Review

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Neutrophils in cystic fibrosis

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Abstract: Cystic fibrosis (CF) lung disease is characterized by chronic infection and inflammation. Among inflammatory cells, neutrophils represent the major cell population accumulating in the airways of CF patients. While neutrophils provide the first defensive cellular shield against bacterial and fungal pathogens, in chronic disease conditions such as CF these short-lived immune cells release their toxic granule contents that cause tissue remodeling and irreversible structural damage to the host. A variety of human and murine studies have analyzed neutrophils and their products in the context of CF, yet their precise functional role and therapeutic potential remain controversial and incompletely understood. Here, we summarize the current evidence in this field to shed light on the complex and multi-faceted role of neutrophils in CF lung disease.

Keywords: CFTR; cystic fibrosis; inflammation; lung; neutrophils; PMN.

Introduction

Neutrophils represent 50–70% of leukocytes in the human blood and serve as key sentinels against invading pathogens. They form the first barrier during host defense and participate in diverse processes of the inflammatory response (Kruger et al., 2015). In the genetic disorder cystic fibrosis (CF), an early and sustained recruitment of neutrophils to the airways occurs in response to pathogens and/or CF-dependent inflammatory mediators (Hartl et al., 2012). In the past decade, numerous studies demonstrated the potential role of neutrophils in the course of CF lung disease, notably through neutrophil-driven compounds, such as neutrophil elastase (NE), whose activity negatively correlates with lung function in CF patients (Tirouvanziam, 2006; Mayer-Hamblett et al., 2007). Gene expression profiling of CF blood and airway neutrophils revealed a modulation of a distinct set of genes involved in chemotaxis, priming and lifespan, suggesting a profound activation of these cells in CF disease (Adib-Conquy et al., 2008). However, despite these intriguing previous findings, the precise regulation and role of neutrophils in the course of CF lung disease remains controversial and poorly defined. In this review, we focus on the complex and evolving role of neutrophils in CF lung disease.

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Biogenesis and lifespan of human neutrophils

Granulopoiesis

In human adults, neutrophils are produced and released from the bone marrow at an estimated rate of ~10^9 cells/kg/day, and thus represent the most abundant immune cells in the circulation (Bugl et al., 2012). Neutrophils originate from hematopoietic stem cells, and downstream, from myeloid common progenitors and myeloblasts. After these successive stages of myelopoiesis, granulopoiesis progresses through pro-myelocyte, myelocyte, meta-myelocyte and band cell formation before mature neutrophils are generated. Fully mature neutrophils encompass three distinct granule types in addition to secretory vesicles, all of which are formed by carefully timed waves of protein biosynthesis and packaging during differentiation (Borregaard et al., 1995). Indeed, a stepwise transcriptional program involving CCAAT/enhancer binding protein (C/EBP) and PU.1 transcription factors has been shown to regulate the successive generation of azurophil (primary), specific

(secondary) and gelatinase (tertiary) granule proteins (Theilgaard-Mönch et al., 2005). Neutrophil granules contain effector proteins required for the recruitment to the site of infection, anti-microbial functions, and the resolution of inflammation. Upon priming and activation, neutrophil degranulation is initiated, starting with secretory vesicles, followed by gelatinase, specific and azurophil granules (the latter are not as easily mobilized to the surface) (Borregaard et al., 2007). Granulopoiesis is a tightly regulated process, which during inflammation can switch to an 'emergency' state. Emergency granulopoiesis leads to an accelerated neutrophil production and egress from bone marrow, inducing systemic neutrophilia (Terashima et al., 1998; Cain et al., 2011).

