural damage and development of bronchiectasis. This further emphasizes the need for identifying and validating noninvasive markers of lung disease that can be used to monitor the presence of lower airway inflammation in young children with CF. It is also important to recognize the regional heterogeneity that occurs in the initial stages of CF lung disease, and performing BAL in one bronchopulmonary segment of a lobe may not accurately represent the changes that are occurring in other lung segments.

BAL is collected from a localized distal bronchopulmonary segment and therefore the information it provides is local. IS on the other hand may collect material from more than one region of the lung, although it likely overrepresents diseased regions (with ongoing mucus impaction). However, it should be noted that regional variability in the origin of IS has been previously suggested as a cause for variability in repeat assessments of inflammatory markers in IS from adults with CF [36]. Unfortunately, we lack experimental evidence at this point to state with certainty whether IS from children and adults with CF collected over repeated bouts of deep expectorations is reflective of multiple areas of the lung or regional. Importantly, IS is not expected to be affected by the dilution effect of serial lavages that are performed during the collection of a BAL sample [31]. Overall, it is important to recognize intrinsic differences

between IS and BAL as we investigate inflammatory markers best suited for disease monitoring via each sampling technique.

Out of the 20 soluble immune mediators selected for analysis, GMCSF was the only one below the detection limit in the majority of IS samples, and IL-18 was the only one with lower levels in IS than in BAL. The other 18 were at equal or greater abundance in IS compared to BAL. This may be explained by the convergence of secretions from distal airways (reflected in BAL) to large airways (reflected in IS) and will benefit IS-based studies by enabling the detection of mediators that may be below detection limits in BAL. Importantly, this pilot study shows that established markers of early inflammation in CF airways are measured robustly in IS. Among these, CXCL8 is one of the most potent neutrophil chemoattractants in CF lung disease [37], along with IL-6 and TNF-α. VEGF-A contributes to vascular permeability [38] and IL-1RA serves as a counter mechanism to IL-1α- and IL-1β-mediated proinflammatory signaling [39]. IL-1α itself was comparable between IS and BAL and is one of the first proinflammatory mediators present in early lung disease [7], which makes its measurement particularly critical.

By flow cytometry, IS neutrophils exhibited significant secondary granule exocytosis compared to visit-matched blood cells, reflecting a similar shift from blood as BAL neutrophils. We previously described significant neutrophil primary granule exocytosis in early CF lung disease, as evidenced by high CD63 and low CD16 expression characteristic of CF airway GRIM neutrophils [19]. Here, we observed a similarly high proportion of GRIM neutrophils in IS and BAL, demonstrating that this key functional shift can be captured from both samples. As a result of primary granule exocytosis, NE is released into the extracellular fluid, which we and others have shown to be associated with symptoms and severity of lung disease [15, 19]. Measurements of surface and soluble NE demonstrated some differences between IS and BAL in

this study. Surface NE trended lower on IS than BAL neutrophils but was greater on IS monocytes/macrophages. In addition, soluble NE activity in IS was significantly lower in IS than in BAL. These findings suggest that NE may be differentially compartmentalized in IS and BAL, and that although sufficient NE is present in IS to be captured on the surface of scavenger cells, its activity in IS may be inhibited by the antiprotease shield, as we previously suggested [19].

Besides phenotypic data of specific cell populations, comparing the presence of each major leukocyte class in the airways can provide important insights into the status of lung disease. Early in the disease, the CF airway lumen is dominated by monocytes/macrophages with occasional T cells. As the disease progresses, neutrophils infiltrate the lumen with T cells largely excluded (reviewed in [40]). As expected in this 2-year-old cohort, monocytes/macrophages were dominant in BAL, and present in equal proportions in IS samples. Neutrophils were present to a lesser degree, which is also typical of early CF airway disease, and were equally represented in BAL and IS samples, too. T cells, however, while representing between 1% and 10% of total live leukocytes in BAL samples, were present at less than 1% in all IS samples. Thus, T-cell numbers appear to dwindle more precociously in IS than in BAL at the onset of CF airway disease, a finding that warrants validation in a larger, longitudinal cohort of patients.

There are limitations to this pilot study. First, this was a single-center cross-sectional study and the number of subjects recruited is small. Second, IS collection from young children requires additional maneuvers (use of high-frequency chest wall oscillation and pharyngeal suctioning) and additional infection control precautions for the caregiver (see details in Supporting Information: Methods). Another limitation lies in the limited volume and cell yield inherent to IS collection in young children with minimal lung disease. These may reduce the practicality of IS collection in routine care of CF children with minimal lung disease. On the

other hand, this highlights the importance of applying sample-sparing/multiplexed and highly sensitive assays like the ones used in this study to the analysis of IS. Of note, to conduct a side-by-side comparison of IS to BAL, we chose to normalize the calculated concentrations of soluble mediators and NE to that of total proteins. This method is likely a better approach than normalizing to volume, considering the possible differences in absorption of saline into the airway tissue for BAL, the variable output per lavage attempt, and the expected differences in density of IS samples. However, we previously noted total protein in BAL increases with PRAGMA-CF % Dis score [19, 41], likely as leukocytes secrete proteins into the lumen. As a result, protein normalization may also normalize in part to the inflammatory burden. Another important limitation of IS collection is the possibility of contamination of the sample by other fluids, including saliva and nasal passage secretions. In this study, we took particular precautions during the collection procedure to minimize such contamination (see Supporting Information: Methods for details). An example is the use of a suction catheter followed by aspiration of phosphate-buffered saline + ethylenediaminetetraacetic acid where we reported the final sample volume rather than mass as is typically done with adult samples. While there was a slight loss of total volume in some samples, this technique did ensure the collection of high-quality sample that was sufficient to perform most or all assays. Finally, all subjects in this study were young children with overall low measures of lung disease measured by CT scans. While we only observed one significant correlation between inflammatory markers and measures of lung disease in the present study (% neutrophils in IS correlating with air trapping), we are following these patients on a longitudinal basis and will be able to reassess correlations over time as more changes appear on repeat CT imaging. Longitudinal data will also be needed to determine if

subjects who experienced aspiration have any differences in trajectories of chest CT scores and inflammatory markers.

In sum, the concomitant recruitment of neutrophils and acquisition of the GRIM phenotype, the disappearance of T cells, and cytokine profile showing both pro- and anti-inflammatory mediators signifies that these patients who have minimal signs of lung damage by chest CT scan may still present with measurable immunological signatures of progressing towards chronic CF lung disease. In this pilot study, we demonstrated that IS can be employed successfully in young children with CF to collect valuable data for multiparameter analysis of early CF lung disease. These findings warrant further testing of IS in disease monitoring efforts and interventional trials in early CF.

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Chapter 5

In vitro modeling of neutrophilic inflammation in lung disease

5.1 At-a-glance commentary

Current scientific knowledge on the subject: There is a growing appreciation for the plasticity displayed by blood neutrophils upon recruitment to stressed tissues and sites of disease such as the airway lumen. Primary human samples are highly valuable in exploring neutrophil plasticity given the shortcomings of immortalized granulocytic cell lines and animal models. However, tissue samples such as airway fluid may be difficult to obtain. To overcome this limitation, our group has developed a modular in vitro model in which primary human blood neutrophils are made to transmigrate across an airway epithelium into fluid from patients, in order to recapitulate airway adaptations that they undergo in either healthy or various pathological contexts.

What this study adds to the field: We illustrate multiple uses of our TM model to investigate airway neutrophil signaling and functions in healthy and CF airway-like settings. These include novel observations related to secretion of the regulatory enzyme acid sphingomyelinase and the type III IFN mediator IL-29 (IFN-λ1) by healthy airway-like neutrophils, and impaired NO production by CF-airway like neutrophils (and monocytes). We also show that blood neutrophils from CF subjects do not have an inherent tendency for transcriptional burst when transmigrated to healthy control chemoattractant, although they rapidly increase intracellular RNA content when transmigrated to CF airway fluid. Finally, we adapt our TM model to test the effect of *Yersinia pestis* culture supernatants, allowing us to generate airway-like neutrophils in the context of pulmonary *Y. pestis* infection circumventing the constraints of working with this select agent.

