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New Research on the Importance of Cystic Fibrosis Transmembrane Conductance Regulator Function for Optimal Neutrophil Activity

Michelle M. White, Fatma Gargoum, Niall Browne, Killian Hurley, Noel G. McElvaney and Emer P. Reeves

Additional information is available at the end of the chapter

Abstract

Despite tremendous recent advances in our understanding of the molecular and cellular basis of cystic fibrosis (CF), there remains a paradox of why recruited neutrophils fail to eradicate bacterial infections in the airways of individuals with CF. The focus of this chapter is on new research authenticating the CF neutrophil as a key player in disease pathogenesis. Studies specifying intrinsic abnormalities due to a lack of cystic fibrosis transmembrane conductance regulator (CFTR) function, along with reports indicating reprogrammed cell activity secondary to chronic bacterial infection and inflammation, will be discussed.

Keywords: cystic fibrosis, cystic fibrosis transmembrane conductance regulator, neutrophil function

1. Introduction

Cystic fibrosis (CF) is a multi-system disease resulting from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). In excess of 1900 CF mutations have been identified thus far [1], leading to misfolding of the CFTR protein and defective chloride (Cl^-) transport across cell membranes. CF affects various organs including

the pancreas, liver, and intestinal tract, but the most severe complications often occur in the lung. CF related symptoms, although variable among patients, are typically present early in life and increase in severity with age despite aggressive therapeutic intervention. CFTR absence or malfunction causes defective ion transport across the epithelium, reduction in pericillary liquid volume, and persistent mucus hypersecretion. As a consequence, mucus accumulates on the airway surface and leads to chronic bacterial infection, exacerbated airway inflammation, and lung injury. Pulmonary inflammation in the CF setting has been observed as early as infancy and there is evidence of structural lung disease present in children with CF as young as 10 weeks old, with 50%–70% of children presenting with bronchiectasis by 3–5 years [2-6]. Additional studies have shown neutrophil-dominated airway inflammation in children with CF [7, 8], and with increasing age elevated levels of proinflammatory mediators including interleukin (IL)-8 [9], tumour necrosis factor-alpha (TNF-alpha) [10], and leukotriene B_4 (LTB_4) [11] serve to escalate the chronic neutrophil presence in the pulmonary circulation. Indeed, key studies have demonstrated that neutrophil-released granule proteins, particularly neutrophil elastase (NE), play a crucial pathological role (Figure 1) [12, 13]. In the healthy lung, neutrophils represent approximately 1% of the inflammatory cells; however, they account for 60–70% of the total cell count in CF bronchial lavage fluid [14, 15] and ~25% of CF children were positive for free NE activity in airway samples at 3 months old [16].
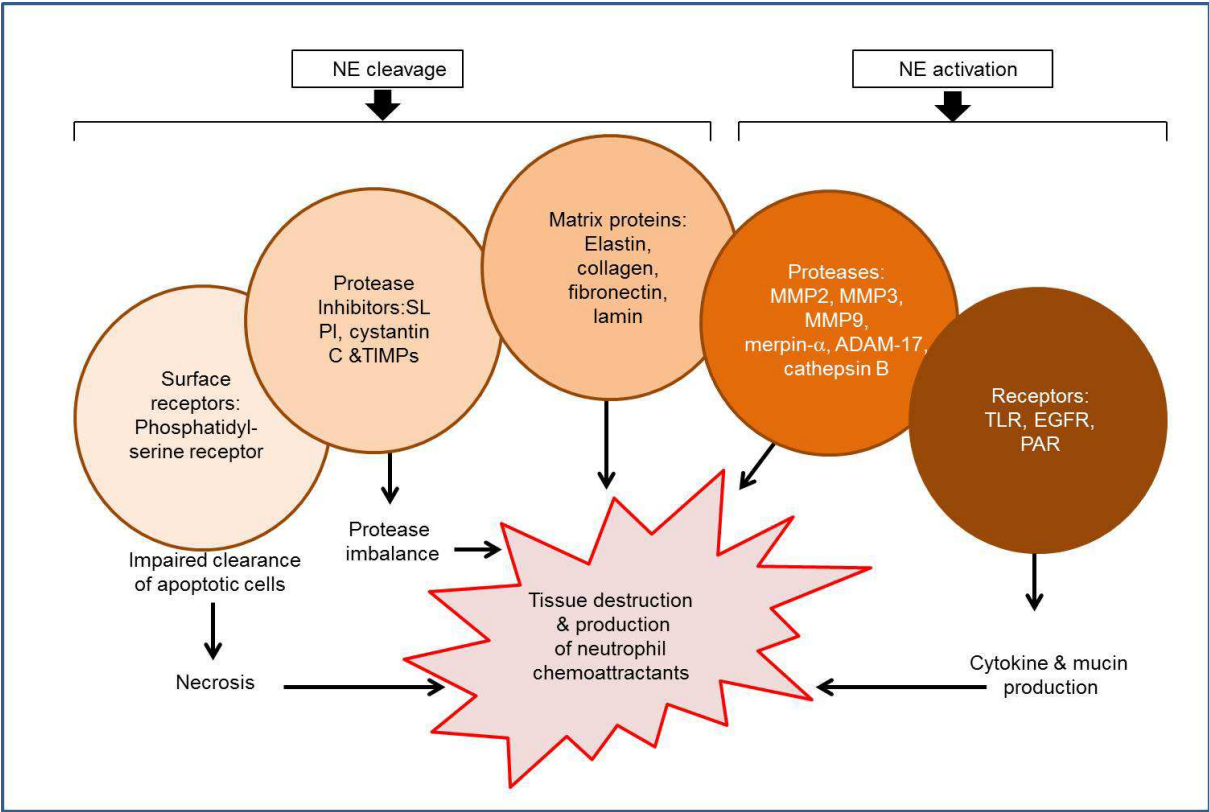
Figure 1. The potential effects of active NE in CF. Neutrophil elastase (NE) has the potential to cleave structural molecules and antiproteases causing an anti-protease imbalance and also activate enzymes and receptors that play a crucial pathological role in CF. Abbreviations used: NE, neutrophil elastase; SLPI, secretory leukocyte protease inhibitor; TIMPs, tissue inhibitors of metalloproteinases; MMP, metalloproteinase; TACE, tumour necrosis factor-alpha-converting enzyme; TLRs, toll-like receptors; EGFR, epidermal growth factor receptor; PAR, protease activated receptors.