Lifespan

Neutrophil homeostasis is dependent on their production (7–10 day long process), egress from the bone marrow, circulation in blood, extravasation and clearance. The granulocyte colony-stimulating factor (G-CSF) modulates progenitor mobilization and differentiation, as well as the release of mature neutrophils (Lieschke et al., 1994; Richards et al., 2003). G-CSF regulates neutrophil egress from the bone marrow by inducing a downregulation of the CXCL12-CXCR4 interaction, which keeps developing neutrophils tethered to the stroma (Semerad et al., 2002; Summers et al., 2010). While retention signals delivered by CXCR4 are downregulated, CXCR2 expression increases, rendering mature neutrophils more sensitive to peripheral chemokine (CXCL8 or IL-8) gradients (Eash et al., 2010; Nauseef and Borregaard, 2014). In absence of inflammation, newly released neutrophils have a short lifespan estimated to last <24 h in humans, with a half-life estimated around 8 h (Dancey et al., 1976). However, this concept is still controversial, since a half-life of 3.8 days, using *in vivo* deuterium labeling, has been proposed for human neutrophils (Pillay et al., 2010; Tofts et al., 2011). Although regulation of apoptosis in neutrophils needs to be further explored, it is an essential process promoting the resolution of inflammation and notably neutrophil clearance or efferocytosis by scavenger macrophages and dendritic cells. Related to CF, Moriceau et al. described that neutrophils collected from heterozygote or homozygote subjects for CFTR mutations, presented delayed apoptosis and lower expression of surface phosphatidylserine and caspase-3 activity than neutrophils from healthy subjects carrying two functional alleles (Moriceau et al., 2010).

Neutrophil functions

Chemotaxis and diapedesis

After leaving the bone marrow, circulating neutrophils migrate towards chemotactic gradients in order to enter into tissues. This process of leukocyte extravasation involve adhesion, tethering and rolling through neutrophil interactions with inflamed endothelial cells via selectins (Borregaard, 2010; Amulic et al., 2012). Subsequent steps of neutrophil diapedesis have been shown to involve β_2-integrins and intercellular adhesion molecules (Kolaczkowska and Kubes, 2013). β_2-integrin-independent migration can occur in microenvironments where blood capillary diameter is smaller than that of the circulating neutrophils (Doerschuk et al., 2000). In CF, early and persistent neutrophil recruitment is a hallmark of airway inflammation. This sustained and non-resolving neutrophil recruitment is believed to be modulated by altered responsiveness to chemoattractants and lipid mediators and/or microbial colonization (Conese et al., 2003; Downey et al., 2009; Hayes et al., 2011).

Killing mechanisms

Once recruited to the site of infection, neutrophils deploy a wide range of removal mechanisms and toxic compounds that together promote pathogen killing. Phagocytosis is the most rapid and major mechanism for pathogen uptake and clearance. It involves the engulfment of microbes through Fc-, complement- and/or pattern-recognition receptor (PRRs)-mediated uptake into a specialized compartment called 'phagosome' (Underhill and Ozinsky, 2002). Once the phagosome is formed, it matures by intracellular fusion with neutrophil secondary and primary granules, thus becoming a phagolysosome. NADPH oxidase subunits get assembled at the phagolysosomal membrane and reactive oxygen/nitrogen species (ROS/RNS) production is triggered, modulating pH levels and proteolytic enzyme activity (e.g. NE) (Jankowski et al., 2002). Myeloperoxidase (MPO), also from primary granules, transforms the hydrogen peroxide produced by NADPH oxidase into the potent antimicrobial hypochlorous acid (HOCl) (Winterbourn et al., 2006). Neutrophil granules can also fuse with the plasma membrane, thereby releasing their content in the extracellular milieu, a process named exocytosis or degranulation. Numerous antimicrobial molecules and proteases can thus be released, especially those contained in secondary

