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**NEUTROPHIL PLASTICITY ENABLES THE DEVELOPMENT OF PATHOLOGICAL MICROENVIRONMENTS: IMPLICATIONS FOR CYSTIC FIBROSIS AIRWAY DISEASE**

By

Camilla Margaroli
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and molecular pathogenesis

Rabindra Tirouvanziam _____
Advisor

Gregory Gibson _____
Committee Member

Joshy Jacob _____
Committee Member

Haydn Kissick _____
Committee Member

Periasamy Selvaraj _____
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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Camilla Margaroli
B.Sc., University of Fribourg, 2013
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An abstract of
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Abstract

**NEUTROPHIL PLASTICITY ENABLES THE DEVELOPMENT OF PATHOLOGICAL MICROENVIRONMENTS: IMPLICATIONS FOR CYSTIC FIBROSIS AIRWAY DISEASE**

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Neutrophils constitute 60% of blood leukocytes and act as one of the first lines of defense against sterile and pathogen-induced inflammation. The peculiarity of their nuclear shape, showing as hypercondensed chromatin with three to four nuclear lobes instead of the canonical nuclear round shape, together with low abundance of cytoplasmic RNA, has led to the paradigm holding these immune cells as short-lived, terminally differentiated and with little opportunity for plasticity. Over the course of the last decade, research has shown that tissue neutrophils can alter their fate to remain metabolically active and avoid rapid cell death. In cystic fibrosis (CF) specifically, airway neutrophils undergo profound functional and phenotypical changes (resulting in the "GRIM" fate) and play a dominant pathogenic role. The work presented in this thesis addresses three key questions related to clinical and basic biology of CF airway disease. First, we show the presence of GRIM neutrophils in the airways of CF infants, at very early stages of disease, prior to chronic microbial infection. We also show a potential role for GRIM neutrophils in modulating the immune response of resident airway macrophages in CF infants. Second, the work presented here demonstrates a strict transcriptional dependency of GRIM reprogramming. This novel mechanism by which neutrophils actively adapt to the CF airway microenvironment contradicts the conventional paradigm holding neutrophils as pre-programmed. Third, we illustrate the use of a new drug delivery system that can be customized to match proteolytic microenvironments and efficiently deliver hydrophobic drugs to diseased airways, including but not limited to CF. In conclusion, this dissertation challenges prevailing paradigms in clinical development of CF lung disease and basic neutrophil biology, and provides insights into mechanisms of neutrophilic inflammation, thus opening opportunities for immunotherapies targeting this key component of airway pathology in CF and other diseases.

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The work presented in this dissertation is the results of collective effort between different centers across the world brought together by the need for a better quality of life for patients born with cystic fibrosis. The friendship and mentorship established with world renown researchers has been an invaluable part of my training.

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# Chapter 1

*Sections of this chapter have been published in the journal*

*Molecular and Cellular Pediatrics*

## 1.1. Introduction

Neutrophils constitute the first line of defense against infection in most organisms. It is estimated that the human body produces $10^9$ neutrophils/kg/day, making them the most abundant leukocytes in bone marrow (BM) and blood. Neutrophils play an important role in protective immunity, which explains the severe pathologies arising upon hereditary or acquired impairment of neutrophil number and function. Blood neutrophils are conventionally thought of as terminally differentiated cells with little license to adapt to conditions within tissues beyond their ability to kill pathogens intracellularly by phagocytosis, or extracellularly by degranulation or release of DNA-based neutrophil extracellular traps (or NETs) in a recently discovered process dubbed "NETosis".

However, in the context of cystic fibrosis (CF) lung disease, neutrophils show complex properties, detailed below, that come in stark contrast with the rigid pre-programmed phenotype generally expected of them and instead emphasize their inherent plasticity. CF is a hereditary, recessive disease that predominately impacts individuals of European ancestry. According to the World Health Organization, its incidence varies between 1 in 2,000 and 1 in 3,500 newborns worldwide. The gene mutated in CF patients encodes the CF Transmembrane Conductance Regulator (CFTR), an ATP-binding cassette family member that regulates the movement of anions, such as chloride, bicarbonate, thiocyanate and glutathione (GSH), across the plasma membrane [1, 2]. So far, more than 1,800 disease-causing mutations have been

identified among CF patients, with the F508Del mutation being the most frequent (~70% of mutated alleles) [3, 4]. Digestive enzyme supplements have noticeably increased CF patients' lifespan, and shifted the main cause of morbidity from nutrient malabsorption due to pancreatic failure, to chronic lung disease [5].

Impaired mucociliary clearance, bacterial infection, and neutrophilic inflammation are all hallmarks of CF lung disease [6, 7]. Among those, neutrophil burden and extracellular activity of the protease neutrophil elastase (NE) in CF airway fluid correlate best with disease progression in CF patients, from infancy to adulthood [8]. The role of neutrophil inflammation in CF pathophysiology has been exhaustively reviewed elsewhere [9-11]. Recent reviews detail the putative role of other immune cells, such as macrophages, in CF lung disease [12-14]. In this thesis, our goal is to direct the attention of the reader to the phenotypic reprogramming process that neutrophils undergo in the context of CF lung disease, and explore potential mechanisms and treatment opportunities afforded by this newly discovered process. Importantly, this new view of neutrophils, which we illustrate in the context of CF, echoes recent findings made in the context of acute infection and sepsis, as well as other chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA), and systemic lupus erythematosus, as well as cancer, where neutrophils also display new, complex phenotypes and effector functions [15].

## 1.2 Neutrophil plasticity in CF lung disease: emergent mechanisms

*Lifespan and aging*

Pulse-chase experiments were conducted recently to measure the lifespan of human neutrophils in blood. Models accounting for the loss of the deuterium label led to estimates of a few hours to up to 5 days [16, 17]. Although their exact lifespan is debated,

it is of general consensus that neutrophils leave the BM with a default pro-apoptotic program that can be inhibited by stimuli received upon migration to peripheral tissues [18, 19]. In CF, there is no experimental data on the precise lifespan of neutrophils in the lung, and this subject remains debated. On one hand, the hostile environment of the CF lung, and notably the presence of bacterial toxins, could induce rapid necrosis of incoming neutrophils [20-22]. On the other hand, neutrophil lifespan may be extended by several factors, such as pro-survival signals from neutrophils, as well as exogenous drugs, and epithelial and microbial inflammatory mediators and metabolites. For example, Sutanto et al. [23] showed that primary epithelial cells from CF infants not only secrete higher levels of inflammatory mediators compared to their healthy counterparts at baseline, but also display an increased production of interleukin-8 (IL-8) in response to human rhinovirus infection. In addition to being a strong chemoattractant [24], IL-8 can delay neutrophil apoptosis [25]. Thus, the CF airway epithelium may contribute to a higher lifespan of neutrophils recruited to the lumen.

To achieve a balanced number of neutrophils in blood, the high daily rate of release of mature young neutrophils into the bloodstream is compensated by the clearance of senescent neutrophils from it. Circadian rhythm is a major factor influencing hematopoiesis in general, and neutrophil turnover in particular [26-28]. From a phenotypic standpoint, developing neutrophils in the BM express the chemokine receptor CXCR4, which acts as a retention signal by binding to its cognate ligand CXCL12 on stromal cells. The release of mature neutrophils into the circulation coincides with the downregulation of CXCR4 expression, and concomitant increase in expression of CXCR2, a receptor for IL-8. However, senescent neutrophils increase CXCR4 expression again [17], which is thought to lead to their return to the BM where they are cleared by resident macrophages. In addition to its role in mediating cell retention in the BM, CXCR4 signaling has been proposed as a direct regulator of

neutrophil lifespan. In mice, CXCR4 expression is increased in neutrophils after migration to the lungs and correlates with increased lifespan [29]. In patients with COPD, neutrophils are present in large numbers within the bronchoalveolar lavage fluid (BALF) [30], and express higher levels of CXCR4 compared to control subjects [31]. Similarly, neutrophils isolated from the sputum of CF patients showed increased surface expression of CXCR4 compared to blood neutrophils [32], and its ligand CXCL12 was detected in some CF sputum samples, suggesting a potential role of this pathway within the CF airway lumen.

In addition to the CXCR4/CXCL12 axis, new insights from the zebrafish model of neutrophil development show that signaling through the oxygen-dependent transcription factor hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) can also significantly delay neutrophil apoptosis [33]. Since affected areas in CF lungs become hypoxic due to mucus impaction and fast oxygen consumption by activated neutrophils [34], it is tempting to speculate that HIF-1$\alpha$ signaling may be triggered in neutrophils present in this pathological microenvironment, affecting their lifespan. Consistent with this notion, significant HIF-1$\alpha$ signaling has been demonstrated in the $\beta$ENaC mouse model of CF lung disease, inducing substantial pro-inflammatory signaling within the epithelium that results in neutrophilic inflammation [35].

Interestingly, it has been suggested that neutrophils in CF patients have an intrinsic increase in lifespan due to the mutation of the cftr gene. Indeed, *ex vivo* experiments on blood neutrophils isolated from healthy controls and CF patients with the F508Del mutation showed delayed apoptosis in the latter [36, 37]. However, these data do not imply increased lifespan *in vivo*. Also, since ongoing treatments can significantly impact neutrophil behavior [38], it is likely that drugs administered to CF patients from whom neutrophils are collected can alter the lifespan of these cells *ex vivo*. Another interesting factor to consider when reflecting on potential influences

exerted onto neutrophil lifespan is that of the resident microorganisms. Indeed, it has been demonstrated that neutrophil biogenesis and aging in mice is controlled, in part, by the gut microbiome [39, 40]. In patients with chronic infections, e.g., CF or COPD, it is likely that the lung microbiome could also play a role in shaping neutrophil lifespan [41], although this notion remains debated [42, 43].

In the context of a normal immune response to an insult, increased neutrophil lifespan can be beneficial for the host, at least temporarily. However, if this response becomes dysregulated, it can promote a perpetuation of neutrophilic inflammation [44]. To this day, many questions relative to the recruitment of neutrophils and their precise lifespan within the CF lung remain unanswered. A key difficulty resides in studying these mechanisms *in vivo*, and in untangling factors intrinsic to CF (compared to other diseases with similar neutrophilic inflammation, such as COPD), and those affected by exogenous drugs. Finally, since neutrophils, the airway epithelium and microorganisms all contribute to the development of CF lung disease, integrative approaches combining signals from all components of this pathological microenvironment are needed to yield better understanding of mechanisms at play.

*Overview of effector functions*

In the course of inflammation, neutrophils recruited from blood cross into tissues and organize themselves in "swarms" to travel to the site of injury. Neutrophil migration responds to gradients of exogenous and autocrine/paracrine chemokines (e.g., IL-8), cytokines (e.g., tumor necrosis factor $\alpha$), as well as bioactive lipids (e.g., leukotriene $B_4$ - $LTB_4$-) [45, 46]. Dynamic expression of specific receptors to these chemokines, cytokines, and bioactive lipids at the surface of neutrophils is critical to their migration. In addition, neutrophils express a plethora of Pattern Recognition

Receptors (PRRs) that allow them to sense and capture signals present in their surroundings in the form of Pathogen-Associated Molecular Patterns (PAMPs) or Danger-Associated Molecular Patterns (DAMPs) [47]. PAMPs (e.g., lipopolysaccharide from gram-negative bacteria) and DAMPs (e.g., extracellular advanced glycation endproducts or adenosine triphosphate) are present in damaged tissues and play a major role in influencing the functional fate of incoming neutrophils, notably by modulating the mobilization of intracellular granules.

Neutrophil granules are designated based on their content and order of production during BM development. Primary or azurophilic granules are formed at the early stages of neutrophil lineage formation in the BM (myeloblast to promyelocyte) and contain the potent proteases neutrophil elastase (NE) and cathepsin G, the chlorinating enzyme myeloperoxidase (MPO), and defensins. Secondary or specific granules arise at the later metamyelocyte stage and are characterized by the presence of lactoferrin, collagenase, carcinoembryonic antigen cell adhesion molecule family members CD66a and CD66b, and the antiprotease cystatin C. Tertiary or gelatinase granules appear at the band cell stage, right before the final segmented stage of neutrophil BM development, and enclose lysozyme and matrix metalloproteinase 9 (MMP9). Secretory vesicles are present only in mature neutrophils and are thought to be produced by endocytosis of surface-expressed proteins, enabling their rapid redeployment at the surface upon activation, as exemplified by the upregulation of PRR surface expression upon priming of blood neutrophils [48, 49]. Mobilization of secretory vesicles, tertiary and secondary granules to the plasma membrane appears to be a default activation path for neutrophils. By contrast, primary granules generally fuse either with the phagosome or the nucleus [50]; the latter being part of the recently discovered NETotic fate of neutrophils. During NETosis, DNA is decondensed, released along with histones, and complexed with

cationic primary granule proteins (chiefly NE and MPO), thus forming extracellular traps endowed with antimicrobial activities [51].

Until recently, it was believed that, due to the high self-harming potential of primary granule enzymes, the content of primary granules was rarely if ever discharged actively in the extracellular environment during the normal course of an inflammatory response. Thus, the massive amounts of NE and MPO present in the pathological milieu of CF and COPD airway fluid were thought to stem from the passive release of primary granules following neutrophil necrosis. However, the discovery of viable neutrophils in the CF lung lumen capable of active primary granule exocytosis has overturned this belief [52]. In these cells, mobilization of primary granules to the plasma membrane is not a passive outcome, but rather a finely orchestrated active mechanism leading to a fate distinct from phagocytosis and NETosis **(Fig. 1.2.1)**. Molecular mechanisms underlying differential primary granule mobilization to the phagosome (phagocytosis), nucleus (NETosis) or plasma membrane (third, and presumably distinct, fate), and whether each of the three described fates is exclusive of the others, are but a few examples of the current mysteries surrounding neutrophil biology that will have to be addressed in future research.

*Focus on neutrophil elastase (NE)*

A major effector of neutrophils with a critical role in CF is NE, a serine protease composed of 218 amino acids. First discovered in 1968 by Janoff and Scherer [53] in the granular fraction of neutrophils, it took 15 more years for the sequence of NE to become known [54]. Upon primary granule release, the majority of NE remains bound to the plasma membrane [55, 56], which enables it to have its catalytic region facing the extracellular environment while concealing its regulatory region, thus making inhibitors less effective. The important pathophysiological role played by NE is

highlighted by the fact that NE-knockout mice are highly susceptible to sepsis induced by gram negative bacteria [57]. In humans, cyclic neutropenia, a genetic disease caused by mutations in the NE-coding ela2 gene, is associated with recurrent troughs in neutrophil production and heightened susceptibility to infections, suggesting a dual developmental and functional role for NE [58, 59].

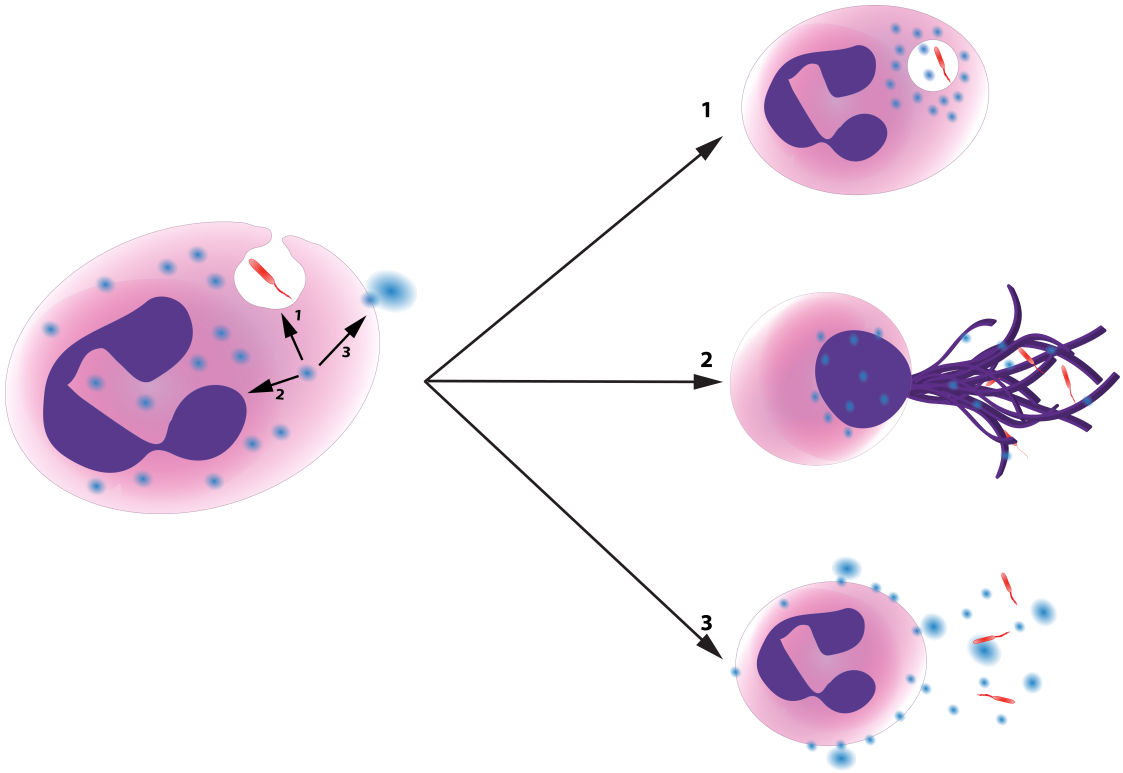



**Figure 1.2.1. Primary granule mobilization and functional fates of human neutrophils.** Recent studies have revealed the existence of multiple functional fates of neutrophils, which lead to different interactions with incoming bacteria (in red). Fusion of primary granules (in blue) to the phagosome drives neutrophils towards the classical phagocytic fate (1), while their mobilization to the nucleus drives them toward the extrusion of DNA-based extracellular traps in a process called "NETosis" (2). By contrast, primary granule fusion with the plasma membrane instead leads to hyperexocytosis and potential reprogramming (3), a third fate that further emphasizes the functional plasticity of human neutrophils.

In CF, increased presence of active NE in the airway fluid of pediatric and adult patients has been correlated with impaired structural tissue integrity, worsening lung function, and decreased body mass index over time [60-63]. In a recent study, detectable NE activity in BALF of 3-month old CF infants was the best predictor of future bronchiectasis development, with a likelihood seven times higher at twelve months and four times higher at the age of three than 3-month old CF infants with no detectable NE in BALF activity [62]. Since BALF is highly diluted (due the way it is collected), it is possible that detection of free NE activity in CF infants happens only after constant NE release has overcome the secreted antiprotease shield present in the airways, thereby crossing a certain pathological threshold. Thus, more sensitive methods for extracellular NE detection are required in order to detect abnormal neutrophilic inflammation before it reaches a critical level and causes significant pathology.

Complementing early work by Owen and colleagues demonstrating NE activity in close vicinity to the plasma membrane of exocytosing neutrophils [55], additional work needs to be performed to determine the exact localization of NE in the extracellular environment within CF and COPD airways *in vivo*. Recently, Schulenburg et al. [64] designed a Förster resonance energy transfer probe specific for NE activity that could serve such a purpose. Potential applications of such probes have been extensively reviewed elsewhere [65]. Due to the wide range of proteins with NE cleavage sites that could potentially serve as NE substrates within pathological environments **(see Table 1.2.1)**, it is hard to predict which of these proteins will be effectively proteolyzed *in vivo*. Among these proteins, one finds both immunological and non-immunological target proteins expressed by neutrophils, T cells, macrophages, and epithelial cells. Further adding to this complex picture, NE can be acquired by neighboring cells following its release by neutrophils [66]. This effectively

extends the number of possible targets of NE-mediated cleavage to include intracellular proteins, which in turn affect signaling in neighboring cells **(Tab. 1.2.2)**.

| **Immunological targets** | |
|---|---|
| Arginase-1 [67] | CD2 / CD4 / CD8 [68] |
| Chemerin [69] | CD14 [70] |
| IL-36 receptor antagonist [71] | CD16 [72] |
| IL-8 [73] | CD43 [74] |
| MMP-9 [75] | CCL3 [76] |
| PAR-1 / PAR-2 [77, 78] | Complement factors [79-82] |
| Pro-IL-1β [83] | CXCL12 [84] |
| Transient receptor potential vanilloid 4 [75] | CXCR1 [85] |
| Tumor growth factor $\alpha$ [86] | IgA [87] and IgG [88, 89] |
| | IL-2 receptor [90] |
| | IL-6 [91, 92] |
| | IL-8 [93] |
| | PAR-3 [78] |
| | Progranulin [94, 95] |
| | TIMP-1 / TIMP-2 / TIMP-3 [96, 97] |
| **Non-immunological targets** | |
| $\alpha 2\beta 3$ integrins [98] | Cadherins [99] |
| EGFR [100] | Elastin [101] |
| ENaC [102, 103] | Ferritin [104] |
| | Fibrin stabilizing factor XIII [105] |
| | Surfactant protein A [106] |
| | Surfactant protein D [107] |
| | Vascular endothelial growth factor [108] |

**Table 1.2.1. Direct targets of NE-dependent regulation.** EGFR: epidermal growth factor receptor, ENaC: epithelial sodium channel, PAR: protease-activated receptor, TIMP: tissue inhibitor of metalloproteinase. Green indicates targets activated by NE-mediated cleavage, while red represents targets inhibited by it.

| Regulatory target | Modulation by NE | Signaling pathway |
|---|---|---|
| β-defensin 2 [109] | Activation | Unknown |
| Cathepsin B [110] | Activation | TLR4 / IRAK |
| CFTR [111] | Inhibition | Calpain |
| *P. aeruginosa* flagellin | Inhibition | Unknown |
| IL-12 p40 [113] | Activation | PAR-2 / EGFR / TLR4 |
| IL-8 [108, 114-116] | Activation | TLRs / MyD88 / IRAK |
| MHC I [117] | Activation | Unknown |
| MMP-2 [110] | Activation | TLR4 / IRAK |
| MUC5AC [118, 119] | Activation | EGFR |

**Table 1.2.2. Indirect targets of NE-dependent regulation and cognate signaling pathways.** EGFR: epidermal growth factor receptor, ERK: extracellular regulated kinase; IRAK-1: IL-1 receptor associated kinase-1; MHC I: major histocompatibility complex I; MIP: macrophage inflammatory protein; PAR-2: protease-activated receptor-2; TLR4: Toll-like receptor 4.

A prototypical example highlighting the impact of unopposed NE activity in a pathological milieu is its ability to activate MMP9, another potent neutrophil protease. Upon concomitant release of primary and tertiary granules, NE can potentiate MMP9 through direct activatory cleavage and/or indirect degradative cleavage of its inhibitor tissue inhibitor of metalloprotease-1 (TIMP-1), leading to increased collagen degradation, tissue damage and bronchiectasis in CF children [75, 120]. Likewise, surface phagocytic receptors CD14 and CD16 on neutrophils found in the lumen of CF patients' lungs are inactivated by NE in autocrine and paracrine fashion [121]. Moreover, antibody-mediated bacterial killing is impaired not only on the receptor side, but also on the opsonization potential of the antibody. As matter of fact, it has been shown that NE can cleave immunoglobulins A (IgA) [87] and G (IgG) [88, 89] near their hinge region.

This leads to the formation of Fab and Fc fragments that are able to bind *separately* to the bacteria and receptors on target cells, thus losing the adaptor function of the antibody [122, 123]. In addition to NE, the CF opportunistic pathogen *P. aeruginosa* also contributes its own elastase activity, which can also cleave IgG [124]. This dual inhibition exerted by NE on antibodies present in the CF airway lumen has implications for the design of vaccine strategies aiming to induce anti-bacterial responses in CF, suggesting that these may be severely limited by the high extracellular NE burden. Another example of effector function modulation by NE is the cleavage of the IL-8 receptor CXCR1, associated with impaired bacterial killing [125]. This may contribute to the infection by opportunistic bacteria such as *S. aureus* and *P. aeruginosa*, which are also hallmarks of CF lung disease [126, 127]. Whether NE-mediated damage is a primary cause of these persistent infections or whether other elements in the CF lung environment also contribute to impaired clearance of these specific bacteria remains a matter of debate [128, 129].

Beyond the failed clearance of these bacteria, their continued adaptation to the CF environment, notably their switch to mucoid and biofilm resistance phenotypes, may also benefit from NE activity. Indeed, NE-mediated signaling can repress flagellin transcription in *P. aeruginosa*, which facilitates biofilm formation [112]. Interestingly, NE-produced fragments of CXCR1 were identified as potential contributors to epithelial activation and release of IL-8 in a Toll-like receptor 2 (TLR2)-dependent manner [85], thus creating a pathological feedback loop of neutrophil recruitment, NE release, CXCR1 cleavage on epithelial cells, and further neutrophil recruitment. Additional contributions to this positive feedback loop come from the NE-mediated transcriptional upregulation of IL-8 via MyD88 / IRAK / TRAF-6 [116] and direct NE-mediated processing of IL-8 in the extracellular milieu [73]. Indeed, IL-8 is produced as a 99-amino acid precursor protein which is proteolytically cleaved at its N-terminus

before release [130]. Once in the extracellular milieu, IL-8 can be further processed by extracellular proteases, such as NE, leading to different bioactive forms that vary from 77 to 69 amino acids in length, with the 72-amino acid form being the most potent [73]. *In vitro* studies also suggest that IL-8 can be ultimately degraded in an NE-dependent manner over time [93] which could serve to balance out the induction of IL-8 production and its post-transcriptional activation by NE.

It is worth mentioning that although exocytosis of primary granules content can be considered a hallmark of CF lung disease, this process is not homogeneously expressed among all neutrophils found in the CF airway lumen. Indeed, our laboratory [32] proposed a subset classification of airway neutrophils based on their surface phenotype, with neutrophils initially migrating into the lumen and expressing low CD63 (limited primary granule exocytosis) and high CD16 expression on their surface; followed by the acquisition of high surface CD63 expression (high primary granule exocytosis), and concomitant loss of surface CD16. This striking phenotypic and functional transition, and its implications for CF pathogenesis are discussed in more details below.

*Impact of CFTR on neutrophil function*

In humans, the impact of CFTR on neutrophil effector functions is unclear, due in part to limitations in research tools. First, animal models, such as CFTR knockout mice, ferrets, pigs, and rats, still have not allowed researchers in the field to adequately recapitulate the natural history of CF lung disease as seen in patients, and particularly the central role played by neutrophils. Second, the CFTR protein is generally expressed at low levels in cells, which, combined with the paucity of reliable anti-CFTR antibodies, has made it difficult to establish the presence of significant CFTR expression in human neutrophils and how it may impact their function [131-133].

To date, several lines of evidence support the notion that neutrophil effector functions are not intrinsically controlled by CFTR mutations. To begin with, the fact that CFTR knockout animal models do not recapitulate neutrophilic lung inflammation as seen in CF patients itself suggests that neutrophil dysfunction in CF patients is due to one or several coinciding mechanism(s) unique to humans, besides CFTR deficiency [134]. Additional support for this idea comes from a xenograft model in which human fetal tracheal tissues were implanted in severe combined immunodeficient mice [135]. In this model, mouse neutrophils (with normal CFTR expression) were recruited to CF but not non-CF xenogratfs, emphasizing the role of the CF airway microenvironment in triggering neutrophil dysfunction. Consistently, a recent study in primary airway epithelial cultures from CF and non-CF infants showed the existence of a pro-inflammatory imbalance at steady state and upon stimulation with a viral insult in the former compared to the latter [23]. Furthermore, restoring CFTR expression in the airway epithelium of CF mice is sufficient to restore normal bacterial clearance therein, which suggests a minimal role for CFTR expression in non-epithelial cells, at least in this model [136]. Intriguingly, selective knockout of CFTR in myeloid cells in another mouse strain led to a basal inflammatory dysfunction that was further accentuated upon infection [137], suggesting that in mice, the impact of CFTR on myeloid cells (including but not limited to neutrophils) may be dependent upon the strain and conditions tested.

From a microbiological perspective, the predominance of a handful of bacterial species in CF lungs *in vivo* suggests that neutrophil-mediated clearance may not be completely abolished in this setting, but rather that distinct evolutionary pressures are at play that are unlikely to be solely driven by CFTR-dependent dysregulation of neutrophil phagocytosis or degranulation. Indeed, a recent study showed that the CF lung pathogen *P. aeruginosa* is resistant to neutrophil-mediated extracellular killing,

a process that is CFTR-independent [138]. Furthermore, if neutrophils in CF patients were intrinsically defective due to endogenous CFTR dysfunction, one would expect evidence of chronic infection and inflammation in organs other than the lungs, which is not the case.

It is also noteworthy that in COPD and non-CF bronchiectasis patients devoid of a hereditary CFTR defect, massive neutrophil transmigration also occurs in the lungs, with subsequent release of primary granules and impaired phagocytosis reminiscent of the picture seen in CF patients [139, 140]. This suggests that a primary defect in CFTR expression is not the root cause of neutrophilic inflammation in these disease contexts. It remains possible, however, that CFTR expression may be intrinsically normal in these patients, only to be downregulated post-translationally due to high extracellular activity of NE, thus affecting neutrophil fate [111]. In CF patients, chronic disease may lead to similar adaptive changes in blood neutrophils. This could account, for example, for the observed dysfunction in blood neutrophils from adult CF patients of Rab27a, a key protein involved in tertiary and secondary granule exocytosis, coupled with the finding that significant improvement in Rab27a function in these neutrophils can brought upon by *ex vivo* treatment with the CFTR potentiator ivacaftor [141]. Proving the existence of an intrinsic defect in neutrophils in CF patients would ultimately require well-controlled data in infants, prior to the advent of chronic disease, a feat that has not been achieved so far. These and other novel approaches and experimental designs will be necessary to further elucidate the etiology of the abnormal neutrophil effector functions that are manifest in CF lungs.

*Immunomodulatory role of neutrophils*

Since the early 1960s, significant heterogeneity among circulating and tissue neutrophils has been recognized, and this notion has gained further traction recently as evidence of divergent immunomodulatory functions by neutrophil subsets are emerging in different pathological contexts [142]. One critical example lies in tumor microenvironments, where neutrophils can display a strong immunosuppressive phenotype, promoting tumor survival [143, 144]. As immunomodulatory cells, neutrophils not only modulate their own kin, but also a variety of other immune and structural cells. To do so, neutrophils use a variety of chemokines and cytokines that regulate other cells acutely and have the potential to induce chronic signaling loops that shape the long-term immune response [145, 146].

In CF, the lumen of the lung is brimming with neutrophils, while it is conspicuously devoid of T cells. The enzyme arginase-1 can be released by neutrophils, as well as M2-polarized macrophages and dendritic cells, leading to depletion of extracellular arginine, which in turn can inhibit T cell activity. Arginase-dependent T cell inhibition is common in tumor microenvironments [147] and upon infection with certain viruses [148]. In CF lungs, arginase-1 is released by neutrophils, making the airway lumen a highly inhibitory milieu for T cells [67]. In addition to the strong inhibitory role played by neutrophil-derived arginase-1, NE can also cleave multiple critical T cell coreceptors, therefore blocking T cell activation (see references [68] and [90], and **Table 1.2.1**). Furthermore, programmed death ligand 1 (PD-L1), a known inhibitor of T cell function in a variety of pathologies and a major target for immunotherapy [149], was also found to be expressed in human airway neutrophils at higher levels than on their blood counterparts (in both CF and healthy subjects), but with a characteristic bimodal expression in CF. In addition, soluble PD-L1 was also

detected in CF, but not healthy, airway fluid [67]. The precise role of cell-associated and soluble PD-L1 on T-cell modulation in CF remains to be fully explored.

Interestingly, the impact of CF airway neutrophils on T-cell function may not be solely inhibitory, since these cells were shown to increase expression of the T-activatory surface receptors major histocompatibility complex II, co-activator CD80 and prostaglandin D2 receptor CD294, further underlining their functional plasticity [121]. Expression of major histocompatibility complex II and CD80 is conventionally thought to be the prerogative of professional antigen-presenting cells, such as dendritic cells and macrophages, while CD294 is a marker for Th2-polarized immune cells in the context of allergy and hypersensitivity reactions [150]. The exact role of these T-activatory proteins on the surface of CF airway neutrophils has yet to be determined, although one can speculate a possible role in skewing T-cell responses that may occur in spite of arginase-1 and NE-dependent inhibition. Indeed, it has been observed that $CD4^+$ T-cells in CF mouse models [151] and human CF airway samples and tissues [152-155] are skewed toward pro-inflammatory Th2/Th17 responses, while inhibitory T-regulatory function is inhibited.

Positive regulation of T cells by neutrophils was also suggested in early stage human non-small cell lung cancer [156], in which tumor-associated neutrophils expressing typical antigen-presenting cell markers were able to induce T-cell activation *ex vivo*. A recent study also shown the importance of neutrophils in promoting a protective Th17 T-cell response upon vaccination against tuberculosis [157]. Since Th17 cells and their product IL-17 create a positive feedback loop for neutrophil recruitment by tissues [158], neutrophil / T cell interplay may be critical to pathogenesis in CF and other relevant diseases. RA is another example of a chronic disease in which neutrophils recruited from blood to the synovium dominate signaling loops to induce a skewed immune response [159].

*Metabolic licensing of neutrophils*

The CF lung lumen is a very peculiar microenvironment in terms of oxygen, and metabolite content. The normal lung lumen is oxygen-rich due to constant breathing activity. However, in diseased areas within the CF lung lumen, neutrophil clusters, bacterial and/or fungal colonies, and inspissated extracellular scaffolds of mucus, DNA and actin can lead to profound oxygen depletion. Local hypoxia can in turn promote inflammation through the release of DAMPs from host epithelial cells [35, 160, 161]. Furthermore, the CF lung environment has a distinct metabolite composition, presumably as a consequence of both CFTR dysfunction and of the chronic presence of neutrophils and microbiota in the lumen. First, it has been suggested that CFTR, although functioning primarily as a chloride and bicarbonate channel [162], can also enable transmembrane flux of the redox intermediates GSH and thiocyanate [163-165]. CFTR is also indirectly involved in the control of neutral amino acid transport across the epithelium [166]. In addition, the CF lung lumen was notably shown to contain abnormal levels of nucleotides [167] and peptides [168].

The composition of the CF airway milieu drives adaptations in neutrophils, and in turn, these adaptations influence this pathological microenvironment. A telling example is that of the redox imbalance that constitutes a hallmark of CF. Local and systemic accumulation of oxidants are believed to impact CF blood neutrophils, which display lower intracellular GSH levels [121]. Meanwhile, reactive oxygen species produced by neutrophils including hypochlorous acid (bleach), a byproduct of the enzyme MPO exocytosed from primary granules at the same time as NE, can quickly and profoundly oxidize the lung microenvironment [169]. Finally, neutrophils can contribute to extracellular GSH catabolism, by expressing at their surface the GSH-metabolizing enzyme gamma-glutamyltransferase [170]. Another example is that of arginine, which neutrophils can deplete from the CF airway lumen by releasing

arginase-1 [171] from their granules [172]. Consequently, low availability of arginine results in decreased nitric oxide production [173] and high levels of arginine degradation products ornithine and polyamines [174].

From an intracellular signaling standpoint, comparative studies conducted in blood and airway neutrophils collected from CF patients *in vivo* showed that these do not differ with regards to their levels of active, phosphorylated forms of the critical intermediate kinases Akt, cJun-related kinase, p38 mitogen-activated protein kinase, p44/42 extracellular-regulated kinase, or of the pro-inflammatory transcription factors, nuclear factor $\kappa$B p65 and signal transducer and activator of transcription 5 [121]. However, CF airway neutrophils had increased levels of phosphorylated forms of effector proteins in the mechanistic target of rapamycin pathway, a major anabolic switch. These included phosphorylated S6 ribosomal protein [121], eukaryotic initiation factor 4E and 4E-binding protein 1 [32]. Additionally, levels of the phosphorylated cyclic AMP-response element binding protein, as well as its upstream sensors, CD114 and receptor for advanced glycation endproducts, and downstream targets, CD39 and CXCR4, which function together as another anabolic switch in cells, were found to be increased in CF airway neutrophils [32]. In aggregate, these results suggest that CF airway neutrophils are licensed by the microenvironment to become anabolic, i.e., to use resources at their disposal to survive and expand their functions.