5.2 Abstract

Background: Airway neutrophils play a key role in homeostasis and disease, notably in the lung. However, primary samples such as BAL or sputum are not always available to study. Our group developed a TM model that mimics airway neutrophil phenotype using primary blood neutrophils recruited through an epithelium into human airway fluid.

Methods: Blood neutrophils were transmigrated to LTB4 as a control chemoattractant, sputum from CF patients, or *Yersinia pestis* culture supernatant. Transmigrated cells were assessed by western blot and flow cytometry, and multiplexed cytokine assay was performed on TM supernatants.

Results: Healthy airway-like neutrophils demonstrated secretion of the regulatory enzyme acid sphingomyelinase, and the type III IFN IL-29 when stimulated with the STING agonist MSA-2. In the CF context, transmigrated neutrophils and monocytes demonstrated reduced intracellular NO compared to those recruited to LTB4, and primary blood neutrophils from CF donors (similarly to those from healthy donors) increased their RNA content only when transmigrated to CF sputum supernatant and not LTB4. Finally, in experiments using culture supernatants from *Y. pestis*, the agent of plague, neutrophil activation following intracellular calcium increase mediated by a TRPM8 channel agonist was blocked by bacterial products, suggesting a potential way by which *Y. pestis* avoids killing.

Conclusion: Our findings highlight novel secreted products of airway neutrophils, with implications for immune signaling in the lung. We also identify a NO signaling defect in CF airway leukocytes which may impair bacteria killing. Finally, we showcase a new in vitro model of pulmonary *Y. pestis* infection circumventing the constraints of working with this select agent.

5.3 Introduction

Neutrophils have an essential role in maintaining homeostasis in the lungs by exerting regulatory functions and responding to infectious challenge as well as environmental insults [1]. However, dysregulated airway neutrophil responses can also lead to lung damage, as evidenced by their key pathological role in CF lung disease [2]. Neutrophils undergo functional reprogramming when recruited to the airways of people with CF in which they display increased lifespan and degranulation [3] but, paradoxically, reduced bacteria killing [4]. Direct analysis of primary airway fluid samples such as sputum and BAL has been essential to unveil live neutrophil adaptations in the lung, but this presents a variety of challenges. Procurement of clinical samples does not always follow a defined schedule, which can be problematic for planning complex experiments. Moreover, samples must be transported to the laboratory for immediate processing to yield robust results in neutrophil-targeted assays, and consistency in data from different centers can still be affected by inherent variability among patient populations. Finally, research depending on use of primary airway samples has been slowed due to concerns with contamination, reduction of clinic visits and transition to telemedicine caused by the COVID-19 pandemic [5].

To overcome some of the challenges imposed by a dependency on fresh clinical samples, our group developed an in vitro model to mimic the adaptations human blood neutrophils undergo when recruited into the airway lumen, and initially used this model to recapitulate CF airway neutrophil defects using blood neutrophils from healthy donors transmigrated to CF airway fluid [6]. In brief, this TM model is based on a polarized and differentiated air-interface culture of human airway epithelial cells grown on a lamina propria-like scaffold to mimic the lung architecture (detailed standard operating procedure published in [7]). By applying readily-

available blood neutrophils from healthy donors to the basal side of the model, the neutrophils migrate through the scaffold and airway cell layer towards the apical compartment containing chemoattractants. By including previously obtained and processed airway fluid samples from given patients in the apical compartment, the neutrophils will reprogram to reflect the in vivo phenotype of neutrophils from that pathological microenvironment [6]. The robustness of this model has been extensively confirmed by applying its use to blood monocytes in a similar manner [8] and was adopted by our collaborators affiliated with the Australian Respiratory Early Surveillance Team for CF [9]. Importantly, this model can be applied to airway diseases besides CF including severe asthma, COPD [6] and ARDS [10]. By using the flexibility of the TM model, we identified novel functions and adaptations of airway-like neutrophils and monocytes that would be very difficult to investigate using primary airway samples.

5.4 Methods

Human subjects and sample processing

Healthy control adults were enrolled and consented through the Cystic Fibrosis Biorepository of Emory University (IRB00018494) for collection of venous blood. Isolation of neutrophils and monocytes was performed by density centrifugation as described in section **2.4**.

Transmigration (TM) model

The preparation of the TM model was conducted as previously described [6, 7], and is provided in section 2.4. In brief, H441 cells were cultured at air-liquid interface on the Alvetex scaffold for two weeks and then the Alvetex membrane was inverted so that the apical side was facing down into the cell culture plate (**Figure 5.4.1**). The wells of the cell culture plate contained either RPMI with 100 nM LTB4 as a healthy control chemoattractant, debris-free CFASN diluted 1:3 in RPMI, or debris-free culture supernatant from *Y. pestis* cultures. Purified cells were then added to the basolateral side and allowed to migrate through the H441 later for 12 hours. Transmigrated cells were harvested by centrifugation at 800 x g for 5 minutes at 4°C and the supernatant was removed for further centrifugation at 3,000 x g. This debris-free supernatant was used for quantification of immune mediators. The cell pellet was washed and resuspended in RPMI for use in downstream assays. Test drugs were added to the apical compartment prior to loading of the cells for TM.

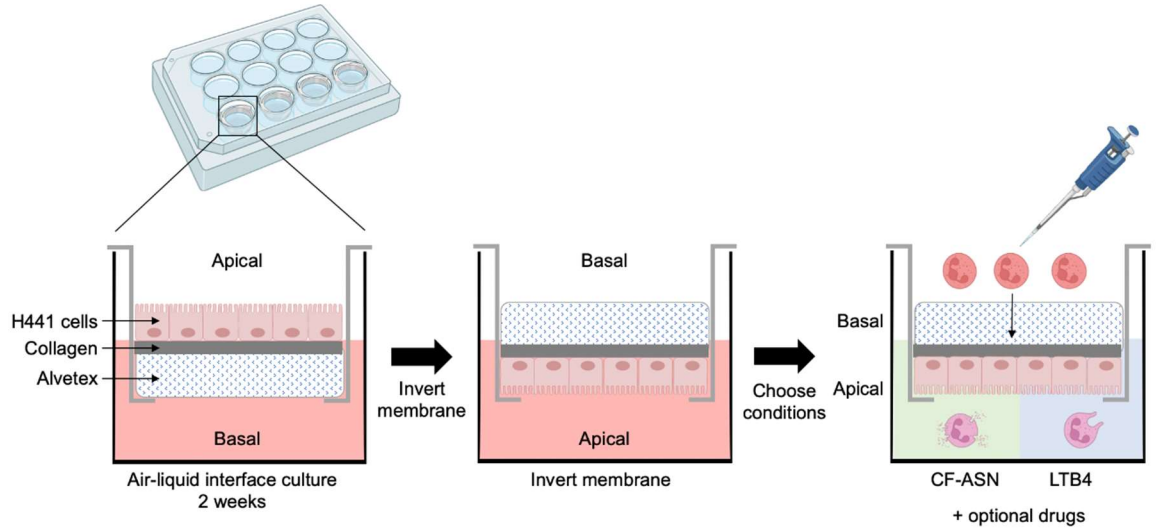

Figure 5.4.1. In vitro TM model. A collagen layer is deposited on top of the Alvetex scaffold and then H441 epithelial cells are seeded and allowed to grow at air-liquid interface for 2 weeks. To perform the experiment, the Alvetex scaffold is removed, inverted, and replaced so that the apical side is now facing down into the culture well. The media in the well is replaced to reflect the desired conditions, using RPMI as a base with added CFASN, LTB4, or other fluids and test compounds. Neutrophils or monocytes isolated from blood are added on top of the inverted scaffold (the basal side) and allowed to migrate through the epithelial monolayer toward the chemoattractants. Transmigrated cells may then be harvested from the culture well.