and primary granules (e.g. defensins, lysozyme, lactoferrin, NE, MPO) (Nathan, 2006). NADPH oxidase can also assemble at the neutrophil surface, which enables ROS/RNS production and release into the extracellular milieu (El-Benna et al., 2008). In the past decade, another neutrophil-killing mechanism has been described, which consists of an extracellular DNA mesh formation that traps and kills microorganisms by bringing them in close physical contact with neutrophil effectors (Brinkmann et al., 2004). This process of extracellular trap formation, or NETosis, can be accomplished with DNA from the nucleus (associated with cell death) or from the mitochondria (Fuchs et al., 2007; Yousefi et al., 2009), both leading to the extracellular localization of histones as well as secondary and primary granule enzymes (all cationic) with anionic DNA (Wang et al., 2009; Papayannopoulos et al., 2010). This emerging mechanism has been considered as a killing modality used by neutrophils in acute and chronic infections. However, pathogen-killing efficacy during NETosis remains controversial since it was shown that *Staphylococcus aureus* released from *in vitro*-induced NETs were still viable (Nauseef, 2012; Parker et al., 2012). This skepticism is further supported by a recent study showing that serine proteases, critical for NETs formation, are not essential for neutrophil-mediated bacterial killing in a patient affected by Papillon-Lefèvre syndrome (Sørensen et al., 2014). Also, several studies have now provided evidence for NETosis or NETosis-like structures in inflammatory lung diseases (Cheng and Palaniyar, 2013). Regarding CF, separate investigations were performed to further corroborate the concept of NET formation in CF airway fluids (Papayannopoulos et al., 2011; Manzenreiter et al., 2012; Dwyer et al., 2014; Rahman and Gadjeva, 2014; Marcos et al., 2015). Young and coworkers demonstrated that peripheral blood neutrophils derived from CF patients did not show an altered NETosis compared to healthy control neutrophils (Young et al., 2011). The latter study also demonstrated that NET-mediated killing depends on the stage of CF lung disease, since NET-mediated killing was effective against bacteria isolated from patients with early, but not late CF lung disease. In some previous reports, diminished phagolysosomal function in neutrophils and macrophages, and impaired bacterial killing have been attributed to CFTR deficiency (Painter et al., 2006, 2008, 2010). In contrast, Young and colleagues have shown a CFTR-independent mechanism of NET-mediated bacterial killing, further supporting the notion that killing efficiency of CF neutrophils is preserved, but possibly affected by the adapted isolates of *Pseudomonas aeruginosa* during disease progression.

Neutrophils in cystic fibrosis

CFTR in neutrophils

Since the first data showing detectable, albeit low, *CFTR* mRNA expression in neutrophils (Yoshimura et al., 1991), only few studies have specifically explored CFTR expression and function in these innate immune cells. However, in the past decade, combined *in vitro* and *in vivo* studies, both in mice and humans, demonstrated that the absence or dysfunction of CFTR in neutrophils impairs bacterial killing and disease outcome (Painter et al., 2006; Bonfield et al., 2012; Ng et al., 2014). These studies also demonstrated the localization of CFTR at the membrane of phagolysosomes, which was shown to be essential for chloride transport and was linked to bacterial protein chlorination during phagocytosis. Additionally, Painter et al. described CF neutrophils as deficient in intraphagolysosomal, but not extracellular HOCl production. Therefore, the defect in intraphagolysosomal chlorination of CF neutrophils associated to the absence of CFTR at the phagolysosomal membrane could be explained by impaired organelle-specific function of the mutated CFTR protein. Moreover, other studies showed that CF neutrophils exhibit a disturbed ion homeostasis (increased cytosolic levels of chloride and sodium and decreased levels of magnesium) associated with an impaired degranulation of secondary and tertiary granules (Pohl et al., 2014). Importantly, this neutrophil defect was partially restored in patients with the G551D mutation by the ion channel potentiator ivacaftor (Zhou et al., 2013; Pohl et al., 2014). Despite these intriguing findings, more investigations on CFTR function in neutrophils are required to precisely understand the role of CFTR in neutrophil homeostasis and effector functions.

Circulating neutrophils

Several studies focused on systemic factors that could alter neutrophil chemotaxis and migration in CF. CFTR dysfunction was found to have no effect on neutrophil transmigration across epithelial monolayers (Pizurki et al., 2000). Alternatively, chemotaxis in CF might be impaired due to differential responsiveness to stimulants or chemoattractants. Previous studies have suggested that the lipoxygenase pathway and resulting leukotrienes are involved in CF lung disease (Saak et al., 1990). Leukotriene B4 (LTB_4) has been further associated with impaired neutrophil chemotaxis in patients

with rheumatoid arthritis, chronic bowel disease and CF (Lawrence and Sorrelli, 1992). Neutrophil chemotaxis is positively regulated by chemokines, prototypically IL-8, the main inflammatory mediator in CF produced by both epithelial and immune cells. Serum protein levels of IL-8 were found highly increased in CF patients when compared to controls (Dean et al., 1993). In addition, NE from CF airway fluid induced IL-8 production by epithelial cells, suggesting a positive feedback loop between NE and IL-8-mediated neutrophil recruitment (Nakamura et al., 1992; Hartl et al., 2007). Likewise, IL-8 receptor inhibition using specific ligand Sch527123 suppressed pulmonary neutrophilia *in vivo,* which further highlights the major role of IL-8 in promoting neutrophil accumulation in CF airways (Chapman et al., 2007). In addition to IL-8, the pro-inflammatory cascade in CF involves increased levels of TNF-α, IL-1α/β, IL-6 and lower levels of IL-10, depending on the patient cohort and lung disease severity (Sagel et al., 2002). Moreover, Corvol et al. demonstrated that CF blood neutrophils, *in vitro*, could spontaneously secrete higher level of IL-8 and lower level of the anti-inflammatory IL-1 receptor antagonist (IL-1Rα) than neutrophils from control subjects (Corvol et al., 2003).