Insights gained from the analysis of intracellular phosphorylation cascades in CF airway neutrophils were confirmed by analysis of nutrient transporter expression. Compared to their blood counterparts, CF airway neutrophils as a whole displayed high expression of the inorganic phosphate transporter PiT1 (necessary for ATP synthesis), and of the glucose transporter Glut1, coinciding with increased glucose uptake [175]. Subset analysis of CF airway neutrophils showed that the degranulation of primary granules typical of the A2 subset was associated with higher expression of

Glut1 and PiT1, and of the other inorganic phosphate transporter PiT2. Expression of CD98, a shared subunit of multiple amino acid transporters, did not differ in CF airway compared to blood neutrophils [32], but that of ASCT2, a neutral amino acid transporter, was highly upregulated in the A2 subset [175]. It is likely that this metabolic surge leads to *de novo* transcription and protein production in CF airway neutrophils, since neutrophils recruited to other pathological environments, e.g., the synovium of RA patients, have shown an ability to increase mRNA output [49, 176, 177].

The combined massive and sustained recruitment of neutrophils from blood into CF lungs (presumably leading to an increased neutrophil production in the BM), and increased metabolic activity of these neutrophils once they have reached the CF airway lumen are expected to impact lung tissue function and systemic metabolism in patients. Indeed, the severity of CF lung inflammation has been correlated not only to decreased lung function [178], but also to decreased body mass index [61], decreased heart function [179], and other cardiovascular complications [180]. Generally, the establishment of a complex microenvironment involving not only the chronic and massive presence of neutrophils, but also large populations of bacteria (and/or fungi) in the airway lumen may increase the metabolic share taken by the lung, at the expense of other organs [181].

The relationship between inflammation, high cellular turnover and increased systemic energy expenditure is not confined to CF, but rather is a common feature of an array of chronic human diseases. For example, lower body mass index due to increased energy expenditure at late cancer stages predicts a higher mortality rate [182]. Understanding mechanisms underlying the anabolic switch in CF airway neutrophils and the interplay between the different actors within the CF lung microenvironment could help identify treatments impacting not only lung disease, but also the overall metabolic balance in patients with various conditions.

*The CF airway microenvironment*

The existence of discrete microenvironments within the human body is not a new concept. This concept is exemplified by the gut mucosa, featuring fine-tuned interplay between the resident flora in the lumen and the immune cells in the lamina propria, with the epithelium as an interface. This microenvironment may become imbalanced in the context of inflammatory bowel disease due to changes in host and microbial functions. Other conditions can lead to the formation of chronic pathological microenvironments in organs that do not normally harbor a prominent population of inflammatory cells and/or associated microbiota. CF, COPD, RA, and several forms of cancer serve as examples of such pathological microenvironments featuring a dominant neutrophilic component.

A key element in the formation of pathological microenvironments is the establishment of tolerance and cooperation between the different players, enabling an acute process to become chronicized. For instance, inflammatory bowel disease is characterized by a massive neutrophil infiltration in the gut [183, 184] and as the disease progresses, neutrophils orchestrate with the gut epithelium the advent of a chronic state. Consequently, adaptive responses are dampened [185], and neutrophils and bacteria coexist in concentric luminal structures termed "casts" [186]. A similar scenario unfolds in CF, where neutrophils interact with the airway epithelium and opportunistic bacteria such as *P. aeruginosa* and *S. aureus* to establish multi-decade colonies, featuring minimal involvement of adaptive immune cells, and stable biofilm structures in the lumen where bacteria and neutrophils coexist [187].

Recent studies suggest that the formation of pathological microenvironments featuring substantial relocation of hematopoietic cells within a peripheral organ can impact other distal organs besides the BM, where hematopoiesis takes place. For

example, Masri et al. [188] have shown that establishment of a lung tumor mass can lead to re-tuning of the liver circadian clock and reprogramming of its nutrient output in order to support the metabolic requirements of the remote lung tumor. Whether the establishment of a pathological microenvironment in the CF lung does not only involve complex local coordination, but also impacts other organs in the body (notably the liver and gut, in order to respond to its metabolic demands) remains to be established. Taken together, evidence from *in vivo* and *in vitro* studies suggest that, at a minimum, the airway epithelium, recruited neutrophils, and bacteria present in the CF microenvironment coevolve over time to enable a somewhat stable, albeit armed and ultimately tissue-damaging, coexistence, as illustrated in **Figure 1.2.2.**

*Treatment opportunities*

The development of drugs aimed to treat CF has mainly focused on fluidifying secretions, regulating microbial burden and, more recently, rescuing mutant CFTR function. The latter approach has paid substantial dividends with the drug ivacaftor for CFTR gating mutants such as G551D and the recent introduction of a triple therapy including ivacaftor, tezacaftor and VX-659 (two CFTR correctors for patients bearing at least one copy with F508Del mutation). However, downsides of this approach are that it is mutation-specific and does not prove efficacious in all patients [189] . Additionally, recent unpublished data suggest that although FEV1 increases following CFTR modulator therapy, the rate of decline in lung function and presence of lung infections are not improved in the long run. So far, only little attention has been paid to the regulation of neutrophil function, since the long-held view has been that these cells die quickly upon migration to CF lungs. Data generated in this thesis project contradict this view and open new avenues for neutrophil-focused therapies in CF.

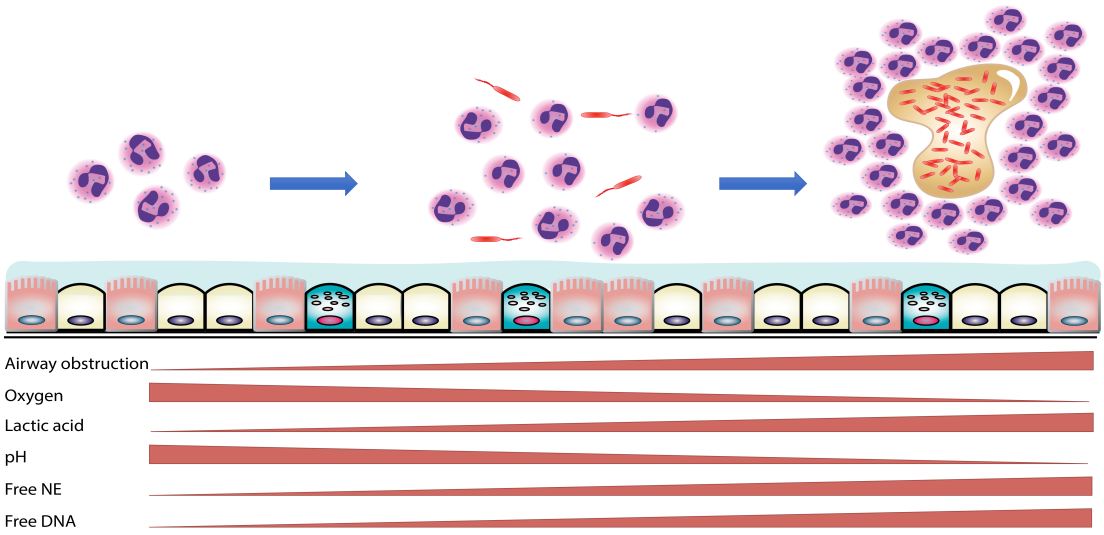



**Figure 1.2.2. Development and evolution of a pathological microenvironment in CF airways.** From birth to adulthood, CF patients undergo stepwise changes to their airway microenvironment. In the first phase (left), neutrophil start to accumulate in the lumen in response to cues from the underlying epithelium, prior to the advent of chronic infection. The second phase (middle), features the stable coexistence of an even more substantial population of luminal neutrophils with planktonic bacteria (in red, drawn with flagellum to exemplify *P. aeruginosa*, the gram-negative pathogen most commonly found in CF patients). In the third phase (right), bacteria switch to a resistant, and generally less virulent and auxotrophic mode of existence, encased in an extracellular molecular scaffold (mucoid or biofilm forms, in yellow), with a very large population of neutrophils organized as a cast around them. Shown under the epithelium are the critical environmental conditions that change during the formation of this pathological microenvironment, including the degree of airway obstruction (increasing), and luminal levels of oxygen (decreasing as neutrophil accumulate and consume it to produce reactive oxygen species) and lactic acid (increasing with neutrophil glycolytic activity), pH (decreasing as lactic acid accumulates), as well the burden of free NE and DNA (both correlated positively with neutrophil presence and active degranulation).

Conventional anti-inflammatory drugs, including ibuprofen and prednisone, have shown beneficial, albeit marginal, effects by slowing down CF disease progression [190, 191]. However, prednisone treatment of CF patients is not common due to important side effects on growth [192]. More recent efforts focused on drugs designed to inhibit neutrophil recruitment to the CF lung, such as BIIL 284, a $LTB_4$ receptor antagonist [193], and SB 656933, a CXCR2 antagonist [194]. Both drugs led to an increase in inflammatory signaling (increased frequency of exacerbations for the former, and increased circulating inflammatory mediators for the latter), suggesting that inhibiting neutrophil recruitment to the CF lung may prove detrimental for patients [195]. In addition to inhibiting neutrophil recruitment into the lung lumen, BIIL 284 was also found to promote apoptosis of neutrophils that had transmigrated [196]. Arguably, focus should now be put on the development of drugs directed toward regulating neutrophil function or aiming to orchestrate their chemotaxis to the lung to attain normal homeostatic levels, rather than blocking their recruitment, which could lead to detrimental, sub-optimal levels of these cells within the lung lumen.

Since NE activity is elevated in CF patients and correlates with disease progression, development of NE inhibitors has also been of prime interest [197, 198]. Unfortunately, due to the high amount of NE present in CF airways, its broad range of substrates, and its compartmentalization as both a free-floating, mucus-associated, and membrane-bound enzyme [55], the design of inhibitors and modes of administration have to be significantly improved to attain therapeutic efficacy [199]. In a recently introduced approach, Forde et al. [200] leveraged NE activity in diseased airways to process a synthetic pro-drug, giving rise to a fully active anti-infectious small peptide. A similar approach could be applied to design NE-activated immunomodulatory drugs. Examples of relevant immunomodulatory drugs includes agents able to: (i) target the organizing stage of neutrophil swarming preceding their transepithelial migration,

which may reduce, as opposed to fully abrogate their recruitment to the lung; (ii) manipulate the metabolism and /or functional fate of neutrophils, to promote phagocytosis while reducing NETosis and degranulation/reprogramming; and (iii) regulate the lifespan of airway neutrophils or interfere with other factors enabling the establishment of a pathological, neutrophil-driven microenvironment in CF lungs.

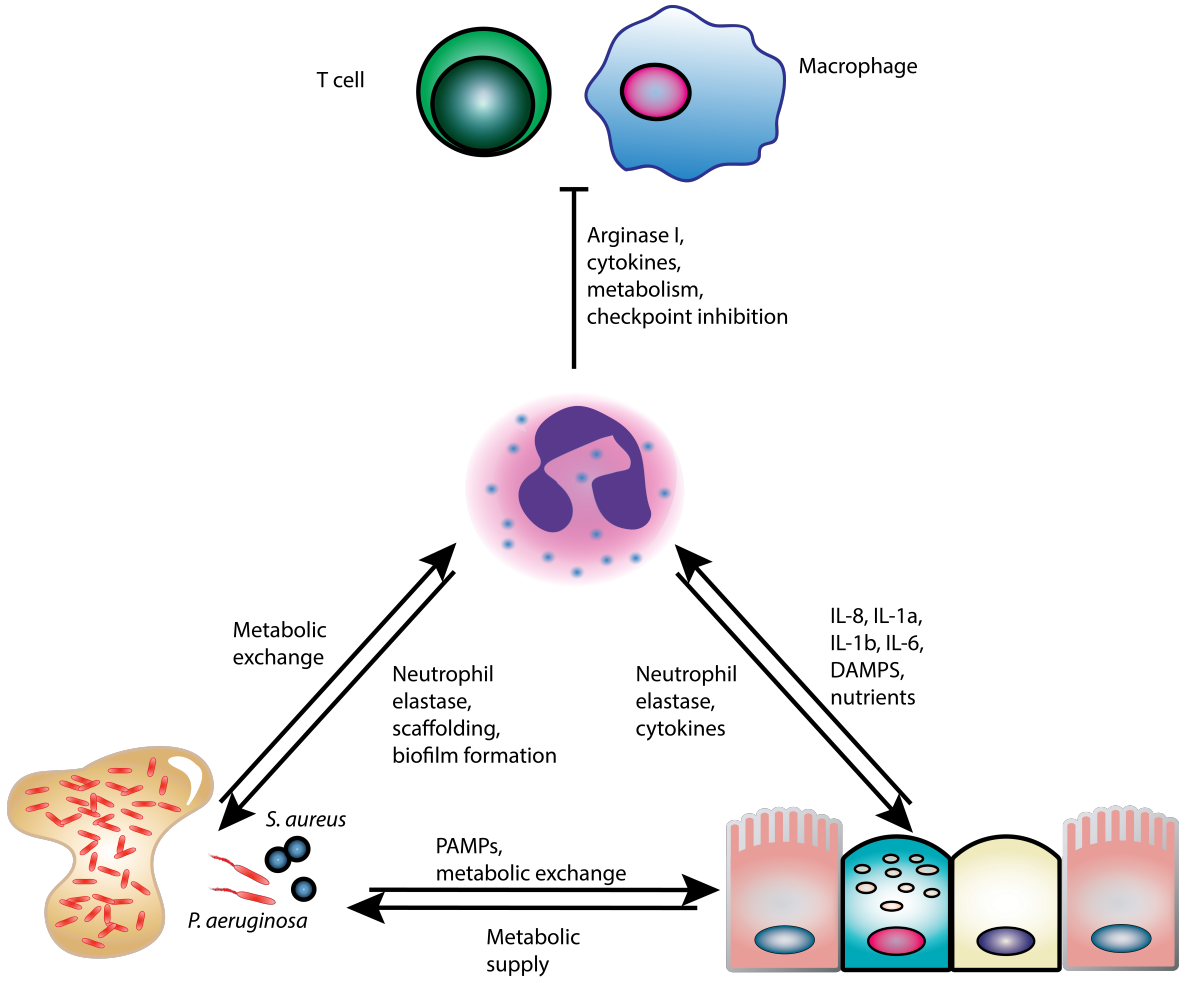
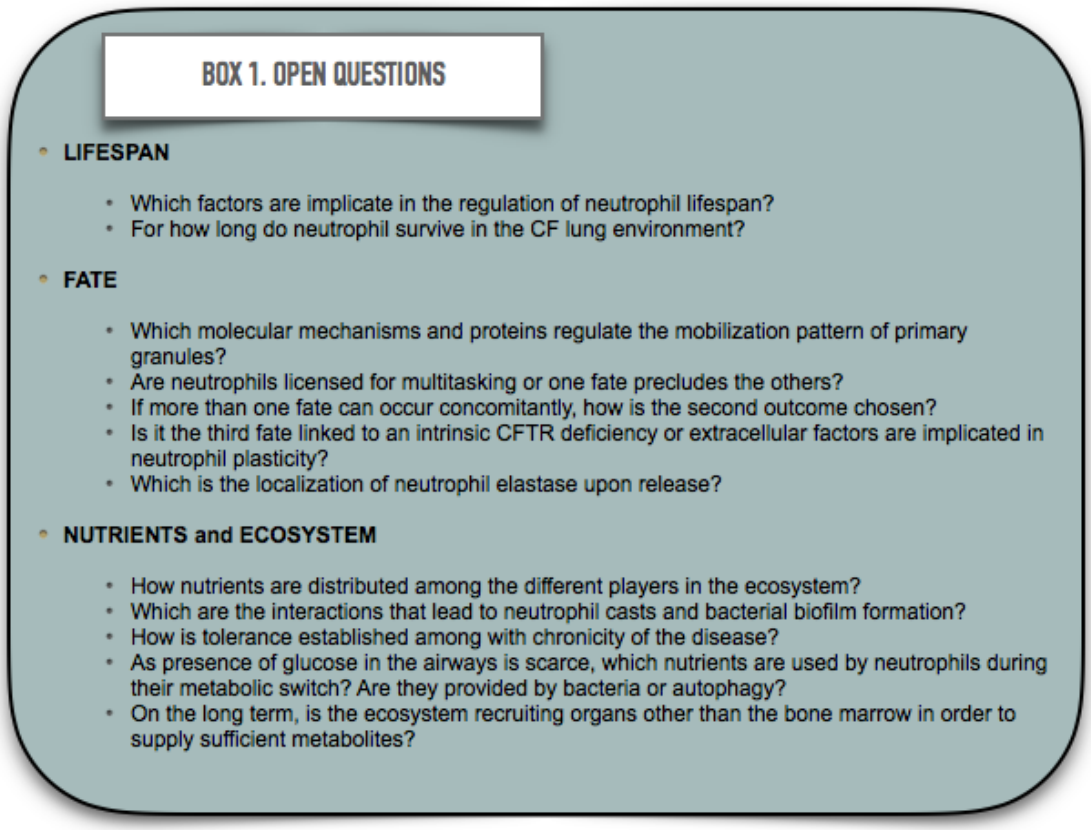



**Figure 1.2.3. Neutrophils as protagonists among contributors to CF airway disease.** Neutrophils (center) establish metabolic and signaling ties with lung epithelial cells (bottom right) and resident planktonic and mucoid/biofilm bacteria (bottom left), while exerting primarily inhibitory control over other immune cells, including T cells and macrophages (top). The result of these complex relationships is the formation of a relatively stable pathological microenvironment within CF airways.

## 1.3 Conclusions

In the last decade, substantial progress has been made in our collective understanding of processes underlying the pathophysiology of CF lung disease. Despite extensive work, much remains to be explored regarding neutrophil functions and plasticity, and their ability to claim a central role in the development of the pathological microenvironment in CF lungs (**Figure 1.2.3**).

Neutrophils currently enjoy renewed interest from basic and clinical researchers, as emerging evidence supports the idea that mechanisms of metabolic and functional plasticity described here are not confined to CF. Therefore, a better understanding of molecular mechanisms underlying neutrophil plasticity and neutrophil-epithelium-microbial partnership should help identify novel targets for treatments aiming to normalize pathological microenvironment development in CF, and similar neutrophil-driven diseases such as COPD, RA, and certain forms of cancer. Expanding our knowledge in terms of crosstalk between metabolic switching, interconnecting pathways and effector functions in neutrophils will be of high value for those and other reasons. To close this introduction, we invite readers to consult **Box 1.3.1**, which lists several of the open questions pertaining to neutrophil plasticity and function that will need to be addressed in the near future.




**Box 1.3.1. Open questions related to human neutrophil fate and their role in the formation of pathological microenvironments.**

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# Chapter 2

## Inflammatory mechanisms in early CF lung disease:

## interplay between neutrophils and macrophages

### 2.1 Elastase exocytosis by airway neutrophils associates with early lung damage in cystic fibrosis children

### 2.2 Airway macrophage PD-1 expression signals neutrophil takeover, infection, and structural lung damage in children with cystic fibrosis

*Sections of theses chapters have been published in the American Journal of Respiratory and Critical Care Medicine and have been submitted for review at the European Respiratory Journal*

## 2.1 Elastase exocytosis by airway neutrophils associates with early lung damage in cystic fibrosis children

### 2.1.1 At-a-glance commentary

*Current scientific knowledge on subject*. Neutrophils are recruited into the airways of individuals with cystic fibrosis (CF), and actively exocytose the primary granule protease elastase, whose free extracellular activity correlates with CF lung damage, notably bronchiectasis. During childhood, free extracellular elastase activity is only measurable in a subset of patients, and the exocytic function of airway neutrophils is unknown.

*What does this study add to the field?* Using flow cytometry, we show that neutrophils actively release elastase in the airway lumen of CF children, prior to the detection of bronchiectasis. In a cross-sectional two-center study, this measure of elastase exocytosis correlated with early structural lung damage measured by chest computed tomography. These findings implicate live airway neutrophils in early CF airway pathogenesis, which should instruct biomarker development and anti-inflammatory therapy in early CF.

### 2.1.2 Abstract

*Rationale.* Neutrophils are recruited to the airways of individuals with cystic fibrosis (CF). In adolescents and adults with CF, airway neutrophils actively exocytose the primary granule protease elastase (NE), whose extracellular activity correlates with lung damage. During childhood, free extracellular NE activity is measurable only in a subset of patients, and the exocytic function of airway neutrophils is unknown.

*Objective.* We sought to measure NE exocytosis by airway neutrophils in relation to free extracellular NE activity and lung damage in CF children.

*Methods.* We measured lung damage using chest computed tomography (CT) coupled with the PRAGMA-CF scoring system. Concomitantly, we phenotyped blood and bronchoalveolar lavage fluid (BALF) leukocytes by flow and image cytometry, and measured free extracellular NE activity using spectrophotometric and Förster resonance energy transfer (FRET) assays. Children with airway inflammation linked to aerodigestive disorder were enrolled as controls.

*Measurements and main results.* CF but not disease control children harbored BALF neutrophils with high exocytosis of primary granules, prior to the detection of bronchiectasis. This measure of NE exocytosis correlated with lung damage (Rho=0.55, p=0.0008), while the molecular measure of free extracellular NE activity did not. This discrepancy may be due to the inhibition of extracellular NE by BALF antiproteases and its binding to leukocytes.

*Conclusions.* NE exocytosis by airway neutrophils occurs in all CF children, and its cellular measure correlates with early lung damage. These findings implicate live airway neutrophils in early CF pathogenesis, which should instruct biomarker development and anti-inflammatory therapy in CF children.

### 2.1.3 Introduction

Cystic fibrosis (CF) is a multiorgan disease caused by recessive mutations of the CFTR gene [1]. Morbidity and mortality in CF relate primarily to progressive lung damage, leading eventually to respiratory failure. Although some studies point to anomalies in prenatal development of large airways in CF [2], the first overt symptoms may be detected months to years after birth [3-5], primarily due to involvement of the small airways [6]. Thanks to newborn screening, CF infants can be diagnosed within a few weeks of birth and monitored closely from a very young age, which has led to improved patient care and outcomes [7]. Among methods for monitoring in early CF are chest computed tomography (CT) scans [8], to assess structural lung damage, and bronchoalveolar lavage fluid (BALF) collection [9], to investigate infection status, as well as cellular and molecular biomarkers [10].

Nearly twenty years of work have revealed that CF children develop signs of lung disease in early in life [5, 11, 12]. Inflammation as evidenced by increased levels of pro-inflammatory mediators and downstream recruitment of blood neutrophils into the airway lumen, occurs within a few weeks after birth [13]. It is still unclear whether early CF airway inflammation is triggered solely by cellular stress linked to abnormal CFTR expression [14], as suggested by several CF models in which infection can be excluded experimentally [15-18]. Early infection is also believed to play a role in this process, possibly as a trigger, and most definitely as an accelerator, of inflammation[19]. Still to be better defined are the roles played in early disease of typical CF-associated pro-inflammatory microorganisms like *Pseudomonas aeruginosa, Staphylococcus aureus*, *Haemophilus influenzae* and *Aspergillus spp.* [20-23] and of oral microflora that may also spread to the airways [24].

Critically, recruitment of blood neutrophils to the airways in early CF disease is marked by the appearance of neutrophil-derived metabolites in BALF [25, 26], and release of the protease neutrophil elastase (NE). A cross-sectional study close to a decade ago showed that free extracellular NE activity in BALF (measured with a conventional spectrophotometric assay) correlated with structural lung damage as assessed with a three-slice CT method [27]. A follow-up longitudinal study showed that 3 month-old CF infants whose BALF was positive for free extracellular NE activity had increased odds of developing bronchiectasis at 12 and 36 months than those for which BALF was negative [28].

Another important issue relates to the mode of release of NE by airway neutrophils, which is normally contained in primary granules in the cytosol [29]. We showed in prior studies that live neutrophils recruited to the airway lumen in CF adolescents and adults are characterized by hyperactive NE exocytosis and the loss of phagocytic receptors, among other anomalies [30-33]. However, it remains unknown whether similar active dysfunction occurs in neutrophils present in the airway lumen of CF children, and how this dysfunction may relate to free extracellular NE activity and structural lung damage. This question is particularly important at a time when novel therapies are slowing the appearance of symptoms in CF patients [34].

To address this question, we performed phenotyping of airway leukocytes from CF children using flow and image cytometry [35]. Concomitantly, we used chest CT scans scored with the sensitive PRAGMA-CF tool recently developed for early disease monitoring [36], and free extracellular NE activity measurement by a Förster Resonance Energy Transfer (FRET)-based method [37-39]. Our findings support the notion that NE exocytosis by airway neutrophils occurs in CF children, and that its cellular measure

correlates with early lung damage. Some of the results of these studies have been previously reported in the form of abstracts [40-43].

### 2.1.4 Methods

*Human subjects and samples*

Data were collected prospectively from 42 CF children (age: 3-62 months) enrolled in disease surveillance programs at Telethon Kids Institute, Perth, Australia and Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands. Measures of NE exocytosis by freshly collected blood and BAL leukocytes, free extracellular NE activity in BALF, inflammatory mediator levels in BALF, and chest CT scans analyzed with the PRAGMA-CF scoring method were sought at the same visit.

Control subjects (N=10) were enrolled among non-CF children less than 6 years of age who were undergoing a bronchoscopy procedure as part of their diagnostic work up in the Aerodigestive Clinic at Emory University. This clinic follows patients with airway disorders (laryngomalacia, subglottic stenosis, tracheomalacia, bronchomalacia), dysphagia, chronic aspiration and patients with complex medical conditions and genetic disorders associated with various malformations. We excluded patients with a tracheostomy, those with significant airway reconstruction (slide tracheoplasty), significant prematurity (<28 weeks gestational age), or those with evidence of diffuse lung disease (childhood interstitial lung disorders). The study was approved by our institutional ethics committees. Consent for collection of blood and bronchoalveolar lavage (BAL) samples was obtained from parents on the day of the procedure. All specimens were stored on ice after collection and during transport to the laboratory for immediate processing. Cell counts and differential for BAL specimen was performed by the clinical pathology laboratory, and culture results were reported

using standard aerobic culture technique by clinical microbiology laboratories at our respective institutions. Demographic data for the primary prospective cohorts of CF and control children are summarized in **Table 2.1.4.1**. Because samples from children were sometimes limiting in terms of cell yield, we list in **Table 2.1.4.2** all CF and control subjects and the assays that were effectively implemented on their samples. In addition, fixed BALF leukocytes were available to conduct confirmatory image cytometry measurement of NE content from another prospective cohort of 10 CF children enrolled in 2015 at Erasmus MC/Sophia Children's Hospital (age: 13-37 months).

*Chest computed tomography (CT) imaging*

Volumetric chest CT scans obtained under general anaesthesia at Princess Margaret Hospital for Children [8] and free-breathing chest CT scans obtained without anaesthesia at Erasmus MC/Sophia Children's Hospital were scored using the Perth-Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis (PRAGMA-CF) method [36]. Total disease (PRAGMA-Dis%), bronchiectasis (PRAGMA-Bx%) and air trapping (PRAGMA-TA%) were reported.

| | ***CF*** | ***Control*** |
|---|---|---|
| ***Sex*** | | |
| *Males, n (%)* | 20 (47.6) | 5 (50.0) |
| *Females, n (%)* | 22 (52.4) | 5 (50.0) |
| ***Age [in months: mean (SD)]*** | 30.82 (23.0) | 21.4 (7.5) |
| ***Genotype*** | | |
| *F508del homozygous, n (%)* | 18 (42.9) | NA |
| *F508del heterozygous, n (%)* | 19 (45.2) | NA |
| *Other, n (%)* | 5 (11.9) | NA |
| ***Infection status (pathogens detected)*** | | |
| *None, n (%)* | 32 (76.2) | 6 (60.0) |
| *One, n (%)* | 7 (16.7) | 2 (20.0) |
| *Two or more, n (%)* | 3 (7.1) | 2 (20.0) |

**Table 2.1.4.1 Demographics of the primary CF and control cohorts.**

| ID | Age | Sex | Site | CFTR mutation 1 | CFTR mutation 2 | Infection | Assay | CT |
|---|---|---|---|---|---|---|---|---|
| 1 | 22.8 | F | P | G85E | unknown | *M. catarrhalis* | FC, NE, C | Yes |
| 2 | 23.64 | M | P | F508 | F508 | *A. spp, HI, PA* | FC, NE, C | Yes |
| 3 | 23.64 | F | P | F508 | F508 | None | FC, NE, C | Yes |
| 4 | 24.36 | M | P | F508 | F508 | *EC, P. lilacinus* | FC, NE, C | Yes |
| 5 | 23.28 | F | P | F508 | R117H | None | FC, NE, C | Yes |
| 6 | 24.24 | M | P | F508 | 1898+1G->A | None | FC, NE, C | Yes |
| 7 | 11.4 | M | P | F508 | F508 | None | FC, NE, C | Yes |
| 8 | 12.48 | M | P | 621+1G->T | I507 | *HI* | FC, NE, C | Yes |
| 9 | 10.92 | M | P | F508 | F508 | None | FC, NE, C | Yes |
| 10 | 11.64 | F | P | F508 | I502T | None | FC, NE, C | Yes |
| 11 | 11.28 | F | P | F508 | F508 | None | FC, NE, C | Yes |
| 12 | 4.44 | F | P | F508 | 1717-1G->A | None | FC, NE, C | Yes |
| 13 | 9.6 | M | P | F508 | F508 | *CMV* | FC, NE, C | Yes |
| 14 | 12.24 | F | P | F508 | R1158X | None | FC, NE, C | Yes |
| 15 | 3.84 | M | P | F508 | F508 | None | FC, NE, C | Yes |
| 16 | 3.72 | M | P | F508 | F508 | None | FC, NE, C | Yes |
| 17 | 7.8 | F | P | F508 | c.3718-2477C>T | *Rhinovirus* | FC, NE, C | Yes |
| 18 | 3.24 | F | P | F508 | F508 | *A. niger, CA, EC* | FC | Yes |
| 19 | 39 | M | R | F508 | G542X | *PA* | FC, NE, C | Yes |
| 20 | 60 | M | R | F508 | A455E | *HI* | FC, NE, C | Yes |
| 21 | 62 | M | R | F508 | A455E | *HI, SA* | FC, NE, C | Yes |
| 22 | 61 | M | R | F508 | R117H-7T | None | FC, NE, C | Yes |
| 23 | 61 | F | R | F508 | F508 | *PA* | FC | Yes |
| 24 | 61 | M | R | E60X | 4015delATTT | *SA* | NE, C | Yes |
| 25 | 61 | M | R | F508 | F508 | *A. fumigatus* | FC, NE, C | Yes |
| 26 | 60 | F | R | F508 | F508 | None | FC, NE, C | Yes |
| 27 | 60 | M | R | F508 | R117H | None | NE, C | Yes |
| 28 | 62 | F | R | F508 | R117H | None | FC, NE, C | Yes |
| 29 | 61 | F | R | F508 | F508 | *HI, SA* | FC, NE, C | Yes |
| 30 | 61 | M | R | F508 | DELE2,3(21 KB) | None | FC, NE, C | Yes |
| 31 | 12 | F | R | F508 | F508 | None | FC, NE, C | Yes |
| 32 | 13 | F | R | Y275X | A559T | None | FC, NE, C | No |
| 33 | 36 | F | R | F508 | N1303K | None | FC, NE, C | No |
| 34 | 62 | M | R | F508 | N1303K | None | FC, NE, C | No |
| 35 | 12 | M | R | F508 | E60X | None | FC, NE, C | Yes |
| 36 | 12 | F | R | F508 | A455E | None | NE, C | No |
| 37 | 60 | F | R | F508 | NK1303K | None | FC | No |
| 38 | 61 | F | R | F508 | F508 | None | FC | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 39 | 36 | F | R | F508 | F508 | None | FC, C | Yes |
| 40 | 12 | F | R | F508 | F508 | None | FC, C | Yes |
| 41 | 13 | M | R | R1162X | R1162X | None | FC, NE, C | No |
| 42 | 12 | F | R | F508 | 1682dup | None | FC, C | Yes |
| 43 | 35 | F | R | R117H | 1857delT | None | IC | Yes |
| 44 | 13 | F | R | F508 | F508 | None | IC | Yes |
| 45 | 13 | F | R | F508 | F508 | None | IC | Yes |
| 46 | 13 | M | R | F508 | 3272-26A>G | None | IC | Yes |
| 47 | 37 | M | R | F508 | F508 | None | IC | Yes |
| 48 | 14 | F | R | F508 | c.3407_3422 del | *PA* | IC | Yes |
| 49 | 37 | M | R | F508 | F508 | None | IC | Yes |
| 50 | 36 | F | R | F508 | F508 | None | IC | Yes |
| 51 | 37 | F | R | F508 | F508 | *A. fumigatus* | IC | Yes |
| 52 | 37 | F | R | F508 | F508 | None | IC | Yes |
| 54 | 25 | F | E | NA | NA | None | FC, NE, C | No |
| 55 | 13 | M | E | NA | NA | None | FC, NE, C | No |
| 56 | 18 | F | E | NA | NA | *Hi, PA* | FC, NE, C | No |
| 57 | 17 | F | E | NA | NA | None | FC, NE, C | No |
| 58 | 37 | M | E | NA | NA | *HI* | FC, NE, C | No |
| 59 | 17 | M | E | NA | NA | None | FC, NE, C | No |
| 60 | 12 | M | E | NA | NA | None | FC, NE, C | No |
| 61 | 24 | F | E | NA | NA | *MRSA, SA* | FC, NE, C | No |
| 62 | 26 | M | E | NA | NA | None | FC, NE, C | No |
| 63 | 25 | F | E | NA | NA | *M. catarrhalis* | FC, NE, C | No |

**Table 2.1.4.2 Details on study subjects and assays.** Age is shown in months. FC: flow cytometry; IC: image cytometry; MI: multiplexed immunoassay; NA: not applicable; NE: neutrophil elastase; MRSA: multidrug-resistant *S. aureus*, SA: *S. aureus*, PA: *P. aeruginosa*, HI: *H. influeanzae*, EC: *E. Coli*, CA: *Candida albicans*, A: *Aspergillus*, CMV: Cytomegalovirus.

*Sample collection and processing*

Blood was collected by venipuncture in $K_2$ EDTA tubes, and the cells were recovered as previously described [33]. Bronchoscopy with BAL was performed in the right middle lobe while under general anesthetic as part of routine clinical management [28]. BAL was retrieved by instillation and aspiration of three aliquots of sterile saline (1 mL/kg up to a maximum of 20 mL). The first aliquot was cultured by clinical microbiologists for the detection of pathogens using standard qualitative culture techniques. Pulmonary infection was defined as colony counts for a specific organism (excluding mixed oral flora) greater than $10^4$ colony-forming units per mL. The second and third aliquots were pooled and a fraction was used to generate cytospin slides for differential leukocyte counts by the clinical pathology laboratory [28]. EDTA was added to the remainder of the pooled second and third BAL fractions (2.5 mM final) to stop cell activation until processing (within an hour of collection). Cells were separated by gentle passing through an 18-gauge needle, followed by centrifugation at 800 g for 10 minutes at 4 °C. The supernatant was depleted of bacteria by centrifugation at 3,000 g for 10 minutes at 4 °C, and stored at -80 °C for downstream assays, which included NE activity assays and a multiplexed assay for inflammatory mediators (IL-1$\alpha$, IL-1$\beta$, IL-1RA, IL-6, IL-8, IL-10, IL-18, IFN$\gamma$, TNF$\alpha$, G-CSF, GM-CSF, M-CSF, MIP-1, MCP-1, ENA-78, I-TAC, TRAIL, IP-10, VEGF, GRO$\alpha$).

*Flow cytometry*

Multiparametric flow cytometry was performed on cells obtained from blood and BALF, as detailed before [33, 35], with some modifications: cells were pre-incubated for 10 minutes on ice with Fc block reagent and Zombie live/dead Aqua dye (Biolegend), followed by staining for 20 minutes on ice with antibodies against various surface

markers, including CD3 (pan T-cell), CD16, CD45 (pan-leukocyte), CD63, CD66b, CXCR2, and CXCR4 (all from Biolegend); neutrophil elastase (Novus Biologicals); Siglec-8 (eosinophils), and Siglec-9 (both from R&D systems). Cells were then washed with PBS-EDTA, and fixed at 4°C with Lyse/Fix Phosflow reagent (BD Biosciences). Prior to acquisition, fixed cells were spun at 800G for 10 minutes at 4 °C, and resuspended in PBS-EDTA. Samples were acquired on a FACS Fortessa (BD Biosciences) or on a FACS LSRII (BD Biosciences). To ensure robust acquisition of flow cytometric data on fresh samples acquired over the course of the study, we used premixes of the antibodies listed above to stain neutrophils and monocytes / macrophages, and employed a stringent calibration method with Rainbow bead-based channel standardization to provide constant output from the flow cytometers [35]. This method enabled robust data acquisition on samples collected over a year in multiple sites. Fluorescence compensation and data analysis were performed using FlowJo V9.9.5 (Treestar).

