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## **Role of Neutrophils in Cystic Fibrosis Lung Disease**

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Massimo Conese, Stefano Castellani,  
Susanna D'Oria, Sante Di Gioia and  
Pasqualina Montemurro

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/67798>

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### **Abstract**

Cystic fibrosis (CF) is a genetic syndrome caused by mutations in the *CF Transmembrane Conductance Regulator (CFTR)* gene. In CF patients, chief morbidity and mortality are due to pulmonary manifestations. CFTR lack/dysfunction brings an altered ion flux through the airway epithelium and ablation of mucociliary clearance, which in turn ensues in colonization and infection by opportunistic bacterial pathogens and subsequent neutrophil-dominated inflammation. This response eventually leads to the damage of the lung tissue. A host of inflammatory mediators attract, activate, and reprogramme neutrophils to survive (avoiding apoptosis) and produce a wealth of proteases and radical oxygen species. The protease/antiprotease imbalance and oxidative stress have multiple downstream effects, including impaired mucus clearance, increased and self-perpetuating inflammation, and impaired immune responses, thus facilitating and fostering bacterial infections. On the other hand, CFTR lack or dysfunction is likely responsible for alterations in neutrophils concerning chemotaxis, phagocytosis, oxidative burst, degranulation, and neutrophil extracellular trap (NET) formation. A good opportunity to reveal new and non-invasive biomarkers of CF lung disease is the evaluation of circulating neutrophils. Indeed, neutrophil responses are now investigated as outcomes of the aetiological therapies in CF, such as hypertonic saline, antiproteases, CFTR correctors and potentiators.

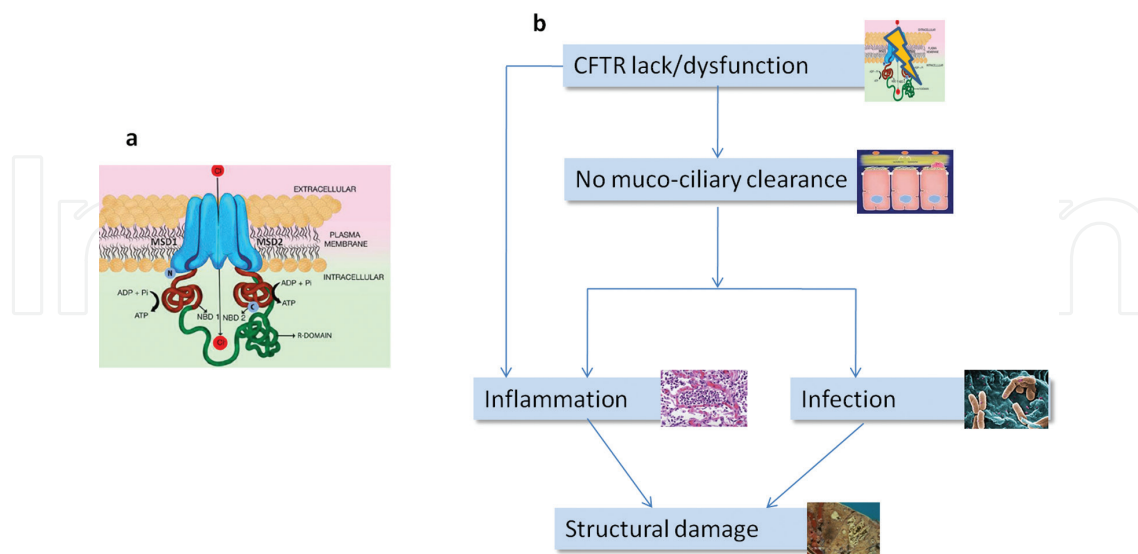
**Keywords:** neutrophils, cystic fibrosis, proteases, NETs, oxidative burst, degranulation, chemotaxis

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## 1. Introduction

Cystic fibrosis (CF) is a rare autosomal recessive disease whose average birth incidence rate is now 2.9/10,000 (i.e. 1/3500) in Europe [1] and prevalence is 100,000 globally [2]. Although CF is a chronic disease affecting many organs, the lung manifestations are still today the major cause of morbidity and mortality of these individuals and are the consequences of an ongoing inflammatory process, which stems either in the absence or in the presence of opportunistic bacterial infections. Lung inflammation and respiratory infections affect the prognosis of CF patients [3, 4]; indeed, they are associated with the progressive destructive changes that are responsible for most of the morbidity and mortality in CF [5]. Over 1000 microbial species (viruses, bacteria, mould, and fungi) have been found in the airways of CF patients [6]. *Staphylococcus aureus* and *Haemophilus influenzae* are the most common pathogens isolated from the sputum in the first decade of life, while *Pseudomonas aeruginosa* is found to dominate numerically in the second and third decades of life [7]. However, according to the Cystic Fibrosis Foundation Registry, *P. aeruginosa* is no longer the most common pathogen cultured in individuals with CF in the USA, and there has been an increase in the prevalence of *S. aureus* and *Stenotrophomonas maltophilia* [8].