Western blot

Acid sphingomyelinase was detected by western blot in transmigrated neutrophils and culture supernatant as described in [11]. In brief, samples were boiled at 95°C and then proteins were separated on a polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and blocked using Licor Intercept buffer. Cellular and secreted forms of acid sphingomyelinase were detected using 1:200 anti-SMPD1 antibody (R&D Systems) overnight at 4°C, then 1:5000 secondary antibody (Licor donkey anti-goat 680) for 1 hour at room temperature.

Soluble immune mediator quantification

A custom array of 20 soluble immune mediators was quantified in debris-free TM culture supernatant using a chemiluminescence assay (U-PLEX, Meso Scale Diagnostics) according to the manufacturer's protocol.

Flow cytometry

Staining of transmigrated cells was conducted as detailed in section 4.4. In brief, wells were washed and pre-stained with Fc block and live-dead stain, followed by antibody staining for surface markers. Measurement of NO and intracellular calcium were performed using diaminofluorescein-FM-diacetate (Thermofisher, catalog #D23844) and Fluo-4 (Thermofisher, catalog #F14201), respectively. Cells were stained with these reagents for 30 minutes at 37°C prior to the pre-stain. Samples with only surface staining were then fixed and acquired within two weeks, but samples stained for NO or calcium were acquired immediately without fixation.

RNA purification and quantification

RNA was isolated from transmigrated neutrophils using a purification kit according to the manufacturer's instructions (Takara Bio USA, #740955.50). In brief, $1.5x10^6$ neutrophils were resuspended in lysis buffer with β-mercaptoethanol and vortexed. The lysate was centrifuged in a filter column and the flow through was discarded. Ethanol was added to the column, mixed by pipet on the filter, and then transferred to a new column. The filter was centrifuged and transferred to a new collection tube. Desalting buffer was added to the filter and centrifuged, and the flow through was discarded. The filter was treated with DNase and washed three times, then a final elution was performed by adding RNase-free water to the column and centrifuging. RNA

concentration and sample quality were measured using a Bioanalyzer (Agilent Technologies, Inc.).

Bacterial culture

50 mL of RPMI 1640 without serum or pH indicator (Corning) was added to flasks. Flasks were inoculated with *Yersinia pestis* CL415 or *Pseudomonas aeruginosa* PAO1 and cultured in a shaking incubator at 200 rpm. *P. aeruginosa* was grown at 37°C, while *Y. pestis* was grown at either 20°C or 37°C. Cultures were transferred to 50 mL conical tubes and spun at 4000 rpm for 10 minutes. The culture supernatant was removed and filtered through a 0.2 μm syringe filter to remove any remaining bacteria. To confirm that no viable cells remained in the supernatant, 10 μL of the media was aliquoted onto an LB agar plate (VWR) and grown at 37°C overnight. This *Y. pestis* strain is select agent exempt due to a 100,000 base pair deletion in the *pgm* locus which encodes essential virulence factors, resulting in attenuation of in vivo replication [12].

Statistical analysis

Non-parametric statistics including the Friedman test with Dunn's test for multiple corrections and Wilcoxon matched pairs signed-rank test were performed using Prism version 9.

5.5 Results

Airway-like neutrophils produce and secrete acid sphingomyelinase

Acid sphingomyelinase converts sphingomyelin to sphingolipids, which have been shown to promote CF lung disease [13]. This enzyme is a known bacterial virulence factor [14] but the early stages of CF lung disease often occur prior to detectable infection. Acid sphingomyelinase can be secreted by lung macrophages and fibroblasts [15]. The lung is a major compartment for neutrophil recruitment and these cells are key contributors to CF lung disease pathogenesis [1]. However, it is unknown whether neutrophils produce acid sphingomyelinase. To tackle this question, we obtained blood neutrophils from healthy control donors and transmigrated them to LTB4. Neutrophils were harvested, washed, and allowed to condition fresh RPMI medium for 4 hours. The cells were then pelleted and we used western blot to measure acid sphingomyelinase. As shown in **Figure 5.5.1**, we detected acid sphingomyelinase in both cell lysate (lysosomal form) and conditioned media (secreted dimer form).

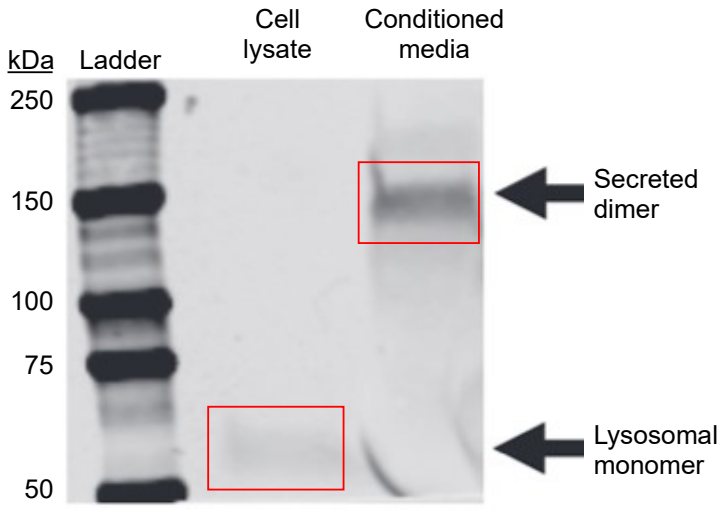

Figure 5.5.1. Airway-like neutrophils produce acid-sphingomyelinase. Healthy donor blood neutrophils (n = 1) were transmigrated to LTB4 for 4 hours, harvested, and incubated to generate conditioned medium. Western blot was used to detect the lysosomal (60 kDa) and secreted (150 kDa) forms of acid sphingomyelinase in the lysate and conditioned medium.

Airway-like neutrophils secrete the type III IFN IL-29 upon STING agonist stimulation

In addition to their well-known role in responding to bacterial infections, neutrophils are also important for antiviral responses [1]. However, excessive neutrophil recruitment during viral infections can lead to acute respiratory distress syndrome [16]. The first class of IFN to be produced in response to viral infection are type III, including IL-29 [17]. IL-29 is mainly produced by epithelial cells, macrophages, dendritic cells, monocytes, and monocyte-derived macrophages and dendritic cells [18, 19] and expression of the receptor, IFNLR1, is restricted primarily to epithelial cells, but also neutrophils [20]. Because neutrophils are among the limited set of cells that express IFNLR1 and are related to the other myeloid cells which produce this cytokine, neutrophils may represent a currently unknown source of IL-29 in the lungs. In contrast to types I and II IFNs, IL-29 can promote early antiviral immunity while limiting neutrophilic

inflammation [17], making it an early, non-inflammatory IFN. Despite the promise of IL-29 as a potential antiviral agent with limited side effects, experimental studies have been limited by the fact that mouse models do not express IL-29 [20]. Because STING signaling is important for mediating type I IFN production by myeloid cells [21], and can also promote production of type III IFN [22], we aimed to determine if activation of STING signaling in human lung-recruited neutrophils can induce production of IL-29 which may serve to limit excessive neutrophil activation.