Beyond cytokines and chemokines, MPO-related oxidative burst activity was shown to be altered in CF blood homozygote and heterozygote neutrophils when compared to controls, which is in line with the role of CFTR in halide transport into phagolysosomes and HOCl formation (Witko-Sarsat et al., 1996; Painter et al., 2006; Zhou et al., 2013). Furthermore, high extracellular chloride concentration was shown to have an impact on neutrophil function through increase of IL-8 synthesis and decrease in *P. aeruginosa* killing *in vitro* (Tager et al., 1998). In addition, Terada et. al. showed that *P. aeruginosa* compounds have the ability to suppress neutrophil respiratory burst (Terada et al., 1999), which could explain the previously described heterogeneity in neutrophil oxidative burst activity among CF patients (Frühwirth et al., 1998). By contrast, a more recent study described that blood neutrophil ROS production, through NADPH oxidase activity, was equivalent in control subjects and ΔF508 homozygote CF patients that were infected with *P. aeruginosa* (McKeon et al., 2010). Taken together, these data suggest that the different oxidative burst phenotypes observed in CF blood neutrophils primarily relate to MPO activity, implying modulation by surrounding pathogens and CFTR expression.

Recruitment of neutrophils from blood to airways can also be modulated through surface adhesion molecules/integrins and other surface receptors. While the expression of the adhesion molecule CD11b was not altered on circulating CF neutrophils, shedding of L-selectin at the cell surface upon stimulation was found to be decreased (Russell et al., 1998). Besides integrins, Toll-like receptors (TLRs) have been studied in CF patients and changes in TLR2, TLR4 and TLR5 expression were showed (Koller et al., 2009). Upon priming and activation, neutrophils in CF displayed a lower expression level of TLR2 when compared to healthy controls. This distinct pattern was associated with an inverse correlation between TLR2 at the neutrophil surface and elevated TNF-α concentration in CF blood (Petit-Bertron et al., 2008). Interestingly, proline-glycine-proline (PGP) and high mobility group box protein-1 (HMGB1), bioactive mediators found at high concentration in CF airways, were identified as non-canonical neutrophil ligands (acting through CXCR2 and TLR4 receptors, respectively) able to modulate neutrophil influx and pulmonary inflammation (Lotze and Tracey, 2005; Weathington et al., 2006; Gaggar et al., 2010). These recently characterized molecules represent potential biomarkers for neutrophilic inflammation in CF lung disease (Reilly et al., 2009; Gaggar et al., 2010; ten Hoeve et al., 2012).

Acidification of the intra- and extracellular milieu is another factor that was shown to regulate neutrophil function and fate. Notably, both the cytosolic pH and the inflammatory microenvironment in CF were identified as critical regulators of neutrophil cell death following phagocytosis (Coakley et al., 2002). Moreover, the observed delay in neutrophil apoptosis in CF (Moriceau et al., 2010) seems to be an acquired effect rather than intrinsic, since incubation of control neutrophils with CF sera could mimic this effect (McKeon et al., 2008). Similarly, blood neutrophils from CF patients and healthy subjects were equally able to induce NETs and kill *P. aeruginosa in vitro,* suggesting that *P. aeruginosa* resistance to neutrophil killing might be dependent on the bacterial phenotype found in CF airways (e.g. mucoid form), rather than on an intrinsic defect in NETosis (Young et al., 2011).