*Image cytometry*

Cells retrieved from the BAL were fixed in Lyse/Fix Phosflow (BD Biosciences) and stored at -80 °C until use. Thawed cells were washed with PBS-EDTA, permeabilized with Perm Buffer I (BD Biosciences) and stained with DAPI (nuclear stain), cholera toxin B (CTB, to distinguish neutrophils) (31), as well as CD63 and NE. Cells were acquired on the Amnis Imagestream X Mark II (EMD Millipore), with 40x magnification and low flow rate/high sensitivity on the INSPIRE software. Brightfield was set on channels 01 and 09, while scattering was set in channel 06. Data were analyzed using the IDEAS software v6.1 (EMD Millipore). Gating of neutrophils and macrophages followed the strategy illustrated in **Figure 2.1.6A**.

*BALF assays for inflammatory mediators and free extracellular NE activity and inhibition*

Levels of 20 inflammatory mediators were measured in BALF using a multiplexed high-sensitivity chemiluminescent assay (U-plex, Meso Scale Diagnostics), per the manufacturer's protocol. Free extracellular NE activity was measured using: (i) a conventional spectrophotometric assay (28) based upon the chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (working range of 0.02-12.0 μg/mL); and (ii) the FRET-based NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described [37-39]. The NEmo-1 probe was also used to assess the potential of native BALF from CF children to inhibit NE activity, as before [38]. Samples were measured in duplicate with repeated measures for values outside the working range.

*Statistical analysis*

Data were tabulated in Excel (Microsoft) and transferred to JMP13 (SAS Institute) and Prism v7 (GraphPad) for statistical analysis. The potential effect of study site was evaluated for all measured variables based on linear models adjusting for age and site, with or without their interactions. Data for those variables found to be significantly affected were corrected for site effects while adjusting for age using a well-established non-parametric empirical Bayesian method [44] typically used for multi-site analysis of data. Downstream statistical analyses were performed on the combined data with the site effect corrected. Data were analyzed using non-parametric statistics, including the Mann-Whitney test for unpaired samples, Wilcoxon signed-rank test for paired outcomes, and Spearman's test for correlations.

### 2.1.5 Results

*Macrophages and neutrophils coexist in BAL from CF and disease control children*

CT scans were successfully conducted on 36 out of 42 CF children and analyzed using the PRAGMA-CF scoring method [36]. This analysis yielded a median value for total disease (PRAGMA-%Dis) of 2.97 (interquartile range -IQR-: 2.2-3.85); for trapped air (PRAGMA-%TA) of 2.28 (0.48-5.06); and for bronchiectasis (PRAGMA-%Bx) of 0.0 (IQR: 0.0-0.26). The fact that bronchiectasis was not detectable or below 1% in the majority of children in this study illustrates their early stage of disease. Airway leukocyte counts by the clinical laboratory showed a predominance of macrophages (median: 83%) over neutrophils (median: 14.5%), and lymphocytes (median: 3.0%). In disease control children, BAL counts showed a predominance of macrophages (median: 83.6%) over lymphocytes (median: 9.0%), and neutrophils (median: 4.35%). These data are consistent with BAL counts assessed in a cohort of 48 normal children aged 3-16 years [45], with macrophages predominant (median: 84%) over lymphocytes (median: 12.5%), and very few neutrophils (median: 0.9%). Absolute BAL counts presented in **Table 2.1.5.1** show that CF children had higher numbers of total leukocytes, macrophages and neutrophils than disease control children enrolled in this study. However, disease control children still presented with significant numbers of BAL neutrophils, consistent with airway inflammation (albeit lower than in CF infants).

| | CF BAL | Disease control BAL | p |
|---|---|---|---|
| **Total leukocytes** | $7.1x10^5$ ($2.6x10^5$ - $1.9x10^6$) | $2.3x10^5$ ($6.2x10^4$ - $4.2x10^5$) | 0.0025 |
| **Macrophages** | $4.9x10^5$ ($1.3x10^5$ - $1.8x10^6$) | $6.7x10^4$ ($4.1x10^4$ - $3.8x10^5$) | 0.0077 |
| **Neutrophils** | $7.0x10^4$ ($8.7x10^3$ - $1.7x10^5$) | $6.1x10^3$ ($1.9x10^3$ - $1.9x10^4$) | 0.0044 |
| **Lymphocytes** | 789.4 (0.0 - $4.2x10^3$) | 668.4 (93.4 - 2.2 $x10^3$) | n.s. |
| **Eosinophils** | 0.0 (0.0 - 0.0) | 0.0 (0.0 - 40.9) | n.s. |

**Table 2.1.5.1. Absolute cell counts in BAL from CF and disease control children enrolled in the study.** Shown are median (interquartile range) values for BAL counts expressed in cells/ml. Differences between CF and disease control children were assessed using the Wilcoxon rank sum test. n.s., not significant.

*Airway neutrophils in CF children show distinct changes consistent with hyperexocytosis*

Flow cytometry was used to gain further insight into airway leukocytes from CF and control children. Matched blood samples collected at the same visit as the BAL procedure were also available for CF and control children (34 out of 42, and 9 out of 10, respectively). Our gating strategy discriminated major leukocyte subsets from blood and BAL **(Figure 2.1.5.1A** and **Figure 2.1.5.2)**, and yielded airway macrophage and neutrophil percentages that were highly correlated with those reported by the clinical laboratory for both CF and control cohorts (Rho>0.7 for all), thus validating our analytical approach.

Next, we assessed surface marker expression on airway neutrophils. We found that all CF children enrolled (age range: 3-62 months) harbored a distinct population of live airway neutrophils which we previously found to be typical of CF airways in

adolescents and adults [33], with increased CD63 expression (reflecting exocytosis of NE-rich primary granules), and decreased CD16 expression (reflecting phagocytic receptor loss) compared to blood neutrophils **(Figure 2.1.5.1A)**. Upregulation of CD66b, reflecting exocytosis of secondary granules **(Figure 2.1.5.1B)** was also consistent with prior data in adolescents and adults with CF [32, 46]. Since CD16 was also significantly downregulated on disease control airway neutrophils compared to blood (albeit to a lesser extent than CF airway neutrophils), this change may not be viewed as specific to CF. By contrast, neither surface CD66b nor surface CD63 were significantly upregulated on airway neutrophils from disease control children, which suggests that hyperactive exocytosis of secondary granules and, most importantly, of NE-rich primary granules may be distinguishing features of CF pathogenesis.

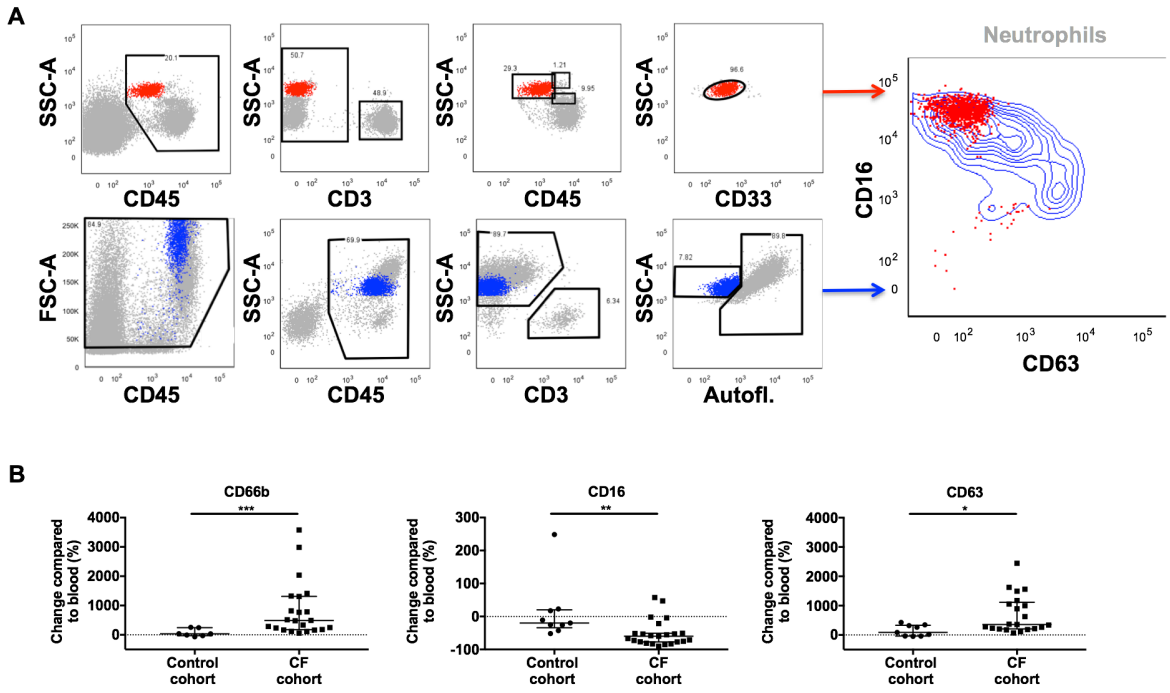


**Figure 2.1.5.1. Flow cytometry gating strategy and neutrophil phenotype.** **A.** After definition of singlets (not shown), blood (red, top left panels) and airway (blue, bottom left panels) neutrophils were gated in four sequential steps, as shown. Autofluorescence in the violet laser-excited 450/50 nm channel (Autofl.) was low in airway neutrophils, and high in airway macrophages. All CF children showed a typical pattern in airway neutrophils with increased surface CD63 (primary granule exocytosis) and decreased surface CD16 (phagocytic receptor) expression (right panel). **B.** Surface expression of CD66b (left panel; control cohort, N=9; CF cohort, N=22), CD16 (middle panel; control cohort N=9; CF cohort N=33), and CD63 (right panel; control cohort N=9; CF cohort N=33) on airway neutrophils are shown in % change compared to matched blood neutrophils. Significant differences in between-group analyses are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

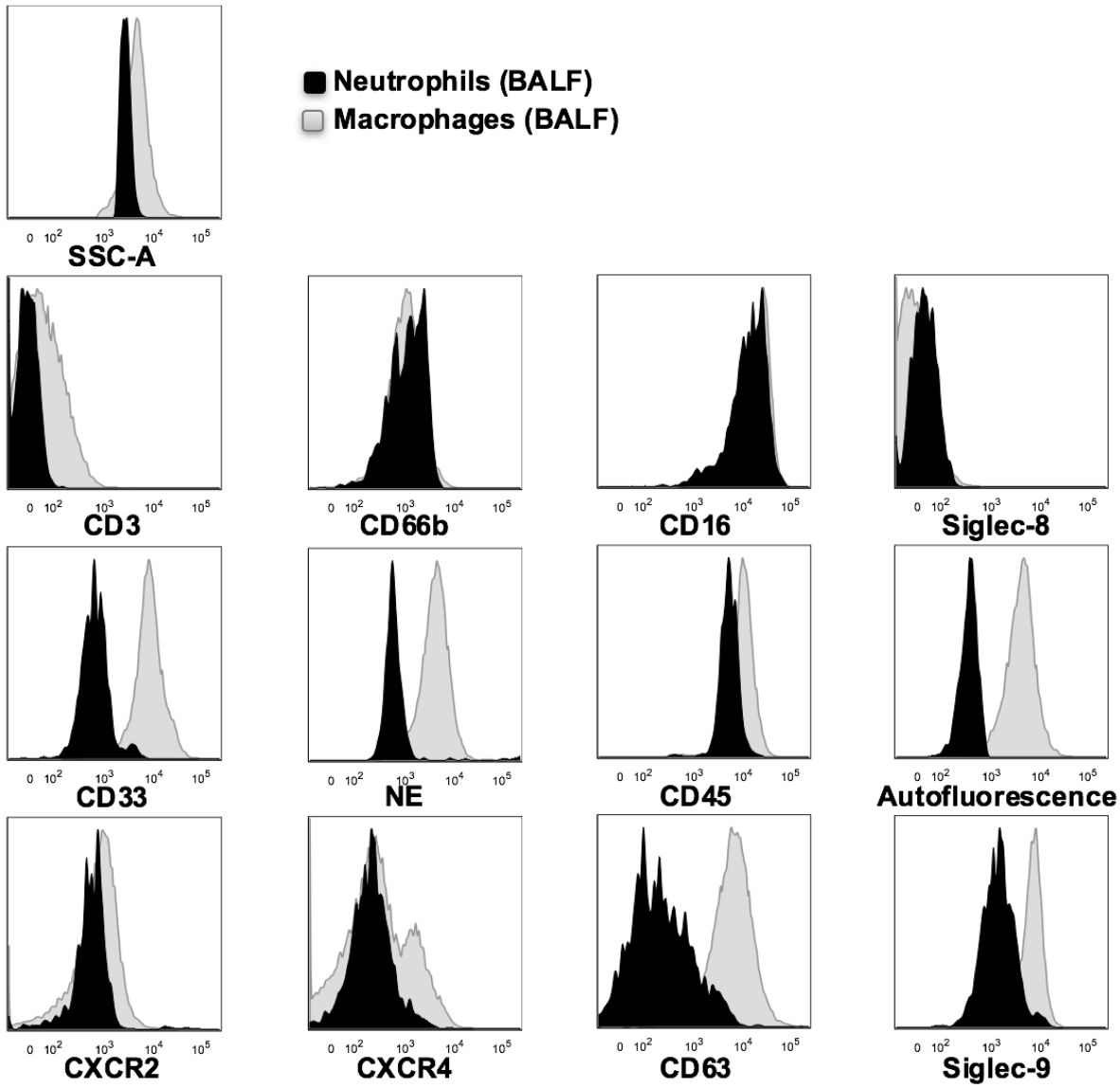


**Figure 2.1.5.2. Multiparameter overlay of airway neutrophils and macrophages gated per our strategy.** Neutrophils and macrophages from CF infant BALF (one representative patient shown here) overlap for several markers typically used to discriminate between neutrophils and monocytes in blood (e.g., CD66b, Siglec 9, CXCR2). However, CD33 and autofluorescence show expected higher expression on macrophages and allow their discrimination upon analysis.

*Cell-based measure of NE exocytosis by airway neutrophils correlates cross-sectionally with structural lung damage in CF children*

The exocytosis of NE-rich primary granules, reflected by airway neutrophil CD63 expression measured by flow cytometry showed a significant positive correlation with structural lung damage (Rho=0.55, p=0.0008), as measured by PRAGMA-%Dis **(Figure 2.1.5.3A)**. By contrast, PRAGMA-%Dis correlates neither with typical measures of inflammation such as BAL neutrophil % or count (not shown), or the levels of 20 inflammatory mediators **(Table 2.1.5.2)**, although we observed a trend toward a positive correlation for interleukin (IL)-8.

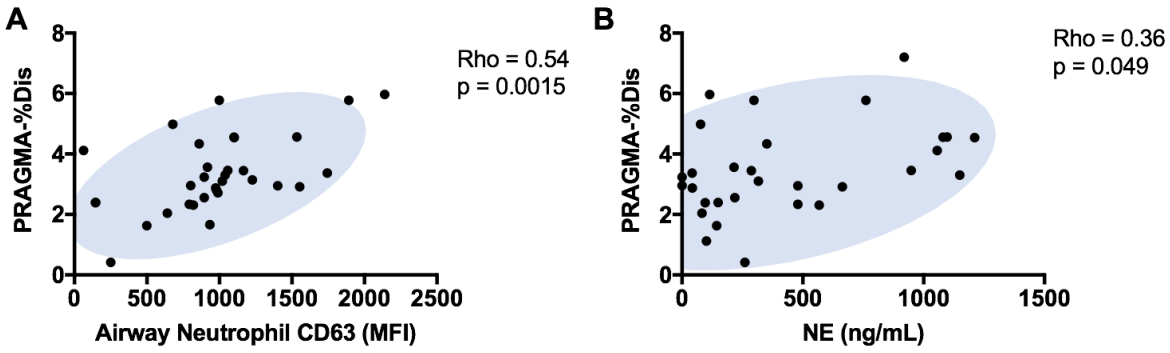


**Figure 2.1.5.3. Correlation of NE exocytosis by airway neutrophils, and free extracellular NE activity with structural lung damage. A.** Correlation of the PRAGMA-%Dis (total disease score) with airway neutrophil CD63 levels (MFI; N=33). **B.** Correlation of the PRAGMA-%Dis score with free extracellular NE activity (interpolated to a concentration of recombinant NE standard expressed in ng/ml; N=29).

Since primary granule exocytosis by neutrophils results in extracellular NE release, we next assessed NE activity in BAL from this cohort. Of 42 patients, 35 had enough BALF for NE measurement. Of those, the conventional spectrophotometric NE activity assay [28] yielded measurable levels in 27 samples. To achieve higher sensitivity, we used a newly introduced FRET method based on the NEmo-1 probe [37-39]. This method yielded measurable NE activity in 33 out of 35 samples. Despite its increased sensitivity, the FRET-based NE activity measure trended but did not correlate significantly with PRAGMA-%Dis **(Figure 2.1.3B)**.

| | ***Rho*** | ***p*** | ***q*** | | ***Rho*** | ***p*** | ***q*** |
|---|---|---|---|---|---|---|---|
| **IL-1*α*** | -0.21 | n.s. | n.s. | **I-TAC** | 0.22 | n.s. | n.s. |
| **IL-1*β*** | 0.21 | n.s. | n.s. | **TNF*α*** | 0.02 | n.s. | n.s. |
| **IL-1RA** | -0.08 | n.s. | n.s. | **VEGF** | 0.21 | n.s. | n.s. |
| **IL-6** | 0.25 | n.s. | n.s. | **G-CSF** | -0.24 | n.s. | n.s. |
| **IL-8** | 0.43 | 0.0135 | n.s. | **GRO*α*** | -0.04 | n.s. | n.s. |
| **IL-10** | 0.18 | n.s. | n.s. | **IP-10** | -0.07 | n.s. | n.s. |
| **IL-18** | -0.17 | n.s. | n.s. | **M-CSF** | -0.21 | n.s. | n.s. |
| **IFN*γ*** | -0.14 | n.s. | n.s. | **MCP-1** | -0.16 | n.s. | n.s. |
| **ENA-78** | -0.08 | n.s. | n.s. | **MIP-1*β*** | -0.05 | n.s. | n.s. |
| **GM-CSF** | -0.15 | n.s. | n.s. | **TRAIL** | 0.09 | n.s. | n.s. |

**Table 2.1.5.2. Correlations of 20 selected inflammatory mediator levels measured in BALF of CF children with PRAGMA-%Dis.** Indicated are respective Spearman Rho, p and q values (significance threshold of 0.05 adjusted for multiple comparisons to 0.0025 using the Bonferroni method). n.s.: not significant.

*Cell-based measure of NE exocytosis by airway neutrophils and free extracellular NE activity are not impacted by infection status in CF children*

We investigated whether airway neutrophil CD63 expression and free extracellular NE activity differed based on infection, as determined by clinical microbiology. Infection status was classified based on the presence of 0, 1, or 2 or more pro-inflammatory pathogens, which were previously identified in studies of CF children to encompass *P. aeruginosa*, *S. aureus*, *H. influenzae*, and *Aspergillus spp.* [21, 22]. Among the 42 CF children included in the present study, both airway neutrophil CD63 **(Figure 2.1.5.4A)** and free extracellular NE activity **(Figure 2.1.5.4B)** were not associated with infection status among CF children. Similar results were observed in disease controls (not shown).

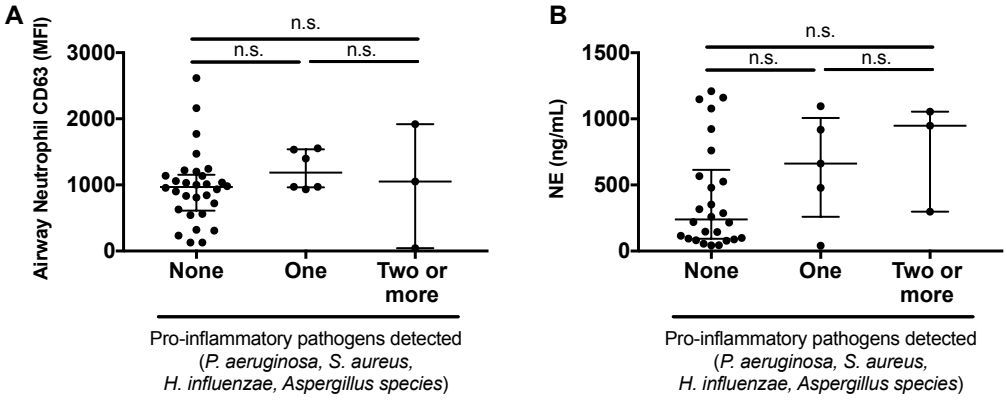


**Figure 2.1.5.4. NE exocytosis by airway neutrophils and free extracellular NE activity are not impacted by infection status. A.** Airway neutrophil CD63, as a measure of NE exocytosis (in median fluorescence intensity, MFI; N=39) is compared between CF children based on infection status (0, 1 or 2 or more pro-inflammatory pathogens detected in BALF). **B.** Free extracellular NE activity (interpolated to a concentration of recombinant NE standard expressed in ng/ml; N=34) is also compared between CF children based on infection status. n.s., not significant.

*Extracellular NE is counteracted by antiproteases and compartmentalizes in airway leukocytes in BALF of CF children*

To explain the discrepancy between airway neutrophil CD63 expression and FRET-based free extracellular NE activity data, we investigated whether the activity of NE released by neutrophils could be counteracted by antiproteases present in the BALF of CF children, and/or compartmentalize in airway leukocytes. The presence of a functional antiprotease shield was assessed using BALF from CF children with high or low level of free extracellular NE **(Figure 2.1.5.5A)**. We observed that the BAL from CF children with low NE status was still able to inhibit the recombinant NE activity, while those with high NE were unable to inhibit recombinant NE, suggesting that the antiprotease shield in CF children is still active at early stages of disease. Using flow cytometry, we found significant surface NE expression in CF children on airway neutrophils and macrophages, with higher levels on the latter **(Figure 2.1.5.5B)**. Finally, to assess whether NE could be found inside of airway leukocytes, we used fixed and permeabilized BAL cells from another prospectively enrolled CF cohort (N=10, age: 13-37 months), and used image cytometry to quantify NE burden **(Figure 2.1.5.6)**. As expected, airway neutrophils contained large amounts of total NE, although sizeable amounts were also detectable in airway macrophages. Because NE biosynthesis is confined to neutrophils, these data suggest that airway macrophages in CF children can bind and internalize extracellular NE released by airway neutrophils.

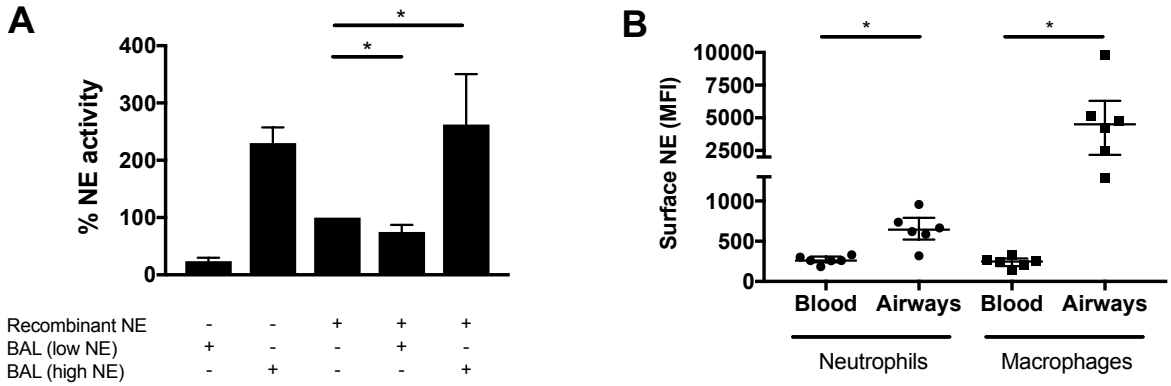


**Figure 2.1.5.5. Free extracellular NE activity in CF children is limited by BALF antiprotease activity and by compartmentalization on airway leukocytes. A.** Activity of recombinant NE was measured in vitro by FRET in the absence or presence of BALF from CF children with either low or high intrinsic NE activity. Results were compared to the unopposed activity of recombinant NE in the absence of BALF (100%). **B.** Surface NE antigen was measured by flow cytometry in CF blood and airway neutrophils and macrophages (N=6). * indicates $p<0.05$.

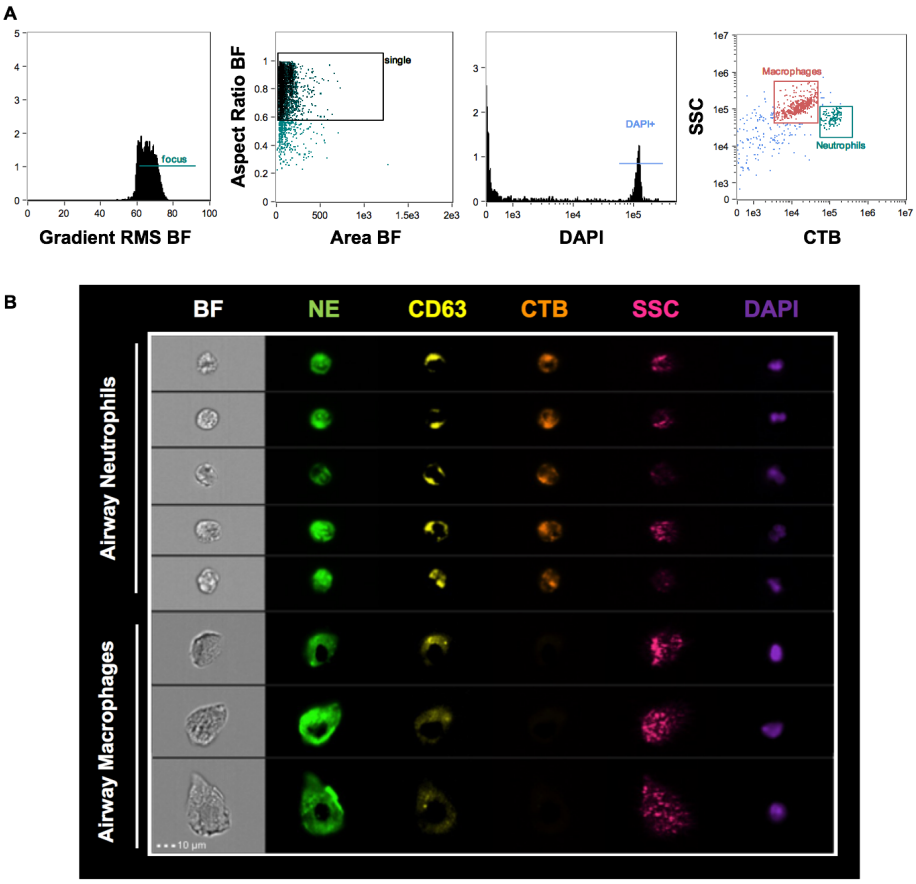


**Figure 2.1.5.6 Image cytometry analysis of BAL immune cells. A.** Gating strategy from left to right: 1) Focused events were gated from the gradient RMS brightfield (BF); 2) Aspect Ratio vs. Area in the BF channel were used to gate single events; 3) Live non-apoptotic cells were gated based on staining with the nuclear dye DAPI expression (diploid); 4) Neutrophils were discriminated from macrophages based on higher level of cholera toxin B (CTB) staining and side scatter (SSC) [31]. Cells contained in each gate were validated based on respective images collected during acquisition. **B.** Gated BAL neutrophils and macrophages (representative examples in top five, and bottom three rows, respectively) yield single-cell data on BF, NE, CD63, CTB, SSC, and DAPI expression (from left to right), confirming significant NE expression in both BAL subsets.

### 2.1.6 Discussion

Through cellular phenotyping, we demonstrate that CF children at all stages of disease harbor a distinct population of airway neutrophils with high active exocytosis of NE-rich primary granules. Airway neutrophil CD63 expression correlated positively with structural lung damage (Rho=0.55), and did not depend on infection status. In contrast, conventional biomarkers of neutrophilic inflammation used in CF individuals to track the severity of lung damage [10], such as BAL neutrophil % and inflammatory mediator levels, did not correlate with lung damage. However, the correlations of IL-8 levels (measured by a multiplexed chemoluminescent assay) and free extracellular NE activity (measured by a sensitive FRET-based assay) with lung damage both trended toward significance.

CD66b, a marker of secondary granule exocytosis was upregulated on airway neutrophils from CF but not non-CF children, compared to blood as we previously observed on airway neutrophils from older CF patients [32, 33]. Also consistent with previous findings in older CF patients, we found that airway neutrophils in CF children downregulated the phagocytic receptor CD16 [33], although similar (albeit lesser) downregulation was seen in disease control children. Together, our findings suggest that neutrophils recruited to the airway lumen of CF children are conditioned to adopt an activated state with distinguishing features (increased CD63 expression). This is consistent with the notion that the CF airway environment can trigger early inflammation, as previously proposed in select mouse models of CF [38, 47, 48], human CF fetal xenografts [15], CF ferrets [18, 49], and CF children [13]. Insights gained from this study of CF children can help calibrate models [50].

Our study confirms that recruitment and dysfunction of neutrophils are key processes in early CF airway disease, whether triggered by infectious or sterile

mechanisms [51]. Our findings have important therapeutic implications, as they highlight the need for efficient leukocyte-targeted interventions [52] to help curb down the progression of structural lung damage in CF children. A prime target for intervention is NE, for which inhibitors [53] should be tested for their ability to block not only extracellular, but also surface-associated forms. Alternative strategies include reducing the number of neutrophils recruited from blood [54, 48] and their ability to exocytose primary granules [55].

The apparent discrepancy between the cellular measure of NE release (airway neutrophil CD63 expression) and the molecular measure of free extracellular NE activity (FRET-based assay) is explained, at least in part, by our findings that NE is present at the surface and inside of airway neutrophils and macrophages, and that the airway antiprotease shield is still able to inhibit NE activity in CF children, at least until its maximum buffering capacity is reached. This finding also suggests that assays for free extracellular NE activity do not reflect the overall burden of NE in CF airways.

The finding of high NE expression on airway macrophages also suggest a role for this subset in controlling the fate of NE. Therefore, it may also be beneficial to design targeted interventions aimed at optimizing airway macrophage function in CF children [56]. Understanding the fate of extracellular NE is critical to correct the protease-antiprotease imbalance that characterizes CF airways [57]. However, targeting NE may not suffice, as similar complex activation and compartmentalization may occur with other proteases such as matrix metalloproteinases 2, 9 and 12, which originate from neutrophils and macrophages [46, 58-60]. Future work should aim to identify the mutual impacts that these proteases have on each other, and points for intervention at molecular and/or cellular levels.

The present study extends prior data [27-28] gained with a less sensitive method for CT scoring, and for free extracellular NE measurement, yielding categorical data (presence/absence) adequate only for odds ratio calculation over time, while the FRET method used here yields continuous data enabling correlation analysis. The recently developed PRAGMA-CF CT scoring method [36] was critical in detecting early disease events in our cohort of CF patients. Indeed, very few children showed evidence of bronchiectasis, including among 5 year-olds. This is in contrast with studies conducted in the past decade, and may reflect improved abilities to preserve the integrity of lung architecture in CF children. This emphasizes the need for sensitive methods able to detect pathological processes occurring in CF airways prior to the detection of bronchiectasis.

The present study also presents several limitations. First, disease controls included in this study had higher numbers of neutrophils in their BAL than healthy children from a prior study [45], but did not include patients with primary ciliary dyskinesia (PCD) or idiopathic bronchiectasis, who may undergo very pronounced neutrophil recruitment to the airways and hence may have represented more appropriate controls. With regards to PCD itself, the onset of neutrophilic inflammation is not known and prior pediatric studies have enrolled patients over 6 years of age [61-63], which exceeds the age range of CF children (5 years and under) enrolled in this study. Further studies investigating BAL of infants with early onset PCD or idiopathic bronchiectasis are needed to shed light on whether these conditions show similar neutrophil exocytosis profiles as infants with CF.

Second, it is important to note that the infection status of CF children in our study used routine clinical microbiological methods, rather than highly sensitive genomics-based methods [23]. Therefore, while we can safely state that the presence of

typical CF proinflammatory pathogens (*P. aeruginosa*, *S. aureus*, *H. influenzae*, and *Aspergillus spp*.) does not impact airway neutrophil CD63 expression at the time of the measurement, we cannot exclude potential impact of prior infections, or low-level presence of atypical organisms [24]. Viral infections may also be at play, although a recent study suggested that CF children do not suffer more frequent viral infections or increased inflammatory responses upon viral infections than non-CF children [64].

Third, it is important to acknowledge that this study is cross-sectional in nature, and while it shows a significant correlation between NE exocytosis, as indicated by NE CD63 surface expression, and early lung damage, which excludes bronchiectasis for all but a handful of patients, and thus does not provide any information on its predictive value for future damage. A follow-up longitudinal study is a clear future direction for our multisite research effort.

Finally, our attempt to generate CT, BALF and BAL leukocyte analyses at the same visit from CF children under 5 years of age sometimes faced limitations of volumes that prevented some downstream analyses. In that context, it will be critical in follow-up studies to choose surrogate endpoints wisely, and airway neutrophil CD63 emerges as a worthwhile candidate in that regard.

## 2.2 Macrophage exhaustion signals neutrophil takeover in early cystic fibrosis airway disease

### 2.2.1 At-a-glance commentary

*Current scientific knowledge on subject.* Recently introduced molecular therapies directly targeting the mutated protein causing cystic fibrosis (CF) have improved patient quality of life. However, these therapies do not fully control inflammatory and infectious complications of CF airway disease, which involve resident macrophages, neutrophils recruited from blood, and pro-inflammatory pathogens. While prior studies identified an early neutrophil response in the airways of CF children, why resident macrophages fail to exert appropriate regulatory and repair functions in this context remains unknown.

*What this study adds to the field.* Our study identifies the exhaustion marker programmed death -1 (PD-1) as a critical receptor on CF airway macrophages. PD-1 expression on airway macrophages increases with airway neutrophil recruitment and activation, and infection with pro-inflammatory pathogens, and tracks with early structural lung damage measured by a sensitive computed tomography tool. Our study identifies for the first time a trackable marker of macrophage dysfunction at a very early stage of CF airway disease.

### 2.2.2 Abstract

*Rationale.* Macrophages are the major resident immune cells in human airways. Therein, they coordinate responses to infection and injury, which may involve the recruitment of blood neutrophils to carry out temporary effector functions, such as bacterial killing and tissue repair. In cystic fibrosis (CF), neutrophils are recruited to the airways shortly after birth, and actively exocytose damaging enzymes prior to chronic bacterial infection, suggesting a potential defect in macrophage immunomodulatory function. Signaling through the exhaustion marker programmed death protein 1 (PD-1) controls macrophage function in cancer, sepsis, and airway infection.

*Objective.* We sought to address the role of macrophage PD-1 in modulating early immune responses in CF children.

*Measurement and main results.* Blood and bronchoalveolar lavage fluid (BALF) were collected from 45 CF children aged 3 to 62 months and analyzed for the expression of PD-1 and its ligands PD-L1 and PD-L2, and the presence of enzymes and immunomodulatory mediators from macrophages and neutrophils. Airway macrophage surface PD-1 levels were correlated with structural lung damage measured by computed tomography, as well as cellular and molecular markers of neutrophilic inflammation. In contrast with prior results suggesting that active neutrophil exocytosis in the airways of CF children occurs independently of infection, we found that macrophage PD-1 expression was associated with infection by pro-inflammatory pathogens.

*Conclusions.* Together, our findings show a role for PD-1 signaling within airway macrophages in children with CF, associating with neutrophil takeover of the inflammatory milieu, infection with pro-inflammatory pathogens, and structural lung damage.