To this end, we transmigrated healthy donor neutrophils to LTB4 in the presence of vehicle control, the STING agonist MSA-2, the EZH2 inhibitor EPZ6438, or combination treatment of MSA-2 + EPZ6438 to assess possible synergistic effect as EZH2 is a known inhibitor of STING [23]. We quantified cytokines in the debris-free TM supernatant and observed significantly higher IL-29 and IL-1β in response to MSA-2 (**Figure 5.5.2**). To determine if this secretion is primarily from the transmigrated neutrophils or the epithelium, we compared IL-29 concentration in supernatant from a neutrophil TM to H441 ALI cultures that were stimulated with the same drugs but no TM was performed. While IL-29 concentration was increased upon treatment with MSA-2 as expected, it was substantially lower than the supernatants from neutrophil TM. We also transmigrated monocytes which are known to secrete IL-29 [18], and observed concentrations similar to or slightly higher than for neutrophil TM. In contrast to this type III IFN signature, type I and II IFNs were mainly below the limit of detection in this model.

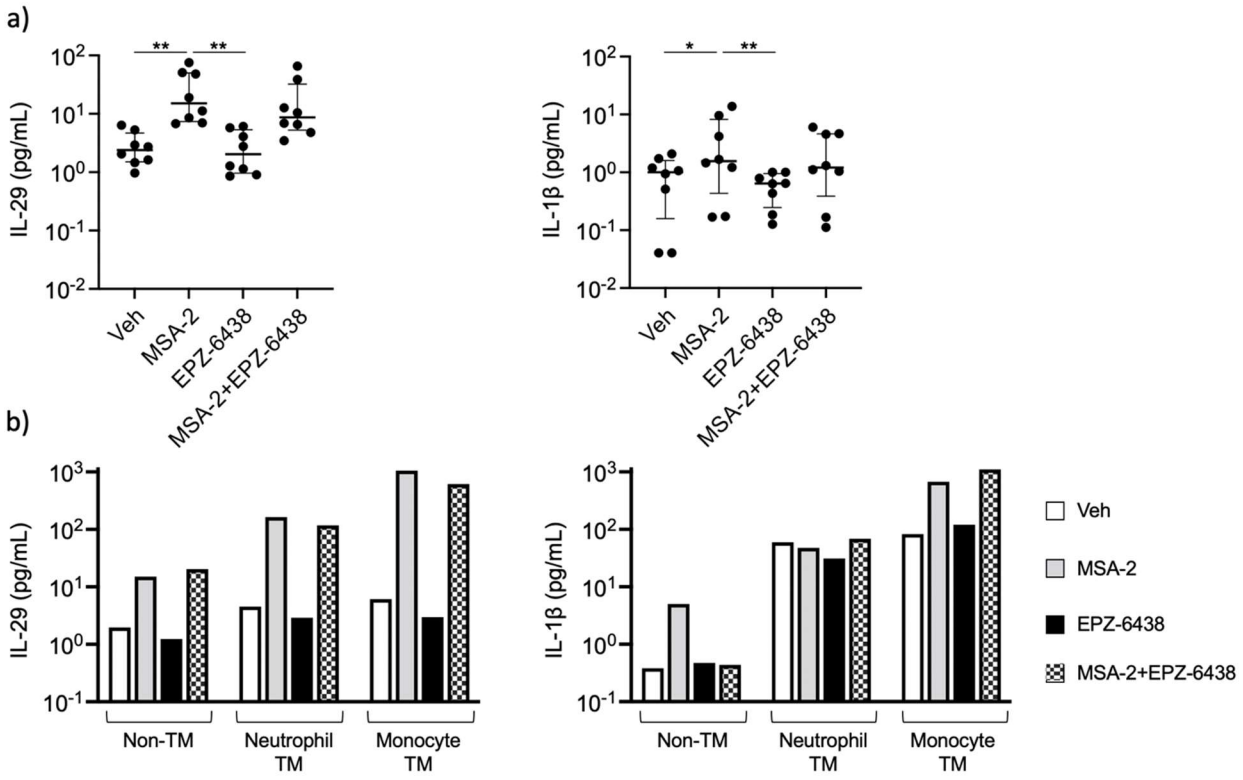

Figure 5.5.2. Airway-like neutrophils secrete IL-29 in response to STING agonist. (a) Blood neutrophils were isolated from healthy donors (n = 8) and transmigrated for 12 hrs to MSA-2 (STING agonist), EPZ-6438 (EZH2 inhibitor), combination treatment, or vehicle control. Cytokines were quantified in the fluid from the apical compartment after removal of transmigrated cells by centrifugation. Groups were compared by the Friedman test with Dunn's test for multiple comparisons, with significant difference indicated as *$p \leq 0.05$, **$p \leq 0.01$. **(b)** To determine the contribution of specific cell types to cytokine production, healthy donor blood neutrophils or monocytes (n = 1) were transmigrated to the same conditions and cytokine concentration was compared to the apical fluid of ALI cultures to which no TM was performed.

CF airway-like neutrophils and monocytes demonstrate impaired NO production

Neutrophils undergo substantial functional and phenotypic reprogramming when recruited to the CF airways including excessive degranulation and loss of phagocytic receptors

[3], as well as reduced ability to kill bacteria [4], which we also showed in monocytes [8]. While a transcriptional burst upon recruitment to the airway environment has been shown to associate with impaired bacteria killing by neutrophils in our TM model [4], there are likely to be multiple factors involved. Previously, we demonstrated that the high level of Arg1 activity in the CF airways is responsible for blocking T-cell activity through the depletion of arginine [24]. Because arginine is a necessary substrate for inducible NO synthase to produce NO, important for its role as a progenitor of downstream RNS [25], we assessed NO production in neutrophils and monocytes using the TM model (**Figure 5.5.3**). Both cell types were transmigrated to LTB4 or CFASN and stained for intracellular NO. Both neutrophils and monocytes demonstrated reduced NO in the CFASN compared to LTB4 TM condition, despite supraphysiological concentrations of L-arginine in the medium. Indeed, the concentration of L-arginine in CF sputum is approximately 10-20 nM [26] while the concentration in RPMI media used in the TM model is 1.15 mM.

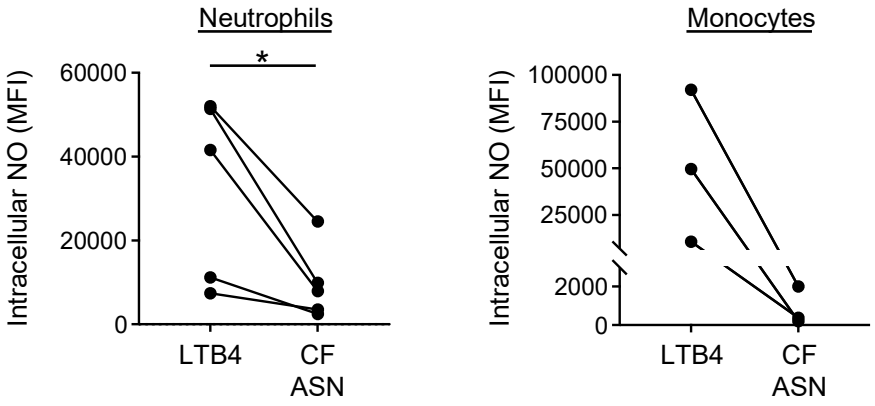

Figure 5.5.3. Recruitment into the CF airway microenvironment impairs NO production in neutrophils and monocytes. Neutrophil (n = 5) and monocytes (n = 3) from healthy donors were transmigrated to LTB4 or CFASN for 12 hours and NO was quantified by flow cytometry. Both cell types showed lower intracellular NO when transmigrated to CFASN compared to LTB4. Statistical comparison was run by the Wilcoxon signed-rank test. Significant difference is shown as *$p \leq 0.05$.