Mutations in the 250-kb *CF transmembrane conductance regulator (CFTR)* gene are responsible for CF, but other environmental and genetic modifiers are thought to play a role in the phenotype of lung disease [9]. The CFTR gene encodes for a chloride channel that is expressed on the apical membrane of epithelial cells residing in organs with absorptive/secretory properties (**Figure 1(a)**). More than 2000 mutations have been identified at the moment ([www.genet.sickkids.on.ca/cftr/](http://www.genet.sickkids.on.ca/cftr/)), which can be classified in six classes (**Table 1**).



**Figure 1.** CFTR structure and CF lung disease. (a) A supposed CFTR structure when inserted in the plasma membrane. CFTR is composed of a two-membrane spanning domain (MSDs), each linked to nucleotide-binding domains (NBD1 and NBD2). Unique to CFTR, NBD1 is connected to the NBD2 by a regulatory domain (R). (b) The pathophysiological cascade of CF lung disease.

CFTR mutation class	Example	Effect on CFTR protein
Class I (stop mutation)	G542X	No expression
Class II (trafficking mutants)	F508del	Very low expression
Class III (low ATP binding)	G551D	Very low function
Class IV (low conductance)	R117H	Low function
Class V (low synthesis)	A455E	Low expression
Class VI (high turnover)	120del23	Low expression

**Table 1.** The six classes of CFTR mutations and their effects at the protein level.

The hallmark of the CF lung disease is a neutrophil-dominated inflammatory response; however, the link between CFTR mutations and the complex inflammatory milieu of the CF lungs is largely still poorly understood. The pathophysiological cascade which leads from the lack/dysfunction of CFTR chloride channel activity to the airway inflammation and infection, and eventually to tissue damage and destruction, is represented in **Figure 1(c)**. In the airways, the low excretion of chloride ions and bicarbonate, along with the hyperabsorption of sodium by the epithelial sodium channel (ENaC) and subsequently of water, contributes to the volume depletion from the periciliary liquid and its acidification. Thus, the loss of CFTR reduces the effectiveness of at least two defences—mucociliary transport and antimicrobial activity [10–12]. This eventually brings the colonization and infection by opportunistic bacterial pathogens and opposing inflammation, which, far from being resolute, seems to be dysregulated, becoming chronic. In this context, polymorphonuclear leukocytes (PMNs) are thought to play a fundamental role on the onset and progression of lung tissue damage. Observational clinical studies made in the past have ascertained that infants with CF do show an airway inflammation prior to overt infection [13], indicating that the inflammatory response is dysregulated a priori before any bacterial infection and also suggesting that CFTR mutations are implicated in this abnormal response (**Figure 1(b)**). This is supported by the findings showing that free and bound airway neutrophil elastase is detected very early in CF infants and predicts the development of bronchiectasis later in life [14]. Furthermore, it has been found that CFTR is involved in some functions of innate immune cells that are diverted by CFTR mutations. We will discuss these evidences in Section 4.

## 2. Recruitment and activation of neutrophils in CF lungs

Neutrophils are the main cell types involved in the first-line defence of many organs, including the respiratory tract. However, they remain in the blood circulation unless they are recruited in the tissue. In the airways, they are marginated along the endothelium of capillaries and are ready to migrate first through the endothelium and then across the respiratory epithelium [15]. Marginated neutrophils are recruited rapidly to sites of inflammation, where their primary role is to kill invading bacteria and certain fungal species through phagocytosis and production of a range of oxygen species within the phagolysosomes and

also by preformed granular enzymes and proteins. Tissue inflammation results in the release of multiple inflammatory mediators and subsequent neutrophil priming. Priming results in a marked change in neutrophil shape and rheology that leads to their increased stiffness and retention within the capillary microvascular bed of the lung [16]. These mediators include an early wave comprised of cytokines, such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and pathogen-associated molecular patterns (PAMPs) such as endotoxin, the ligand of Toll-like receptor (TLR)-4, followed by a late wave of chemoattractants and growth factors including IL-8, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and granulocyte-macrophage colony-stimulating factor (GM-CSF).