### 2.2.3 Introduction

Resident macrophages play a key role in tissue homeostasis and responses to infection and injury [65]. In the airways, macrophages quickly adapt to variations in oxygen pressure, inflammation, and the presence of pathogens [66]. Education of airway macrophages at birth and thereafter shapes long-term responses and the likelihood to mount aberrant responses to later insults [67]. In cystic fibrosis (CF), airway epithelial cells and macrophages display intrinsic defects due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In particular, mucin hyperconcentration and dehydration can alter airway macrophages [59], which may contribute to abnormal immune regulation, and subsequent recruitment and activation of neutrophils, with ensuing lung damage [68-70]. In itself, CFTR dysfunction in CF airway macrophages may result in impaired phagocytosis and pathogen clearance [71-73]. 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.

Programmed cell death protein-1 (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 [74]. Comparatively, the role of PD-1 in macrophages is less well described. In blood macrophages 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 [75-77]. Recently, PD-1 signaling was also found to mediate extrinsic control of resident macrophages by recruited immunosuppressive leukocytes in a model of airway infection [78].

We previously showed that neutrophils recruited to CF airways undergo reprogramming therein, leading to complex functional changes [31, 33, 79-81]. Among those

is the acquisition of immunosuppressive functions [32], including expression of the PD-1 ligand, PD-L1 [30]. It is unknown if PD-1 signaling is active in CF airway macrophages or not, and if so, how it relates to disease symptoms. Findings here suggest that PD-1 upregulation may signal macrophage exhaustion, bacterial infection, neutrophil takeover, and early lung damage in children with CF.

### 2.2.4 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) 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). The study was approved by relevant Institutional Ethical Review Boards at each site. Details on chest CT [36] appear in the methods section 2.1.4, while a summary of demographic information (**Table 2.2.4.1**), and a detailed tally of assays performed on each sample (**Table 2.2.4.2**) can be found below.

| | CF pediatric cohort (N=45) | Non-CF disease control pediatric cohort (N=10) |
|---|---|---|
| **Age in months, mean (SD)** | 30.4 (22.3) | 21.4 (7.5) |
| **Sex** | | |
| Males, n (%) | 22 (48.9) | 5 (50.0) |
| Females, n (%) | 23 (51.1) | 5 (50.0) |
| **Genotype** | | |
| F508Del homozygous, n (%) | 20 (44.4) | NA |
| F508Del heterozygous, n (%) | 20 (44.4) | NA |
| Other, n (%) | 5 (11.2) | NA |
| **Infection status (pathogens detected)** | | |
| None, n (%) | 34 (75.6) | 6 (60.0) |
| One, or more n (%) | 11 (24.4) | 4 (40.0) |

**Table 2.2.4.1. Summary of patient demographics.**

| ID | Age | Sex | Site | CFTR mutation 1 | CFTR mutation 2 | Infection | Assays | CT |
|---|---|---|---|---|---|---|---|---|
| 1 | 22 | F | P | G85E | unknown | *M. catarrhalis* | FC, E, C | Yes |
| 2 | 23 | M | P | F508 | F508 | *A. spp, HI, PA* | FC, E, C | Yes |
| 3 | 23 | F | P | F508 | F508 | None | FC, E, C | Yes |
| 4 | 24 | M | P | F508 | F508 | *EC, P. lilacinus* | FC, E, C | Yes |
| 5 | 23 | F | P | F508 | R117H | None | FC, E, C | Yes |
| 6 | 24 | M | P | F508 | 1898+1G->A | None | FC, E, C | Yes |
| 7 | 11 | M | P | F508 | F508 | None | FC, E, C | Yes |
| 8 | 12 | M | P | I507 | 621+1G->T | *HI* | FC, E, C | Yes |
| 9 | 10 | M | P | F508 | F508 | None | FC, E, C | Yes |
| 10 | 11 | F | P | F508 | I502T | None | FC, E, C | Yes |
| 11 | 11 | F | P | F508 | F508 | None | FC, E, C | Yes |
| 12 | 4 | F | P | F508 | 1717-1G->A | None | FC, E, C | Yes |
| 13 | 9 | M | P | F508 | F508 | *Cytomegalovirus* | FC, E, C | Yes |
| 14 | 12 | F | P | F508 | R1158X | None | FC, E, C | Yes |
| 15 | 3 | M | P | F508 | F508 | None | FC, E, C | Yes |
| 16 | 3 | M | P | F508 | F508 | None | FC, E, C | Yes |
| 17 | 7 | F | P | F508 | c.3718-2477C>T | *Rhinovirus* | FC, E, C | Yes |
| 18 | 3 | F | P | F508 | F508 | *A. niger, EC, CA* | FC | Yes |
| 19 | 39 | M | R | F508 | G542X | *PA* | FC, E, C | Yes |
| 20 | 60 | M | R | F508 | A455E | *HI* | FC, E, C | Yes |
| 21 | 62 | M | R | F508 | A455E | *HI, SA* | FC, E, C | Yes |
| 22 | 61 | M | R | F508 | R117H-7T | None | FC, E, C | Yes |
| 23 | 61 | F | R | F508 | F508 | *PA* | FC | Yes |
| 24 | 61 | M | R | E60X | 4015delATTT | *SA* | E, C | Yes |
| 25 | 61 | M | R | F508 | F508 | *A. fumigatus* | FC, E, C | Yes |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 26 | 60 | F | R | F508 | F508 | None | FC, E, C | Yes |
| 27 | 60 | M | R | F508 | R117H | None | E, C | Yes |
| 28 | 62 | F | R | F508 | R117H | None | FC, E, C | Yes |
| 29 | 61 | F | R | F508 | F508 | *HI, SA* | FC, E, C | Yes |
| 30 | 61 | M | R | F508 | Dele2,3(21KB) | None | FC, E, C | Yes |
| 31 | 12 | F | R | F508 | F508 | None | FC, E, C | Yes |
| 32 | 13 | F | R | Y275X | A559T | None | FC, E, C | No |
| 33 | 36 | F | R | F508 | N1303K | None | FC, E, C | No |
| 34 | 62 | M | R | F508 | N1303K | None | FC, E, C | No |
| 35 | 12 | M | R | F508 | E60X | None | FC, E, C | Yes |
| 36 | 12 | F | R | F508 | A455E | None | E, C | No |
| 37 | 60 | F | R | F508 | NK1303K | None | FC | No |
| 38 | 61 | F | R | F508 | F508 | None | FC | No |
| 39 | 36 | F | R | F508 | F508 | None | FC, C | Yes |
| 40 | 12 | F | R | F508 | F508 | None | FC, C | Yes |
| 41 | 13 | M | R | R1162X | R1162X | None | FC, E, C | No |
| 42 | 12 | F | R | F508 | 1682dup | None | FC, C | Yes |
| 43 | 23 | F | E | F508 | F508 | None | FC, BK | No |
| 44 | 34 | M | E | F508 | R553X | None | FC, BK | No |
| 45 | 18 | M | E | F508 | F508 | *HI* | FC, BK | No |
| 46 | 25 | F | E | NA | NA | None | FC, E, C | No |
| 47 | 13 | M | E | NA | NA | None | FC, E, C | No |
| 48 | 18 | F | E | NA | NA | *HI, PA* | FC, E, C | No |
| 49 | 17 | F | E | NA | NA | None | FC, E, C | No |
| 50 | 37 | M | E | NA | NA | *HI* | FC, E, C | No |
| 51 | 17 | M | E | NA | NA | None | FC, E, C | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 52 | 12 | M | E | NA | NA | None | FC, E, C | No |
| 53 | 24 | F | E | NA | NA | *MRSA, SA* | FC, E, C | No |
| 54 | 26 | M | E | NA | NA | None | FC, E, C | No |
| 55 | 25 | F | E | NA | NA | *M. catarrhalis* | FC, E, C | No |

**Table 2.2.4.2. Detailed list of assay implementation by patient (1-45: CF; 46-55: non-CF disease control).** BK: in vitro bacterial killing assays; C: multiplexed cytokine assay; E: enzyme activity assays; F: female; FC: flow cytometry; M: male, MRSA: multidrug-resistant *S. aureus*, SA: *S. aureus*, PA: *P. aeruginosa*, HI: *H. influeanzae*, EC: *E. Coli*, CA: *Candida albicans*, A: *Aspergillus*.

*Human sample collection and processing*

Blood and BALF were collected from CF children (N=45) and non-CF disease controls (N=10). Blood was collected in $K_2$-EDTA tubes by venipuncture, cells and plasma were separated by centrifugation and the cellular fraction was used for flow cytometry. BALF was collected under general anesthesia using sterile saline as per the standard clinical procedure previously described [18]. BALF was then mechanically dissociated on ice in presence of 2.5 mM EDTA, cells were recovered after an 800 g, 10 minutes, 4°C centrifugation, washed and used for downstream assays. BALF supernatant was further spun at 3,000 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 were performed by the clinical pathology laboratories at the Erasmus MC/Sophia Children's Hospital and Emory University/Children's Healthcare of Atlanta. Infectious status was determined by clinical microbiology using standard aerobic culture techniques at each respective institution, and classified for presence of either one or several of the pro-inflammatory pathogens *P. aeruginosa, S. aureus, H. influenzae,* and *Aspergillus spp.* [31]. 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.

*Flow cytometry and SPADE analysis*

Multiparametric flow cytometry analysis of whole blood and BALF cells was standardized across study sites as before [80], with additional details provided in the method section 2.1.4. Analysis and compensation were performed in FlowJo V9.9.5 (FlowJo, LLC), and clustering analysis and group comparisons in SPADE V4.0 [82].

*Assays for soluble mediators in BALF*

BALF cytokines were quantified using a 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-1 respectively (Sirius Fine Chemicals SiChem GmbH), as described [37].

*Ex vivo bacterial killing assay*

Co-incubation of bacteria and leukocytes (1:1), was performed in presence or absence of PD-1 blockade. PD-1 blockade was accomplished using 1 $\mu$g/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 [83]. Overnight cultures of pro-inflammatory bacteria *P. aeruginosa* (strain PAO1) and S. *aureus* (Xen29) were sub-aliquoted and grown to reach the exponential growth phase. Bacteria were then incubated in RPMI, supplemented with 10% FBS, on an end-over-end rotating wheel for 30 minutes at 37°C. Fresh BALF leukocytes ($10^5$ cells) were resuspended in RPMI, 10% FBS and incubated at 37°C, 5% $CO_2$ for 15 minutes. Co-incubation of bacteria and

leukocytes, 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% $CO_2$. Bacterial killing capacity was calculated using colony forming units (CFU), with the bacteria plus RPMI and 10% FBS condition set as 100% survival.

*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 [80]. Predictor screening analysis was performed using bootstrap forest partitioning in JMP13, while simple linear fitting modelling was performed in R.

### 2.2.5 Results

*PD-1 expression is increased on airway macrophages, independently of lipidation*

To assess the role of PD-1 signaling in early CF airway inflammation, blood and bronchoalveolar lavage fluid (BALF) from 45 children with CF, aged 3 to 62 months, and 10 age-matched non-CF disease control children with aerodigestive disease, prone to aspiration-induced inflammation, were analyzed by flow cytometry (**Table 2.2.4.1**). In both cohorts, PD-1 was upregulated on airway cells compared to their blood counterpart (**Figure 2.2.5.1A-C**). Moreover, PD-1 expression on airway macrophages was higher than on airway neutrophils and T cells, by 10- and 17-fold in children with CF (**Figure. 2.2.5.1D**), and by 10- and 18-fold in children without CF, respectively (**Figure 2.2.5.1E**). While PD-1 expression was not significantly different between the two cohorts, a subset of eight children with CF and one without CF displayed higher PD-1 expression on airway macrophages.

Next, we assessed which factors could contribute to a higher PD-1 expression. Uptake of oxidized lipids and the subsequent inability to efficiently metabolize them can be quantified by a lipidation index, which correlates to a dysfunctional state in macrophages [84], as described in atherosclerosis [85] and several lung conditions, including aspiration- and infection-induced inflammation and CF [86, 87]. Here, we defined infection by the presence of one or several of four pro-inflammatory pathogens, namely *P. aeruginosa, S. aureus, H. influenzae,* and *Aspergillus spp*. [88], at the time of BALF collection. To determine if lipidation could be responsible for the high PD-1 expression observed in airway macrophages, BALF samples from 22 children with CF and 9 age-matched non-CF disease control children were further analyzed by microscopy.

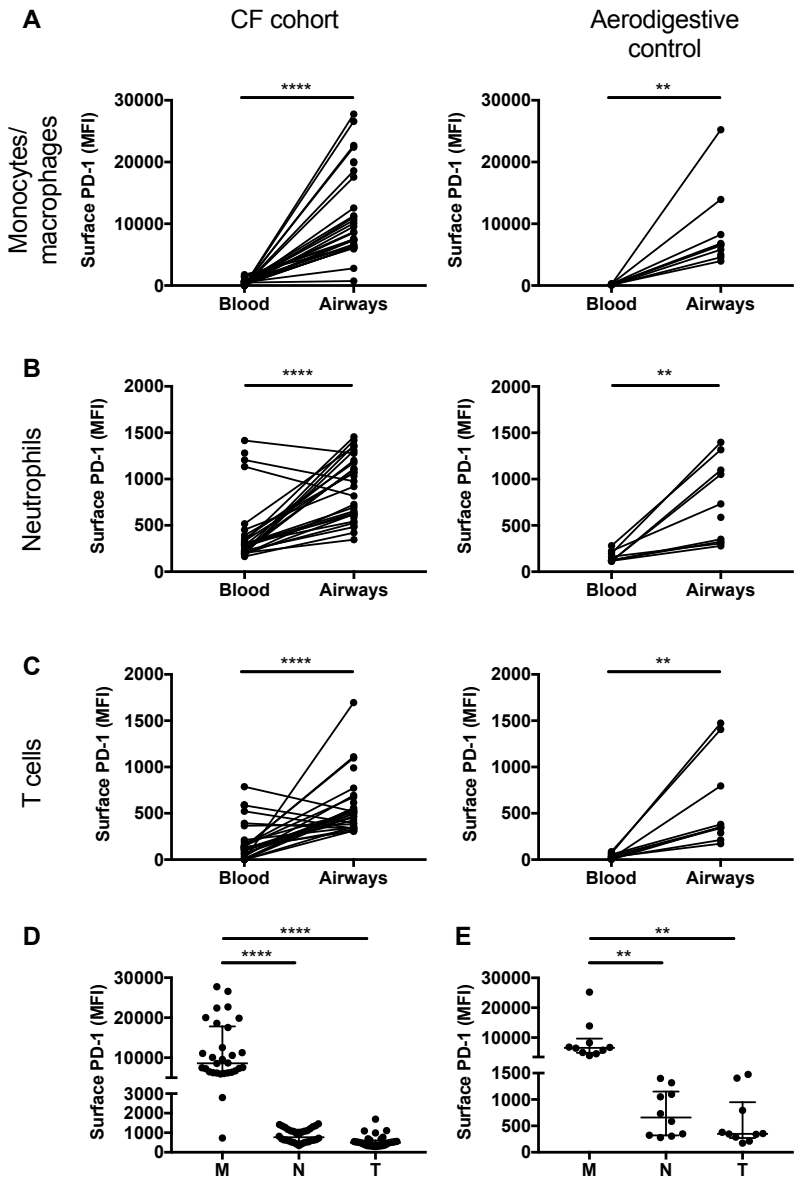


**Figure 2.2.5.1. PD-1 expression is increased in airway leukocytes.** Flow cytometry analysis of blood and BALF leukocytes from CF and non-CF disease control children shows higher PD-1 expression on airway compared to blood macrophages **(A)**, neutrophils **(B)**, and T cells **(C)**. PD-1 expression on macrophages was higher than on airway neutrophils and T cells in children with CF **(A)** and age-matched non-CF disease control children **(B)** (N=45 and 10, respectively, 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< 0.01, and **** for p<0.0001.

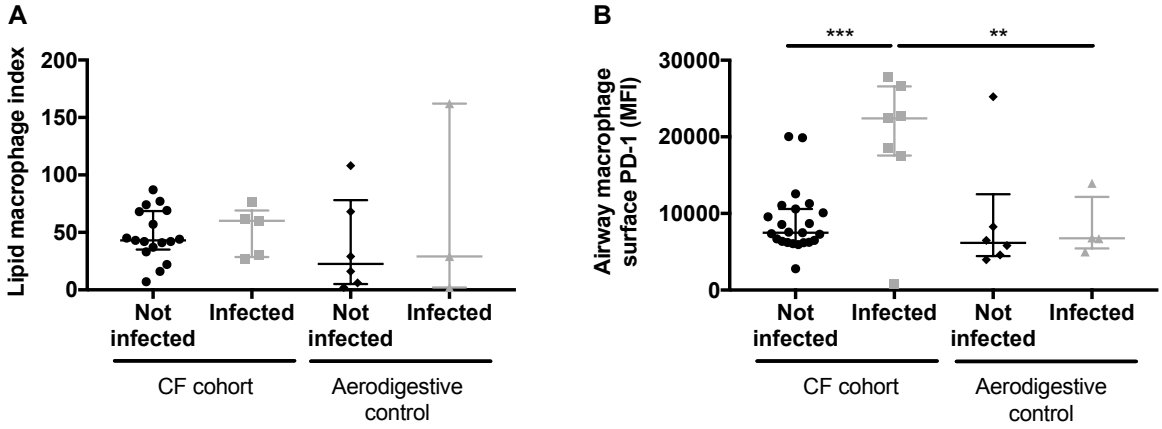


**Figure 2.2.5.2. PD-1 expression on CF airway macrophages associates with airway infection, but not lipidation**. In CF or non-CF disease control children, infection by pro-inflammatory pathogens was not related to airway macrophage lipidation, as measured by Oil Red O stain on cytospin slides **(A)**. 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) **(B)**. Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as ** for p< 0.01, *** for p<0.001, and **** for p<0.0001.

We found that the two cohorts did not differ in their lipidation index (**Figure 2.2.5.2A**), even in the presence of infection, while PD-1 expression was significantly increased on CF compared to non-CF disease control airway macrophages in the presence of infection (**Figure 2.2.5.2B**). Moreover, higher PD-1 expression on CF macrophages compared to non-CF did not results in differences in the airway macrophage absolute count (**Figure 2.2.5.3**), while it was significantly higher on CF than in non-CF disease control BALF in absence of infection, consistent with previous studies [89]. These results suggest that PD-1 expression on airway macrophages from children with CF is not influenced by lipidation.

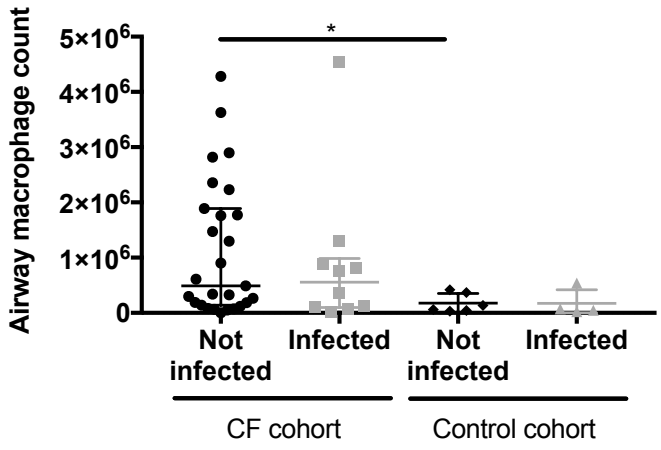


**Figure 2.2.5.3. Airway macrophage count is higher in children with CF than in disease control children in the absence of infection**. Presence of infection by pro-inflammatory pathogens, measured by clinical microbiology cultures, did not coincide with changes in absolute macrophage counts in children with CF or in disease control children. However, increased counts were observed in absence of infection in CF compared to disease control children.

*PD-1 expression on CF airway macrophages is associated with age, and infection status*

To investigate which factors other than lipidation and CFTR mutation could contribute to high PD-1 expression on CF airway macrophages, we measured soluble and cell-bound inflammatory mediators in BALF and combined them with clinical variables in a bootstrap forest partitioning analysis (N=42 patients included). The main predictors for PD-1 expression on airway macrophages were patient age, infection with pro-inflammatory pathogens, and neutrophil mediators. Since the likelihood of infection increases with age and correlates with enhanced neutrophil recruitment to CF airways [90, 91], we next fit 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. 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 ($p<0.001$). After adjusting for age, the effect of infection on PD-1 expression remained significant ($p<0.001$). These findings confirm that age increases the risk of infection, and that both age and infection impact PD-1 expression on airway macrophages in children with CF (**Figure 2.2.5.4A**).

*PD-1 expression on CF airway macrophages correlates with structural airway damage and airway neutrophil burden*

To determine the clinical significance of the PD-1 pathway (including PD-1 and its ligands PD-L1 and PD-L2) in early CF disease, we assessed the relationship between PD-1 and clinical variables. No difference was observed in the level of PD-1 expression between patients bearing F508Del homozygous vs heterozygous genotypes (**Figure 2.2.5.5A**), or males vs. females (**Figure 2.2.5.5B**). In a subset of 24 children with CF, for whom flow cytometry data and date-matched CT scans were acquired, the total

score of structural lung damage (%Dis) ranged between 0 and 5%, and the score for bronchiectasis (%Bx) ranged from 0 and 2.1%, reflecting the very early stage of disease in this cohort. Among all molecules of the PD-1 pathway measured on all BALF subsets (macrophages, neutrophils, and T cells), only PD-1 expression on airway macrophages correlated with both %Dis (Rho = 0.51, p = 0.01, **Figure 2.2.5.5C**) and %Bx (Rho = 0.46, p = 0.03). In that cohort, PD-1 expression on airway macrophages did not correlate with absolute macrophage count (Rho = 0.03, p = n.s.) but it did correlate negatively with airway macrophage percentage (Rho = -0.57, p = 0.003, **Figure 2.2.5.5D**), and positively with airway neutrophil absolute count (Rho = 0.50, p = 0.01) and percentage (Rho = 0.51, p = 0.009, **Figure 2.2.5.5E**).

*PD-1 expression on CF airway macrophages increases uniformly upon infection*

Next, we assessed whether the increase in PD-1 expression on airway macrophages in children with CF positive for infection was due to specific upregulation on a discrete subset. Clustering analysis with the sequential pattern discovery using equivalence classes (SPADE) algorithm [82], revealed that PD-1 expression was upregulated uniformly comparing infected and non-infected subgroups. This suggests 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 (**Figure 2.2.5.5F**).

Interestingly, among all surface markers included in our cytometry panel, PD-1 emerged as the most significant feature discriminating between infected and non-infected children with CF ($p<0.0001$). None of the other surface markers listed as significant discriminators between the two groups reproduced the uniform pattern observed with PD-1, although some of them, like PD-L1 (**Figure 2.2.5.4B**), showed differences between groups in discrete macrophage subsets. Together, these data

suggest that concomitant with the presence of pro-inflammatory pathogens, CF airway macrophages uniformly show an exhausted phenotype, measurable by higher PD-1 expression.

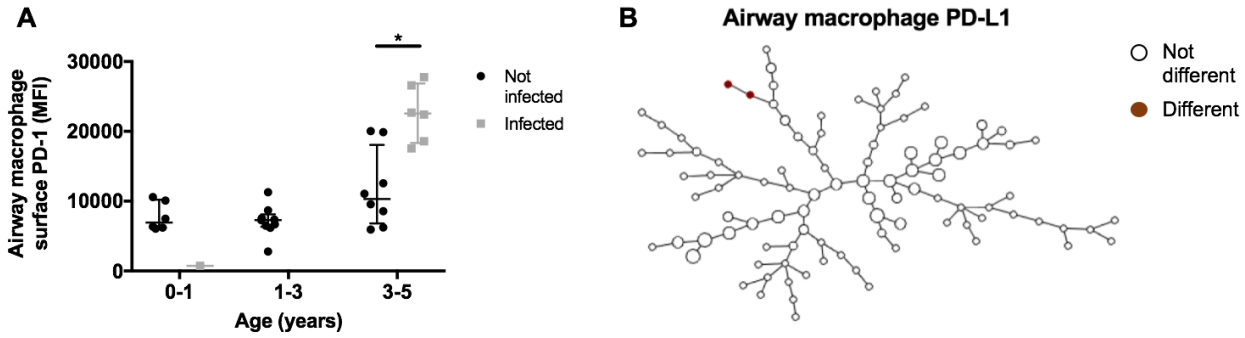


**Figure 2.2.5.4. PD-1 expression on CF airway macrophages is associated with age and infection status**. **(A)** PD-1 expression on CF airway macrophages increases with age and infection. Data are represented as median and interquartile range. Statistical analyses were performed by the Wilcoxon rank sum test, * $p<0.05$. **(B)** SPADE analysis of BALF macrophages shows that surface expression level of PD-L1 differs between infected and non-infected groups only in a few subsets (dark red).

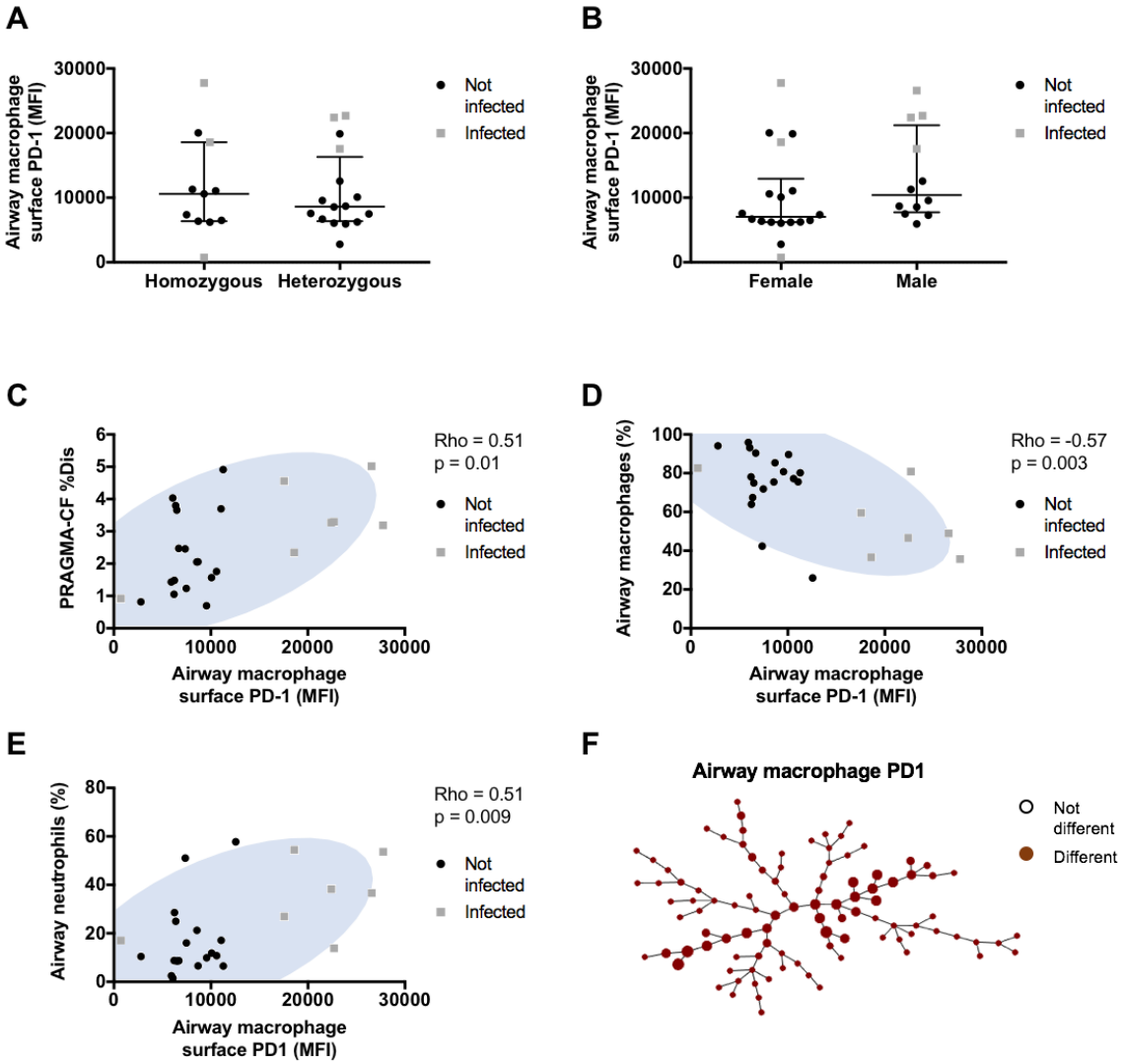


**Figure 2.2.5.5. PD-1 expression on airway macrophages correlates with lung disease in CF children.** PD-1 expression on CF airway macrophages is not dependent upon CFTR genotype of CFTR mutations **(A)** or sex **(B)**, but is associated with PRAGMA-CF %Dis (correlation shown as Spearman Rho and p-value) **(C).** PD-1 expression on airway macrophages correlated negatively with airway percentage **(D)** and positively with airway neutrophil percentage **(E)**. SPADE analysis of CF airway macrophages shows that PD-1 modulation across all macrophage subsets in infected CF children (dark red) **(F)**. Data were analyzed by the Wilcoxon rank sum test.

*Increased PD1 expression on CF airway macrophages coincides with neutrophil takeover*

Inflammation in CF airways is linked to pathological interactions between the airway epithelium, resident macrophages, neutrophils recruited from blood, mucus, and colonizing pathogens [92]. Among neutrophil-derived BALF mediators, elastase (NE), interleukin (IL)-8, and granulocyte colony-stimulating factor (G-CSF) also correlated with PD-1 expression on airway macrophages (**Figure 2.2.5.6**), which confirms correlations seen with airway neutrophil burden (above) and suggests that CF airway macrophage exhaustion coincides with intensifying neutrophilic inflammation.

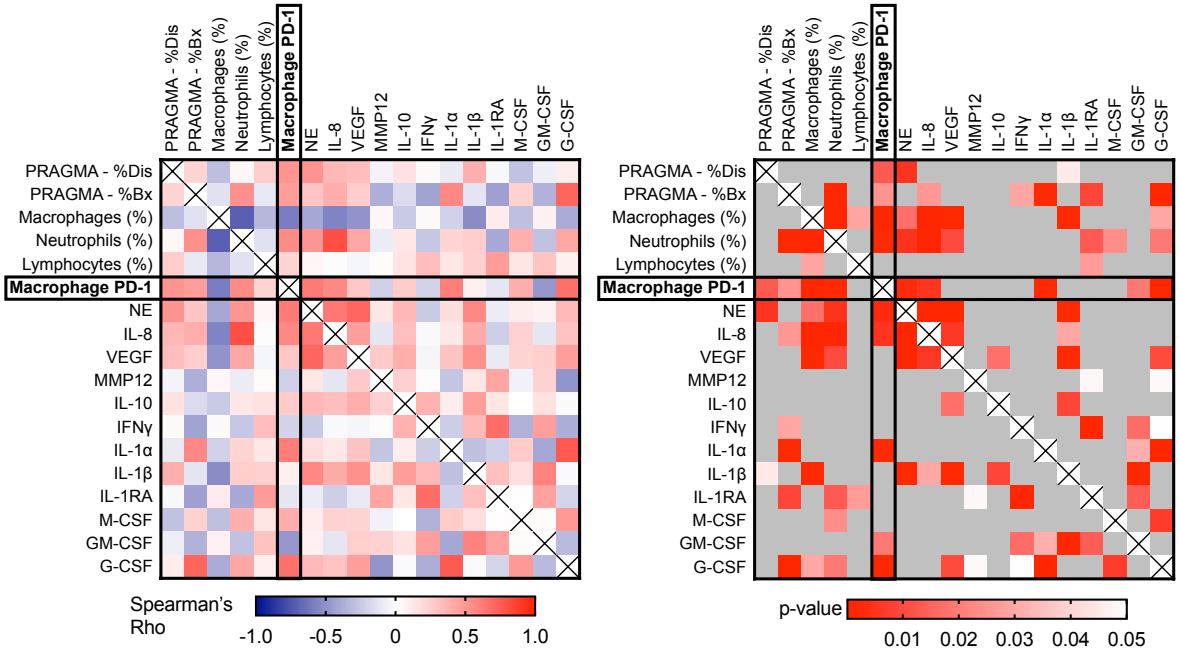


**Figure 2.2.5.6. PD-1 expression on CF airway macrophages is associated with neutrophilic inflammation.** PD-1 expression on CF airway macrophages correlates with neutrophil-derived mediators. Correlations are shown as coefficient strength of Spearman Rho (left panel) and significance levels of p-values (right panel, with grey indicating $p > 0.05$).

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 is not different between infected and non-infected groups [80]. However, upon infection with pro-inflammatory pathogens, neutrophils are increasingly recruited to the airways, resulting in the extracellular accumulation of neutrophil-derived mediators. Among those, NE, IL-8, vascular endothelial growth factor, and IL-1 and CSF families of mediators were significantly increased in patients positive for infection (**Figure 2.2.5.7A-C**). By contrast, no difference was observed between infected and non-infected groups in macrophage-derived regulatory proteins such as IL-10, interferon gamma, and matrix metalloproteinase 12 (**Figure 2.2.5.7D**).

Considerable overlap was observed between infected and non-infected groups in inflammatory mediator levels, suggesting that a high state of activation is also observed in patients without detectable infection with pro-inflammatory pathogens. Indeed, extracellular NE (Rho = 0.62, p = 0.02) and IL-8 (Rho = 0.48, p = 0.04) correlated with PD-1 expression on airway macrophages, in the absence of infection **(Figure 2.2.5.8)**. Interestingly, IL-1$\alpha$, a mediator previously linked to hypoxic responses in the airway epithelium [16, 93], was also positively correlated with PD-1 expression, suggesting that both hypoxic epithelial responses and neutrophilic inflammation may relate to the apparent exhaustion of airway macrophages in children with CF.

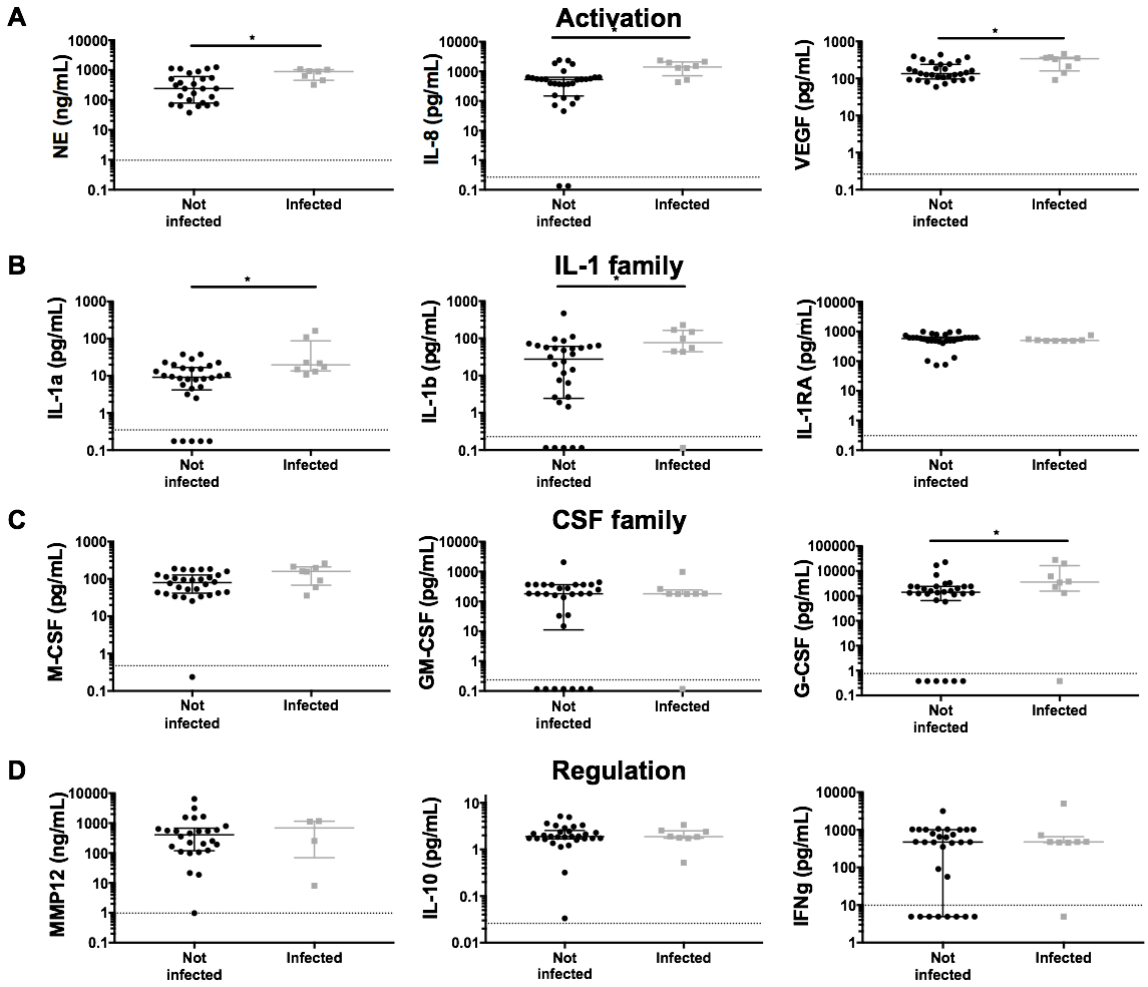


**Figure 2.2.5.7. BALF mediators associated with airway macrophage surface PD-1 expression are also modulated upon infection by pro-inflammatory pathogens**. BALF mediators are grouped into functional categories and compared between groups of CF children relative to the infection status by pro-inflammatory 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 (*$q<0.05$).