Transcriptional burst in CF airway neutrophils is dependent on the airway microenvironment

A hallmark of CF airway neutrophils in vivo is increased RNA content compared to their blood counterparts [4]. Using our TM model, we demonstrated that healthy control blood neutrophils also had higher RNA content when transmigrated to CFASN, demonstrating that this effect was driven by exposure to the diseased microenvironment [4]. However, it remains unknown whether CF donor neutrophils have an inherent tendency for transcriptional burst when recruited to any airway environment, such as that of a healthy lung. To answer this question, we isolated neutrophils from the blood of a CF patient and transmigrated them to LTB4 or CFASN. After harvesting the cells, we quantified RNA in the transmigrated populations as well as non-transmigrated purified neutrophils from the donor. Using the blood population as a baseline, we

observed a greater than 10-fold increase in total RNA for the CFASN-transmigrated cells compared to blood, while LTB4-transmigrated cells showed no increase (**Figure 5.5.4**).

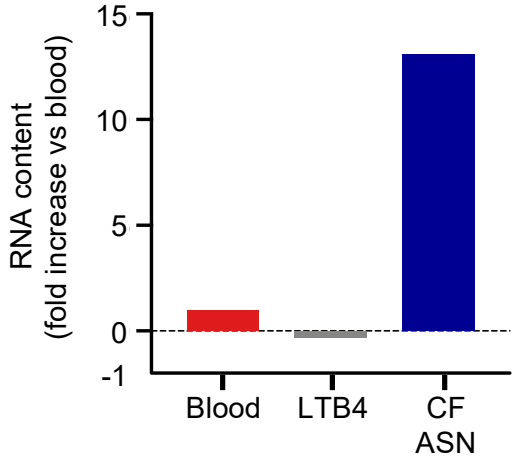

Figure 5.5.4. Transcriptional burst in CF subject neutrophils is dependent on conditions of the microenvironment. CF donor neutrophils (n = 1) were isolated from blood and transmigrated to LTB4 or CFASN. RNA was isolated from transmigrated cells and compared to non-transmigrated neutrophils purified from blood. Blood cells were used as the baseline to calculate fold change in RNA content.

Transmigration to Y. pestis culture supernatant impairs airway neutrophil calcium flux

Y. pestis is the causative agent of the fatal pulmonary disease pneumonic plague. Mounting an early neutrophil response is particularly important for controlling this infection [27], and virulence factors of *Y. pestis* have evolved to block neutrophil recruitment and activation [28]. There are considerable challenges in working with a biosafety level-3 select agent pathogen such as *Y. pestis*, including special approvals and use of attenuated strains [29]. Experimental studies of *Y. pestis* pathogenesis can be conducted with mouse models, but there are considerable differences between mouse and human neutrophils [30].

As a potential alternative to mice, we adapted our TM model to allow for the study of airway neutrophil responses exposed to *Y. pestis* virulence factors. Our TM model allows for experiments to be conducted in any laboratory following culture of the bacteria in BSL-3. Owing to the thermal regulation of *Y. pestis* virulence factors [31], we used the cell-free bacterial culture supernatant from *Y. pestis* cultures grown at low and high temperature in the apical compartment, and culture supernatant from *P. aeruginosa*, LTB4, and CFASN as controls. *Y. pestis* culture supernatants effectively recruited neutrophils into the apical compartment of the TM model, with efficiency comparable to CFASN (**Figure 5.5.5a, left**). By contrast, *P. aeruginosa* culture supernatants were less effective. The supernatant from high-temperature *Y. pestis* cultures promoted secondary granule exocytosis (demonstrated by surface CD66b expression) comparably to CFASN, while supernatant from low-temperature *Y. pestis* cultures induced even higher secondary granule release and CD66b expression (**Figure 5.5.5a, right**).

Calcium signaling is important for coordination of the essential antimicrobial functions in neutrophils such as phagocytosis and degranulation [32]. We used icilin, a potent agonist of the cation channel transient receptor potential melastatin-8 (TRPM8), to promote calcium signaling during TM. Neutrophils transmigrated to LTB4 or *P. aeruginosa* culture supernatant in the presence of icilin had increased calcium levels, but not neutrophils transmigrated to either *Y. pestis* culture supernatants (**Figure 5.5.5b, right**).

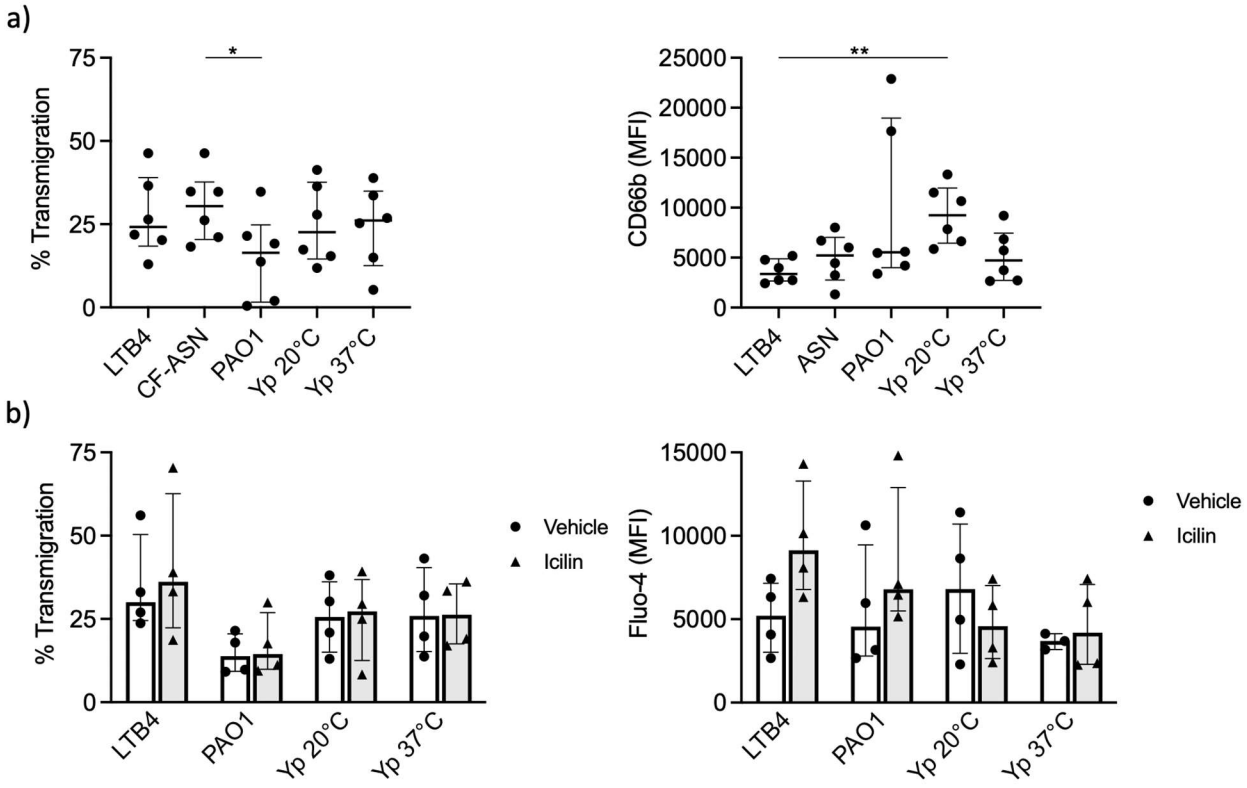

Figure 5.5.5. *Yersinia pestis* culture supernatants induce neutrophil recruitment and block calcium. **(a)** Culture supernatants from *Y. pestis* grown at 20°C and 37°C were used to recruit neutrophils (n = 6 donors) into the apical compartment of the TM model (**see Figure 5.4.1**) and compared to supernatant from the well-characterized PAO1 strain of *P. aeruginosa*, as well as LTB4 and CFASN as additional controls. *Y. pestis* culture supernatants efficiently recruit neutrophils and induce activation. **(b)** Calcium flux in transmigrated neutrophils (n = 4 donors) was assessed in cells recruited to LTB4 or supernatants from PAO1, *Y. pestis* low temperature, or *Y. pestis* high temperature cultures in the presence or absence of the TRPM8 agonist icilin. Activating the calcium-permissive TRPM8 channel increased intracellular calcium in cells transmigrated to LTB4 and PAO1 supernatant, but not the *Y. pestis* supernatants. Groups were compared by the Friedman test with Dunn's test for multiple comparisons, with significant difference indicated as *$p \leq 0.05$, **$p \leq 0.01$

5.6 Discussion

Neutrophils are a much more transcriptionally and functionally adaptable and versatile leukocyte subset than previously believed, with many recent studies calling to attention the plastic changes undergone by neutrophils when recruited to the lung [1, 33]. The physical act of migration into the lung environment is an important aspect of the reprogramming process, as we showed in the development of our TM model that incubation of blood neutrophils in airway fluid alone (without TM) was insufficient to fully recapitulate the CF airway phenotype [6].