The CF airway microenvironment

CF airway disease is characterized by the chronic presence of bacteria and fungi and a massive and sustained neutrophil recruitment to the lungs (Davis et al., 1996). In response to whole pathogens or distinct pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), both epithelia and immune cells, induce a pro-inflammatory cascade with high secretion of cytokines and chemokines (Bonfield et al., 1999). This pro-inflammatory non-resolving process results in bronchiectasis leading to a progressive decline in respiratory function and destruction of the lung tissue

(Hartl et al., 2012; Rieber et al., 2014). The primary genetic defect in CFTR function causes changes in Cl^- and Na^+ conductance leading to airway surface liquid (ASL) dehydration, increased thickness of the mucus layer and impairment of mucociliary clearance (Matsui et al., 1998). These primary dysfunctions have been associated with increased bacterial attachment to the epithelium and the development of hypoxia, favoring the growth of anaerobic pathogens and inflammation of the airways (Worlitzsch et al., 2002). Early bacterial infection is mainly caused by *S. aureus* and *Haemophilus influenzae*. This picture evolves and changes over time, with more than 80% of CF teenagers showing chronic *P. aeruginosa* infection (Tingpej et al., 2007). Colonizations and/or infections with other 'emerging bacteria', such as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cepacia* increase upon patient aging, but are less frequent overall (Hartl et al., 2012). Adaptation of bacteria to CF airways is achieved through production and secretion of exopolysaccharides, which are essential to biofilm formation. Particularly, alginate is secreted by *P. aeruginosa* when bacteria acquire a mucoid resistant phenotype associated with the loss of virulence factors and motility (Costerton, 1999; Singh et al., 2000). Biofilms have been associated with increased resistance to antibiotic treatment, protection from phagocytosis and other anti-bacterial responses mounted by recruited neutrophils (Meluleni et al., 1995; Jesaitis et al., 2003), which renders mucoid *P. aeruginosa* difficult to eradicate. In addition, CF airways are enriched in elastolytic proteases that have been involved in biofilm clearance deficiency through the inactivation of neutrophil lactoferrin (Rogan et al., 2004). In addition to bacteria, mycobacteria, yeast and filamentous fungi, such as *Aspergillus fumigatus*, colonize the airways of CF patients. The latter is responsible for a complex allergic syndrome, termed allergic bronchopulmonary aspergillosis (ABPA) (Stevens et al., 2003; Moss, 2010). Besides bacteria and fungi, respiratory viral infections have been associated with a higher decrease in lung function and higher probability of hospitalization in CF patients (Hiatt et al., 1999; van Ewijk et al., 2005). Therefore, the chronic inflammation characteristic of the CF airway disease is linked to persistent infection- and neutrophil-driven tissue damage (Rieber et al., 2014).

Airway neutrophils: hallmark of CF lung inflammation

Inflammation is observed early in the course of the disease and several studies showed higher IL-8 levels and significant neutrophil recruitment prior to overt infection in both CF infants and CF human fetal xenografts (Tirouvanziam et al., 2000, 2002; Rosenfeld et al., 2001; Armstrong et al., 2005). Nevertheless, the presence of significant inflammation before infection in CF airways remains a controversial hen-egg issue. CF airway disease is characterized by oxidative stress. Therefore, the ability of neutrophils in producing ROS was assessed, and flow cytometry analysis showed that airway neutrophils could not generate as much ROS as their blood counterpart (Houston et al., 2013). This lack of function could be explained by a spontaneous release of ROS directly upon entry into CF lungs, in concordance with the potential impact of CFTR on neutrophil MPO activity and respiratory burst (Witko-Sarsat et al., 1996). In response to pathogens, notably *P. aeruginosa*, attracted airway neutrophils release their powerful anti-microbial compounds, such as NE or MPO. NE burden in CF has been associated with the presence of IL-8 and TNF-α and the use of blocking antibodies to these inflammatory mediators abrogated NE secretion from CF blood neutrophils (Taggart et al., 2000). IL-8 secretion in CF airways stimulates recruitment and activation of blood neutrophils, notably through CXCR1 and CXCR2. However, the abundance of neutrophil proteases, mainly NE, in CF lungs cleaves and impairs CXCR1, which leads to bacterial killing deficiency and sustained inflammation (Hartl et al., 2007). The chronic inflammation induces an imbalance between proteases and their inhibitors, responsible for the early proteolytic stress in CF airways (Birrer et al., 1994). The activity of NE negatively correlates with CF lung function and is associated with decreased immune responses (Mayer-Hamblett et al., 2007; Griese et al., 2008). NE also cleaves elafin and the secretory leucoprotease inhibitor (SLPI), both involved in anti-microbial and anti-inflammatory responses (Guyot et al., 2008; Weldon et al., 2009). The capacity of neutrophils to combat and eradicate infections in CF has been investigated for a long time. Morris and colleagues demonstrated that CF airway neutrophils had a lower phagocytic capacity than CF blood neutrophils and both blood and airway neutrophils from healthy subjects, suggesting an extrinsic effect of the inflammatory environment in CF lungs (Morris et al., 2005). Neutrophils in CF airways expressed moderate levels of the LPS receptor TLR4, but remarkably high amounts of TLR5, involved in recognition of bacterial flagellin (Koller et al., 2008, 2009). Levels of free DNA in the CF airway microenvironment were found to correlate with lung function (Marcos et al., 2015). However, besides NETosis-derived DNA other sources of extracellular DNA (such as neutrophil necrosis) probably also contribute to this finding.