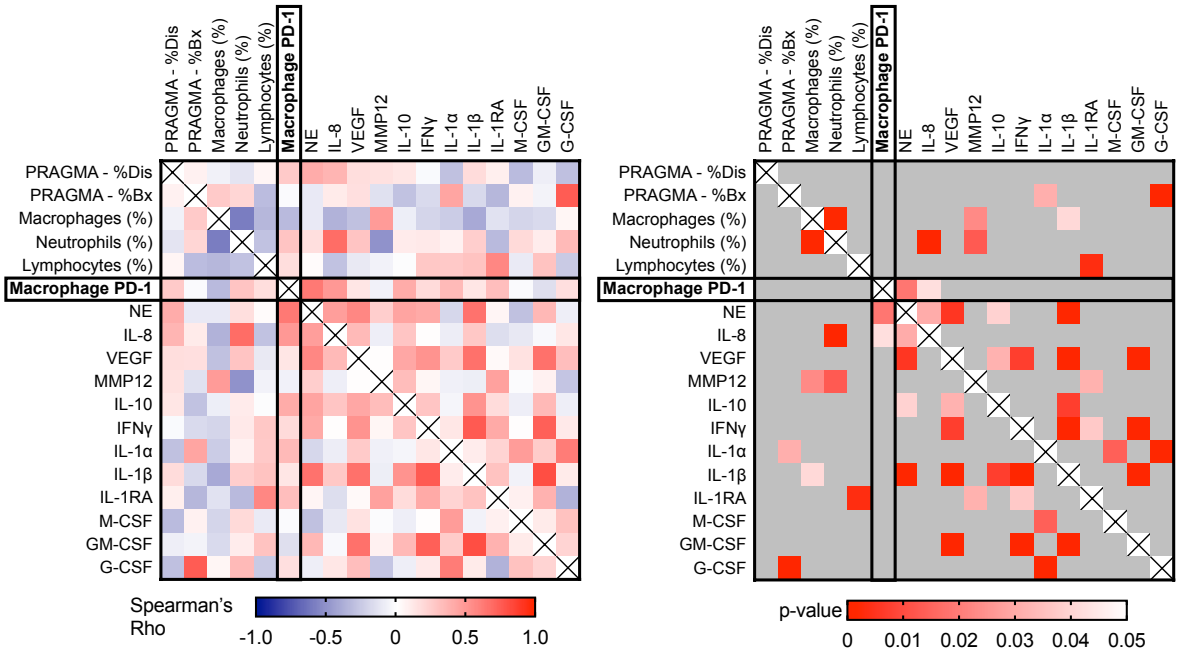


**Figure 2.2.5.8. PD-1 expression on CF airway macrophages is associated with neutrophilic inflammation in non-infected patients.** PD-1 expression on CF airway macrophages correlated significantly with neutrophil-derived mediators of inflammation. Correlations are shown as coefficient strength of Spearman Rho (left) and significance levels of p-values (right, grey p>0.05).

*Airway immune cells express PD-1 ligands*

We sought to determine whether PD-1 on BALF macrophages may be ligated by its conventional ligands, PD-L1 or PD-L2 [74]. Analysis of blood and airway cells showed enhanced expression of PD-L1 on airway macrophages (**Figure 2.2.5.9A**), neutrophils (**Figure 2.2.5.9B**), and T cells (**Figure 2.2.5.9C**), compared to their respective blood counterparts. PD-L2 expression was increased on airway neutrophils compared to blood (**Figure 2.2.5.9B**), but did not differ between airway macrophages (**Figure 2.2.5.9A**) and T cells (**Figure 2.2.5.9C**) and their respective blood counterparts. These data suggest CF BALF leukocytes express PD-1 ligands, making PD-1 ligation on airway macrophages plausible.

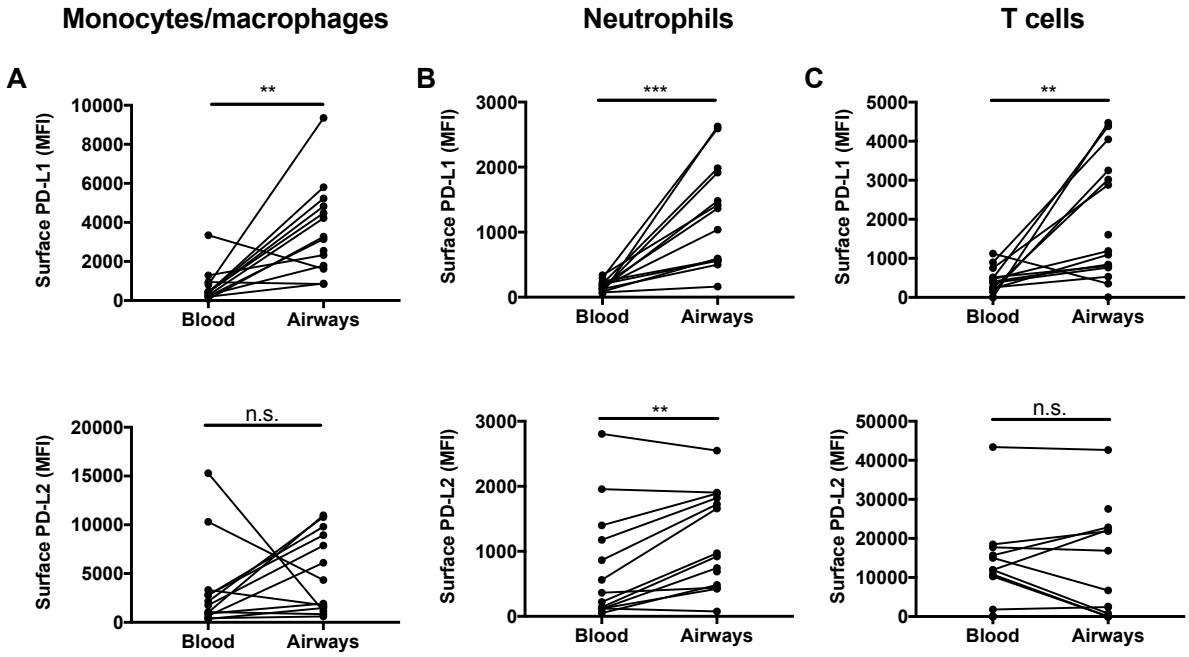


**Figure 2.2.5.9. PD-L1 and PD-L2 expression is modulated on BALF leukocytes from CF children.** Airway macrophages **(A)**, neutrophils **(B)** and T cells **(C)** show increased PD-L1 expression (top panels) compared to their blood counterparts, while PD-L2 expression (bottom panels) was increased on airway neutrophils, but not airway macrophages and T cells, as compared to their blood counterparts. Data are presented as median fluorescence intensity -MFI-. Each patient is represented as a single point and matched samples are connected by a line. Statistical analyses were performed using the Wilcoxon signed rank test, with significance levels indicated as ** for $p<0.01$, **** for $p< 0.0001$, and n.s., not significant.

*PD-1 blockade in CF BALF short-term cultures increases bacterial killing*

Finally, we sought to determine whether PD-1 on BALF leukocytes was active and influencing cell behavior. To this test this eventuality, we designed a short-term culture assay in which fresh BALF leukocytes from CF children were gently isolated and incubated with planktonic forms of the prototypical CF pro-inflammatory pathogens *P. aeruginosa* and *S. aureus*. Bacterial killing was assessed in the presence or absence of PD-1 blockade using a combination of a PD-1 blocking antibody and treatment with SHP099, an inhibitor of the PD-1-associated phosphatase SHP2. Interestingly, bacterial killing improved upon PD1 blockade (**Figure 2.2.5.10A-B**), suggesting that surface PD-1 is active upon in BALF leukocytes from CF children and that blocking its signaling modulates bacterial killing.

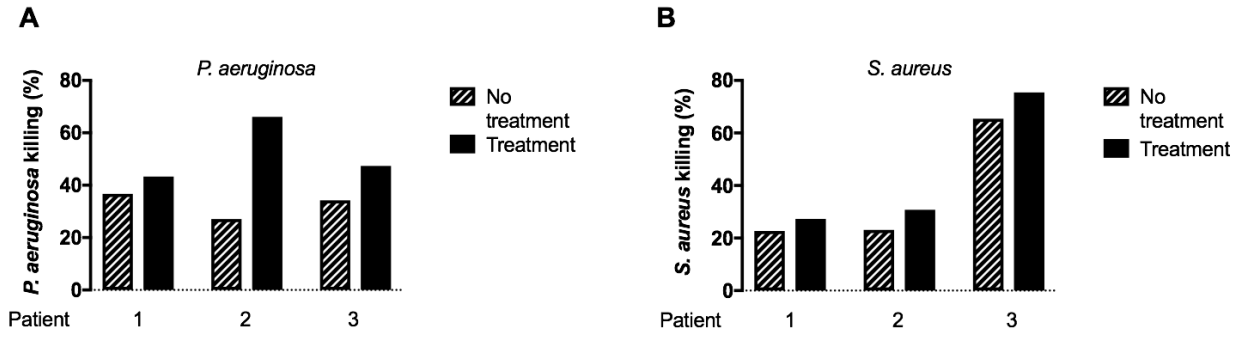


**Figure 2.2.5.10. PD-1 blockade augments bacterial killing in short-term CF BALF leukocyte cultures.** BALF leukocytes from three CF children were exposed to combined treatment with anti-PD1 antibody and a SHP2 inhibitor, while co-incubated with *P. aeruginosa* **(A)** and *S. aureus* **(B)**. Killing was determined by CFU counts. Treatment effectiveness was calculated by subtracting the effect of treatment on bacteria alone (incubation control without BALF leukocytes). Each bar corresponds to a patient, where the dashed bar corresponds to cells and bacteria alone, while the black bars represent killing with treatment.

### 2.2.6 Discussion

Tissue-resident macrophages are key players in integrating homeostatic and stress-signaling pathways and translating these into appropriate immune responses [94]. The exhaustion marker PD-1 is known to play a critical role in controlling macrophage responsiveness in several chronic and acute diseases, suggesting that this pathway is not disease-specific [75, 77, 78]. Findings presented here support the notion that early neutrophilic inflammation in CF airways, characterized by active NE exocytosis and other pathological responses independent of infection, modulate PD-1 expression on airway macrophages, and that this process is amplified upon infection by pro-inflammatory pathogens, ultimately leading to structural damage (**Figure 2.2.6.1**).

Immune responses in CF airways depend on close interactions between the CF epithelium, which shows enhanced pro-inflammatory signalling [93, 95], resident macrophages and recruited neutrophils, mucus, and colonizing pathogens, which evolve over the course of the disease [92, 96]. Macrophages and neutrophils in particular collaborate and modulate each other during responses [97, 98], by dynamically sharing released factors such as NE [99], implementing bacterial clearance [100], and orchestrating gene expression [101]. Here, we demonstrated that apparent macrophage exhaustion in the airways of CF children coincides with intensifying neutrophilic inflammation, reflected by enhanced extracellular levels of NE and IL-8.

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 that its intensity is not influenced by a patient's infection status [80]. This is in apparent contrast with the increase in airway macrophage PD-1 expression identified here, which seems to become further pronounced after infection. This study does not delineate whether enhanced PD-1 expression on airway macrophages is a cause or consequence of the

progressive neutrophil takeover and presence of pro-inflammatory pathogens in that pathological microenvironment. Indeed, a limitation in both this and prior [80] 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.

Using short-term cultures to circumvent the low cell yield of BALF from CF children at this early stage of disease, we provide proof-of-concept evidence for the use of PD-1 blockade to reinvigorate bacterial killing in the airways. 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 metabolic activity [102], including autophagy [103], which controls pathogen clearance by macrophages [104].
Further studies are also needed to determine if PD-1 expression on airway macrophages in CF children itself causes an inability to clear pathogens, or if increased expression of PD-1 on these cells are caused by an increased presence of neutrophils, along with these pathogens, and/or intrinsic effects of CFTR deficiency of macrophages. While it has been previously shown that CFTR mutations can affect in vitro responses of monocyte-derived macrophages [105], less is known about CFTR function in resident airway macrophages.

Macrophages have previously been proposed as potential therapeutic targets for CF airway disease [106]. 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 [107]. 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 dampen progressive neutrophil takeover observed in the early phase of pro-inflammatory pathogen infection and lung damage in CF children.

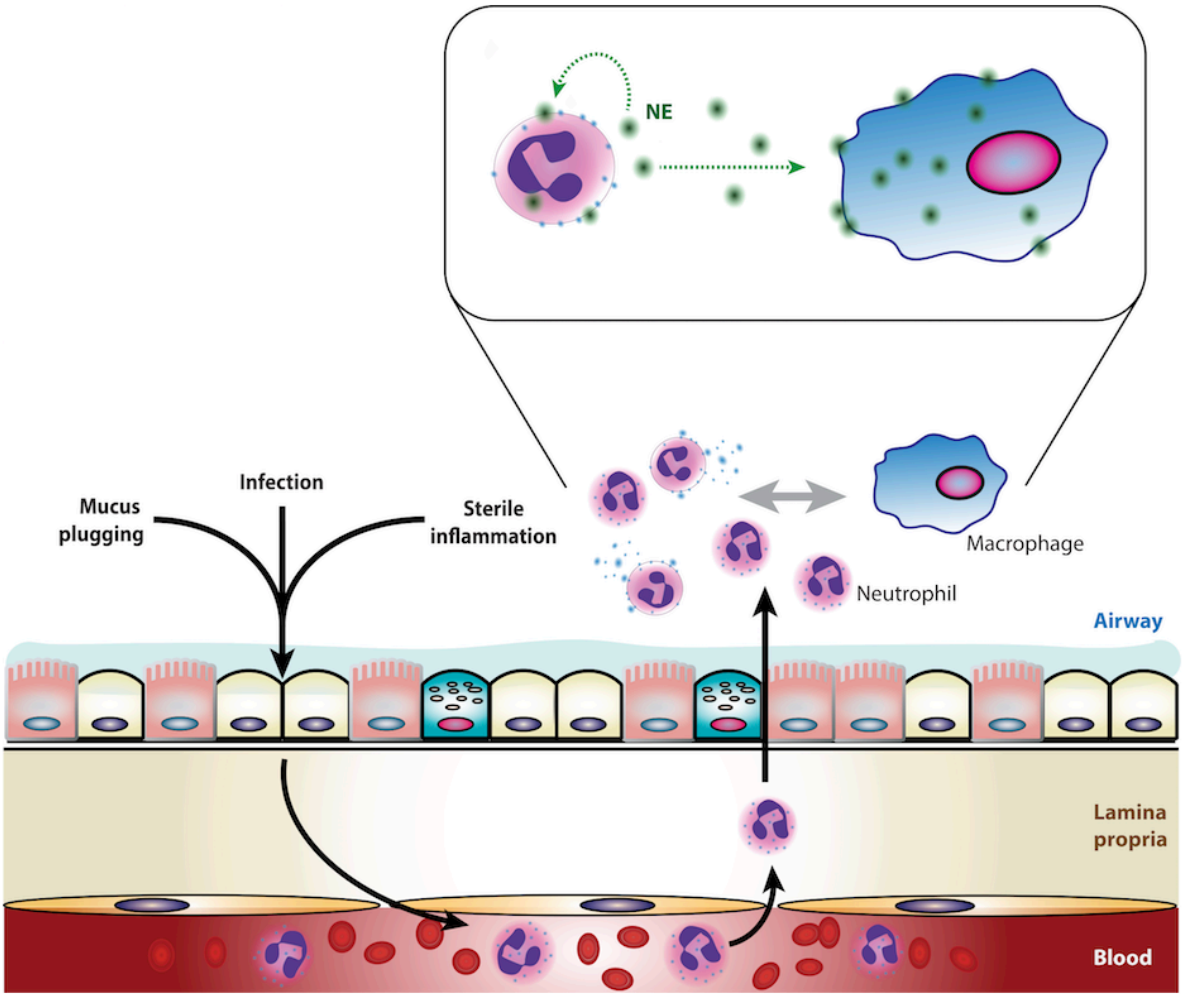


**Figure 2.2.6.1. Model for neutrophil recruitment and interplay with macrophages.** At the earliest stage of CF lung disease, small airway epithelial cells respond to local stressors (mucus plugging, infection, sterile inflammation) by recruiting blood neutrophils, through the lamina propria, and in the lumen. In all CF children irrespective of age and infection, and prior to the onset of bronchiectasis, a significant fraction of airway neutrophils releases NE into the lumen, after which NE is recaptured at the surface of neutrophils and macrophages, with internalization possibly ensuing. Accumulation of NE is strongly associated with the upregulation of the exhaustion marker PD-1 in macrophages.

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# Chapter 3

## Transcriptional reprogramming drives neutrophil plasticity in the cystic fibrosis airways

*Sections of this chapter will be submitted for publication at Science Immunology*

## 3.1 At-a-glance commentary

*Current scientific knowledge on subject*. Neutrophils are thought to be pre-programmed and short-lived with very little opportunity for plasticity. In cystic fibrosis airway disease, they dominate the inflammatory response and they display phenotypic and functional changes. Hallmarks of this newly discovered phenotype, dubbed GRIM, include active release of primary granules, immune modulation and inhibition of the adaptive immune response, impaired killing of bacteria, and metabolic licensing with activation of anabolic pathways. However, the mechanisms supporting neutrophil adaptation to the CF airway microenvironment remain poorly understood.

*What does this study add to the field?* By taking advantage of an *in vitro* transmigration model that allows the pathological conditioning of neutrophils mimicking their *in vivo* airway phenotype, we show that the acquisition of the GRIM phenotype by airway neutrophils in CF is dependent upon *de novo* transcription. These findings challenge the paradigm holding neutrophils as short-lived and transcriptionally silent cells and open avenues for novel immunotherapy targeting live airway neutrophils in CF.

## 3.2 Abstract

*Rationale.* Circulating neutrophils are often considered short-lived, pre-programmed, and terminally differentiated. In cystic fibrosis, a hallmark of airway disease is chronic neutrophil inflammation, characterized by functional and phenotypic changes (GRIM phenotype) which associates with loss of lung function and prolonged bacterial infections. Among the pathways differentially regulated between blood and airway neutrophils, the latter show a metabolic switch towards anabolism, including nucleotide synthesis.

*Objective.* We sought to determine whether neutrophil adaptation to the CF airway microenvironment depends on transcriptional reprogramming.

*Methods.* Blood and airway (GRIM) neutrophils were isolated from patients with CF and analyzed by flow cytometry and their transcriptional profile was determined by microarrays. In addition, blood neutrophils were conditioned in an *in vitro* transmigration model of CF airway inflammation and analyzed by RNA sequencing, untargeted proteomics and flow cytometry. To determine the contribution of *de novo* transcription to the acquisition of the GRIM phenotype, *in vitro* transmigrated neutrophils were treated with the transcriptional inhibitor $\alpha$-amanitin.

*Results.* CF airway (GRIM) neutrophils in vivo and in vitro displayed increased RNA content and a unique transcriptional profile compared to their matched blood counterpart and to transcriptional profiles of bone marrow neutrophil precursors. Changes in gene transcription were mirrored by presence of corresponding proteins. Blockade of transcription restored bacterial killing and prevented release of primary granules by GRIM neutrophils.

*Conclusions.* These findings show that neutrophil plasticity contributes to disease progression in CF and that the pathological conditioning and GRIM phenotype of CF airway neutrophils is dependent upon *de novo* transcription and translation.

## 3.3 Introduction

Neutrophil development after birth occurs in the bone marrow over the course of 10-14 days [1] with cells of the neutrophilic lineage within the bone marrow classified as stem cell, mitotic, and post-mitotic pools [2]. Maturation beyond the myeloblast occurs over five stages of differentiation with progressive chromatin condensation and generation of four granule subsets and secretory vesicles. This step-wise maturation is finely tuned by several transcription factors, such as C/EPB$\alpha$, C/EPB$\varepsilon$, PU.1, CDP, Gfi-1, and the retinoic acid receptor [1, 3-6], culminating in the formation of terminally differentiated mature neutrophils that are released into the blood stream.

Upon presence of inflammatory stimuli, neutrophil interaction with soluble factors, such as cytokines or pathogen-related molecules, as well as migration into tissues, induce neutrophil priming, a pre-activated state that features several phenotypic and functional changes [7,8]. These changes are mediated by defined intracellular signaling pathways and physical adaptation dictated by the passage through the tissue, both of which may lead to modification of the chromatin state in neutrophils, and transcription of new genes [9, 10].

In cystic fibrosis (CF), airway neutrophils display a unique phenotypical adaptation to the airway microenvironment, dubbed the "GRIM" phenotype, which includes active of primary granule release [11-14], immunomodulatory activities toward other immune subsets [15] and metabolic licensing [16, 17], including increased glycolytic capacity and activation of the mammalian target of rapamycin (mTOR) anabolic

pathway, a master regulator of cellular metabolism and nucleotide synthesis [18-20]. Therefore, we investigated whether neutrophil adaptation to the CF airway microenvironment, outlined by the acquisition of the GRIM phenotype, was dependent upon *de novo* transcription. To address neutrophil transcriptional reprogramming in CF, we analyzed freshly isolated blood and airway neutrophils from CF patients and exploited an *in vitro* transmigration model that recapitulates the pathological conditioning of CF airway neutrophils toward the GRIM phenotype [21].

## 3.4 Methods

*Human subjects and sample collection*

Blood was obtained from healthy donors and age-matched patients with CF by venipuncture in $K_2$-EDTA tubes. Airway neutrophils and sputum supernatant were obtained as previously published [12]. Briefly, expectorated sputum from CF patients was solubilized with 2.5 mM final ice-cold PBS-EDTA and gently passaged through an 18.5 G needle. Airway neutrophils were recovered after an 800 g, 10 minutes, 4°C centrifugation, while the CFASN clear supernatant was collected following an additional 3,000 g, 10 minutes, 4°C centrifugation.

*In vitro transmigration*

Blood neutrophils were isolated using the density gradient Polymorphprep following manufacturer protocol [22]. Neutrophil purity and viability was assessed using flow cytometry. Purified neutrophils were loaded in the *in vitro* transmigration model [21] as follows: 1) transmigration towards the neutrophil chemoattractant LTB4 (100 nM, Sigma) (transmigrated but not reprogrammed neutrophils); and 2) transmigration using the CF airway supernatant (CFASN) generated from sputum as apical fluid, (transmigrated and reprogrammed GRIM neutrophils). Neutrophils were then

collected at 1, 2, 4, 6 and 10hr post-transmigration and used for downstream assays. Blockade of transcription was performed using neutrophils transmigrated for 2 hours, washed cells were incubated in RPMI for 8 hours with or without the transcriptional blocker $\alpha$-amanitin (Sigma) at 1 $\mu$g/mL per 1 million cells. Cells recovered after incubation were assessed for their bacterial killing capacity and their phenotype determined by flow cytometry.

*Neutrophil elastase activity*

Free extracellular NE activity was measured using the FRET-based NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described in method section 2.1.4. Samples were measured in duplicate with repeated measures for values outside the working range.

*Extracellular vesicles*

Extracellular vesicles (EVs) were pulled down from neutrophil supernatant following transcriptional blockade and from sputum supernatant from patients with CF. Briefly, streptavidin-coated beads (SBI) were incubated for two hours on an end-over-end rotating wheel with biotinylated anti-CD66b antibody as per manufacturer protocol. Antibody-coated beads were incubated overnight with cell supernatants on an end-over-end rotating wheel at 4°C. Bead-bound EVs were washed with 2.5 mM final PBS-EDTA and stained for the EV marker ExoFITC (SBI) and surface neutrophil elastase (Novus Biologicals).

*Flow cytometry*

Blood, purified and transmigrated neutrophils were stained following the protocol presented in the methods section 2.1.4 with the following cellular markers and dyes:

CD63, CD66b, CD15, CD16, Live/dead zombie dye (Biolegend), total RNA content was quantified by SytoRNA (Thermofisher). Cells were acquired on a LSRII cytometer (BD Biosciences) and results were analyzed by FlowJo v.9.9.5 (TreeStar). Acquisition of EVs was performed on the Cytoflex S (Beckman) and analysis was conducted using FlowJo v.10.4.2 (TreeStar).

*Microarrays*

Blood and sputum neutrophils were sorted from patients with CF (N=7) as previously described [17]. Total RNA was extracted from sorted fractions, mRNA was amplified and cDNA generated (WT-Ovation Pico RNA Amplification System, NuGen). Next, cDNA from sorted neutrophils from sputum and blood sample pairs were then labelled with Cy3 and Cy5, respectively, to generate two-color cDNA GE microarray data by competitive hybridization (GE2-v5, Agilent), per manufaturer's instructions. Data from RNA profiling were normalized, transformed and analyzed using GO terms.

*RNA sequencing*

RNA was isolated from purified (blood >98% purity) and transmigrated (LTB4 or CFASN, >98% purity) neutrophils using the Nucleospin RNA isolation kit (Clontech, Takara Bio) following manufacturer protocol. RNA quality and concentration were quantified using a Bioanalyzer (Agilent technologies, Inc.) and only samples with RIN>8.0 were used for sequencing. Libraries were prepared using the TruSeq RNA Single Indexes Set B kit (Illumina) and AmPure beads (Beckman) and sequenced as paired end (100bp) by high seq rapid run (Illumina) with an aim of ~20 million reads per sample. Analysis of transcriptomics data was performed using hisat2, DeSeq2, PCA and GSEA.

*Fluidigm*

48 target genes, including NE, MPO, MMP9 and arginase-1, were assessed using a Fluidigm array, and multiplexed qPCR was performed on mRNA from blood and CF sputum neutrophils as previously described [15]. mRNA levels are shown as threshold cycles (Ct), with 30 cycles being the lower detection limit.

*Proteomics*

Proteins were extracted from purified (blood >98% purity) and transmigrated (LTB4 or CFASN, >98% purity) neutrophils after cell lysis, followed by reduction and alkylation, and digestion with trypsin. The samples were separated into ten fractions using high-pH reverse phase high-performance liquid chromatography (HPLC, pH=10). These fractions were analyzed by liquid chromatography / tandem mass spectrometry (LC/MS/MS, LTQ Orbitrap Elite). The spectra were searched against database with 10 ppm precursor mass tolerance and 0.5 Da product ion mass tolerance using the SEQUEST algorithm. Protein clustering used DAVID bioinformatics.

*Bacterial killing*

Overnight cultures of pro-inflammatory bacteria *P. aeruginosa* (strain PAO1) were sub-aliquoted and grown to reach the exponential growth phase. Bacteria were then incubated in RPMI, supplemented with 10% FBS, on an end-over-end rotating wheel for 30 minutes at 37°C. Transmigrated neutrophils ($10^5$ cells) were resuspended in RPMI, 10% FBS and incubated at 37°C, 5% $CO_2$ for 15 minutes. Co-incubation of bacteria and leukocytes was performed at a multiplicity of infection of 0.1, in RPMI, 10% FBS, on an end-over-end rotating wheel for 30 minutes at 37 °C, 5% $CO_2$. Bacterial killing capacity was calculated using colony forming units (CFU), with the bacteria plus RPMI and 10% FBS condition set as 100% survival.

*Statistical analysis*

Data transferred to JMP13 (SAS Institute) and Prism v7 (GraphPad) for statistical analysis and graphing, respectively. Transcriptomic analysis for differential gene expression was assessed using DESeq2 in R v3.5.2. The dataset used in the comparison between the in vitro transmigrated neutrophils and the developmental stages in the bone marrow was COMBAT corrected before calculating the PCA.

## 3.5 Results

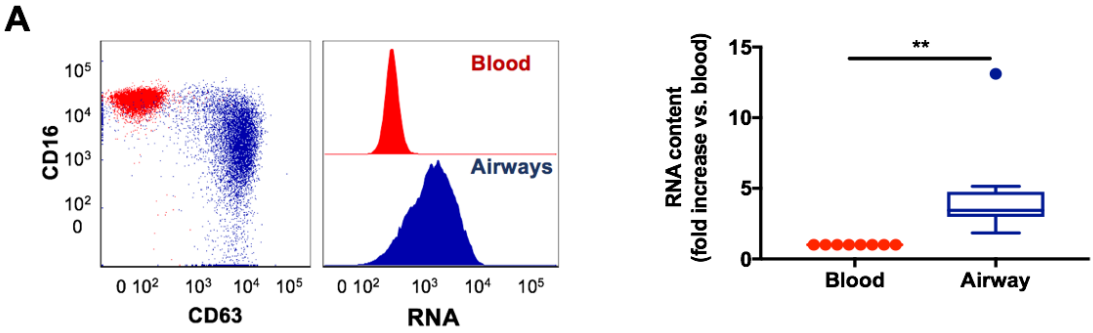
*CF airway GRIM neutrophils display profound transcriptional changes in vivo*

To determine whether acquisition of the GRIM phenotype was related to changes in the transcriptional profile, blood and sputum neutrophils from eight adult patients with CF were analyzed by flow cytometry. Airway neutrophils displayed characteristic increased release of primary granules, measured by surface CD63, and loss of the phagocytic receptor CD16. In addition to these previously described changes, airway neutrophils showed a median 3.5-fold increase in total RNA content compared to their matched blood counterpart **(Figure 3.5.1A)**.

Interestingly, the transcriptional profile of airway neutrophils mirrored their functional adaptation to the CF airway microenvironment **(Figure 3.5.1B)**. Genes coding for anabolic pathways and production of new proteins were upregulated compared to their matched blood counterpart, while immune canonical signatures and cell death pathways were downregulated. Together these findings suggest that *de novo* transcription and translation are integrating parts of the pathological conditioning of neutrophils by the CF airway microenvironment leading to the acquisition of the GRIM phenotype.

*In vitro-produced CF airway GRIM neutrophils recapitulate transcriptional changes observed in vivo*

To study the contribution of *de novo* transcription to the acquisition of the GRIM phenotype, we used a transmigration *in vitro* model that recapitulates the *in vivo* airway neutrophil phenotype in CF [21]. Neutrophils isolated from blood were transmigrated for 10 hours towards CFASN or LTB4 (transmigration control) **(Figure 3.5.2A)**. Similarly to what we observed in the airway neutrophils *in vivo*, neutrophils transmigrated *in vitro* to CFASN showed surface loss of CD16, and increases in surface CD63 and total RNA content (the latter by over 10-fold). Meanwhile, neutrophils transmigrated to LTB4 did not differ from blood neutrophils except for a 2-fold increase in total RNA content **(Figure 3.5.2B and C)**. Moreover, each condition showed a unique transcriptional profile **(Figure 3.5.2D)**, suggesting a two-hit model where neutrophils are primed by the passage through the epithelium and then further conditioned by the CF airway milieu to become GRIM.




**B**

| UPREGULATED | | | DOWNREGULATED | | |
|---|---|---|---|---|---|
| GO# | | p-value | GO# | | p-value |
| 0006415 | Translational termination | 6.6 E-98 | 0002376 | Immune system process | 5.8 E-20 |
| 0006414 | Translational elongation | 1.5 E-95 | 0009611 | Response to wounding | 1.0 E-15 |
| 0003735 | Structural constituent of ribosome | 6.8 E-84 | 0043067 | Positive regulation of PCD | 1.0 E-9 |
| 0071822 | Protein complex organization | 2.3 E-40 | 0042981 | Positive regulation of apoptosis | 3.9 E-7 |
| 0071843 | Cellular component biogenesis | 1.0 E-11 | 0004721 | Protein phosphatase activity | 2.4 E-3 |

**Figure 3.5.1. CF airway GRIM neutrophils display transcriptomic changes *in vivo*. (A)** Blood (red) and sputum (blue) neutrophils from patients (N=8) with cystic fibrosis (CF) were analysed by flow cytometry. Airway neutrophils displayed the characteristic loss of the phagocytic receptor CD16 and release of primary granules, measured by surface CD63. Increased total RNA content compared to their matched counterpart was measured by median fluorescence intensity. **(B)** RNA analysis of sorted cells from blood and sputum of patients with CF (N=7) was performed by microarray competitive binding with matched blood and airway RNA. Results are shown as median and interquartile range, statistical analysis was performed by Wilcoxon matched-pairs signed rank test, **p<0.01.

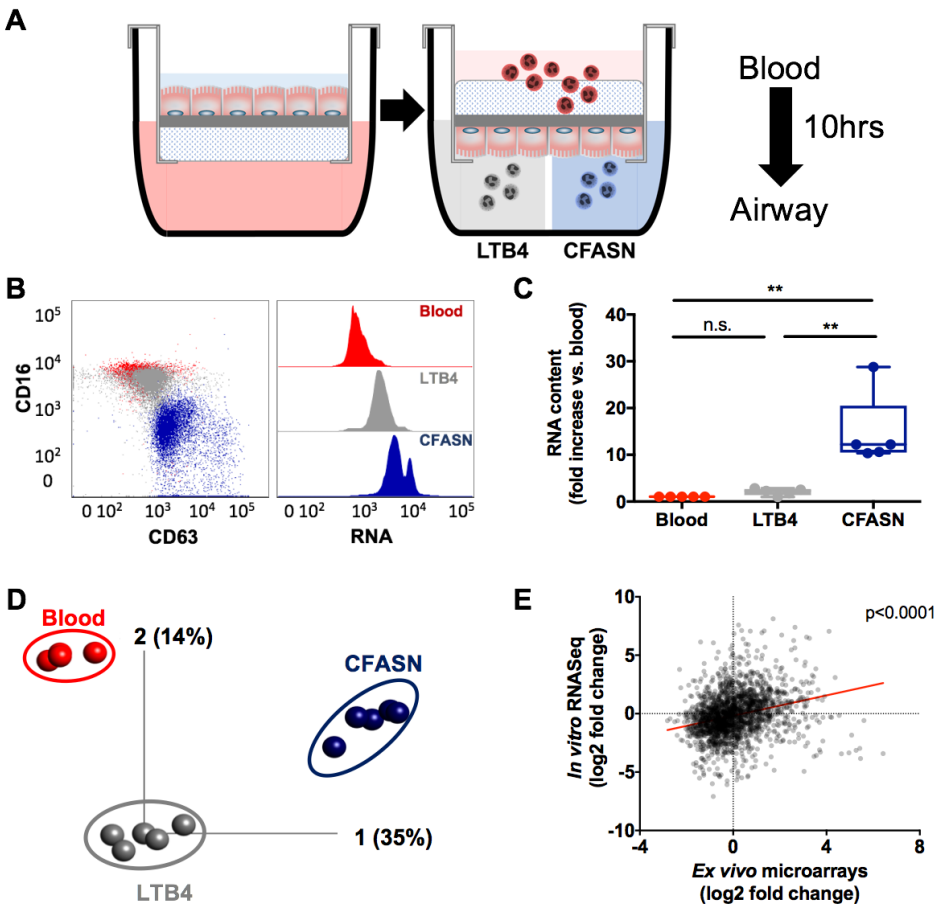



**Figure 3.5.2. *In vitro*-produced CF airway GRIM neutrophils mimic changes observed *in vivo*. (A)** Schematics of the *in vitro* transmigration model. **(B)** Blood neutrophils (red, N=5) were transmigrated *in vitro* using airway supernatant from the sputum of patients with CF (CF ASN, blue) and the chemoattractant leukotriene B4 (LTB4) as a transmigration control (grey). Flow cytometry analysis revealed an increase in RNA content in CF ASN airway neutrophils compared to matched blood neutrophils, which was confirmed by quantification by bioanalyzer **(C)**. **(D)** PCA analysis revealed a distinct gene expression pattern in the three conditions. **(E)** Correlation of gene expression obtained by RNASeq (*in vitro*) and microarrays (*ex vivo*) between CF ASN/airway neutrophils to matched blood neutrophils was assessed by Spearman's Rho. Results are shown as median and interquartile range, statistical analysis was performed by Wilcoxon matched-pairs signed rank test, **p<0.01.