In this study, we used our TM model to generate airway-like neutrophils in the context of a healthy lung environment using only LTB4 as a chemoattractant, CF airway disease CFASN to recruit and reprogram neutrophils, and pulmonary bacterial infection using bacterial culture supernatant. These experiments allowed us to interrogate specific aspects of neutrophil responses in each setting by controlling the entire process from naïve blood neutrophil to airway-conditioned neutrophils. Such dynamic, controlled studies are not possible with human primary airway neutrophils in vivo, nor with mouse models, which show major differences in neutrophil biology compared to humans [30].

We obtained novel data on the secretion of two proteins by airway-like neutrophils, including acid sphingomyelinase, which can promote CF lung disease through production of sphingolipids [13], and IL-29, a type III IFN important for the early stages of antiviral signaling in the lung [17]. The ability of sphingomyelinase to inhibit CFTR activity [34] further implicates neutrophil activity in CF airway disease, but may also have relevance to other chronic airway diseases. COPD has similar pathology to CF, but occurs independently of mutations in *cftr*. However, functional impairment of the CFTR protein may play a role in COPD pathogenesis [35]. People with COPD are susceptible to both bacterial infections and neutrophilic

inflammation, so worsened symptoms in COPD linked to CFTR dysfunction may be further exacerbated by inhibition of CFTR activity due not only to bacterial sphingomyelinase [34], but also neutrophil-derived acid sphingomyelinase (**Figure 5.5.1**).

Our discovery of a robust secretion of IL-29 by airway-recruited neutrophils upon stimulation by the STING agonist MSA-2 suggest a potential new avenue for therapeutic targeting of the innate immune system during viral infections. Type III IFNs are produced early in the course of viral infection and can prevent overt neutrophilic inflammation while maintaining an effective antiviral response [17]. Stimulation with a STING agonist in our TM model selectively increased IL-29 secretion by neutrophils while type I and II IFNs were present only in very low concentrations or undetectable. This finding suggests that STING agonism early in the course of viral infection may provide clinical benefit by enhancing innate antiviral immunity via promotion of crosstalk between epithelial cells, monocytes, and resident neutrophils while limiting destructive neutrophilia. This may prove particularly beneficial for infections that can progress to neutrophil-driven ARDS such as with RSV in young children and severe acute respiratory syndrome coronaviruses (SARS-CoV) 1 and 2 in adults [36]. These preliminary findings require additional validation with in vitro modeling of lung infection, as we have showed with SARS-CoV-2 [37], and in vivo models to determine if these changes can improve host viral clearance and fitness while avoiding inflammation.

In CF, a key attribute of airway neutrophils is a transcriptional burst that associates with an active repression of bacteria killing, consistent with our prior findings that blocking RNA polymerase activity in CFASN-recruited neutrophils and in short-term culture of fresh sputum neutrophils restored their bactericidal activity [4]. In that study we showed that TM to other chemoattractants, including airway fluid from COPD patients, also induced an increase in RNA

content albeit not to the high levels observed in CF. We therefore questioned if the ability of CFASN to induce such a drastic transcriptional burst was related to a greater tendency at baseline of CF subject neutrophils to increase RNA content recruited to a lung environment, be it healthy or diseased. By obtaining neutrophils from a CF donor and transmigrating those cells to either LTB4 or CFASN, we demonstrated that it was the microenvironment driving this effect and not an inherent difference in naïve neutrophils at baseline, since CF blood neutrophils transmigrated to LTB4 had no increase in RNA production.

Following on a study demonstrating the role of Arg1 in blocking airway T-cell activity through depletion of arginine [24], we investigated the use of arginine by myeloid cells. This amino acid is required by inducible NO synthase for production of NO, an important precursor of essential RNS. Because we do not yet fully understand the mechanisms involved in reduced bacteria killing by CF airway neutrophils [4] and monocytes [8], we investigated if there was an impairment of NO production in these cells. While we did observe reduced intracellular NO in both cell types when naïve blood neutrophils and monocytes were recruited in our TM model to CFASN vs LTB4 control conditions, further studies are needed to determine if this effect can specifically be attributed to Arg1 activity. It is also unclear if this lack of NO has meaningful impact on bacteria killing deficiency since both neutrophils and monocytes produce a plethora of other bactericidal molecules [38, 39].

Yersinia pestis is the causative agent of pneumonic plague, a pulmonary disease that causes severe illness and death if the infection is not controlled quickly. As such this organism is strictly regulated for laboratory use, and typically requires use of attenuated strains [12]. An early and robust neutrophil-driven response to pulmonary *Y. pestis* infection is essential for host survival, but several *Y. pestis* virulence factors can delay and dysregulate neutrophil recruitment

and degranulation [28]. We have previously adapted the TM model to study infection of primary airway cells by common CF-related pathogens [9], as well as SARS-CoV-2 [37], so we sought to determine if we could create a model of neutrophil airway conditioning in the context of pneumonic plague while minimizing our direct handling of the bacteria and gain new insights into the mechanisms by which *Y. pestis* interferes with lung neutrophil responses.

We previously showed that in addition to using primary airway fluids, media conditioned by transmigrated neutrophils could itself be used to recruit monocytes in the TM model and induce PD-1 expression [40]. As a surrogate for bacterial infection, we cultured *Y. pestis* and *P. aeruginosa* and obtained cell-free supernatant of those cultures. Although the *Y. pestis* strain we used was attenuated, as an extra precaution all bacteria were grown in a separate laboratory that routinely works with pathogenic organisms and then the supernatants were transported to our lab for use in the TM model. Because the supernatants were able to efficiently recruit neutrophils and induce degranulation without any added chemoattractants or stimulants, this system may be applicable to use with strains that have stricter biosafety requirements when live cells could not be cultured in certain laboratory settings.

Preliminary findings presented here suggest a new mechanism by which *Y. pestis* may antagonize neutrophil effector mechanisms that was not observed for *P. aeruginosa*. A calcium surge is essential to numerous neutrophil functions including diapedesis, phagocytosis, and exocytosis [32]. TRPM8 is a cation channel through which neutrophils uptake Ca^{2+} ions, and addition of the TRPM8 agonist icilin in our TM model increased intracellular calcium in airway-recruited neutrophils using control media with LTB4 and supernatant from *P. aeruginosa* culture. However, supernatants from both low- and high-temperature *Y. pestis* cultures decreased the effect of icilin. Although *Y. pestis* is known to secrete different virulence factors in a

temperature-dependent manner [31], in this application we did not observe a difference in neutrophil recruitment or calcium content between the two supernatants and more work is needed to determine how these different virulence factors may specifically impact neutrophil recruitment and Ca^{2+} transport. These questions are indeed important for physiologically-relevant modeling of pneumonic plague, as culturing the bacteria at 37°C reflective of body temperature while the temperature in the airways is generally lower, ranging from 32-35°C [41]. Even lower temperatures should be considered for physiological relevance, as pneumonic plague spread rapidly in the cold climates of northern Europe during the Bronze Age where routine inhalation of cold air may have resulted in airway temperatures as low as 20°C [41].