Despite advances in our understanding of the molecular and cellular basis of CF, there persists an enigma as to why recruited neutrophils fail to eradicate bacterial infections in the lung. Support for the hypothesis that a genetic defect gives rise to dysregulated neutrophil responses in CF is shown by a study demonstrating upregulation of genes coding for both chemokines and proteins involved in signal transduction in CF [17]. A recent study has demonstrated that CXC chemokine receptors 1/2 (CXCR1/CXCR2) haplotypes in CF modulate antibacterial neutrophil functions against *P. aeruginosa* [18]. In addition, CF circulating neutrophils display an altered toll-like receptor (TLR) expression when compared to blood neutrophils from healthy subjects, including reduced expression of TLR2 [19, 20]. A recently published study identified IFRD1 (interferon-related developmental regulator 1), a histone-deacetylase-dependent transcriptional co-regulator expressed during terminal neutrophil differentiation, as a genetic modifier of CF disease severity. Neutrophils isolated from IFRD1 deficient mice exhibited impaired oxidative burst, bacterial clearance, and cytokine production leading to excessive bacterial burden and chronic infection of the lung [21]. Furthermore, levels of IFRD in bronchial epithelial cells with the *ΔF508* mutation were significantly reduced but could be rescued by treatment with glutathione suggesting down-regulation of IFRD1 expression in response to oxidative stress [22].

The focus of this chapter is on new research authenticating the CF neutrophil as a key player in disease pathogenesis. Studies published in the previous 10 years specifying intrinsic abnormalities due to a lack of CFTR function, along with reports indicating reprogrammed cell activity secondary to chronic bacterial infection and inflammation will be discussed. Our review of the literature was carried out using the MEDLINE database (from 2005 to the year 2015), Google Scholar, and The Cochrane Library databases using several appropriate generic terms.

2. The debate on CFTR expression by neutrophils

The year 2015 marks the 26th anniversary of the identification and isolation of the *CFTR* gene from epithelial cells [23]. This initial discovery was vital in the development of CFTR-targeted pharmacotherapy and resulted in an explosion of research aimed at identifying the expression of *CFTR* mRNA transcripts and protein levels in epithelial cells and non-epithelial cells alike. Although CF is a pleiotropic disease that affects multiple organs, disease progression is most pronounced in the airways and therefore researchers initially studied the expression of *CFTR* in epithelial cells found within the lungs. In this regard, Yoshimura and colleagues (1991) demonstrated the expression of *CFTR* mRNA transcripts in human bronchial epithelial cells [24], and Trapnall *et al*. demonstrated equal expression of *CFTR* mRNA transcripts from isolated respiratory tract epithelial cells from healthy controls and homozygous and hetero-zygous *ΔF508* patients with CF [25]. The level of research at this stage indicated that the expression of the *CFTR* was epithelial cell specific, yet the conclusions drawn from a further study by Yoshimura suggested that the *CFTR*-gene had characteristics of a house-keeping gene, suggesting that it may be present in other cell types [26]. This latter manuscript prompted researchers to examine *CFTR* mRNA transcripts in other cell types and in late 1991, Yoshimura