Lastly, to validate the relevance of the RNA profile obtained by RNA sequencing on the *in vitro* samples, we compared the fold-change between airways and blood with the microarray data obtained from the *in vivo* samples **(Figure 3.5.2E)**. Despite the difference between the two platforms, we found a significant correlation between the two conditions, suggesting that changes observed *in vitro* are conserved in the *in vivo* neutrophils.

*The CF airway microenvironment imprints a unique transcriptional and proteomic profile upon recruited neutrophils*

To discern between priming and CF ASN-induced transcriptional changes, we compared genes in the LTB4 and CF ASN neutrophils that were up- or downregulated at least by a log2 higher than 2 or lower than -2 compared to blood. Of that gene pool, 1,407 genes were upregulated in LTB4 and CF ASN, while 1,221 were downregulated in both conditions. However, some transcripts showed unique changes with CF ASN having 3,602 uniquely upregulated genes and 3,148 uniquely downregulated genes **(Figure 3.5.3A)**. Among those, only 3,417 were significantly upregulated and 1,731 significantly dowregulated compared to the LTB4 condition **(Figure 3.5.3B)**. Moreover, metabolic and immunological changes previously described at the protein and functional levels, were mirrored in the transcriptional signature of neutrophils transmigrated to CF ASN, but not those transmigrated to LTB4. Among those, genes of the glycolytic and mTOR pathways and genes previously identified in blood myeloid-derived suppressor cells in cancer patients [23] were highly enriched in the former **(Figure 3.5.3C)**.

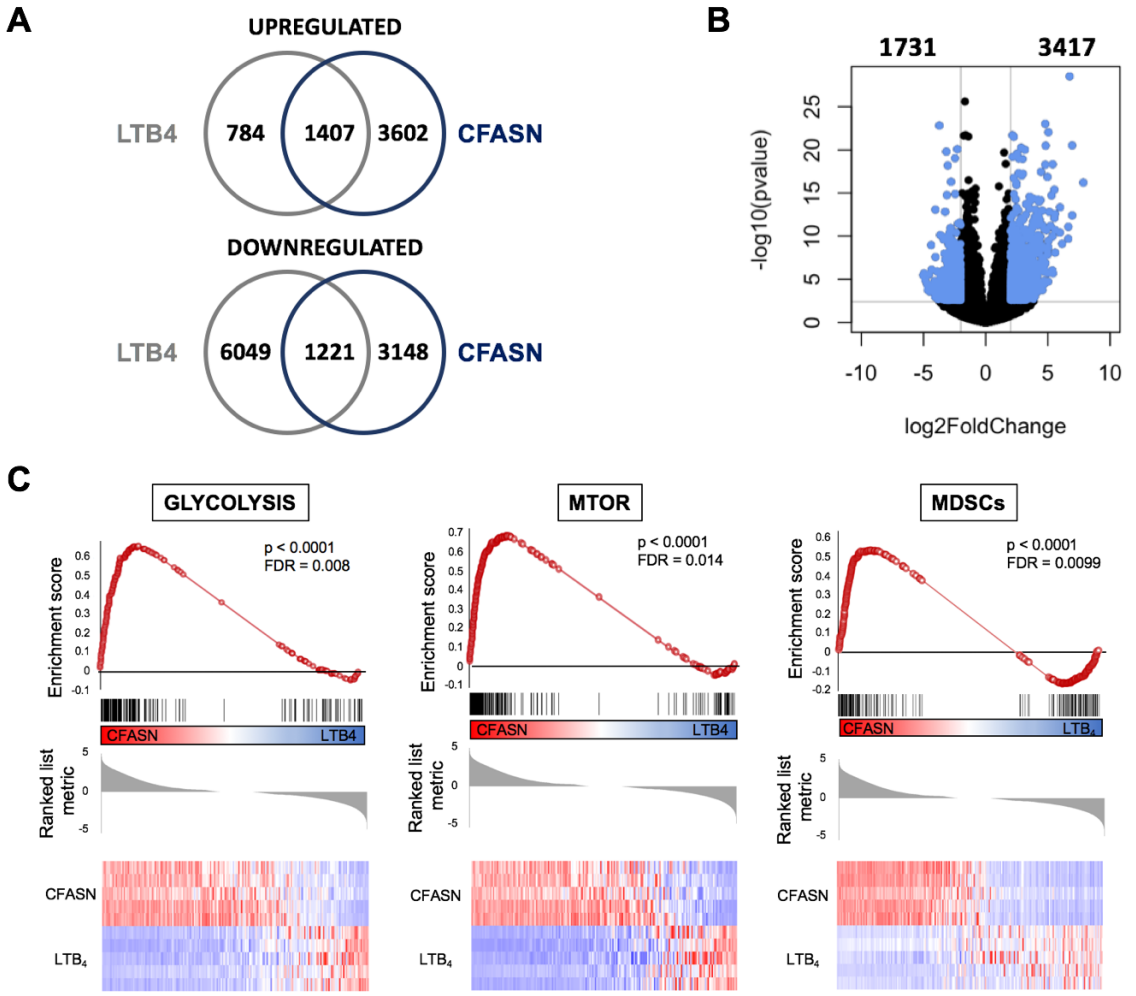



**Figure 3.5.3. The CF airway microenvironment induces unique transcriptional signatures in recruited neutrophils.**

**(A)** Differential gene expression of upregulated and downregulated genes was determined comparing transmigrated conditions to blood with log2 gene expression greater than 2 and smaller than -2, respectively. **(B)** Comparison between CF ASN and LTB4 transmigration conditions showed 3,417 genes significantly upregulated in the CF ASN and 1,731 genes significantly upregulated in the LTB4 condition. Significant genes are shown in light blue and defined as log2 greater or lower than 2 or -2, respectively, and a p-value less than 0.01. **(C)** GSEA analysis showed enriched mTOR and glycolytic metabolic pathways in the CF ASN compared to LTB4, as well as changes in the immunological profile; MDSCs: myeloid-derived suppressor cells.

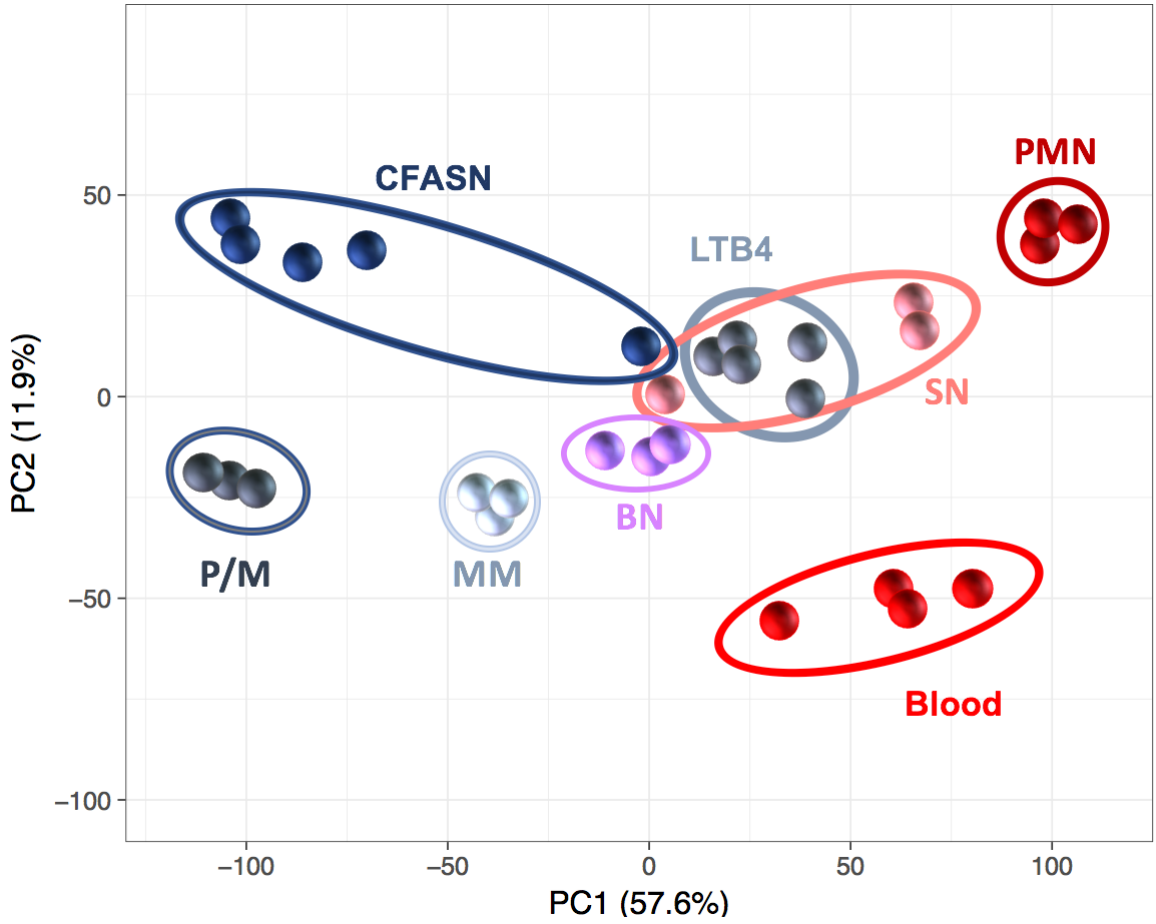



**Figure 3.5.4. The transcriptional profile of CF airway GRIM neutrophils does not match that of developmental subsets of human BM neutrophils.**

The transcriptome of CF airway neutrophils generated in the *in vitro* transmigration model was compared to the profiles of human BM neutrophil subsets published by Grassi et al [24]. The transcriptome profile of CF airway neutrophils did not mirror any of the gene signatures representative of the different developmental stages. P/M: promyelocyte/myelocyte; MM: metamyelocyte; BN: band neutrophil; SN: segmented neutrophil; PMN: mature polymorphonuclear neutrophil; Blood: blood neutrophils from *in vitro* transmigration; LTB4: *in vitro* transmigrated neutrophils towards LTB4; CF ASN: CF airway GRIM neutrophils.

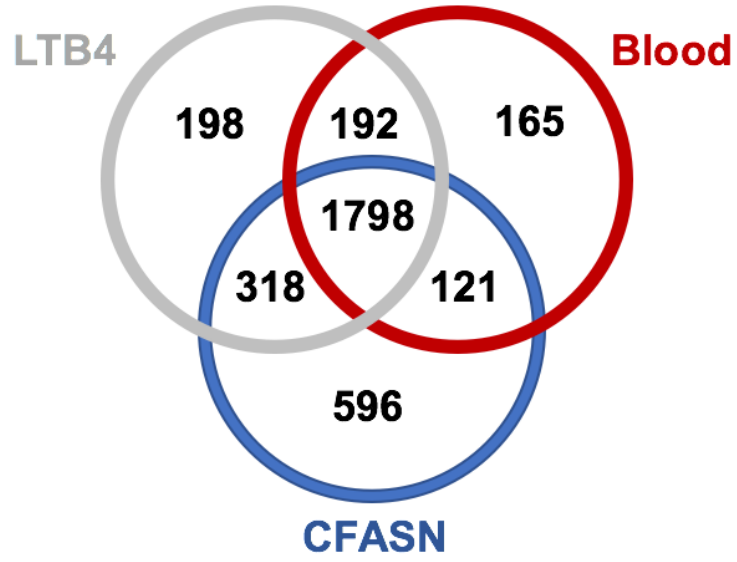
Next, we determined whether the unique transcriptional program observed in CF airway GRIM neutrophils resembled any of the neutrophil developmental stages in the bone marrow. To this end, we used principal component analysis (PCA) to compare the transcriptomic profiles of our blood neutrophils, and neutrophils transmigrated to LTB4 and CF ASN (GRIM) to those defined by Grassi et al. [24] for every developmental stage of the neutrophil lineage in the human BM. Considering PC1, the profile of neutrophils transmigrated to CF ASN resembled those of promyelocyte/myelocyte, metamyelocytes and band neutrophils. However, neutrophils transmigrated to CF ASN showed a unique modulation profile on PC2 **(Figure 3.5.4)**. Meanwhile, the profile of neutrophils transmigrated to LTB4 was similar to that of segmented neutrophils for both PC1 and PC2. Of note, profiles of blood neutrophils from our dataset and of mature PMNs from Grassi et al. [24] did not match even after data correction with the COMBAT algorithm was applied to compensate for study differences, suggesting inter-donor variability related to age, gender or other variables that could not be assessed based on currently published data.

Lastly, we sought to determine whether the transcriptional profile matched the proteomic landscape. Untargeted proteomics of blood neutrophils, and neutrophils transmigrated to LTB4 and CF ASN showed a core of 1,798 common proteins, some of which were shared only between two conditions, and a pool of proteins unique to each condition **(Figure 3.5.5A)**. Pathway enrichment analysis of the unique proteins revealed overlap with respective transcriptional signatures, with blood neutrophils enriched for canonical immunological pathways and cell death proteins, and neutrophils transmigrated to CF ASN matching the observed transcriptional activation of immunomodulatory and anabolic pathways **(Figure 3.5.5B)**.

Together, these findings support the hypothesis that neutrophil conditioning after recruitment into the CF airway environment and subsequent acquisition of the

GRIM phenotype are dependent upon *de novo* transcription and translation. Furthermore, they highlight the unique pathological properties of the CF airway supernatant, a necessary component for the acquisition of the GRIM phenotype.

**A**




**B**

| Blood | LTB4 | CFASN |
|---|---|---|
| Apoptosis | IL-1 signaling | TORC1 signaling |
| Complement | Endocytosis | TLR3 signaling |
| FcγR mediated phagocytosis | Steroid biosynthesis | MHC I mediated antigen processing |
| | Integrin signaling | Antigen processing: ubiquitination and proteasome degradation |

**Figure 3.5.5. Proteomic profiling of *in vitro* transmigrated neutrophils.**

Blood and neutrophils transmigrated *in vitro* for 10 hours (N=3) were lysed and the extracted proteins were analyzed by LC-MS untargeted proteomics. **(A)** Proteins present in at least two repeats were used to generate the Venn diagram, while unique proteins for each condition were used to determine pathway enrichment by GSEA **(B)**. Pathways listed showed an FDR less than 5% and a p-value less than 0.01.

*CF airway GRIM neutrophils lack de novo expression of effector granule proteins in vivo and in vitro*

Active release of primary granules is a hallmark of CF airway GRIM neutrophils. Presence of extracellular neutrophil elastase (NE) and myeloperoxidase (MPO) are among the best correlates of lung function and structural damage in children and adult patients with CF [14, 25-27]. In addition to extracellular NE, we observed an increase in cell-associated NE in CF airway GRIM neutrophils compared to their matched blood counterpart *in vivo* [14] **(Figure 2.1.5.5)**. To further investigate this question, we investigated whether GRIM neutrophils were producing NE and other effector proteins through *de novo* transcription by cross-referencing our results with those obtained in a comprehensive proteomic study of human neutrophil granules [28]. Most of the upregulated transcripts in neutrophils transmigrated to CF ASN compared to blood belonged to proteins related to the structure, transport and docking of granules to their target compartment **(Table 3.5.1)**, while most of the effector proteins contained in the granules themselves were downregulated **(Figure 3.5.6A)**. Next, we investigated whether the transcripts of certain downregulated effector proteins were present in neutrophils transmigrated to CF ASN and LTB4 *in vitro* **(Figure 3.5.6B)**. Apart from matrix metalloprotease 9 (MMP9), the levels of NE, MPO and arginase-I were below the detection limit and several samples had undetectable transcripts. To confirm the relevance of these results, we quantified the levels of the same transcripts by fluidigm in blood and airway neutrophils sorted from patients with CF. Similarly to the *in vitro* results, the levels of NE, MPO, arginase-I and MMP9 were below the limit of detection in CF airway GRIM neutrophils *in vivo* **(Figure 3.5.6C)**.

These findings show that accumulation of primary granule proteins in the airway fluid of CF patients, and within airway neutrophils themselves is likely not

caused by *de novo* transcription, but rather by uptake of proteins discharged by waves of GRIM neutrophils and accumulated in the airway lumen over time. This prompted us to investigate whether there were specific clusters of genes that were not transcribed in CF airway GRIM neutrophils **(Figure 3.5.7)**. Interestingly, we found that defined areas of the chromatin did not show any transcriptional activity, suggesting that the transcriptional burst seen in neutrophils transmigrated to CF ASN is regionally controlled, rather than dependent upon a global switch from hetero to euchromatin.

| Upregulated | Downregulated |
|---|---|
| **Secretory vesicles** | |
| Coiled-coil domain-containing protein 68 | Vimentin |
| Galectin-3 | ABri/ADan amyloid peptide |
| Hypoxia up-regulated protein 1 | GEM-interacting protein |
| NADH dehydrogenase iron-sulfur protein 5 | Leu-rich repeat ser/thr-protein kinase 2 |
| Beta-actin-like protein 2 | Engulfment and cell motility protein 1 |
| AIR carboxylase | Monocyte differentiation antigen CD14 |
| Granzyme A-activated DNase | Eosinophil peroxidase |
| Tubulin gamma-1 chain | Cystatin-A |
| Alpha-enolase | Protein S100-A9 |
| 14-3-3 protein epsilon | Allograft inflammatory factor 1 |
| Hydrophobic protein HSF-28 | Sortilin-related receptor |
| Prohibitin | Matrix metalloproteinase-25 |
| NADH dehydrogenase 1$\alpha$ subcomplex sub 8 | IgG-Fc fragment rec transporter $\alpha$-chain |
| Cytosolic thyroid hormone-binding protein | Fc-gamma RIII-beta |
| Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A | Ser/thr-protein phosphatase 2A 55 kDa regulatory subunit B $\beta$ |
| Cytochrome c | RAS guanyl-releasing protein 2 |

| | |
|---|---|
| 49 kDa TATA box-binding protein-interacting protein | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase $\beta$-2 |
| Immediate early response 3-interacting protein 1 | Toll-like receptor 8 |
| Probable saccharopine dehydrogenase | Protein S100-A8 |
| 48 kDa TATA box-binding protein-interacting protein | Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1 |
| Small ubiquitin-related modifier 3 | Sialic acid-binding Ig-like lectin 9 |
| Eukaryotic translation elongation factor 1 $\varepsilon$-1 | Tubulin alpha-1A chain |
| Phenylalanyl-tRNA synthetase $\alpha$ chain | |
| Aflatoxin B1 aldehyde reductase member 2 | |
| Stomatin-like protein 2 | |
| Transmembrane protein 14C | |
| 14-3-3 protein $\gamma$ | |
| Aspartyl-tRNA synthetase, cytoplasmic | |
| 13 kDa differentiation-associated protein | |
| Small nuclear ribonucleoprotein F | |
| Eukaryotic translation initiation factor 3 sub E | |
| Uracil-DNA glycosylase | |
| Nucleolin | |
| SPRY domain-containing protein 4 | |
| Coatomer subunit $\gamma$-2 | |
| Aldehyde dehydrogenase 10 | |
| Mesenchymal stem cell protein DSCD75 | |
| UMP-CMP kinase 1 isoform a | |
| Cytochrome c oxidase polypeptide VIc | |
| DNA primase 58 kDa subunit | |

ATP synthase protein 8

| | |
|---|---|
| Adenosylhomocysteinase | |
| UPF0451 protein C17orf61 | |
| L-lactate dehydrogenase B chain | |
| annexin IV | |
| Coiled-coil domain-containing protein 56 | |
| Phospholipid scramblase 1 | |
| Cytosolic malate dehydrogenase | |
| Guanine nucleotide-binding prot sub $\beta$-2-like 1 | |
| CDGSH iron sulfur domain-containing protein 1 | |
| Placental ribonuclease inhibitor | |
| Small nuclear ribonucleoprotein E | |
| Reactive oxygen species modulator 1 | |
| Actin, cytoplasmic 2 | |
| Protein DJ-1 | |
| Nef-binding protein 1 | |
| NADH-ubiquinone oxidoreductase chain 3 | |
| NADH dehydrogenase 1$\alpha$ subcomplex sub 5 | |
| Eukaryotic translation initiation factor 3 sub M | |
| 17-beta-hydroxysteroid dehydrogenase 4 | |
| NADH dehydrogenase 1 $\alpha$ subcomplex sub 4 | |
| Eukaryotic translation initiation factor 3 sub A | |
| Clathrin interactor 1 | |
| Signal recognition particle 9 kDa protein | |
| **Ficolin-rich granules** | |
| Desmoplakin | CD85 antigen-like family member D |

| | |
|---|---|
| Suprabasin isoform 1 precursor | Apoptotic protease-activating factor 1 |
| Peroxiredoxin-4 | Semaphorin-4A |
| Phosphoribosylaminoimidazole-succinocarboxamide synthase | Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat sub B |
| 26S proteasome non-ATPase regulatory sub 14 | Neutrophil cytosol factor 1B |
| Ectonucl. pyrophosphatase/phosphodiesterase 4 | Cathepsin S |
| Inosine phosphorylase | Vascular non-inflammatory molecule 2 |
| Phenylalanine--tRNA ligase $\beta$ chain | Integrin $\beta$-2 |
| Heat shock 84 kDa | Hemoglobin subunit $\alpha$ |
| Proteasome subunit beta type-5 | Neutrophil cytosol factor 4 |
| L antigen family member 3 | Arachidonate 5-lipoxygenase |
| Cytosolic thyroid hormone-binding protein | Heat shock 70 kDa protein 6 |
| 26S protease regulatory sub 7 | Protein-arginine deiminase type-4 |
| T-complex protein 1 sub $\delta$ | Catalase |
| T-complex protein 1 sub $\gamma$ | Bridging integrator 2 |
| RAN, member RAS oncogene family, isof CRA_c | Carabin |
| T-complex protein 1 subunit $\zeta$ | Ficolin-1 |
| Glutathione S-transferase P | Hexokinase-3 |
| Integrin $\beta$ | C3b/C4b receptor |
| Cullin-associated, neddylation-dissociated prot 1 | Fibrinogen-like protein 2 |
| Alkyldihydroxyacetonephosphate synthase | LAMA-like protein 1 |
| Adenine phosphoribosyltransferase | |
| Inosine-5'-monophosphate dehydrogenase 2 | |
| Small nuclear ribonucleoprotein Sm D1 | |
| Cystatin-B | |
| Cyclophilin A | |

| | |
|---|---|
| Nucleoside diphosphate kinase | |
| Translin | |
| Proteasome sub $\beta$ type-2 | |
| Phosphodeoxyriboaldolase | |
| T-complex protein 1 sub $\beta$ | |
| Fructose-bisphosphate aldolase | |
| 26S proteasome non-ATPase regulatory sub 11 | |
| Euk translation initiation factor 3, sub E i. p. | |
| T-complex protein 1 sub $\varepsilon$ | |
| Cathepsin H | |
| Elongation factor 2 | |
| Phosphatidylethanolamine-binding protein 1 | |
| 26S protease regulatory sub 6A | |
| ADP-ribosylation factor-like protein 8B | |
| 26S proteasome non-ATPase regulatory sub 2 | |
| 26S proteasome non-ATPase regulatory sub 3 | |
| ADP-ribosylation factor 4 | |
| CCAAT-binding transcription factor I sub A | |
| Transmembrane protein 50B | |
| Heat shock 70 kDa protein 8 | |
| ATP-dependent DNA helicase 2 sub 1 | |
| Cyclophilin E | |
| ATPase family AAA domain-containing prot 3B | |
| Dynein light chain 1, cytoplasmic | |
| Cathepsin D | |
| Adapter-related protein complex 2 $\beta$sub | |

| | |
|---|---|
| proteasome 26S non-ATPase sub 8 | |
| Proteasome sub $\alpha$ type-6 | |
| Proteasome sub $\beta$ type-6 | |
| Calcium-activated neutral proteinase 1 | |
| Glucose-6-phosphate isomerase | |
| Macropain $\zeta$ chain | |
| **Tertiary granules** | |
| ATPase H(+)-transporting lysosomal prot 2 | Neurobeachin-like protein 2 |
| Cytoplasmic FMR1-interacting protein 1 | Glucose transporter type 3, brain |
| TRPM2 protein | Hemoglobin subunit beta |
| Cytosolic NADP-isocitrate dehydrogenase | Beta-thromboglobulin |
| **Secondary granules** | |
| Urokinase-type plasminogen activator | Chitinase-1 |
| Catenin gamma | Kaliocin-1 |
| C-type lectin domain family 5 member A | Lactoferrin |
| Neutrophil gelatinase-associated lipocalin | Truncated lactoferrin |
| C3a anaphylatoxin chemotactic receptor | Fructose transporter |
| Sulfhydryl oxidase 1 | ATPase class I type 8B member 4 |
| G-protein coupled receptor 84 | ANO6 protein |
| Malectin | ADP-ribosyl cyclase 2 |
| Fatty acid transport protein 2 | C-type lectin domain family 4 member D |
| Membrane-associated progesterone receptor component 1 | Cathelicidin antimicrobial peptide precursor |
| Protein tyrosine phosphatase, receptor type, B isoform a | Protein tyrosine phosphatase, non-receptor type 6 |
| Pentaxin-related protein PTX3 | Choline transporter-like protein 2 |

| | |
|---|---|
| Erythrocyte band 7 integral membrane protein | Leukocyte-associated immunoglobulin-like receptor 1 |
| Vesicle-associated membrane protein 8 | EF-hand Ca-binding domain 4B isoform a |
| Interleukin enhancer-binding factor 2 | Cysteine-rich secretory protein 3 |
| CD59 antigen, complement regulatory protein | Myeloid cell surface antigen CD33 |
| $\gamma$-glutamyl hydrolase | CD11 antigen-like family member A |
| Antileukoproteinase | C-type lectin domain family 12 member A |
| Nitrilase homolog 2 | CD20 antigen-like protein |
| Pyridoxal kinase | Ras-related protein Rab-37 |
| Small nuclear ribonucleoprotein Sm D2 | Folate receptor 3 |
| C-type lectin domain family 8 member A | Peptidoglycan recognition protein 1 |
| | Hydrogen voltage-gated channel 1 |
| **Primary granules** | |
| Microsomal glutathione S-transferase 1 | Lymphoid-restricted membrane protein |
| FYN-related kinase | Eosinophil-derived neurotoxin |
| Carcinoembryonic Ag-related cell adhesion 6 | Bactericidal permeability-increasing prot |
| Complement C3 (Fragment) | Cathepsin G |
| Annexin A2 | Neutrophil elastase |
| Synaptic vesicle membrane prot VAT-1 homolog | Protein unc-13 homolog D |
| Alpha-L-fucosidase I | Glioma pathogenesis-related protein 1 |
| Glycogen phosphorylase, brain form | Ribonuclease T2 |
| T-complex protein 1 subunit beta | Eosinophil cationic protein |
| Hepatocellular carcinoma-associated prot TB6 | PCD-associated speck-like prot with CARD |
| Protease serine 2 preproprotein | Protein-arginine deiminase type-2 |
| Magnesium transporter protein 1 | Myeloperoxidase |
| $\alpha$-L-fucoside fucohydrolase 2 | Synaptogyrin-1 |

| | |
|---|---|
| Cellular repressor of E1A-stimulated genes 1 | Grancalcin |
| Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa sub | Calcineurin-like phosphoesterase domain-containing protein 1 |
| $\alpha$-1-antichymotrypsin | Adenosine deaminase CECR1 |
| T-complex protein 1 sub $\theta$ | Arginase-1 |
| PA2G4 protein | 1,4-$\beta$-N-acetylmuramidase C |
| Inosine-5'-monophosphate dehydrogenase | Myeloid cell nuclear differentiation antigen |
| NADH dehydrogenase [ubiquinone] 1 sub C2 | |
| $\beta$-hexosaminidase $\alpha$ chain | |
| Beta-1,4-galactosyltransferase 1 | |
| Sorting and assembly machinery comp 50 hom. | |
| 1-acylglycerophosphocholine O-acyltransferase | |
| 26S proteasome non-ATPase regulatory sub 1 | |
| FAS-associated factor 2 | |
| Prostaglandin E synthase 2 | |
| Aspartylglucosaminidase | |
| 1-Cys peroxiredoxin | |
| Serpin B3 | |
| CD63 antigen | |
| Cerebroside sulfate activator protein | |
| Surfeit locus protein 4 | |

**Table 3.5.1. Changes observed in granule protein transcripts.** The reference list of proteins was retrieved from Rørvig et al. [28]. Log2 fold change was calculated comparing neutrophils transmigrated to CFASN to matched blood. Proteins not shown here had an absolute fold change less than 2.

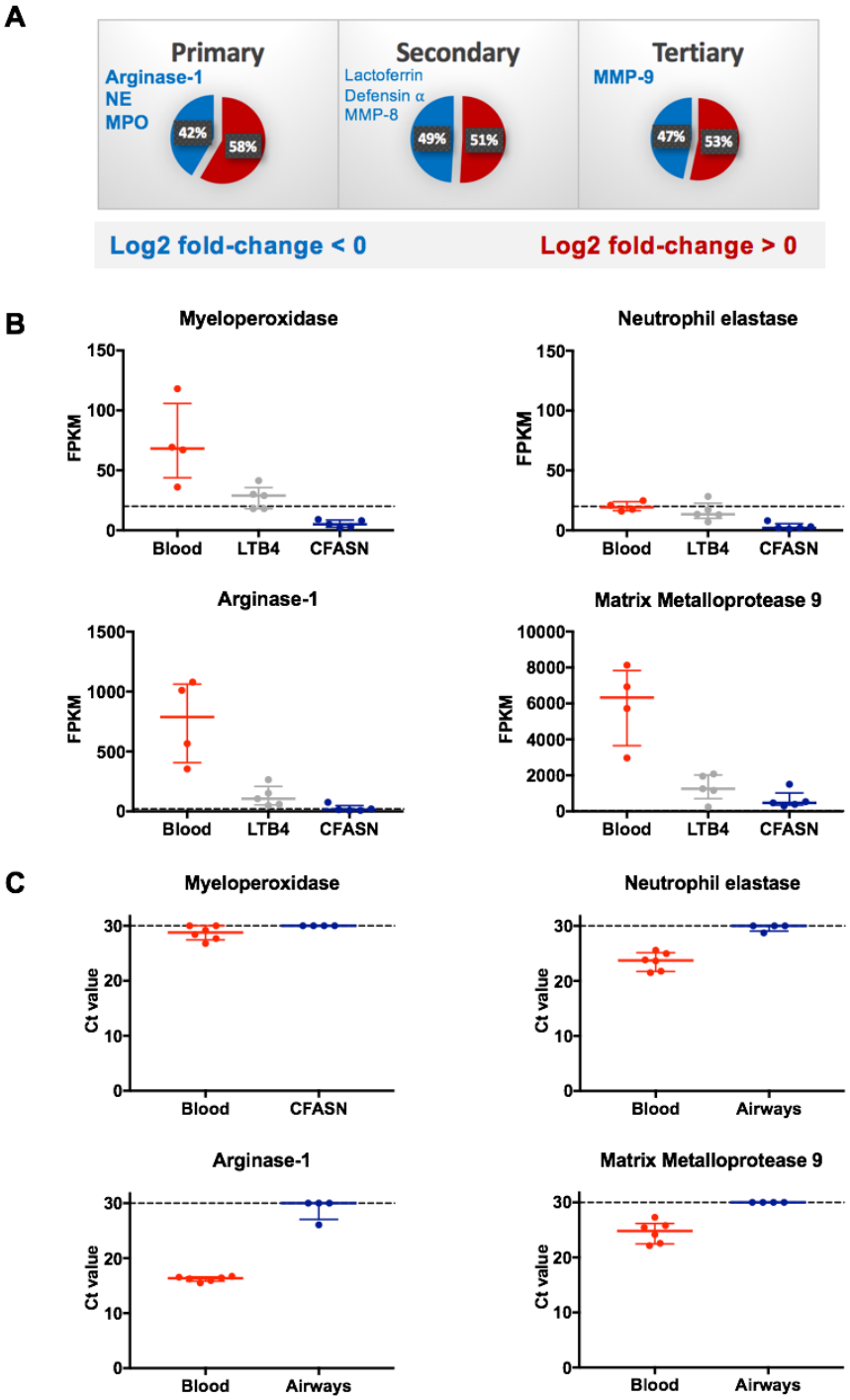



**Figure 3.5.6. CF airway GRIM neutrophils lack *de novo* expression of main granule effector proteins. (A)** Overview of upregulated and downregulated granule proteins in CF airway GRIM neutrophils compared to matched blood. RNA of effector granule proteins measured by RNASeq *in vitro* **(B)** and by Fluidigm *in vivo* **(C)** is not detectable in CF airway GRIM neutrophils. Dotted line represents lower limit of detection (20 FPKM for RNASeq, and 30 Ct for Fluidigm).

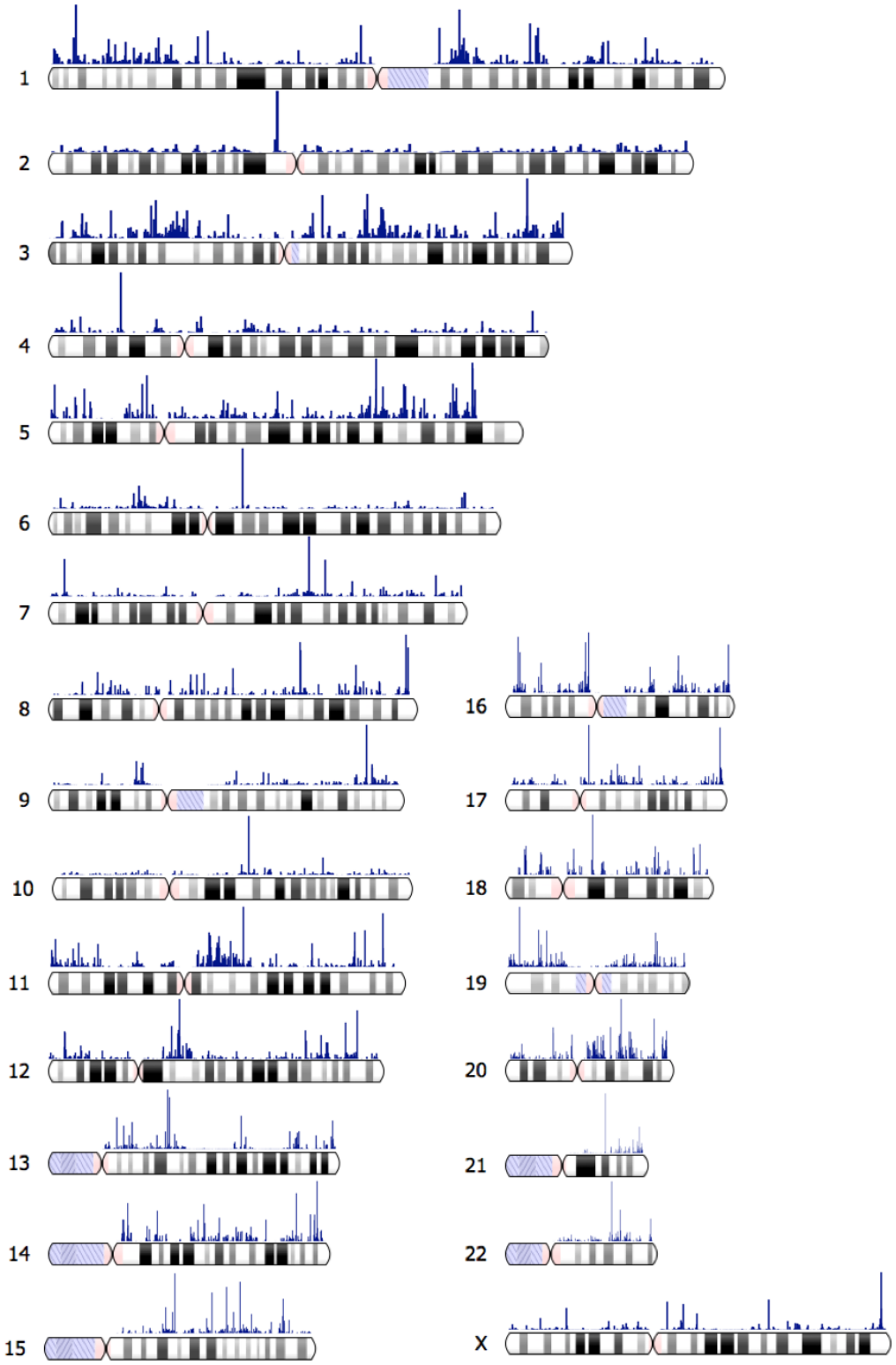



**Figure 3.5.7. Genomic arrangement of transcripts detected in CF airway GRIM neutrophils *in vitro*.** Histograms represent RNA counts of transcripts detected by RNASeq in neutrophils transmigrated to CF ASN at their respective chromosomal positions. Note that distinct areas of the chromatin appear silent.