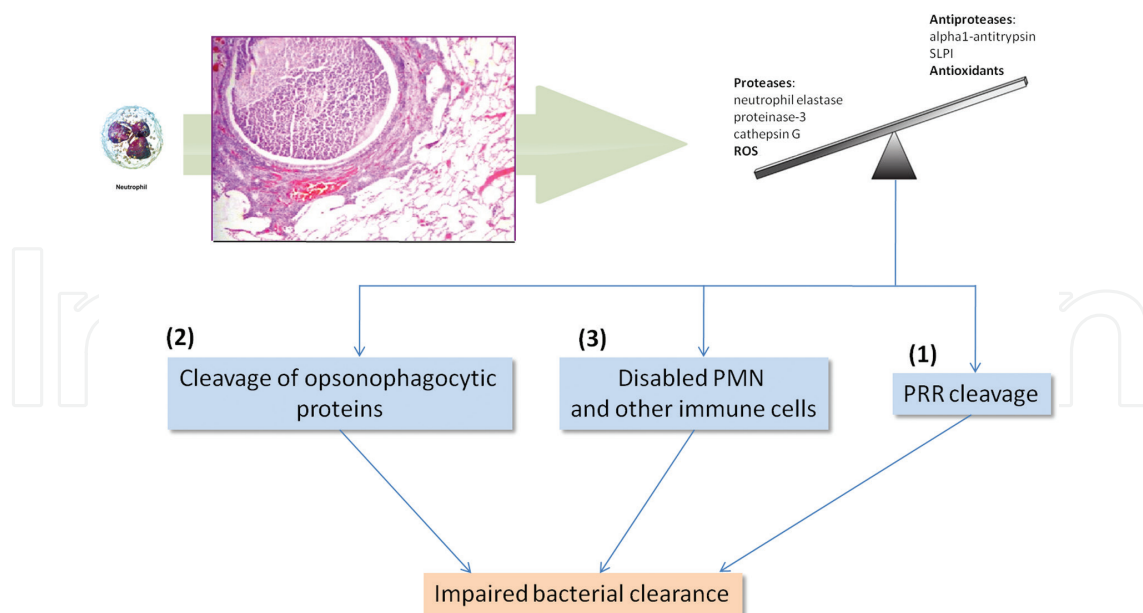
In the airways, macrophages and epithelial cells are the main cell types which sense the pathogens and secrete a wealth of factors both inducing priming and full activation of neutrophils, as well as their extravasation. Upon exposure to bacteria, respiratory epithelial cells release reactive oxygen species (ROS) as an innate anti-infective mechanism, together with several anti-microbial peptides such as human beta-defensins (hBD-1/2/4) and cathelicidins (LL-37). The major pro-inflammatory cytokines (e.g. IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) are initially expressed and released by surface epithelial cells of the conductive airways, which also release chemokines directed to recruit neutrophils (e.g. IL-8, GRO- $\alpha/\gamma$ ) [17–22]. Besides the phagocytosis of inhaled pathogens and apoptotic cells, alveolar macrophages (AMs) play an important role in orchestrating innate immune defences by releasing inflammatory mediators. One of the important regulatory functions of AMs may be to dampen immune responses [23] so that dysfunction of AMs in CF could be related to increased inflammation. Both airway epithelial cells and AMs have been shown to be dysfunctional in CF, contributing to the onset and progression of chronic lung disease [24, 25]. This is reflected by the high burden of cytokines, chemokines, and other mediators found in the airway secretion of CF patients [26]. The CF airways contain massive amounts of cytokines and chemoattractants for neutrophils such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-17, IL-33, LTB<sub>4</sub>, C5a, high-mobility group box 1 (HMGB1), proline-glycine-proline (PGP), and N-acetyl PGP [27–32]. For example, TNF- $\alpha$  enhances the neutrophil oxidative capacity, the granule release, and, with IL-1 $\beta$ , induces the priming of neutrophils [33]. The concentration of IL-8 in bronchoalveolar lavage (BAL) fluid is generally elevated and often correlates with the number of neutrophils in the airways [34]. It is thought that both extrinsic (e.g. microbes) and intrinsic (e.g. *CFTR* mutations) contribute to the alterations of the respiratory epithelium and AMs, ensuing in a hyper-inflammatory state and defect in immune defence.

Besides chemokines, such as CXCL8 (IL-8) [35], and lipid products, such as LTB<sub>4</sub> [36], other mediators have also been recently implicated in the recruitment of neutrophils into the CF airways. UDP-glucose levels are abnormally elevated in lung secretions from CF patients and from a mouse model of CF/chronic bronchitis, the  $\beta$ ENaC-Tg transgenic mouse [37]. Moreover, instillation of UDP-glucose into mouse lung resulted in robust accumulation of neutrophils in BAL. Levels of damage-associated molecular patterns (DAMP), HMGB1, were found elevated in CF sputum and in BAL from  $\beta$ ENaC-Tg transgenic mouse and shown to be chemotactic for neutrophils [38]. Upon activation, neutrophils secrete matrix metalloproteinases (MMP)-8 and -9, which perform an initial digestion of collagen from the macromolecule's size. Subsequently, neutrophils release prolyl endopeptidase (PE), a serine protease

previously only known to be a processor of neuropeptides. PE performs the final digestion of collagen to the tri-peptide PGP, which, upon binding to the same receptors as IL-8, CXCR1 and CXCR2, acts as a neutrophil chemoattractant and activator [39]. Thus, release of this peptidic collagen fragment provides a positive feedback mechanism that contributes to persistent neutrophilic inflammation in the CF lung [40]. During the adaptive immune response phase, neutrophils are recruited to the lung via the IL-23/IL-17A axis. Dendritic cells, activated by bacterial antigens, produce IL-23, which, in turn, binds to IL-23 receptor on T cells and stimulates them to produce IL-17A. This cytokine induces granulopoiesis via the induction of G-CSF and neutrophil recruitment via induction of chemotactic mediators such as IL-8. Both IL-23 and IL-17A have been found at high levels in the sputum from CF patients in acute exacerbation [41] and in stable condition [42], amplifying the extravasation and activation of neutrophils already induced by the innate immune response.

Once extravasated, neutrophils locate all along the CF bronchial tree and particularly in segmental bronchi, where they preferentially locate at the level of the lamina propria and in the lumen [43]. In this position, they are already activated and try to phagocytose microbes (e.g. *P. aeruginosa*) which have adapted to the hypoxic environment by producing an exopolysaccharide called alginate [44]. This frustrated phagocytosis leads to neutrophil hyperactivation which is more harmful than protective.