New paradigm of airway neutrophils

An early, massive and sustained recruitment of blood neutrophils into airways characterizes CF lung disease. The prevailing view is that incoming CF airway neutrophils: (i) are unable to clear infection; (ii) all undergo necrosis; (iii) release passively their granule contents and other toxic material such as oxidants, actin and DNA (Coakley et al., 2002). Earlier studies, analyzing neutrophil-associated factors in CF airway fluids therefore suggested that neutrophils are passive actors in the disease process (Walker et al., 2005). However, several recent studies assessing CF blood and airway samples collected from patients *ex vivo*, showed that CF neutrophils, still viable, maintained oxidative burst activity, ROS production and granule release, leading to oxidative stress in both systemic and airway compartments (Tirouvanziam et al., 2006). Importantly, this substantial fraction of live neutrophils found within CF patients' sputum displayed phenotypical characteristics reflecting mobilization of secretory vesicles, tertiary granules and secondary granules (based on surface CD11b and CD66b expression) when compared to their blood counterparts. However, a cardinal feature of CF is that these live airway neutrophils also express very high levels of CD63 at their surface, a marker indicating highly active exocytosis of primary granules by these cells and the concomitant active release of their toxic content, including NE and MPO, in the extracellular milieu. In CF airways, this process is correlated with the decrease in neutrophil surface expression of phagocytosis-associated receptors CD14 and CD16, both of which are targets for cleavage by extracellular NE (Tirouvanziam et al., 2008). Despite a major portion of necrotic neutrophils in CF airways, these recent evidences highlight the potential contribution of live neutrophils probably undergoing active degranulation, instead of just passively releasing granule content upon necrosis.

This neutrophil activation profile was further characterized by an up-regulation of the cAMP response element binding protein (CREB) pathway, another indicator of an active stress response. Indeed, in addition of higher level of phosphorylated CREB, CF airway neutrophils displayed increase surface expression of the receptor for advanced glycation endproducts (RAGE, a component of oxidant signaling) and the ecto-nucleoside triphosphate diphosphohydrolase (CD39, an enzyme hydrolysing extracellular ATP and regulating neutrophil responses), respectively activator and effector of this axis (Huttunen et al., 2002; Pulte et al., 2007). Moreover, in the same studies, airway fluids collected from CF patients presented lower levels of the decoy receptor (sRAGE) as well as an increase in S100A12 (one of the RAGE ligands) and purine concentrations, which both correlated with neutrophils count in CF lungs (Makam et al., 2009). These data suggest that these cells not only remain alive but also mount an active response to the stress conditions (high extracellular oxidant, DAMPs and ATP levels) reigning in CF airways.