*Acquisition of the CF airway GRIM phenotype depends on time-dependent transcriptional reprogramming of neutrophils*

To better understand the dynamics of the acquisition of the GRIM phenotype, we performed a kinetic assay using the *in vitro* transmigration model and collected airway neutrophils after 1, 2, 4 and 6 hour of transmigration towards CF ASN. We observed that the GRIM phenotype developed in a time-dependent manner, with stepwise increase of total RNA content **(Figure 3.5.8A)**, release of primary granules **(Figure 3.5.8B)** and loss of the phagocytic receptor CD16 (not shown), suggesting a co-regulation of transcription and degranulation. To further investigate this question, we transmigrated neutrophils for 2 hours into CF ASN and then treated them with the transcriptional blocker *α*-amanitin. As expected, the total RNA content was reduced upon treatment **(Figure 3.5.9A)**, showing the effectiveness of the blockade. Furthermore, we observed a concomitant reduction in surface CD63 **(Figure 3.5.9B)**, suggesting a decreased release of primary granules, which was confirmed by quantification of NE in the extracellular milieu **(Figure 3.5.9C)**.

Next, since CF ASN-transmigrated, treated neutrophils did not release primary granule extracellularly to the same extent as non *α*-amanitin treated ones, we questioned whether this would affect their bacterial killing capacity. To this end, we incubated both sets of cells with *P. aeruginosa*, a common CF pathogen, and assessed the killing activity of the airway neutrophils. Surprisingly, the killing capacity of CF ASN transmigrated neutrophils increased from 40% to 95% upon *α*-amanitin treatment, while the LTB4 transmigrated neutrophils remained stable at 65% killing capacity whether exposed to *α*-amanitin treatment or not, suggesting that the effects of *α*-amanitin were confined to CF ASN transmigrated neutrophils and did not affect bacterial growth *per se* **(Figure 3.5.9D)**.

Together, these results suggest that *de novo* transcription is key to driving the neutrophil adaptation to the CF airway milieu and actively inhibits their bacterial killing capacity.

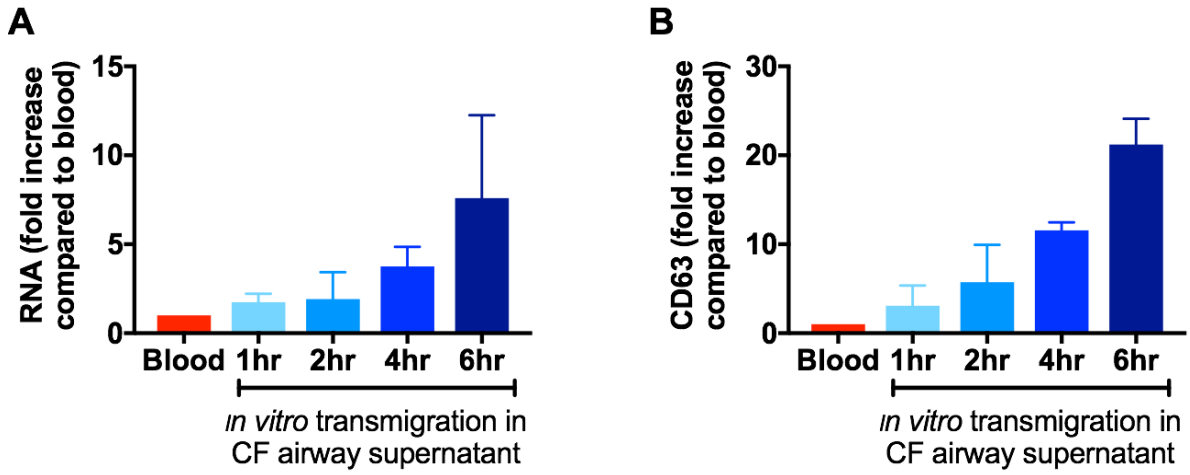



**Figure 3.5.8 Transcriptional reprogramming of CF airway neutrophils and acquisition of the GRIM phenotype are time-dependent.**

Blood neutrophils (red) were transmigrated in vitro towards CF ASN and collected at 1, 2, 4 and 6 hours post transmigration (N=5 independent experiments). Total RNA content **(A)** was assessed by Bioanalyzer, while surface expression of CD63 **(B)** was quantified by flow cytometry.

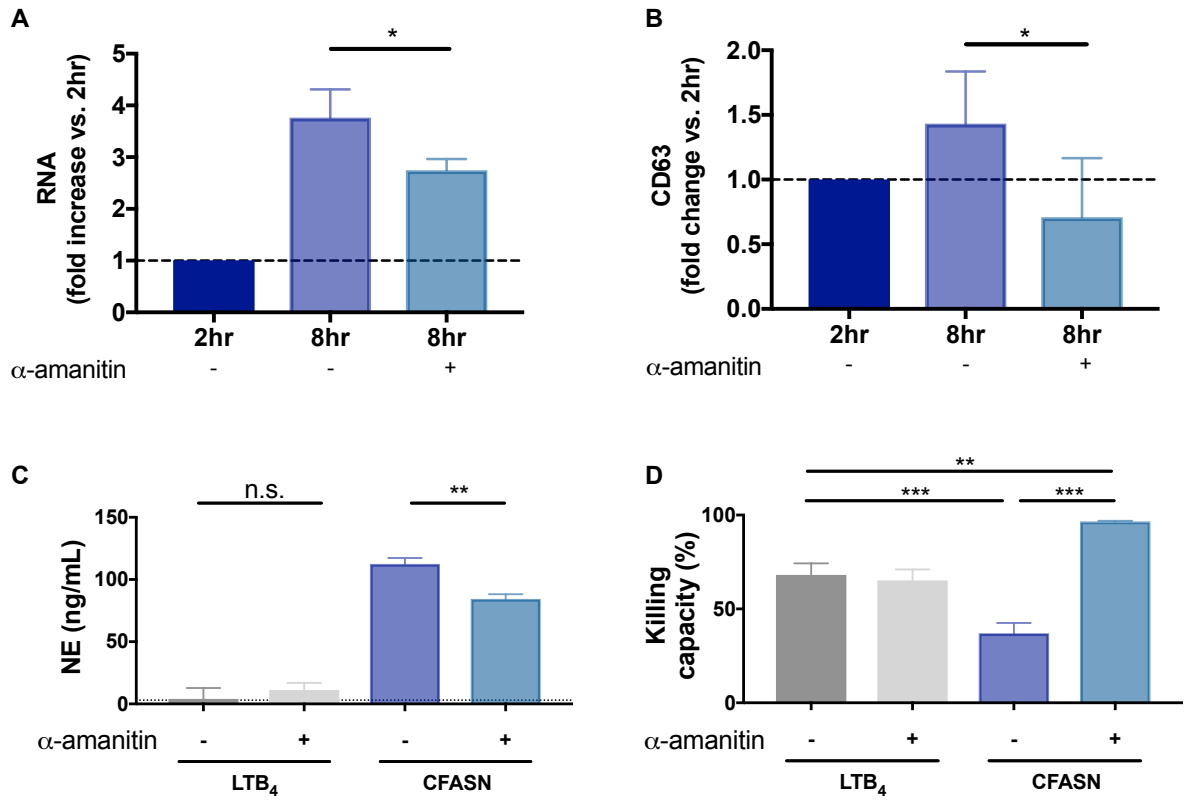



**Figure 3.5.9 Functional adaptation of neutrophils to the CF airway microenvironment critically depends upon *de novo* transcription.** Neutrophils were transmigrated *in vitro* for 2 hours towards LTB4 or CF ASN and subsequently incubated for 8 hours with or without the transcriptional blocker *α*-amanitin. CF ASN transmigrated neutrophils treated with *α*-amanitin showed reduced amounts of total RNA content **(A)**, and a reduced release of primary granules **(B)** concomitant with lower presence of NE in the extracellular milieu **(C)**, as well as increased killing of *P. aeruginosa* **(D)**. Results are shown as median and interquartile range, statistical analysis was performed by Wilcoxon matched-pairs signed rank test, *p<0.05, **p<0.01, ***p<0.001, n.s.: not significant.

*Transcriptional blockade of CF airway GRIM neutrophils modulates production of pathological extracellular vesicles*

Accumulation of NE in the airways of CF patients is thought to be one of the main drivers for tissue damage and loss of lung function [14, 27, 29]. Recently, we showed that extracellular NE compartmentalized on neutrophil-derived extracellular vesicles (EVs) in chronic obstructive pulmonary disease (COPD) patients was insensitive to inhibition by tissue anti-proteases, enabling it to exert unopposed proteolytic activity [30]. To investigate whether similar impact of NE-rich neutrophil-derived EVs is at play in CF, we assessed whether their NE burden in patient sputum was associated with lung function *in vivo*, and whether their production was dependent upon *de novo* transcription *in vitro*. Indeed, the amount of NE on CF sputum EVs was negatively correlated with lung function (N=20 patients, Rho = -0.61, p = 0.04, **Figure 3.5.10A**). Moreover, treatment of CF ASN transmigrated neutrophils with $\alpha$-amanitin decreased the number (not shown) and the amount of NE on EVs (**Figure 3.5.10B)**, suggesting that transcriptional reprogramming of GRIM neutrophils controls the production and release of pathological, NE-rich EVs in CF airways.

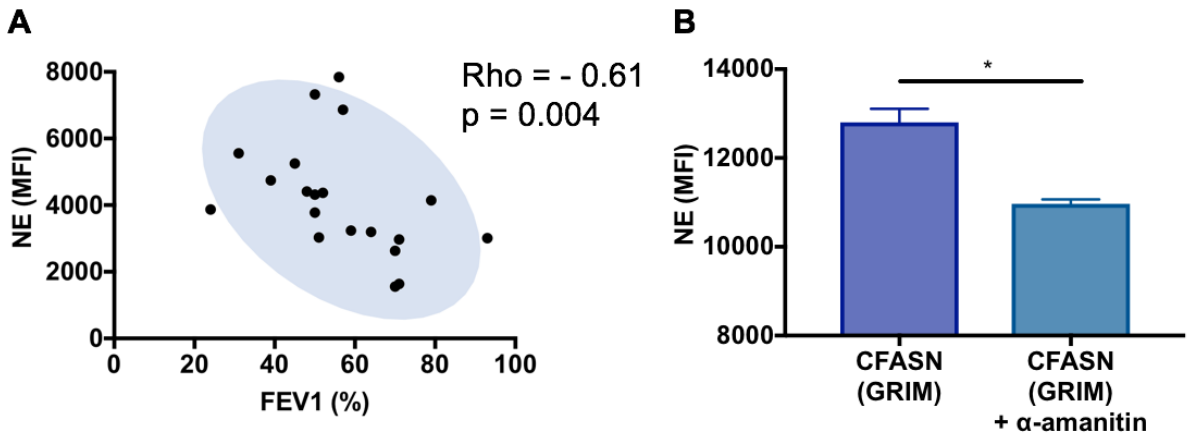



**Figure 3.5.10 Production of pathological NE-rich EVs by CF airway GRIM neutrophils relies on *de novo* transcription.** Neutrophil-derived EVs were pulled down from sputum of patients with CF **(A)** or from the supernatant from the *in vitro* transmigrated neutrophils **(B)**, and analyzed by flow cytometry for surface NE. Correlation with lung function (measured as forced expiratory volume in 1 second - FEV1, N=20 patients) was assessed by the Spearman test. In vitro data were analyzed using the Wilcoxon matched-pairs signed rank test, * $p < 0.05$.

## 3.5 Discussion

Neutrophil adaptation to the CF airways microenvironment relies on profound changes at the metabolic and functional levels, including activation of the mTOR pathway, active release of primary granules, immune modulation and decreased bacterial killing [15-17, 21]. The findings presented here show that the reprogramming observed in CF airway neutrophils depends upon *de novo* transcription, a process previously shown to be central for the formation of neutrophil extracellular traps [31].

Accumulation of extracellular NE in the airways remains a major challenge in the prevention of bronchiectasis and preservation of lung function in patients with CF [29, 32]. Moreover, compartmentalization of NE at the surface of EVs leads to unopposed proteolytic activity, and in turn, significant lung damage [30]. Interestingly, our findings

show that release of primary granule contents in the extracellular milieu and the production of NE-rich EVs both depends upon transcriptional reprogramming of airway neutrophils, highlighting novel mechanisms regulating their function.

The observed lack of *de novo* transcription of NE and other granule effector proteins suggests that accumulation of these enzymes in CF airways is due to waves of recruitment of blood neutrophils into the lung lumen, following by active granule protein release and accumulation, followed by reuptake. This finding is in concordance with previously published studies showing absence of transcription factors key ot granule biogenesis, and of corresponding messenger RNAs in mature neutrophils [33-35]. Furthermore, despite the transcriptional burst observed in airway neutrophils, the absence of specific transcripts encoding for granule effector proteins normally expressed at early stages of BM development supports the notion that GRIM reprogramming is not linked to overall chromatin derepression, but rather to tighly orchestrated reopening of certain areas of the chromatin. Focused follow-up studies of GRIM neutrophil epigenetics are needed to shed light into this question.

Lastly, transcriptional differences in blood neutrophils between healthy donors and patients with CF have been observed [36]. However, findings presented are in line with previously published work [21] and suggest that the origin and status of blood neutrophils bear minimal impact on the acquisition of the GRIM phenotype. Rather GRIM reprogramming is driven by *de novo* transcription under the dominant influence of factors present in CF airway microenvironment. The identification of such factors is a major focus of ongoing investigations, which are outside of the range of this study.

In conclusion, this study xemonstrates the ability of human neutrophils to actively adapt to the airway microenvironment after transmigration, challenging the conventional paradigm that holds these cells as short-lived, terminally differentiated,

and with little opportunity for plasticity. Future studies investigating the time-dependent switch between hetero- and euchromatin states will provide new insights in basic neutrophil biology and open opportunities for host-directed immunotherapies targeting the critical, and yet intractable neutrophilic component of fatal CF disease.

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# Chapter 4

## Targeting airway neutrophilic inflammation:

## exploiting a novel protease-activated drug delivery system

*Sections of this chapter will be submitted for publication in Nature Medicine*

## 4.1 At-a-glance commentary

*Current scientific knowledge on subject*. Pulmonary delivery of therapies targeting inflammation remains a current challenge. The physical and aerodynamic processes of the lung and the presence of resident macrophages impose special requirements onto delivery methods. In addition, many potential treatments feature hydrophobic drugs, further complicating administration to the airways. Novel approaches using nanoparticles as a delivery vehicle for hydrophobic drugs have not met expectations, and some of them have been shown to carry systemic effects, including renal clearance and accumulation. Lastly, in an inflammatory setting nanoparticles or drugs alone can be negatively impacted by the highly proteolytic environment of inflamed airways.

*What does this study add to the field?* We developed a novel multi-stage nanoparticle-in-microgel (N-in-M) delivery platform that fits the physical and aerodynamic requirements of the lung toward lower airway delivery. This platform is not negatively impacted, but rather exploits the proteolytic environment of inflamed airways. By incorporating into an elastase-activated N-in-M a hydrophobic drug inhibiting the release by activated lung neutrophils of elastase itself, we demonstrate efficient, self-limited delivery of this potent neutrophil-targeted drug to the airways, leading to resolution of acute lung inflammation.

## 4.2 Abstract

Pulmonary drug delivery presents unique opportunities to target lower airway inflammation, which is often characterized by neutrophilic infiltration yet lacks specific therapies. To achieve therapeutic efficacy in pulmonary drug delivery, difficult challenges must be overcome such as drug hydrophobicity, oxidative and proteolytic microenvironments, mucociliary and macrophage-dependent clearance. Here, we present a novel drug delivery platform able to reach the lower airways and target neutrophilic inflammation. We designed a self-regulated nanoparticle-in-microgel system, whose activation relies on presence of extracellular neutrophil elastase, and loaded the nanoparticles with a hydrophobic drug targeting neutrophil degranulation. Successful pulmonary delivery of the drug promoted resolution of the inflammatory response by dampening neutrophil degranulation, pro-inflammatory cytokines and presence of pathological extracellular vesicles. Together, these results showcase a new platform that overcomes most challenges presented in pulmonary drug delivery and allows customization to match the pathological imprinting in a variety of airway diseases.

## 4.3 Introduction

Polymorphonuclear neutrophils (PMNs) play a central role in the pathophysiology of several disorders, such as rheumatoid arthritis[1], systemic lupus erythematosus[2], acute respiratory distress syndrome (ARDS)[3], asthma[4], chronic obstructive pulmonary disease (COPD)[5], and cystic fibrosis (CF)[6]. Importantly, although PMNs have been considered short-lived and with little opportunity for plasticity, recent studies have challenged this dogma, particularly in chronic inflammatory diseases [7-10], suggesting prolonged PMN lifespan and active,

orchestrated release of proteolytic mediators, thus opening new therapeutic opportunities. Contrasting with the known role of PMNs and PMN-derived mediators in shaping the clinical outcomes in disease, there is a clear lack of therapies and delivery methods allowing specific targeting in highly proteolytic microenvironments.

One of the hallmarks of PMN-driven inflammation lung diseases, such as ARDS, CF, COPD, is the highly proteolytic microenvironment created by the release of neutrophil elastase (NE) and metalloproteinases (MMP), such as MMP-9[11]. The presence of these proteases in the airway milieu has been linked to progression of structural lung damage[3,12,13]. Targeting of neutrophilic lung disease is of significant therapeutic importance, with potential for broad clinical impact[14]. Unfortunately, to date, the ability to directly target specific areas of the lung with increased proteolytic activity has remained limited.

Site-specific delivery of therapies to the lungs could potentially increase therapeutic efficacy and reduce off-target effects. However, delivery to the respiratory airways is complicated, requiring particles between 0.5-5 μm. Larger particles deposit in the mouth and throat, while smaller particles are exhaled[15]. Particles with a geometric diameter between 1-5 μm are readily phagocytosed by lung macrophages, resulting in rapid clearance [16,17]. Furthermore, particles smaller than 300 nm are required for effective intracellular delivery[18,19]. To tackle this size issue, we designed a nanoparticle-in-microgel (N-in-M) multi-stage particle formulation. A swellable polyethylene glycol (PEG) based microgel provides the appropriate aerodynamic size in the polymer's relaxed state and geometric size in the swollen state, while a crosslinked elastase-degradable peptide incorporated into the microgel backbone allows for the rapid degradation of the microgel in the presence of NE to release the nanoparticles. By using a multi-stage particle system, the microgel can provide the needed aerodynamics to deposit in the airways and degrade to release therapeutic

loaded nanoparticles. These nanoparticles not only protect the encapsulated drug from high levels of proteolytic enzymes and allow for efficient endocytosis but also allow for the effective delivery of small hydrophobic molecules into the airway. The polymers used for both the nano and micro portion of the formulation were chosen due to their biodegradable properties.

Nexinhib20 has been previously shown to be a potent PMN exocytosis inhibitor via regulation of the intracellular target Rab27a that not only regulates exocytosis but also inhibits the up-regulation of the adhesion molecules CD11b and CD66b[20]. However, the poor solubility of Nexinhib20 in water is a significant disadvantage to therapeutic translation. We encapsulated Nexinhib20 by single emulsion into a poly(lactic-co-glycolic acid)-poly(vinyl) alcohol (PLGA/PVA) nanoparticle that was then encapsulated into our NE-degradable microgels for pulmonary delivery modulating PMN-driven inflammation.

## 4.4 Methods

*PLGA Nanoparticle Fabrication*

Nanoparticles were fabricated using a single emulsion solvent evaporation method. 0.8-1 mg of Nexinhib20 (Cayman Chemicals), 0.2 mg of DiR (ThermoFisher), and 130 mg of Poly (D,L-lactide-co-glycolide) (PLGA RG502H, Sigma) was dissolved in 2 mL of ethyl acetate. The PLGA mixture was then added to 4 mL of 2.5 % w/v poly(vinyl) alcohol (PVA; Sigma) and sonicated at 65% for 10 min on ice using a Sonics Vibra-Cell VCX130 with CV18. The emulsion was then added to 50 mL of 0.3 % w/v PVA solution and magnetically stirred for 5 hours. The nanoparticles were centrifuged at 8000 g for 10 minutes, pellet discarded, and nanoparticles in the supernatant were then washed in DI water by centrifuging at 100,000 g for 30 minutes. After the second DI rinse, the nanoparticles were resuspended in 5 mL 1% w/v trehalose solution, snap frozen in LN,

lyophilized for 48h, and stored at -20 C. Nanoparticles were resuspended at 1 μg/mL in water and run on a NanoSight to determine the mean particle size; Nexinhib20 DiR PLGA nanoparticles mean size was 145.6 +/- 3.3 nm D90: 196.5 +/- 8.9 nm; DiR only PLGA nanoparticles mean size was 177.2 +/- 4.3 nm D90 265 +/- 9.5 nm. Nanoparticles (100 mg/mL) were degraded in DMSO to measure Nexinhib20 concentration by absorbance (λ = 274); Nexinhib20 was loaded at 1.48 ug/mg of PLGA formulation (21% encapsulation efficiency).

*Nano-in-Micro Fabrication*

Microgels were fabricated as previously reported with slight modifications to the polymer mixture. A 20 % w/v solution of equimolar amounts of di-sulfhydryl elastase peptide, CGAAPVRGGGGC (Chi Scientific), and 4-arm PEG maleimide (10kDA, Laysan Bio) were dissolved in PBS. 1 μL of 10 mg/mL DyLight 488-maleimide or DyLight 650 4x PEG-maleimide and nanoparticles were added to the PEG macromer solution was added to mineral oil (1% v/v surfactant: Span 80/Tween 80, HLB = 5) and homogenized on a PRO Scientific D Series homogenizer for three minutes at 4,000 rpm in a 35-40C water bath. After several centrifuge washes microgels were then run on an Accuri to determine the % of microgels positive for PLGA nanoparticles and microgel/mL concentration and sterilized by UV. Nanoparticles incorporated were either 10 mg of the PLGA formulations or fluorescent carboxylated polystyrene beads; 60 nm Dragon Green (Bangs Laboratories) or 100 nm blue fluorospheres (ThermoFisher).

*In vitro transmigration and microgel uptake*

PMNs were isolated from health donor's blood, per an approved Emory University Institutional Review Board protocol, using Polymorphprep (Alere Technologies) as

per manufacturer protocol. Purified neutrophils were then loaded on the transmigration chamber; transmigration and following incubations were performed using 100 nM of LTB4 (EMD Millipore) in RPMI or CF ASN prepared as previously published[21]. The uptake of fluorescent microgels was then assessed 10 hours post transmigration by directly incubating PMNs recruited apically with microgels at a 1:10 ratio (PMN:microgel) in either CF ASN or RPMI for 30 and 60 minutes and quantified using flow cytometry.

*Flow cytometry*

Cells from *in vitro* and *in vivo* assays were analyzed by multiparametric flow cytometry analysis as previously published[31]. Briefly, cells were pre-stained with Fc block and Zombie live/dead dye (Biolegend) for 10 minutes on ice, followed by staining for 15 minutes on ice with antibodies against the following markers: CD45, Ly6G, Ly6C, MHCII, CD11b, F4/80, CD206, CD11c, Siglec F, CD63 (Biolegend). Cells were washed with PBS-EDTA (250mM), fixed with Lyse/Fix PhosFlow reagent (BD Biosciences) and acquired on a FACS LSRII (BD Biosciences) and on a Cytoflex S (Beckman Coulter). Robust acquisition of samples over time was ensured by the utilization of stringent calibration methods employing rainbow bead-based laser and channel standardization. Data analysis and fluorescence compensation were performed with FlowJo V9.9.5 and V10.4.2 (Treestar). PMNs were gated as CD45+Ly6G+CD11b+ cells, and macrophages as CD45+F4/80+MHCII+Ly6G- cells, **Figure 4.4.1**.

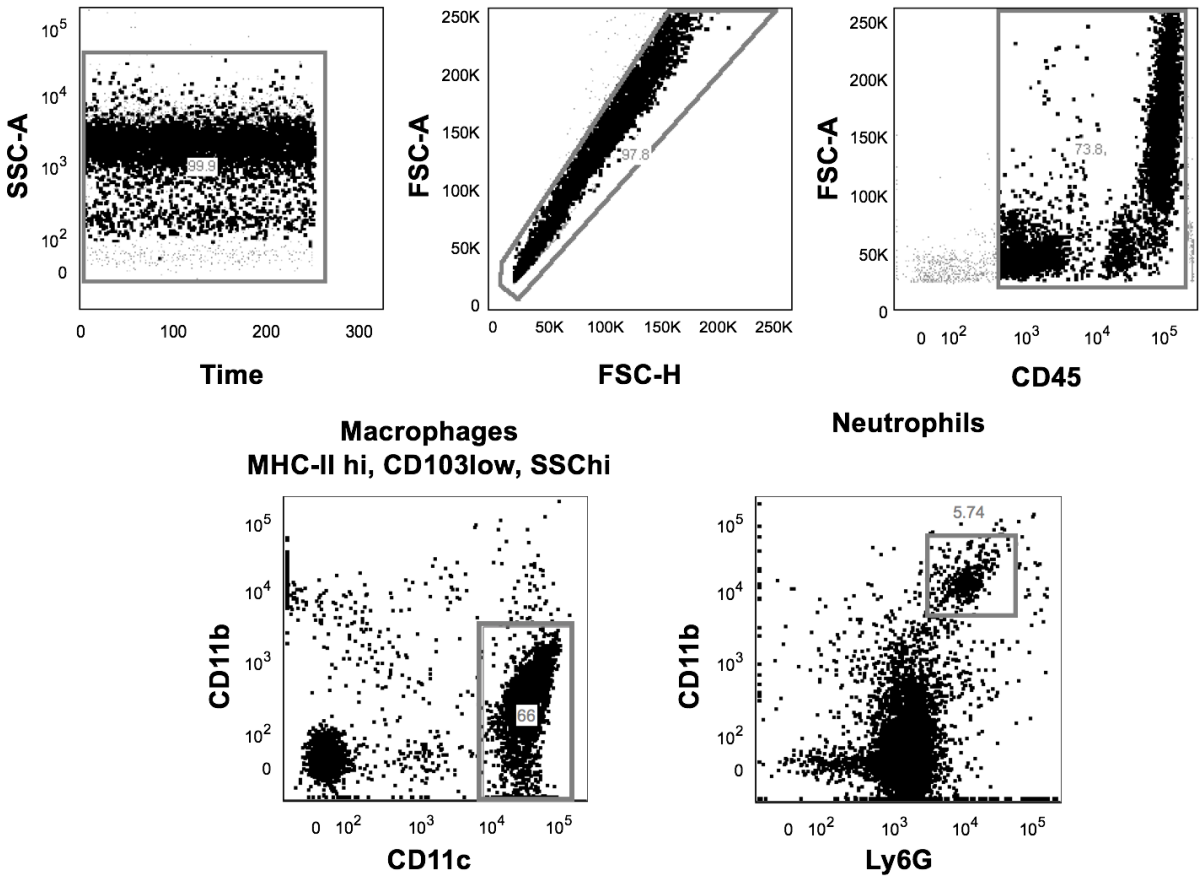


**Figure 4.4.1**. **Flow cytometry gating strategy**. After singlet gating, PMNs were gated as CD45+Ly6G+CD11b+ while macrophages were gated as CD45+ F4/80+ MHCII+Ly6G-.

*Extracellular vesicles (EVs) analysis*

EVs were isolated from the BAL of mice using bead-based pulldown with annexin V, as per manufacturer protocol (SBI). Isolated EVs were stained with the EV marker ExoFITC (SBI) and anti-mouse NE (R&D systems). EVs were analyzed by flow cytometry on a Cytoflex S (Beckman Coulter).

*ELISA*

NE and MMP9 concentrations were measured by ELISA in the BAL of mice as per manufacturer protocol (R&D Systems). Cytokines from the plasma and BAL were

measured using a multiplex chemoluminescent ELISA platform per manufacturer protocol (Meso Scale Diagnostic, V-PLEX mouse cytokines 19 analytes).

*Microscopy*

N-in-M were imaged on a PerkinElmer UltraVIEW VoX spinning disk confocal with a Hamamatsu C9100-23b back-thinned EM-CCD and Nikon 100x NA-1.45 oil objective; lung sections were imaged with the Nikon 40X NA-1.3 oil objective. Size was measured in Matlab using imfindcircles; this does cap the lower size limit measured. Degradation was measured by kinetic imaging every minute using a Biotek Lionheart at 37C using cell traps from the Lu Lab to hold the microgels in place[32].

*Animal Studies*

Studies were performed according to protocols approved by the IACUC at the University of Alabama Birmingham. 5-week-old female C57BL/6 littermates (Jackson Labs) were maintained in a pathogen-free facility, given sterile water and food *ad libitum*. Mice were treated with either saline or 100 μg of LPS then for the fluorescent uptake study N-in-M formulation was delivered 2 h later and for the Nexinhib20 study 1 h after injection. The time for N-in-M injection was shortened since at 2 h post LPS mice had significant difficulty in aspirating the particle formulation. Mice used for IVIS were given a low alfalfa feed diet to reduce fluorescence background.

*IVIS Imaging*

Excised lobes were imaged on an in vivo imaging system (IVIS) and analyzed by LivingImage software (Xenogen).

*Immunofluorescent staining*

Left lung lobes were fixed in 4 % paraformaldehyde at 4 ºC overnight, allowed to settle in 30 % w/v sucrose for 24-48 h at 37 ºC and then frozen in OCT by a dry ice isopentane mixture. 10 μm sections were sliced and stored at −80 ºC until stained. Slides were washed in 1x TBS for 5 minutes, blocked for 2 h at room temperature in 5 % goat serum with 0.5 % Triton X, incubated at 4 ºC overnight with the primary antibody, 1:100 dilution MPO (ThermoFisher) in 5% goat serum. Slides were then washed and stained with secondary, 1:250 dilution Goat anti-Rabbit Alexa Fluor 555 (ThermoFisher) with 5 μg/mL DAPI for 1 h at room temperature. Then samples were mounted using Prolong Gold. Average background of each channel from the secondary stain was used to linearly adjust the contrast equally across all images.

*Statistical analysis*

Data were analyzed in Graphpad; a Shapiro-Wilk test for normality for parametric data a two-sided one-way Anova a two-way ANOVA with post-hoc Tukey's test with adjusted P value for multiple comparisons. For non-parametric data Kruskal-Wallis with Dunn's multiple comparison test with adjusted P value was used.

## 4.5 Results

*N-in-M degrade in presence of neutrophil elastase (CF ASN) resulting in uptake of nanoparticles by activated PMNs.*

Targeting PMNs *in vivo*, particularly in highly proteolytic microenvironments, is limited by our ability to effectively deliver drugs to these cells. The multi-stage N-in-M particle delivery system we designed allows controlled release of nanoparticles in regions of the lung with high concentration of proteolytic enzymes, specifically NE

**(Figure 4.5.1A-B)**. The swollen microgel is at 3.2 μm of mean diameter (8.0 μm range), while encapsulated nanoparticles are under 200 nm. To test the degradation of the multi-stage system crosslinked with an NE-cleavable peptide, we imaged the N-in-M as they were exposed at 37°C to physiologic levels of NE in airway supernatant from CF patients (CF ASN). Within 30 minutes of exposure to NE-rich CF ASN, 95 % of the microgels had degraded **(Figure 4.5.1C)**, which shows that our system quickly releases nanoparticles once the microgel formulation is delivered to protease-rich airways.

Next, we assessed delivery of our N-in-M system to PMNs *in vitro*. This was accomplished in a PMN transmigration model[21], which uses the relevant biological milieu, i.e., CF ASN, as the apical fluid and has been shown previously to recapitulate the *in vivo* phenotype of PMNs found in CF patients' airways. We measured uptake by these airway PMNs of 60 nm fluorescent carboxylated polystyrene nanoparticles encapsulated within the NE-degradable microgels, and observed that the degradation of the microgels in the presence of CF ASN increased the percentage of PMN positive for the nanoparticle by 5-10% at 60 minutes, regardless of the transmigration milieu (chemoattractant control consisting of leukotriene B4 -LTB4, or CF ASN). When both PMNs transmigration and the N-in-M exposure occurred in the context of CF ASN, uptake of fluorescent nanoparticles by PMNs was increased **(Figure 4.5.1D)**. Together, these data indicate that for efficient uptake of nanoparticles by airway PMNs, the NE-responsive microgel must degrade first. The additive effect between the acquired PMN phenotype, imprinted by the transmigration condition, and the incubation milieu with the N-in-M system suggests that targeting of airway PMNs by the N-in-M system would be more efficient in pathological settings (CF ASN) rather than during the normal course of inflammation (LTB4).

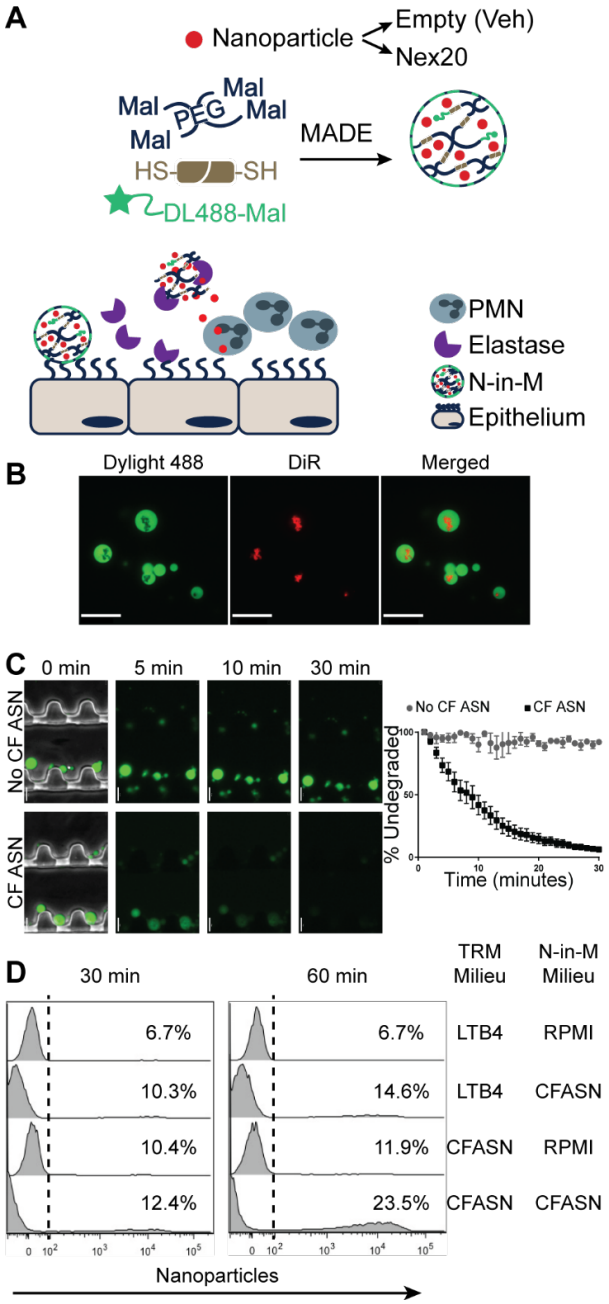


**Figure 4.5.1. NE-responsive N-in-M degrade in CFASN releasing fluorescent nanoparticles for endocytosis by PMNs. (A)** Schematic of the N-in-M system **(B)** DiR loaded PLGA nanoparticles encapsulated in DyLight (DL) 488 labeled microgels. **(C)** The N-in-M system degrades in the presence of high NE concentration (CFASN) within 30 minutes; scale bar = 10 μm, mean ± SEM, N = 3 (no CFASN), N = 6 (CFASN). **(D)** PMNs that transmigrate (TRM) in the CFASN milieu readily show nanoparticles uptake.