In the following subsections, we shall revise the main features of neutrophil physiology and how these are modified in the CF airway microenvironment (**Figure 2**).



**Figure 2.** The role of neutrophils in maintaining inflammation and respiratory infections. The increased burden of neutrophils in the CF airways is the hallmark of the mucus plugs contained in the bronchioles lumen. From this location, PMNs secrete proteases and reactive oxygen species that overwhelm antiproteases and antioxidants, respectively, ensuing in various effects: (1) cleavage of pattern recognition receptors (PRR), (2) cleavage of opsonophagocytic receptors, and (3) disabling PMNs themselves and other immune cells. All these alterations facilitate bacterial infections.

## 2.1. Activation

Neutrophils recruited from the blood into the CF airway environment undergo marked functional changes. They express high levels of markers conventionally found on long-lived antigen-presenting cells (APCs), including class II molecules of the Major Histocompatibility Complex (MHC), the costimulatory molecule CD80, and the chemoattractant receptor of Th2 cells (CD294), all of which suggest profound reprogramming [45]. CF airway neutrophils present marked increases in glucose, amino acid, and phosphate transporters as compared with blood neutrophils [46], indicating that metabolic adaptation of neutrophils occurs as they are recruited to CF airways. However, these changes are not equal for all neutrophil subsets found in CF airways.

## 2.2. Apoptosis and resolution of inflammation

Apoptosis is a physiological process necessary for the clearance of inflammatory cells. Neutrophils are short-living cells which undergo apoptosis at the end of the inflammatory response, attracting macrophages which eventually ingest apoptotic cells in a process called *efferoctosis*. The removal of apoptotic cells is relevant to avoid secondary necrosis and the release of pro-inflammatory mediators that disrupt tissue homeostasis [47]. In CF, the lung disease is characterized by an altered balance of pro- and anti-inflammatory mediators. Studies have shown that CF airways are deficient in several anti-inflammatory molecules, including IL-10 and lipoxin-A<sub>4</sub> (LXA<sub>4</sub>) [48]. IL-10 inhibits the pro-inflammatory activities of cytokines, chemokines, and transcription factors and induces neutrophil apoptosis [49]. Not surprisingly, IL-10 knockout mice inoculated with *P. aeruginosa* that was embedded in agarose beads, in order to mimic a chronic *Pseudomonas* infection, had more drastic weight loss, greater neutrophil infiltration, larger inflammatory exudate of the lungs, and higher concentrations of pro-inflammatory cytokines in BAL compared to wild-type mice [50, 51]. Lipoxins are arachidonic metabolites generated by a lipoxigenase transcellular pathway involving neutrophils with epithelia, endothelia, monocytes, and platelets. In particular, LXA<sub>4</sub> acts to down-modulate acute inflammation by inhibiting neutrophil transmigration induced by LTB<sub>4</sub> and IL-8 and stimulating macrophage phagocytosis of apoptotic PMNs [52, 53]. LXA<sub>4</sub> levels have been found to be reduced in BAL fluid from CF patients, along with a significant suppression of LXA<sub>4</sub>/neutrophil ratios [54, 55].

It seems for a number of reasons that neutrophils are resistant to apoptosis when they have extravasated into the CF airways; for example, it has been suggested that the oversecretion of cytokines might be responsible of apoptosis inhibition of airway neutrophils. The release of G-CSF or GM-CSF by epithelial cells, stimulated by *S. aureus* or *P. aeruginosa*, inhibits apoptosis of CF neutrophils [56], suggesting that increased expression of cytokines by CF airway cells not only induces neutrophil response but also enhances their survival, perpetuating an inflammatory process. Also, it has been described that PMNs from CF patients showed delayed constitutive and TNF- $\alpha$  or GM-CSF-induced phosphatidylinositol 3-kinase (PI3K)-dependent apoptosis [57]. CF airway neutrophils also undergo strong activation of CREB and mTOR's pro-survival pathways [58]. Moreover, it has been postulated that delayed phosphatidylserine externalization and mitochondria depolarization might be responsible for delayed

apoptosis of CF neutrophils [59]. In another study [60], neutrophils isolated from CF patients showed enhanced survival and upregulation of p21/Waf1, a cyclin-dependent kinase inhibitor and partner of proliferating cell nuclear antigen (PCNA). As also suggested by *in vivo* studies in p21(-/-) mice with *P. aeruginosa* lipopolysaccharide (LPS) challenge, p21/Waf1 is involved in the apoptotic response occurring during the resolution of inflammation [60]. In order to dissect the early phases of interaction between CF neutrophils and airway epithelial cells, it was found in co-culture experiments that a high number of non-apoptotic airway PMNs adhered to the CF airway epithelium in the presence of elevated levels of IL-6 and IL-8 [61], indicating another mechanism involved in enhanced inflammatory responses in airways of CF patients. Finally, independent of the sensitivity to apoptosis of CF cells, it has been shown that clearance of apoptotic cells by efferocytosis is defective in CF due to elastase-mediated degradation of macrophage phosphatidylserine receptors and that accumulation of such cells may contribute to ongoing inflammation [62].