Further investigations of CF airway neutrophil physiology characterized unconventional activation of these cells. As compared to blood, live CF airway neutrophils presented increased phosphorylation of elF4E, 4E-BP and S6 ribosomal protein, three canonical targets of the mammalian target of rapamycin (mTOR) (Makam et al., 2009). Activation of this specific anabolic pathway, combined with the high level of extracellular metabolites as glucose and amino acids (Barth and Pitt, 1996; Baker et al., 2007) suggest a metabolic modulation within CF airway neutrophils. Therefore, additional studies described that neutrophils migrating into CF airways can adapt to this enriched milieu by positively modulating their G-CSF receptor (CD114) (Makam et al., 2009) and their metabolite transporters, notably for glucose (GLUT1 or SLC2A1) and inorganic phosphate (PiT1 or SLC20A1) when compared to blood neutrophils. Interestingly, by looking directly into airway subsets, CF airway neutrophils displayed further regulation profile in PiT2 (or SLC20A2, another inorganic phosphate transporter) as well as in amino acid transporter (ASCT2 or SLC1A5), involved in the supplementation of essential amino acids regulating mTOR (Nicklin et al., 2009; Laval et al., 2013). Taken together, up-regulation of the anabolic pro-survival mTOR pathway and changes in surface receptors suggest that neutrophils homing to CF lungs undergo a concerted set of reprogramming processes. Moreover, live CF airway neutrophils showed increased levels of unusual neutrophil receptors, generally associated with antigen-presenting cell function (MHCII, CD80) and T-cell modulation (CD294, inhibiting apoptosis and mediating chemotaxis) (Tirouvanziam et al., 2008). More recently, CF airway neutrophils, with full mature phenotype, were described to release arginase 1, activate it through proteolytic cleavage associated with primary granule exocytosis, which deprive the milieu in arginine and actively modulate T-cell function. This study supports an active inhibition of T-cell response due to neutrophil activity in CF airways (Ingersoll et al., 2015). Thus, as shown in Figure 1, the pathological events occurring in CF airways appear to trigger specific reprogramming of these cells, suggesting that neutrophils are not just passive short-lived and catabolic cells, but rather stay viable and actively contribute to airway disease (Tirouvanziam,