*N-in-M delivered to acute LPS-induced lung injury model degrade, delivering fluorescent nanoparticles to airway PMNs.*

Next, we tested the efficacy of the N-in-M multi-stage particle system in an *in vivo* inflammation model characterized by high levels of extracellular NE[22]. The N-in-M system consisting of microgels labeled with the near IR dye DyLight 650 loaded with representative blue (365/415) 100 nm Fluorosphere nanoparticles was delivered intratracheally to mice treated with saline or LPS. The radiant efficiency in the excised lungs, corresponding to the fluorescence intensity of the microgel dye at 1, 6, and 22 h after N-in-M delivery (3, 8, 24 h post LPS), showed a significant reduction at 6h post N-in-M delivery **(Figure 4.5.2A)**, revealing a faster microgel degradation in mice treated with LPS than in saline-treated controls.

Concurrent with microgel degradation, we observed a reduction over time of blood PMN activation, as well as PMN recruitment to the airways, and subsequent release of NE-rich granules (reflected by surface CD63), leading to increased amount of the soluble NE in the bronchoalveolar lavage (BAL) **(Figure 4.5.2B-C)**. These data suggest that the short-term clearance of the microgels during the LPS treatment is preceded by and dependent upon the degradation of the NE-sensitive peptides crosslinking it. Notably, the population of BAL PMNs double positive for the microgel and nanoparticles showed increased surface CD63 expression, suggesting that fusion to the plasma membrane of NE-rich granules and release of their content in the extracellular milieu is concomitant with increased uptake of soluble material. Lastly, analysis of the distribution of microgel and nanoparticles uptake showed that airway PMNs at 1 and 6 h post-delivery are primarily positive for nanoparticles alone, suggesting that microgels are degraded, allowing the PMNs to endocytose the nanoparticles **(Figure 4.5.2D)**, showing the efficacy of the N-in-M system for nanoparticle delivery to airway PMNs in a protease-rich environment *in vivo*.

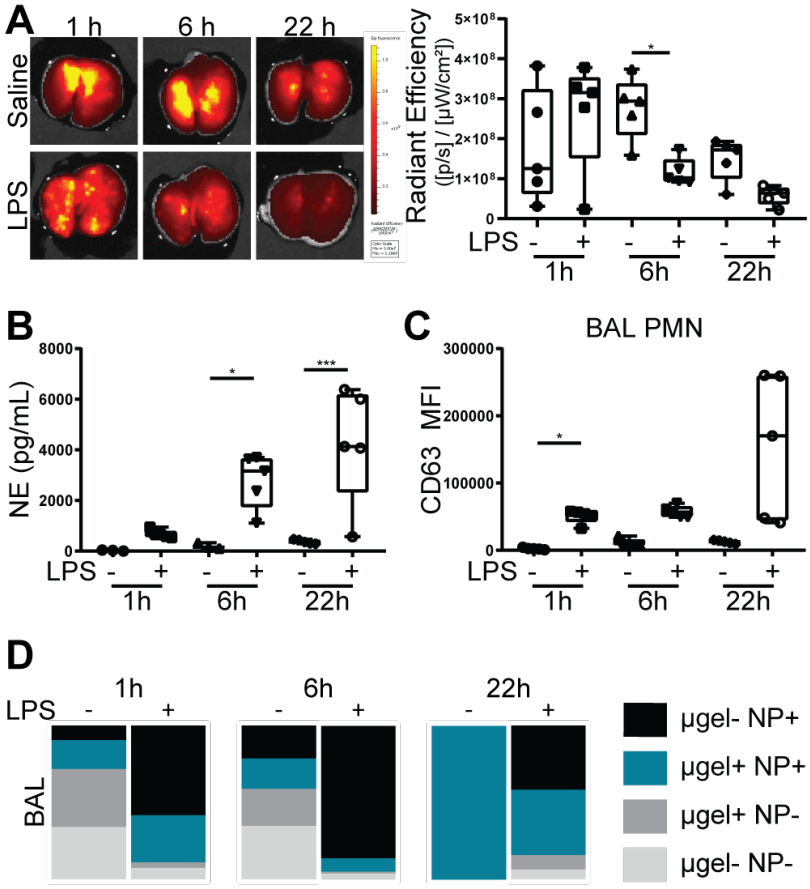


**Figure 4.5.2. N-in-M in an acute neutrophilic inflammation model. (A)** Representative IVIS images of excised mouse lungs with corresponding measures of radiant efficiency for 1, 6, and 22 h. **(B)** Increase in BAL elastase levels for the LPS treated mice. **(C)** Increased CD63 expression on BAL PMNs. **(D)** Percentage of BAL PMNs positive for Nanoparticle only, N-in-M, Microgel only or negative for both. *p < 0.05, ** p < 0.01, *** p < 0.001 within time points indicated.

*N-in-M delivering Nexinhib20 attenuates neutrophilic inflammation in vivo.*

To determine whether the N-in-M system could not only effectively deliver nanoparticles to PMNs but also modulate inflammation *in vivo*, Nexinhib20- and DiR-carrying PLGA nanoparticles were loaded into the NE-degradable microgels (N-in-M/Nex); with DiR, a near IR fluorescent dye serving as a tracer for nanoparticle uptake. To assess if the N-in-M system with PLGA influenced the immune response, additional control groups for both saline and LPS-treated mice were treated with a vehicle DiR-only PLGA nanoparticles inside the NE-degradable microgels (N-in-M/Veh).

While there was no detectable sign of neutrophilia or neutropenia in blood **(Figure 4.4.3A),** N-in-M/Nex reduced the percentage **(Figure 4.4.3B-E)** and mean total number of PMNs in the lung lumen and lung parenchyma compared to the LPS-treated control group, by 13- and 4.3-fold respectively. These results, in addition to the detection of DiR dye in airway PMNs **(Figure 4.4.3F)**, suggests successful delivery and endocytosis of the drug to PMNs by the N-in-M system. Next, we investigated whether the reduction in PMN in the airways was reflective of a different inflammatory milieu. Analysis of BAL and plasma cytokines revealed that important chemoattractants implicated in neutrophil and macrophage recruitment, KC/GRO and MIP-2 **(Figure 4.4.4A-D)**, and proinflammatory cytokines, such as TNFα, IL-6 and IL-1β **(Figure 4.4.4E-J)**, were reduced by treatment with N-in-M/Nex locally and systemically.

To determine whether N-in-M/Nex could influence not only PMN numbers, but also their phenotype and function, active granule release was assessed by ELISA and flow cytometry. Despite similar PMN recruitment in LPS alone controls, in N-in-M/Veh alone controls, and in N-in-M/Veh LPS-treated mice, MMP9 levels in the latter

showed a 28-fold increase compared to the saline treatment group, and a 5-fold increase compared to the LPS alone group **(Figure 4.4.5A)**. Delivery of Nexinhib20 abrogated this response in N-in-M/Nex mice, supporting the effectiveness of this novel delivery system. Most strikingly, concomitant with increase nanoparticle uptake by PMNs, surface CD63 expression on BAL PMNs **(Figure 4.4.5B)**, reflective of NE release, was significantly lower for the Nexinhib20 formulation than all the other treatment groups. Likewise, N-in-M/Nex treatment lowered the amount of extracellular NE **(Figure 4.4.5C)** and presence of NE-rich extracellular vesicles (EVs) **(Figure 4.4.5D)**, a pathological NE conformation resistant to the lung antiprotease shield and previously shown to induce emphysema[23].

Together, these results highlight the first discrete method for N-in-M therapeutic targeting of pathogenic, NE-releasing PMNs directly within sites of inflammation in the lung, thus providing critical proof-of-concept observation of therapeutic efficacy of this novel drug delivery method.

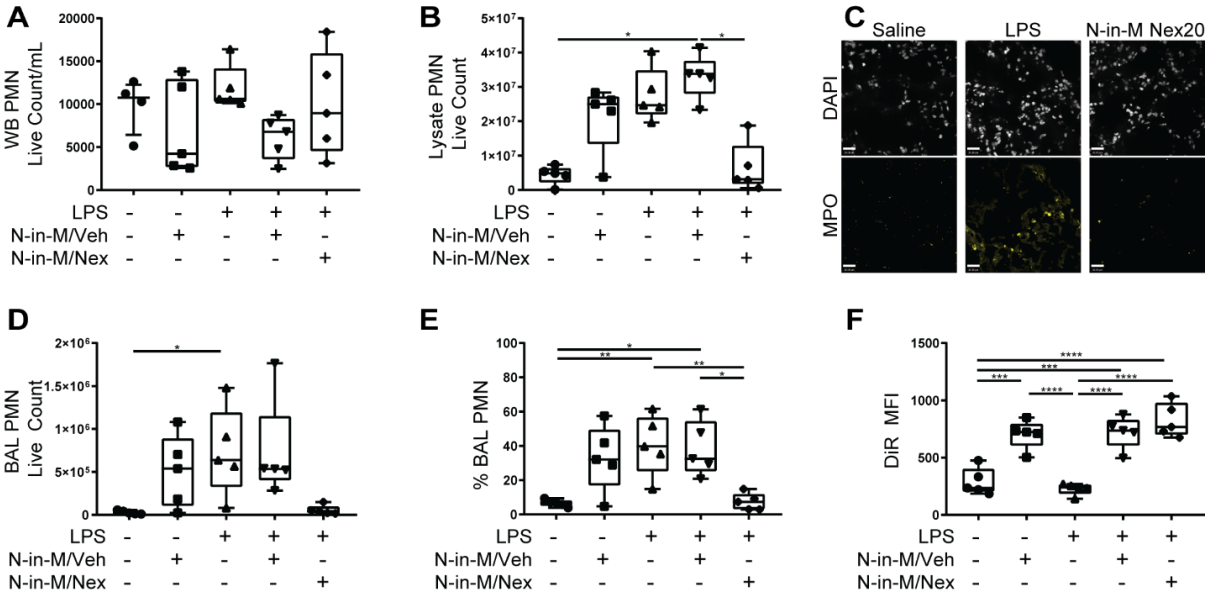


**Figure 4.5.3. N-in-M delivery of Nexinhib20 attenuates neutrophilic inflammation.** **(A)** Blood showed no detectable signs of neutrophilia or neutropenia. **(B)** Lysate absolute neutrophil counts and presence of **(C)** myeloperoxidase (MPO) positive PMNs in the tissue were decreased upon treatment with Nexinhib20. In the BAL, the expected increase in **(D)** PMNs absolute counts and **(E)** percentage with LPS treatment were abrogated in the Nexinhib20 treatment group and were concomitant with **(F)** the uptake of the N-in-M system measured by DiR nanoparticle fluorescence.

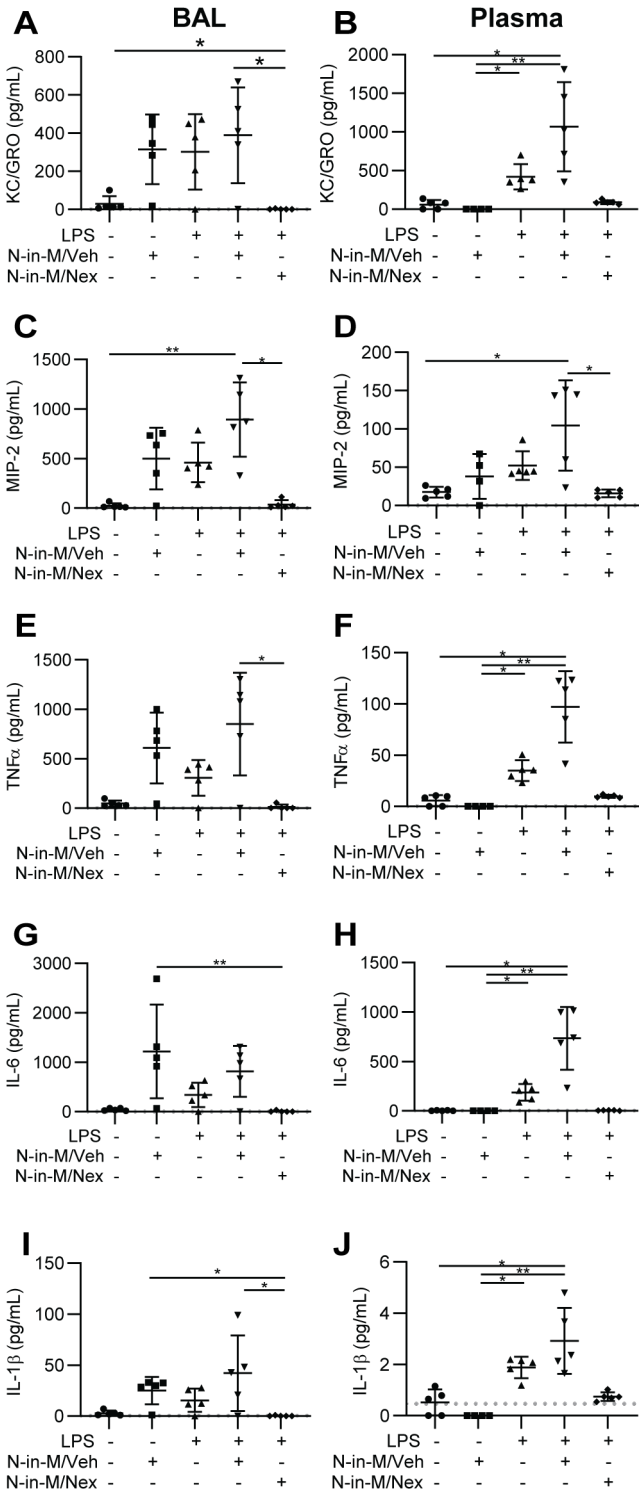


**Figure 4.5.4. Delivery of Nexinhib20 reduces inflammatory mediators in the lung.** Pro-inflammatory cytokines in the BAL and plasma were measured using a multiplex chemiluminescent multiplex ELISA **(A-J)**. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 between groups indicated.

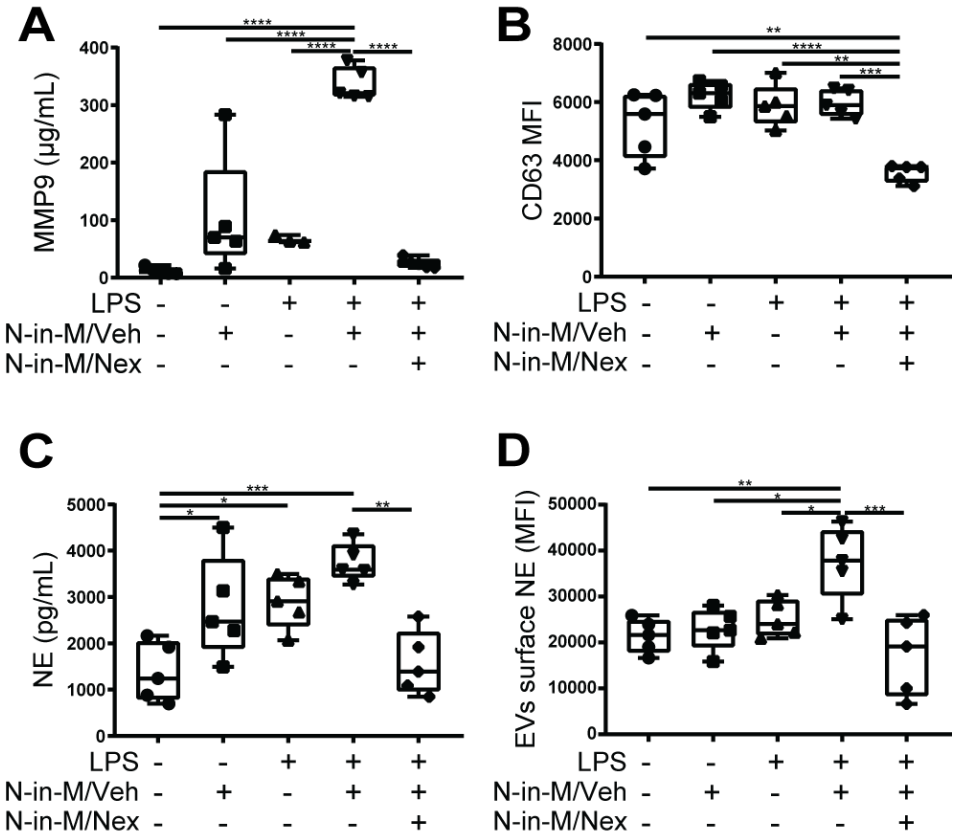


**Figure 4.5.5. Delivery of Nexinhib20 reduces PMN degranulation in the lung. (A)** Treatment with Nexinhib20 reduced active release of primary granules by BAL neutrophils, measured by surface CD63. **(B)** Soluble MMP9, **(C)** soluble NE, and **(D)** EV-bound NE measured in the BAL show that the significant increases given by LPS and control PLGA N-in-M were abrogated by Nexinhib20 delivery. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ between groups indicated.

## 4.6 Discussion

Intractable lung inflammation as seen in patients with ARDS, COPD, and CF is characterized by sustained and non-resolving PMN recruitment to airways, followed by active release of proteases in the extracellular milieu[24,25]. Accumulation of these PMN-derived proteases, such as NE and MMP9, leads to the saturation of the antiprotease shield and unopposed proteolytic action, resulting in structural lung damage and decline in lung function[26,27]. To date, standard of care relies on the prescription of anti-inflammatories delivered systemically or on inhaled corticosteroids, which do not target the neutrophilic component of the inflammatory response. The development of improved therapies targeting neutrophilic airway inflammation is hampered by the hydrophobic nature of most drugs and by the difficulty to deliver drugs to PMNs into the airways. Furthermore, the highly proteolytic microenvironment in diseased areas of the lungs represents an additional challenge to the effective delivery and preservation of the efficacy of drugs [28-30]. However, pulmonary drug delivery offers an alternative administration route that allows for site-specific delivery of therapeutics for respiratory airway disease. Also, efficient localized delivery to the airways can reduce the overall dose needed and potential systemic toxicity of drugs.

Our findings establish a novel and specific delivery method for hydrophobic drug administration to airway PMNs. The protease-sensitive design of the N-in-M system not only allows the microgel to provide the needed aerodynamic size for deposition and geometric size to avoid rapid clearance (< 1 h), but it exploits the high proteolytic microenvironment for controlled release of drug-loaded nanoparticles. Furthermore, encapsulation of the drug in the nanoparticle shields it and preserves its therapeutic potential. As a proof-of-concept, we demonstrated the effective delivery of the highly hydrophobic drug Nexinhib20 utilizing the NE-sensitive N-in-M system to

airway PMNs, leading to the reduction in exocytosis, NE release, and downstream inflammation.

While the therapeutic platform presented here was designed to be effective in presence of NE, it presents ample opportunities for customization as the protease-sensitive peptides embedded in the microgel can be tailored to plethora of cleavage sites specific to other enzymes. Moreover, the N-in-M system can be made to accommodate virtually any drug. Here, we chose to include Nexinhib20, as its main target is actually exocytosis and downstream NE release. Therefore, we accomplished efficient NE-dependent delivery of a drug targeting the machinery responsible for the release of NE itself, which led to the resolution of inflammation, eventually preventing further degradation of new microgels (self-limiting anti-inflammatory therapy). In conclusion, our findings establish the clear potential of N-in-M delivery systems to effectively target inflammation in pulmonary disorders characterized by unrelenting neutrophilic influx activation. Importantly, this platform can provide an appropriate mechanism to strategically deliver a variety of therapeutics in the lung and other organs with high proteolytic burden (e.g., inflamed gut, joint, and solid tumors).

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# Chapter 5

## Summary and perspectives

## 5.1 Summary of thesis findings

Neutrophil recruitment to the airways is a hallmark of CF lung disease. In addition to their chronic recruitment into the airways, neutrophils display profound changes in their phenotype, dubbed GRIM, including active of primary granule release [1, 2], reduced bacterial clearance [3], metabolic changes following activation of the anabolic mTOR pathway [4, 5], increased pinocytic activity [3], and immunomodulatory functions toward other immune subsets [6].

The findings presented in this thesis showcase significant advances in clinical and basic science. First, we illustrated the presence of GRIM neutrophils in the airways of infants with CF, as early as 3 months of age, and their relationship with macrophage exhaustion even in absence of infection (**Chapter 2**). Second, we defined a new mechanism used by airway neutrophils to adapt to the CF airway microenvironment that challenges the current paradigm holding these cells as preprogrammed and terminally differentiated after they leave the BM (**Chapter 3**). Third, we developed a new efficient delivery system to target neutrophil-driven lung inflammation that lacks systemic adverse effects (**Chapter 4**). Implications for our current collective knowledge and perspectives opened by these findings are discussed below.

## 5.2 Clinical implications and perspectives

As of today, the main cause of morbidity and mortality in patients with CF is lung disease. Development of lung damage occurs over time and includes appearance of bronchiectasis (thickening of the bronchi and bronchiolar walls), air trapping due to accumulation of mucus, bacteria and immune cells, and atelectasis (loss of lung volume caused by partial collapse), leading to hypoxia and loss of lung function [7-9]. Therefore, active prevention and modulation of the biological events responsible for lung pathology is crucial in patients with CF.

A major pathological path leading to lung damage is the chronic recruitment of neutrophils, followed by the active release of damaging mediators, such as NE. Our laboratory has now firmly established the role of a new acquired phenotype of neutrophils, dubbed GRIM, in this pathological process [10, 11]. Early detection of neutrophilic airway inflammation is therefore crucial to better adapt clinical management of the patients. Here, we showed that presence of GRIM neutrophils occurs shortly after birth, with some patients bearing these cells in their airways as early as 3 months of age. However, current clinical practices for the detection of airway inflammation and lung damage in infants with CF rely on invasive procedures, including bronchoscopy and bronchoalveolar lavage (BAL) performed under general anesthesia, and computed tomography (CT) scans that deliver low, but significant doses of ionizing radiations to generate detailed structural images of the lung.

Future studies are needed to address several technical issues and biological questions opened by our findings. First, the validation of less invasive techniques against the current standards will be necessary before their implementation. To this end, biomarkers measured in the BAL fluid could be replaced by those in less invasive samples such as induced sputum (IS), and/or exhaled breath condensate (EBC). We previously showed that presence of methionine sulfoxide (MetO) in the BAL of infants with CF correlated with lung damage and presence of myeloperoxidase, another enzyme contained in the primary granules that is required for the synthesis of MetO [12]. Thanks to its chemical nature, MetO could be a good candidate EBC biomarker for the assessment of neutrophilic inflammation and lung damage. Likewise, other bacterial or host-derived byproducts could be assessed to determine presence and levels of infection and inflammation in the CF airways. Furthermore, cells and soluble mediators recovered from IS could be phenotyped and quantified using the same analytical techniques that we and others have successfully applied to BAL. Lastly,

current efforts are directed at reducing the amount of radiation delivered to the patients during CT scans, and both magnetic resonance imaging (MRI) and lung clearance index (LCI) techniques are assessed as potential alternatives to CT scans [13]. Moreover, while CF disease is unevenly distributed among the small airways (with healthy bronchioles adjoining diseased areas), it currenlty is quantified by a global score (PRAGMA-CF) that does not report regional differences. To begin with, a scoring method able to discriminate between the different lobes of the lung could match more precisely local areas probed by BAL and thereby derive lobe-specific values for disease biomarkers. While less invasive, the newer techniques described above come with their own challenges and limitations. For example, the yield of IS samples in infants can be highly dependent upon the skills of the medical practitioner conducting the collection. EBC is easier to collect, but results in high dilution of biomarkers and is subject to potential contamination from the environment. With regards to LCI, it is based on the inhalation of a mixture of trackable gases, which must be proven safe for the patients and reliable for clinical assessments [14-16]. Lastly, the choice between MRI or CT scans may be affected by access to equipment, the ability to implete newer, improved metrics, as well as healthcare costs.

The two studies presented in **Chapter 2** show presence of GRIM neutrophils, exhaustion of resident macrophages, and their relation to disease state in a very young CF patient population. These data are cross-sectional and thus do not follow progression of lung disease within each patient. To better define factors implicated in the loss of structural integrity and function of CF lungs, longitudinal studies using similar multiparametric phenotyping of the airway milieu and immune cells as used in our cross-sectional studies will be needed. Ideally, such longitudinal studies will identify a multivariate score of clinical and biological measures able to predict CF lung disease progression of and help develop evidence-based personalized patient care.

*Introduction of CFTR modulators and correctors*

With the recent introduction of highly efficient molecular therapies aimed at modulating CFTR, the anion channel that is dysfunctional in CF, changes in the pathological airway microenvironment will likely occur [17, 18]. Early reports on the effect of ivacaftor, the first of these molecular therapies to be introduced, show an increase of 10-15% in lung function after administration. However, the rate of lung decline, presence of pro-inflammatory pathogens and inflammatory markers remain mostly unaffected, suggesting an acute therapeutic effect but less clear long-term benefits [19-21]. Furthermore, costs associated with these new therapies are predicted to amount to several billion dollars in additional healthcare costs [22, 23]. Therefore, the development of alternative approaches targeting the inflammatory component of the disease is needed. To this end, the discovery of the transcriptional dependency in GRIM neutrophils (**Chapter 3**) offers a window of opportunities for RNA-based therapies, which could be delivered by the novel system presented in **Chapter 4**. Finally, the approval of these new drugs for the pediatric population is unlikely oto ccur before a decade and their effect on the inflammatory component of the disease will have to be thoroughly assessed. Thus, studies presented here focused on advanced phenotyping tools to characterize the early development of CF airway disease can be leveraged to determine the effectiveness of future treatments when applied to pediatric CF cohorts.

## 5.3 Basic scientific implications and perspectives

*Inflammatory mechanisms of early CF*

The inflammatory microenvironment in the airways of adult patients with CF is dominated by neutrophils. Our studies in young CF children suggest that this

neutrophil-driven inflammatory response is initiated where macrophages and T cells are still present in the lung lumen. Over the course of early CF lung disease, this cellular heterogeneity is lost and neutrophils become the most abundant inflammatory subset in CF airways. This shift between immune cell populations is likely regulated by soluble factors and cell-cell interactions [24-27]. Findings presented in **Chapter 2** suggest a role for NE and for the PD1 signaling axis in the interplay between neutrophil and macrophage populations. However, while our results imply an inhibitory relationship between these two cell types, future studies will be needed to untangle the mechanisms at play and define signaling pathways at play in both cell types. Moreover, we previously showed that in the context of the CF airway microenvironment, T cells are inhibited by extracellular arginase-1 secreted by airway neutrophils. While inhibition by arginase-1 accounts for 60% in the inhibition of proliferation seen upon T-cell exposure to CF ASN, pathways responsible for the remaining 40% of inhibition remain to be identified [6]. Neutrophil-driven exclusion of T cells from the CF airway lumen may be a protective mechanism to avoid autoimmune complications that may derive from the accumulation of proteolyzed and oxidized autoantigens in the CF lung lumen [28, 29]. T-cell exclusion may also lead to suboptimal production of high affinity antibodies to CF pathogens, which depends on T cell-mediated B cell activation [30]. Future studies addressing these questions will be needed.

In addition to changes in the immune cell profile observed in CF airways, releaseds oluble mediators also play an important role in shaping this ecological landscape. As an example, enzymatic activity of arginase-1 affects the production of reactive nitrogen species by resident macrophages [31] and fuels bacterial growth by providing key nutrients such as polyamines [32]. Interestingly, recent studies suggest that infants with CF are less likely to spend extended times in a hospital due to viral infection compared to infants without CF [33], leading to the notion that the immune

response in the CF lung may be polarized towards anti-viral rather than an anti-bacterial state. In our in vitro transmigration model (**chapter 3**), GRIM neutrophils showed decreased capacity to clear bacteria and transcriptional enrichment in the TLR3 pathway, which regulates sensing of viral byproducts. Furthermore, other preliminary data from our group show that GRIM neutrophils efficiently clear respiratory syncytial virus (not shown), supporting a potential shift in their anti-microbial response. Of note, the rewiring of immune responses towards viruses and concomitant loss of bacterial clearance, if true, would not be unique to CF. For example, secondary bacterial infections following influenza virus exposure pose a major threat to the health of the host due to faulty immune polarization [34-37].

Lastly, findings presented in **Chapter 2** suggest the induction of a neutrophilic response prior to the clinical detection of pro-inflammatory pathogens in CF infants. The ontogeny of this response remains to be determined, however, recent findings suggest that neutrophils may be recruited to the CF airways upon hypoxic stimulation of the epithelium by so-called mucus "flakes" [38]. Future studies focused on identifying key components of the CF airway fluid leading to the acquisition of the GRIM phenotype are needed. These studies will be key in developing host-directed therapies optimizing neutrophilic responses tailored to bacteria or viruses.

*Acquisition of the GRIM phenotype*

The compact chromatin state neutrophil display in blood and the inability to culture these cells *ex vivo* contributed to the conventional view that these cells are short-lived, terminally differentiated and with little opportunity for plasticity. Findings presented in this thesis (**Chapter 4**) strongly challenge this notion. However, while we showed profound transcriptional reprogramming in CF airway GRIM neutrophils, future studies will be necessary to precisely map rearrangements

of the chromatin and dynamics of *de novo* transcription in these cells. Identification of the key steps that allow the divergence between LTB4- and CF ASN-transmigrated neutrophils will help advance our basic understanding of neutrophil plasticity and identify new therapeutic targets. Lack of expression of certain genes suggests a tightly regulated access to certain areas of the chromatin in reprogrammed neutrophils. While most epigenetic markers can be modified to transition from a silent to an active chromatin state [39], it has been shown that certain regions of the chromatin can be irreversibly silenced [40, 41]. Identifying the factors leading to the closure of those regions, even during a transcriptional burst, could be useful for the development of therapeutically induced closed chromatin regions in cells of interest. In certain cancers, for example, such tools could help modulate tumor aggressiveness.

It is important to note that neutrophils collecterd from CF airways in vivo display significant heterogeneity dictated in large part by the time spent in the airways. Additional preliminary findings extending those presented in **Chapter 4** suggest differential amounts of RNA content among GRIM neutrophils, with the CD16+ CD63+ subset showing the highest content (not shown). In future studies, analysis of single GRim neutrophils by RNA sequencing and epigenetic profiling will provide a deeper level of understanding of chromatin and mRNA dynamics shaping neutrophilic responses in the CF lung.

*Role of EVs*

As previously mentioned, acquisition of the GRIM phenotype by CF airway neutrophils is likely to occur independently from intrinsic CFTR-dependent defects, but it rather depends on exposure to components of the CF airway fluid. Preliminary data suggest that the transition from primed airway neutrophil to the GRIM state in neutrophils is dependent upon presence of extracellular vesicles (EVs) in the CF ASN

**(Figure 5.4.1)**. The ability of EVs to modulate cell behavior has been previously shown in a variety of homeostatic and pathological conditions [42-44]. EVs contain coding and non-coding RNAs, which can modulate the epigenetic and transcriptional poise of target cells[45-47], as well as specific proteins and lipids acting as signaling molecules and affecting target cell behavior [48]. Moreover, it has been recently shown that interactions between eukaryotes and prokaryotes rely in part on the exchange of EVs [49, 50]. The content of host and bacterial EVs of CF ASN and the relative impact of these EV subtypes on the development of the GRIM phenotype and of disease pathogenesis clearly warrants further study.

## 5.5 Conclusion

In conclusion, the body of work presented in this thesis provide strong impetus for future studies. From a basic scientific standpoint, we showed presence of GRIM neutrophils in CF airway as early as 3 months of age, and identified their relationship to progressive macrophage exhaustion and structural lung damage. Moreover, our findings provide a starting point for exploration of the transcriptional reprogramming occurring in these cells. From a translational standpoint, drug screening on in ivtro produced GRIM neutrophils will lead to the identification of novel host-directed approaches targeting airway inflammation. Such approaches could help circumvent issues with CFTR-directed therapies, and the development of antibiotic resistance by the pathogens colonizing CF lungs. Beyond CF, our preliminary data showing the presence of transcriptionally active GRIM-like neutrophils in other inflammatory syndromes further broadens the potential impact of the new findings presented here.

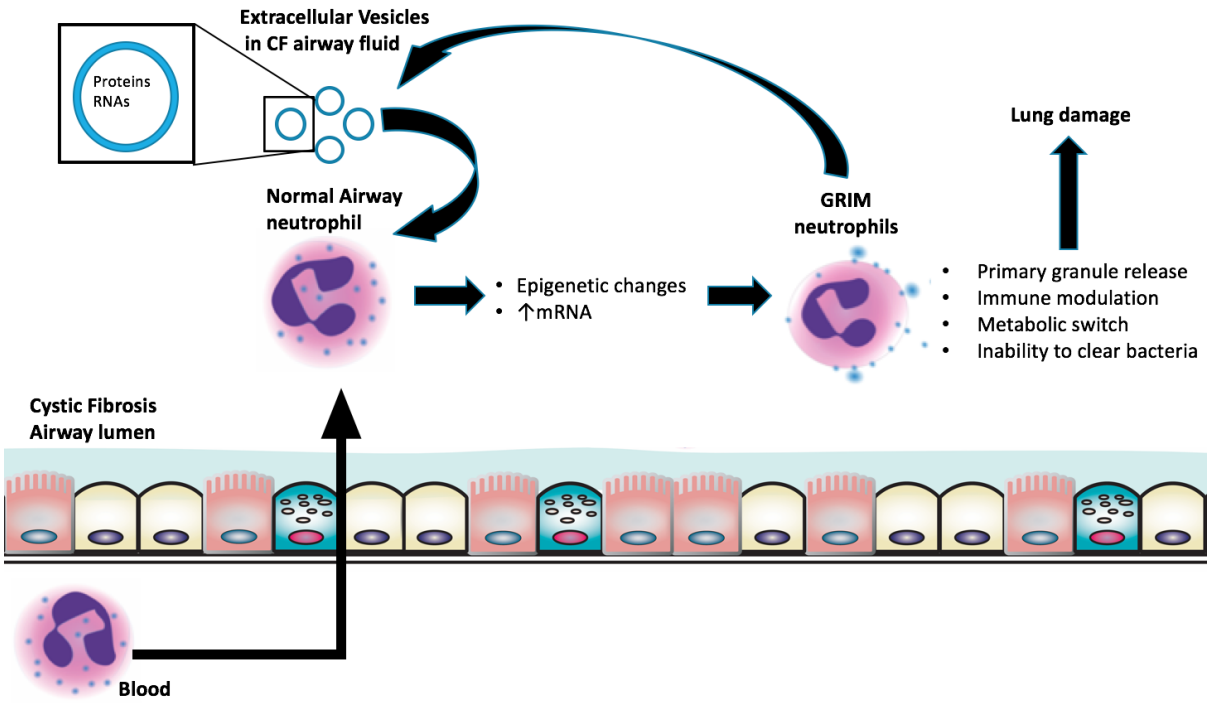


**Figure 5.4.1 Working model of the events leading to the acquisition of the GRIM phenotype by CF airway neutrophils.** Blood neutrophils are recruited to the small airways through epithelial stimulation (sterile inflammation). Migration into the airway lumen leads to their priming (as seen in LTB4-transmigrated neutrophils), after which, upon encounter of extracellular vesicles in the CF airway microenvironment, primed airway neutrophils initiate transcriptional and epigenetic changes typical of the GRIM phenotype. The result of the burst in *de novo* transcription leads to active release of primary granules which is linked to lung damage. As a potential path responsible for the perpetuation of neutrophilic inflammation, GRIM neutrophils produce and release pathological EVs, which will in turn condition the new wave of recruited cells into becoming GRIM.

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