### 2.3. Phagocytosis, oxidative burst, and degranulation

In cystic fibrosis, there is a tendency for bacterial colonization that may be due to dysfunction of phagocytosis. Airway neutrophils of CF patients showed a blunted phagocytic capacity and a reduced expression of cell surface recognition receptors, namely TLRs, leading to impaired bacterial killing [63]. Recent studies have demonstrated that CF neutrophils display an absence or dysfunction of CFTR at the level of phagolysosomes [64]. Likely due to this defect, CF neutrophils are impaired in chlorination of engulfed pathogens due to defective hypochlorous acid (HOCl) production [65].

One of the major mechanisms through which neutrophil phagocytosis kills pathogens entrapped inside the phagolysosomal vacuole is the release of high quantities of ROS [66]. The activation of the nicotinamide adenine dinucleotide phosphate oxidase (NOX2) in the neutrophils induces the production of superoxide anion and consequently the other ROS. Excessive activation of the neutrophil NOX2 results in exaggerated ROS release in the external surroundings, which increases the oxidative damage to tissues [67]. Furthermore, the inflammatory response can be enhanced by imbalance created by excessive release of pro-oxidative and impaired release of anti-oxidative molecules. While some authors have reported that ROS production by CF blood PMNs can be higher than or identical to that of healthy controls [68, 69], others have demonstrated that ROS generation varied according to the infecting pathogen [70] or to the method employed to detect respiratory burst activity [71]. For example, it has been shown that an extracellular polysaccharide of non-mucoid *P. aeruginosa* strain (Psl) inhibits opsonization and reduces ROS production by neutrophils [72]. Montemurro et al. [73] have established that CF blood neutrophils at the baseline are characterized by a higher ROS release as compared with controls PMNs and that the antibiotic therapy does not change this pattern. Nevertheless, ROS production is reduced in airway neutrophils compared to blood neutrophils that have different ROS oxidant activity profiles [74].

Neutrophils are identified by the presence of cytoplasmic primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules as well as the secretory vesicles [75]. Focusing on granules, neutrophils abundantly express a cell-type specific set of neutrophil serine pro-



teases, namely cathepsin G, proteinase 3, and neutrophil elastase (NE), which are stored in the azurophilic granules. Also, myeloperoxidase (MPO) is stored in primary granules. Secondary granules are characterized by the presence of lactoferrin and cathelicidins, such as hCAP-18, while tertiary granules are enriched with gelatinase, an old name for MMPs, in particular MMP-9.

A dysregulated neutrophil degranulation capacity in CF has been shown. Neutrophils obtained from CF patients have an increased capacity to release primary granule contents such as MPO and NE [76]. In the airways, CF neutrophils undergo active exocytosis of primary granules, leading to a massive release of enzymes (e.g. NE, MPO) that damage the airway tissue and perpetuate inflammation [45]. On the other hand, Pohl et al. [77] have demonstrated that blood neutrophils obtained from CF patients can release less secondary (lactoferrin and hCAP-18) and tertiary (MMP-9) granule components compared with cells obtained from healthy individuals. The dysfunction of CFTR channel in neutrophils results in the deactivation of the GTP-binding protein Rab27a and in an impaired granule exocytosis. Interestingly, hypoxia, which is a hallmark of the CF bronchiolar environment, augmented neutrophil degranulation and possibly enhanced damage to respiratory airway cells in a hypoxia-inducible factor (HIF)-independent but PI3K $\gamma$ -dependent mechanism [78].

#### 2.4. NETosis

The neutrophils are the first immune cells to achieve the site of injury or infection and are key players in microbial killing, because they are equipped with three main anti-bacterial weapons: phagocytosis, release of ROS, and granule release. Aside from these traditional mechanisms, neutrophils are also able, upon activation, to release DNA fibres decorated with anti-microbial proteins or neutrophil extracellular traps (NETs) to immobilize and to kill bacteria. NETs are composed of chromatin fibres coated with anti-microbial proteins, such as histones, NE, MPO, and  $\alpha$ -defensins [79–82]. Moreover, NETs and their associated molecules are able to directly induce epithelial death, and massive NET formation has been reported in several pulmonary diseases including CF [83]. NETs are present in excess in CF sputum and the normal host defence functions become pathological [84]. CF patients with poor pulmonary functions presented higher levels of NETs compared to patients with mild lung disease, and the G protein-coupled receptor (GPCR) CXCR2 mediates NOX2-independent NET formation [85]. Histones and protease-coated DNA structures are released by neutrophils in response to respiratory bacteria (whole cells or virulence factors such as LPS, pilus, pyocyanin) or to inflammatory mediators (IL-8, interferon type I [IFN I], C5a) [86]. The exotoxin pyocyanin, a virulence factor of *P. aeruginosa*, enhances NET formation and requires NOX2 for its action [87]. Another pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF), is able to stimulate NET release by promoting mitogen-activated protein kinase and thus exacerbating the inflammation [88]. Finally, *P. aeruginosa* triggers the release by lung epithelial cells of the eicosanoid heptoxilin A3, a neutrophil chemoattractant that induces NETosis [89]. Besides, MPO and NE expressed on NET fibres may induce the degradation of proteins of the connective tissue and of endothelial heparan sulphate proteoglycan at the site of inflammation [90, 91], contributing to lung pathology of CF patients. Furthermore,

there is growing evidence of NET escape by pathogens. NET release might be inhibited by down-regulation of inflammatory responses, or NET degradation might be induced by bacteria, including *H. influenzae*, by deoxyribonuclease [92]. Also *P. aeruginosa*, a very mutable bacterium, is able to acquire resistance to NET-mediated killing [93].