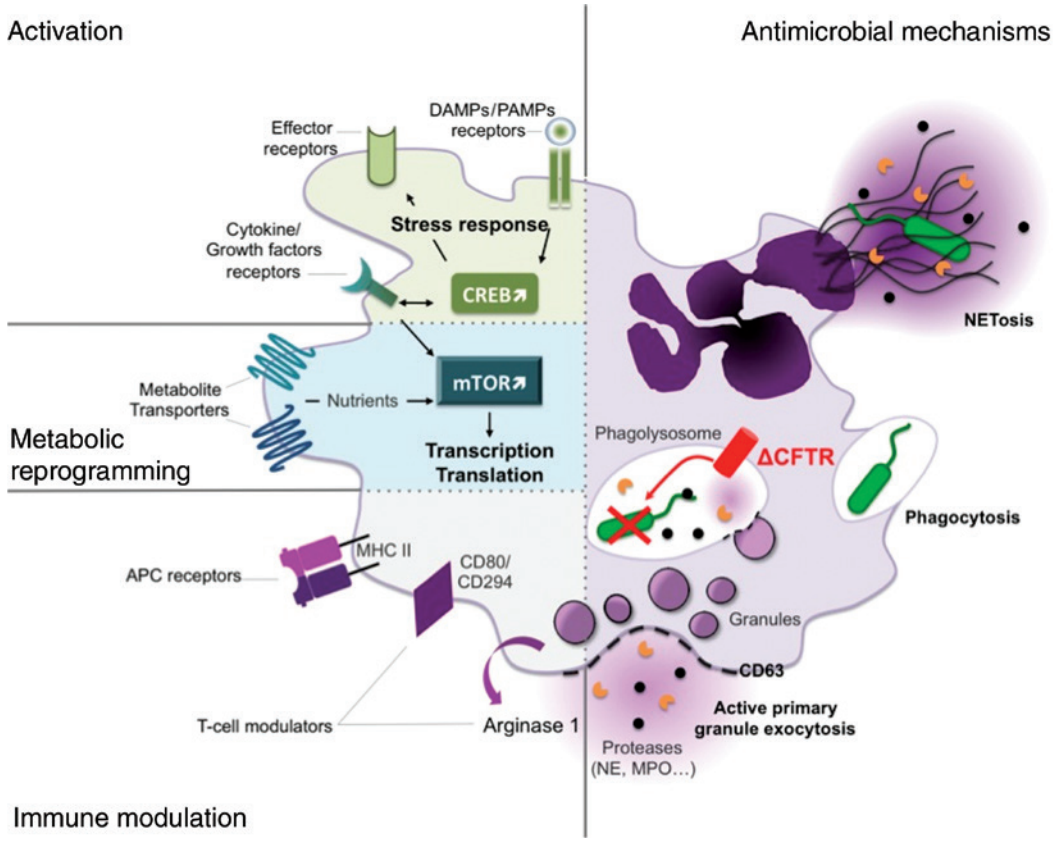

Figure 1: Revised paradigm for neutrophil dysfunction in CF airways.
By opposition to the conventional paradigm, this concept proposes that a fraction of upcoming neutrophils in CF airways are not passive and necrotic but rather stay alive. Therefore, these neutrophils organize a functional response to stress and display metabolic reprogramming and immune modulation concomitantly with active antimicrobial mechanisms. Regarding new discoveries about CFTR role in neutrophils, particularly in phagolysosomes, we underlined its potential impact on engulfed bacterial killing while the protein is mutated (ΔCFTR).

2006). Collectively, these results support a new/revised paradigm of neutrophils in CF airway disease, in which not only CFTR in epithelial cells and pathogens are substantially involved, but also neutrophils actively shape the course of disease.

Neutrophil-based targeting strategies and their implication for CF lung disease

Anti-inflammatory drugs that show clinical benefits in CF (steroids, ibuprofen, macrolides) probably interfere substantially with neutrophil activation, a concept deserving more appreciation and dedicated investigation.

Some limited therapeutic compounds acting on neutrophil inflammation have been evaluated for the treatment of the CF lung disease. Notably, the use of the oral antioxidant *N-acetylcysteine* showed beneficial effects in CF lung disease through reduction of NE activity, associated with increased or stable lung function (Tirouvanziam et al., 2006; Conrad et al., 2015). More specifically, therapies targeting neutrophil proteases using aerosolized protease inhibitors as AAT (α-1 antitrypsin) or SLPI were tested in clinical trials in CF patients. Despite the absence of substantial lung function improvements, the overall outcome of these studies was decreased neutrophil counts, IL-8 levels and NE activity, as well as an improvement in bacterial killing capacities (McElvaney et al., 1992; Birrer et al., 1994; Griese et al., 2007; Hartl et al., 2007). Finally, a more recent investigation evaluated the effect of directly targeting neutrophil recruitment by the use of a CXCR2 antagonist in a randomized, double blind clinical trial, involving 146 CF patients. The authors described a reduced neutrophil activation in CF sputum, notably through decreased neutrophil counts and NE and MPO activities when the inhibitor was used daily at the dose of 50 mg. While these anti-inflammatory

results were encouraging, CXCR2 inhibition has no effect on lung function. Moreover, the associated increase in blood inflammatory markers upon CXCR2 inhibition warrants additional studies to fully understand the mode of action and therapeutic potential of this new treatment strategy in CF (Moss et al., 2013).

Although inhibiting inflammation in CF has been envisaged for a long time, directly targeting neutrophils remains a controversial and evolving concept. Given the emerging concept of neutrophil heterogeneity and plasticity (Makam et al., 2009; Laval et al., 2013; Kruger et al., 2015), neutrophil phenotype-specific targeting approaches could be reasonable, but require further preclinical and clinical studies.

Conclusions

The view on neutrophils is changing: while traditionally neutrophils were regarded as short-lived, passive and non-specific cellular infiltrates, recent studies highlight their adaptive abilities, reprogramming, heterogeneity, and plasticity, supporting their active role in CF lung disease. New neutrophil phenotypes, such as T-cell suppressive granulocytic myeloid-derived-suppressor cells (MDSCs) (Rieber et al., 2013; Ingersoll et al., 2015) broaden the repertoire of neutrophil versatility in CF and underscore their importance beyond direct pathogen killing. While the surrounding pro-inflammatory CF microenvironment substantially affects neutrophil phenotype and function, the intrinsic CFTR defect also seems to modulate neutrophil homeostasis and granule release (Pohl et al., 2014). Collectively, CFTR function in neutrophils has been associated with granule exocytosis and phagocytosis, as well as sustained neutrophilic lung inflammation in response to infection (Painter et al., 2006; Bonfield et al., 2012; Ng et al., 2014; Pohl et al., 2014). Intriguingly, the CFTR potentiator ivacaftor was found to modulate leukocyte activation in CF patients (Pohl et al., 2014; Bratcher et al., 2016). In summary, the paradigms of neutrophils in CF are constantly evolving and become more complex, emphasizing the need for further basic studies to better understand this seemingly simple, yet highly versatile innate immune cell.

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