There are several ways in which the TM model can be further developed or adapted for other scientific and clinical settings. The H441 cells used as a source of human airway epithelial cells in the model are a human small airway (club) cell line, however substitution of primary airway cells as we have previously done [9] could be advantageous for certain experiments. Additionally, incorporation of additional cell types, recently demonstrated using endothelium, for example [42], will continue to increase the physiological relevance of the TM model. Treatment of CF has advanced tremendously in recent years due to expanded approval for use of existing CFTR modulatory therapies for younger age ranges, as well as introduction of new combination therapies compatible with the most common CFTR mutations [43]. Future studies using the TM model should investigate baseline changes in neutrophils from patients on highly effective modulators, to determine if TM of neutrophils to sputum from patients on modulators long-term vs newly prescribed or untreated patients display any differences, for example. Combination of CFTR modulators and novel immunomodulatory agents such as MSA-2 may also be tested.

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Chapter 6

Summary and perspectives

6.1 Summary of findings

CF lung disease is characterized by massive recruitment of neutrophils, which become dominant in this environment as they adopt the pathological GRIM fate. Macrophage and T-cell populations are suppressed by this massive influx of neutrophils, leading to irreversible and destructive (albeit slow) neutrophilic inflammation, as normal control mechanisms in the airways are overwhelmed [1]. Understanding the intricate relationships between these cell types in the lungs, as well as their unique contributions to health and disease, is essential for developing new immunomodulatory treatments, as well as repurposing existing drugs for improved care of CF lung disease and similar chronic neutrophil-dominated airway conditions [2].

The research presented in this dissertation contributes new data to the CF community with important implications for both clinical and basic science. First, we demonstrated that the PD-1 pathway is involved in functional exhaustion of airway macrophages early in the course of lung disease. Expression of this receptor on airway macrophages was found to correlate with lung disease, and we were able to enhance bacteria killing by primary airway leukocytes through antagonism of this signaling pathway (**Chapter 2**). Second, we identified signatures of T-cell activation during early-life APEs which offers a rare opportunity to study T cell responses in the airway before complete exclusion from this environment [3]. Neutrophils were not found to be highly present in the airways during these inflammatory episodes, but we identified increased concentrations of neutrophil chemoattractants in BAL and increased frequencies of neutrophils in circulation, as well as elevated G-CSF in plasma, providing new insights into why APEs accelerate lung function decline (**Chapter 3**). Third, we demonstrated that collection of induced sputum from young children with CF, which is minimally invasive and already part of routine care, can provide data on soluble and cellular markers of inflammation comparable to that of

invasive BAL procedures. Because material collected by sputum induction may be derived from different regions of the airways while BAL is collected from a specific lobe, we show that the early stages of inflammation may not be detected only in those lobes which are showing signs of disease (**Chapter 4**). Finally, we used an in vitro TM model to generate airway-like neutrophils and monocytes reflective of healthy and diseased states using readily-available healthy donor blood. This model allowed us to make new observations about airway leukocyte function in a controlled in vitro setting without the potential confounding effects of primary samples or in vivo models (**Chapter 5**).

6.2 Implications for CF lung disease pathogenesis

The critical role of neutrophilia in CF lung disease inception, even before detectable infection and mucus obstruction, is now widely accepted [4, 5]. However, the exact causes of sterile neutrophilic inflammation are not yet completely understood. While hypoxia is believed to play a role in the early recruitment of neutrophils to the CF airways via activation of stress pathways in epithelial cells [6], there is likely direct involvement of resident immune cells, as well. Tissue-resident macrophages are widely present in the lungs and have important roles such as clearing debris and pathogens and tissue remodeling [7]. They are also essential for controlling neutrophil recruitment into tissues upon cell death and injury [8]. However, macrophages in the CF lung exhibit numerous defects, not all of which can be attributed to CFTR expression [9].

In **Chapter 2** we investigated the role of the PD-1 pathway in exhaustion of airway macrophages, coinciding with neutrophilia, loss of bacterial killing, and advancement of lung

disease. This may be one mechanism by which resident macrophages lose their crucial ability to manage neutrophil influx, although other factors are likely involved. We observed decreased amounts of intracellular NO in monocytes transmigrated to CFASN vs LTB4 (**Figure 5.5.3**). NO is an important regulatory molecule for its role in post-translational modification of proteins needed for a variety of cellular functions, as well as for generating downstream RNS important for bacteria killing [10]. Impairment of monocyte/macrophage function in the airways likely reduces the barriers to neutrophil recruitment, which may also be promoted by other factors such as early-life APEs.

APEs are a major cause of hospitalizations for people with CF. Occurrence of APEs during childhood is known to accelerate lung function decline and is the main risk factor for future occurrences, but the role of the host immune system remains unclear. In **Chapter 3,** we demonstrated that APEs during childhood may accelerate neutrophilia through several means, including increasing amounts of circulating neutrophils, possibly linked to elevated G-CSF in plasma, and elevated secretion of chemoattractants in the airways. While macrophages were still the predominant cell type in the airways at this stage, and there was evidence of active T-cell responses (**Chapter 3**), these observations may foreshadow accelerated recruitment of neutrophils to the airway after resolution of an APE, possibly explaining why these acute events can lead to a long-term decline in lung function.

6.3 Expanding therapeutic options for CF lung disease

Lung disease is the main cause of morbidity and mortality in people with CF, and development of CFTR modulator therapies has focused on the goal of increasing lung function. However, CFTR potentiators and correctors do not solve the problem of inflammation even when lung function does improve. There are few therapeutic options that address the inflammation component of lung disease, including DNase and ibuprofen [11]. Recently, a phase 3 clinical trial showed that preventative azithromycin treatment as a potential anti-inflammatory and anti-infective in infants with CF failed to have any effect on lung infections but did reduce inflammatory markers including CXCL8 and NE activity [12]. This widely-used antibiotic may therefore find use as a repurposed anti-inflammatory treatment, which could be useful in delaying lung disease if implemented after APEs in young children (**Chapter 3**).

Further development of therapeutics to target inflammation should consider the complicated interactions between major leukocyte subsets in the lungs. If immune-directed therapies show success in limiting or even reversing neutrophilic inflammation in CF, it will be important to monitor if macrophages and T cells are able to regain their respective roles in the airway microenvironment, or if persistent distal modulation by neutrophils, such as through secretion of extracellular vesicles (EVs) [13], will continue to exert an immunomodulatory effect on these other populations.

Improving anti-infective treatment options may also benefit from using existing compounds in innovative ways. In **Chapter 2** we showed that PD-1 expression on macrophages correlated with CT-based measures of lung disease and that blocking signaling through this pathway improved bacteria killing. PD-1 blockade is not conventionally viewed as a relevant therapy for CF, but with targeted delivery of drugs specifically to diseased regions of the

airways, as we have previously shown [14], repurposing of existing compounds using new delivery mechanisms may be able to deliver much-needed therapeutic options addressing multiple aspects of CF lung disease.

6.4 Future directions in CF lung disease research

Our collective understanding of the inflammatory mechanisms contributing to CF lung disease has been greatly advanced by the direct analysis of clinical samples. However, such samples are becoming more difficult to obtain. The invasive nature of BAL collection limits its use to only clinically-indicated procedures, greatly reducing the ability to conduct longitudinal studies. Additionally, more efficient CFTR-directed therapies are slowing the progression of disease and changing the frequency of clinic visits for patients with CF. With the study presented in **Chapter 4,** we demonstrate the utility of IS collection for tracking airway inflammation. Future studies driven by IS collection will allow for more frequent collection of samples during stable periods as well as during the course of an APE. These studies may be able to detect small changes in the immune poise of the lung such as concentrations of IL-1α and CXCL8 and acquisition of the GRIM phenotype, which would not be easily observed with yearly (or less frequent) BAL collections.