## 2.5. Cytokine production and immune regulation

As already pointed out above, there are many synergistic mediators which prime, activate, and attract neutrophils in the CF airways. Neutrophils also contribute to the CF airway environment by producing mediators that are pro-inflammatory and modify the function of other immune cells. CF airway neutrophils were found to increase TLR-4 expression on their surface and produce excessive IL-8 at the baseline, while failing to increase secretion in response to LPS or repress it in response to IL-10 [94]. Neutrophils in the sputum and blood of F508del CF subjects at the time of pulmonary exacerbation were found to express IL-17 RNA and protein as well as IL-23 receptor [95]. These investigators also showed a positive correlation between percent-IL-17-producing neutrophils and the total sputum activity of NE and MMP-9 and that IL-17 was absent following antibiotic treatment. IL-17 production by neutrophils may therefore contribute to tissue damage in the lungs of patients with CF.

Neutrophilic myeloid-derived suppressor cells (MDSC) are innate immune cells that are functionally characterized by their potential to suppress T- and natural killer (NK)-cell responses. Circulating neutrophilic MDSC have been found to be increased in patients with CF infected with *P. aeruginosa* as compared with age-matched healthy control subjects, their percentages correlating with lung function in those patients [96]. Further studies have revealed in an in vivo animal model of respiratory infection that *P. aeruginosa* triggers the recruitment of neutrophilic MDSC into the pulmonary compartment and enhances their suppressive capacity towards T cells [97]. Interestingly, they also showed that MDSC obtained from *Cftr*<sup>-/-</sup> mice were generated and recruited as in wild-type mice but were impaired in suppressing T-cell proliferation compared to their *Cftr*<sup>+/+</sup> counterpart cells. Thus, neutrophils contribute to the escape of *P. aeruginosa* from the adaptive immune response, and *CFTR* mutations may contribute to the bacterial infection.

## 3. Neutrophils and the effect of *CFTR* mutations

While bacteria and their products, cytokines and chemokines, are important triggers of neutrophil activation in CF airways, it is an emerging picture that a primary *CFTR* defect in cells of the innate immune system, including neutrophils, monocytes, and lymphocytes, contributes significantly to CF lung pathology [24]. Pharmacologic inhibition of *CFTR* and genetic mutation (F508del) in murine neutrophils activated the nuclear factor kappa-light-chain enhancer of activated B cells (NF- $\kappa$ B) and increased macrophage inflammatory protein-2 (MIP-2) and TNF- $\alpha$  production, as compared to non-inhibited and control neutrophils. Interestingly, under LPS challenge, neutrophil-depleted wild-type mice reconstituted with F508del neutrophils displayed a more severe lung inflammation in comparison with neutrophil-depleted

wild-type mice reconstituted with wild-type neutrophils [98]. Altogether, these data strongly indicate that the lack of functional CFTR could result in excessive NF- $\kappa$ B activation in neutrophils and therefore propagate a hyper-inflammatory response.

CF neutrophils have a reduced phagocytic activity [19, 99] and defects in the respiratory burst, attributed to disrupted chloride transport to the phagolysosome [65, 100–102]. While wild-type CFTR is transported to neutrophil phagosomes, the F508del protein is not targeted efficiently to these organelles [64], explaining why a correct chlorination of phagosomes in CF does not occur and hence the bactericidal defect. A still debated question is, however, the CFTR expression in neutrophils. Morris and colleagues, although found a defect in iC3b-mediated phagocytosis, did not detect CFTR in circulating and airway neutrophils by either immuno-labelling or a Western blot [99]. Others found that CFTR expression was limited or undetectable in neutrophils by flow cytometry and also that no role for CFTR in neutrophil-mediated phagocytosis was observed [103]. On the other hand, Zhou and colleagues found CFTR at the phagosome level, although a lentiviral-expressing system was used to achieve high protein levels. It might be that CFTR, expressed in hematopoietic stem/progenitor cells [104, 105], is down-regulated to low levels during neutrophil maturation, which is nevertheless sufficient for neutrophil phagocytic and killing activities. The lack/dysfunction of CFTR in the bone marrow may lead to an irreversible functional defect. In this context, it is worth mentioning that knocking out CFTR in the myeloid compartment of mice resulted in poor survival, increased inflammation with recruitment of neutrophils, elevated cytokine production, and inability to resolve infection upon challenge with *P. aeruginosa*-loaded agarose beads to mimic a chronic pulmonary infection [106].

#### 4. Disabling neutrophils and other immune cells in CF airways

Excess neutrophil recruitment to the lungs results in the discharge of their destructive weapons not only directed to kill pathogens (see Section 2) but also to damage the lung and airway tissue. A large number of mediators produced by neutrophils, mainly oxidants and proteases, escape from neutrophils during cell death and phagocytosis. NE, a serine protease capable of digesting several substrates including structural proteins, is a direct mediator degrading elastin, which drives towards bronchiectasis and bronchomalacia [18]. Importantly, NE is associated with lung function decline [107]. In the lung, the main protease inhibitors, the prototypical  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) secreted by hepatocytes and secretory leukoprotease inhibitor (SLPI) produced by the respiratory epithelium in bronchi and bronchioles, are designed to oppose free proteases and prevent their deleterious effects. These protease inhibitors are eventually overwhelmed by the protease burden in the lung and degraded by bacterial and human NE. It has been documented that despite normal antigenic concentrations of  $\alpha$ 1-AT and SLPI in children with CF, the majority of  $\alpha$ 1-AT and SLPI were complexed and/or degraded [108]. In addition, CF airways are exposed to ROS ( $O_2$ ,  $H_2O_2$ , HOCl), derived mainly from the host's immune response. This oxidative stress exacerbates pulmonary deterioration and advances bronchiectasis in patients with CF [109]. Similar to the protease/antiprotease balance, antioxidants produced by airway epithelial cells (reduced glutathione [GSH] and thiocyanate [ $SCN^-$ ]) are overwhelmed by the burden of oxidants in the CF airways. Activated

neutrophils are also capable of oxidizing glutathione by HOCl [110], contributing to GSH deficiency in CF airways. Hypochlorous acid is also able to oxidize calprotectin thereby inhibiting its ability to sequester manganese and zinc ions and consequently to limiting the growth of *S. aureus* and *P. aeruginosa* [111]. Moreover, it has been documented that ROS suppresses CFTR function [112] and that NE degrades CFTR [113], further worsening the CF pathophysiologic vicious cycle.

An important role in the degradation of structural proteins in CF airways is played synergistically by serine proteases, such as NE, proteinase 3, and cathepsin G [114]. In cystic fibrosis, neutrophil activation and degranulation result in the excessive release of proteinase 3, cathepsin G, and NE into the extracellular medium as active enzymes. Part of these serine proteases are exposed at the cell surface of immune cells and are important as modulators of the inflammatory response. Proteinase 3 has been shown to convert IL-8 to more potent, amino-terminally truncated forms [115], indicating that neutrophil proteases released in the inflamed lung convert IL-8 to enhance its chemotactic activity. Besides serine proteases, neutrophil-derived metalloproteinases, including MMP-8 and MMP-9, have also been involved in CF lung disease and chronic neutrophilic inflammation [116]. NE contributes to MMP-9 activation early in CF disease as the ratio of active/pro-enzyme MMP-9 was found to be higher in the presence of free neutrophil elastase activity, but not infection, and active MMP-9 was associated with progression of bronchiectasis [117]. In the context of CF, it is important to recall that neutrophil proteases increase mucin secretion in the airways and reduce ciliary beat frequency, contributing to the impairment in mucociliary clearance [118, 119], induce airway epithelial cells to produce neutrophil chemoattractants [120], and activate the apical epithelial sodium channel ENaC [121].

Unopposed serine proteases and metalloproteinases are responsible for degradation of soluble pattern recognition receptors (PRRs). NE proteolytic activity present in the CF sputum has been shown to degrade the prototypic long pentraxin PTX3, explaining the low levels of this PRR in CF airway secretions [122]. Released cathepsin G upon neutrophil activation degrades both components of the extracellular matrix and the surfactant protein A, a peptide that facilitates bacterial clearance by alveolar macrophages [123]. MMP-9 cleaves the pulmonary collectin surfactant protein D (SP-D) more efficiently than NE; this cleavage causes SP-D to no longer be able to agglutinate bacteria and affects SP-D's innate immune functions, as bacteria are no longer efficiently phagocytosed by alveolar macrophages *in vitro* [124].

High levels of neutrophil proteases further worsen the immune response by disabling immune cell functions. NE has several potential roles in disabling neutrophils including cleavage of opsonophagocytosis proteins, such as iC3b, complement receptor 1 (CR1) and C5a receptor [125–127], the chemokine receptor CXCR1 [128], and TIM3 receptor leading to decreased galectin-9/TIM3 interactions [129]. Overall, the loss of these proteins is responsible for suboptimal local neutrophil priming and bacterial clearance. PMN-derived cathepsin G also thwarts efficient phagocytosis by macrophages, resulting in the cleavage of receptors and causing inefficient opsonization and impaired bacterial killing [18]. Cathepsin G cleavage of serum amyloid P component (SAP) renders it anti-opsonic, as evidenced by the increased binding of SAP to *P. aeruginosa* LPS and inhibition of phagocytosis *in vitro* [130], thus sequestering bacteria within the lung and potentially contributing to persistent infections in CF. Cathepsin G also interferes with removal of neutrophilic apoptotic bodies, since it mediates the degradation of

the macrophage phosphatidylserine receptors with failure to resolve inflammation because of the lack of efferocytosis [62, 131]. Also, NK cells and lymphocytes are disabled by neutrophil serine proteases. Cathepsin G determines a proteolytic cleavage of NKp46, a crucial activating receptor expressed on NK cells, an effect also determined by the CF sputum [132]. NE cleaves T-cell receptors CD2, CD4, CD8, and CD14, impairing monocyte activation and also blocking dendritic cell maturation and antigen presentation [133, 134].