Still, continued development of in vitro models to drive mechanistic studies of CF lung disease (**Chapter 5**) will be an important complement to increased disease monitoring capabilities. With the development of an in vitro TM model in our laboratory [15] we now have the ability to induce airway-like phenotypes in neutrophils and monocytes from blood. Moreover, pathological lung phenotypes can be imprinted on naïve blood myeloid cells from

healthy donors upon TM into airway fluid from patients, which enables the mass-production of airway leukocytes and downstream analyses and drug testing. Although the lung is a complex organ with a great variety of immune response mechanisms [5], this model allows us to set pre-determined conditions to interrogate specific components of airway leukocyte physiology (**Chapter 5**).

A major opportunity for use of this model will be in investigating how innate immune cells respond to new therapies that are becoming available for people with CF. This can be performed using the sputum from patients receiving new CFTR modulator therapies to determine changes in functional reprogramming of healthy donor cells recruited to this fluid, or by using blood cells from these patients to determine if their baseline poise is altered after beginning new therapies. Blood cells from patients receiving CFTR modulator therapies could even be transmigrated to the patient's own sputum in an effort to recreate that individual's lung environment, which may be made even more physiologically accurate using their primary cells [16]. These efforts would support personalized medicine in CF, an approach that is gaining momentum due to heterogeneity of disease pathogenesis among patients and the necessity to expand on available treatment options [17].

Another important application is obtaining proof-of-concept data that would be difficult, if not impossible, to obtain in patient-based studies. One example is our finding that inhibition of transcription using the RNA polymerase inhibitor α-amanitin restores bacteria killing in CF airway-like neutrophils [18]. While the toxicity of this compound prevents its use in humans, this finding reveals a core concept about CF neutrophil biology that may inform future trials using drugs with different toxicity profiles. In addition to reversing aspects of the GRIM phenotype, some attributes may be harnessed for advantageous purposes. CF neutrophils rapidly secrete EVs

which promote inflammation via activation of the inflammasome in epithelial cells [13]. The TM model can be used to mass-produce airway-like neutrophils [19] for studies seeking to use EV secretion as a mechanism for delivering compounds to other cells [20]. People with rare *cftr* mutations are most likely to be ineligible for the currently available modulator therapies and would greatly benefit from an increased ability to screen and optimize new compounds for treatment of neutrophil-driven lung disease (**Figure 6.5.1**) [21].

6.5 Conclusion

In conclusion, the research presented in this dissertation provides new knowledge about the intricate relationship between neutrophils, macrophages, and T cells in the human lung with regards to CF airway disease. We identified a new pathway involved in functional exhaustion of airway macrophages which is not likely to be completely resolved by CFTR modulator therapies. We also presented data suggestive of an active role for T cells in the course of early-life APEs which also precedes future neutrophilia. As the landscape of CF changes with the introduction of new treatments, improved monitoring of the airways will be required. To address this need, we demonstrated that collection of IS from young children with CF yields comparable outcomes to BAL and may even offer increased sensitivity for some inflammatory measurements. Finally, we employed an in vitro TM model to deepen our understanding of neutrophil and monocyte functions in healthy vs CF airway settings. We also adapted this model to use for studies of severe pulmonary bacterial infections, demonstrating the broad applications of this technology. These findings will support continued efforts for basic and translational studies of CF lung disease, with implications for other acute and chronic pulmonary conditions.

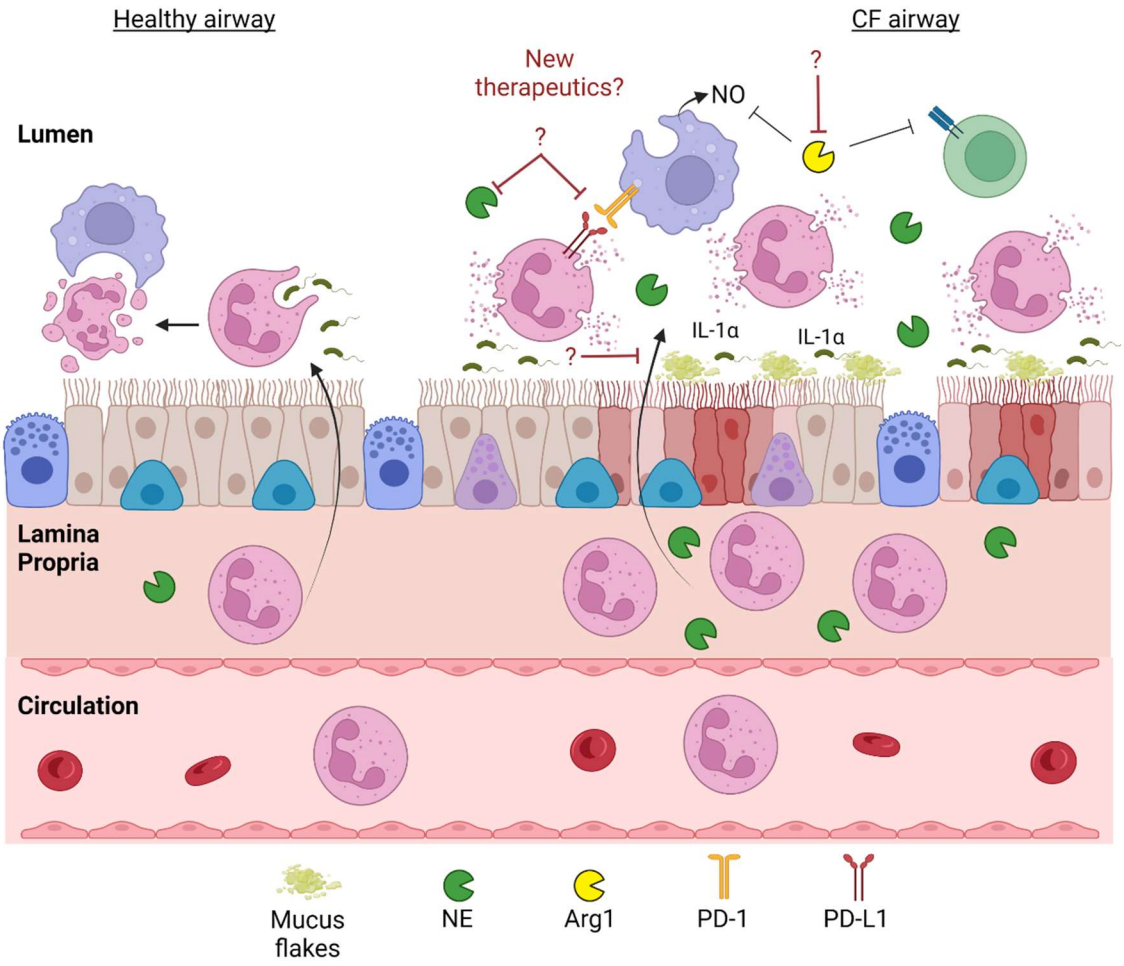

Figure 6.5.1. The central role of neutrophils in healthy and diseased airways. Neutrophils are regularly recruited to healthy airways to clear debris and pathogens, but quickly apoptose and are removed by scavenger cells. In the airways of people with CF, deposition of mucus flakes can result in patches of hypoxia. This may instigate neutrophilia via secretion of IL-1α by epithelial cells, and recruited neutrophils acquire the pathological GRIM phenotype. GRIM neutrophils modulate the airway microenvironment through several mechanisms. Secreted NE causes structural damage in the airways, while Arg1 impairs T cell activation and may contribute to reduced NO production by myeloid cells. Macrophage exhaustion may be induced via PD-1 signaling, with possible receptor ligation from neutrophils and/or their secreted products such as EVs. These interactions suggest the need for new immune-directed therapeutics in the treatment of CF lung disease.

6.6 References

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