## 5. Neutrophils as biomarkers of CF lung disease

The mainstays of CF lung disease management are commenced early in infancy and presently include chest physiotherapy to remove mucus plugs from the airways and antibiotic therapy to control infections [12]. Other therapeutic approaches such as hypertonic saline, finalized to increase mucociliary clearance, should be corroborated by efficacy data [135]. Recombinant human DNase (Dornase alpha) is a strong mucolytic which improves lung function [136] but is given to CF infants only on indication due to its cost [137]. The recent breakthrough in CF, represented by the use of CFTR-correcting therapies, is a milestone in the clinical management of these patients. Ivacaftor (Kalydeco®, Vertex Pharmaceuticals, USA) is a CFTR potentiator given successfully to patients with class III gating mutations. This drug not only improves lung function and normalizes sweat chloride in children above 6 years of age [138], but its efficacy has also been proven in preschoolers [139].

At whatever age, the control of therapeutic efficacy of medications is granted by functional respiratory tests. However, more specific and sensitive assays are urgently needed to monitor the halt in the progression of lung disease, especially now that we entered the era of personalized medicine in CF [140]. Neutrophils, the main cell type involved in the onset and progression of CF lung disease, are clearly an interesting target in this context and are being evaluated for such a purpose. The best indication that neutrophils and their products are sensitive biomarkers of CF lung disease comes from the clinical data about NE. Sputum NE levels have been validated as the most predictive biomarker of lung decline and reduced survival [107, 141], being, however, of no utility in non-expectorating young children. Being easy to isolate from the peripheral blood, circulating neutrophils are more at hand to being studied. Conese et al. [142] analysed blood neutrophils by microarray gene expression in 10 CF patients, homozygous for the F508del mutation, given a course of parenteral antibiotics for an acute exacerbation, before and after therapy. mRNAs of three genes were found downregulated in CF patients before therapy and returned to 'healthy' levels after therapy: phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*), hydrogen voltage-gated channel 1 (*HVCN1*), and  $\beta$ -arrestin 1 (*ARRB1*). Recently, we validated neutrophil *HVCN1* mRNA as a biomarker following the treatment of seven CF patients, homozygous or heterozygous for class III mutations, with ivacaftor, confirming that its expression levels are lower as compared with healthy controls before therapy, while they are increased after CF patients were treated for 6 months (Guerra et al., submitted). Overall, these data strongly indicate that *HVCN1* mRNA level is a neutrophil biomarker sensitive to therapy. In another study [77], ivacaftor treatment resulted in normalized ion homeostasis and corrected Rab27a activation as well as degranulation in blood neutrophils

obtained from six CF patients with the genotype *F508del/G551D*. In line with these findings, extracellular *Pseudomonas* killing by CF neutrophils obtained from CF patients during treatment was significantly increased. Activated CD11b was investigated as a marker of neutrophil activation and whether it was downregulated by ivacaftor treatment in five patients with *F508del/G551D* and *G551D/N1303K* genotypes [143]. A cytofluorimetric assay showed that activated CD11b on PMNs was significantly higher at baseline in the CF patients compared to controls. However, after treatment, this marker was not significantly different from healthy controls, suggesting that ivacaftor treatment results in a decrease, towards normalization, of the activation status of blood neutrophils *in vivo*.

## 6. Conclusion

CF neutrophils display a number of abnormalities including increased survival, hyperactivation with increased protease and ROS production, defects in phagocytosis, and increased NET formation. Altogether, these neutrophil anomalies are derived from an intrinsic CFTR defect and are compounded by bacterial products. The unbalanced protease/antiprotease ratio in favour of proteases is responsible, together with excess oxidative stress, for the structural damage of CF airways and for secondary defects in an innate immune response as well as a skewed adaptive immune response. The neutrophil protease production is thus one of the main targets for therapy today to be explored. CF neutrophils can be also envisaged as a biomarker of therapies. The sensitivity to therapy of neutrophil genes is worthy of further investigation in the clinical setting. A higher number of patients are needed for studies aimed to consider neutrophils and their products as predictors of acute exacerbation and follow up.

## Acknowledgements

This work was supported by the Italian Ministry of Health (Law 548/93). Stefano Castellani is a researcher funded by Intervento Cofinanziato dal Fondo di Sviluppo e Coesione 2007–2013—APQ Ricerca Regione Puglia ‘Programma regionale a sostegno della specializzazione intelligente e delle sostenibilità sociale ed ambientali—Future In Research’.

## Author details

Massimo Conese<sup>1\*</sup>, Stefano Castellani<sup>1</sup>, Susanna D’Oria<sup>2</sup>, Sante Di Gioia<sup>1</sup> and Pasqualina Montemurro<sup>2</sup>

\*Address all correspondence to: [massimo.conese@unifg.it](mailto:massimo.conese@unifg.it)

1 Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy

2 Section of General Pathology, Department of Biomedical Sciences and Human Oncology, University of Bari, Bari, Italy

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