demonstrated the expression of low levels of *CFTR* mRNA transcripts in non-epithelial cells, including T-lymphocytes, neutrophils, monocytes, and alveolar macrophages, all of human origin [26]. This was the first record of *CFTR* gene expression in a multitude of immune cell types, and the authors suggested an important role for CFTR Cl⁻ transport. In agreement, it was later demonstrated that alveolar macrophages from *CFTR* deficient mice retained the ability to phagocytose and generate an oxidative burst, but exhibited defective killing of internalized bacteria due to impaired acidification of the phagosome. Of interest, in this later study CFTR protein was not detected in either murine or human neutrophils [27] and further studies have shown CFTR independent phagosomal acidification in macrophages [28, 29]. Nevertheless, the expression of *CFTR* mRNA transcripts in human macrophages was confirmed by Del Porto, who published that the bactericidal capabilities of macrophages was CFTR dependent, indicating an important functional role for the CFTR protein in these immune cells [30].

Up to this point, no connection between abnormal neutrophil function and the expression of CFTR protein in human neutrophils had been made, and whether or not neutrophils express functional CFTR was still the topic of great debate among leading scientists and clinicians [5, 8, 13, 14], with relevance for the development of CFTR-targeting pharmacotherapy. This is emanating from worldwide laboratories equally committed to the pursuit of knowledge on the cause for impaired neutrophil activity in CF and also the potential consequence of the loss of functional CFTR Cl⁻ channels. Indeed, there is still great controversy as to the true nature of dysregulated neutrophil activity in CF and, for example, in 2010, McKeon and colleagues could only detect low levels of *CFTR* mRNA transcripts in human neutrophils by either reverse transcriptase polymerase chain reaction (RT-PCR) or real time PCR methods of amplification [31]. The authors could not detect CFTR protein expression in membrane or cytosolic fractions, or cell lysates from human neutrophils by Western blot analysis, suggesting that human neutrophils do not express CFTR protein and that the dysregulated neutrophil function in CF was due to the inflammatory status of the individual [31]. Similarly, a study by Morris *et al.*, (2005) who investigated altered phagocytosis of neutrophils in CF due to cell priming, did not detect CFTR protein in human neutrophils by Western blot analysis [32].

Over the years, a number of reasons for not detecting CFTR protein in neutrophil cell fractions by Western blot analysis have been put forward and include the susceptibility of CFTR protein to degradation, a lack of reliable anti-CFTR antibodies, and also boiling of cell fractions prior to electrophoresis. As these issues were addressed researchers have detected CFTR protein in human neutrophils and have established functional roles for membrane associated CFTR. In line with this concept, in 2006, Painter and colleagues demonstrated the presence of CFTR protein in human neutrophil membrane phagolysosomes by confocal microscopy, and also verified the expression of *CFTR* at a mRNA level, and at a protein level by Western blot analysis [33]. Interestingly, in this same study results demonstrated the expression of CFTR protein by Western blot analysis in a human myeloid cell line (HL-60 cells) differentiated into neutrophil-like cells [33]. This is in contrast to the study by Yoshimura *et al.*, who could not detect *CFTR* mRNA transcripts in HL-60 cells [24]. Further studies have identified *CFTR* mRNA transcripts in differentiated HL-60 cells and demonstrated CFTR protein localisation to the phagocytic vacuole, strengthening the similarities between HL-60 cells and human neutrophils [34].

Furthermore, expression of CFTR protein was confirmed by Pohl *et al*. (2014) in healthy control neutrophils and levels were severely impaired in neutrophils isolated from people with CF carrying the *ΔF508* or *G551D* mutation. By flow cytometry, this latter study also demonstrated that the percentage of CFTR positive cells significantly increased post phorbol ester stimulation [35], indicating upregulation of CFTR to the plasma membrane post activation. Interestingly, bacterial lipopolysaccharide (LPS)-stimulated murine neutrophils also demonstrated increased CFTR protein expression when compared to unstimulated cells, suggesting a role for CFTR in bacterial mediated neutrophil cell activation [36].

3. The role of CFTR in neutrophils: oxygen dependent and independent mechanisms of microbial killing

3.1. Oxygen dependent killing

The focus of this chapter will now turn to studies that have reported impaired neutrophil activity due to a lack of CFTR function. Of major relevance to bacterial killing in CF, reported defective killing of microbes due to conditions that prevail within the vacuole of phagocytosing neutrophils will be discussed. The process of neutrophil mediated bacterial clearance can be divided into two main processes: those that are oxygen independent and those that are oxygen dependent. With regards to oxidative mechanisms of microbial killing, the first indications that oxygen plays a role in the functionality of neutrophils was first discovered by Baldridge and Gerard [37]. By exposing canine neutrophils to bacteria they observed a significant increase in oxygen consumption [37], and later it was revealed that this increase in oxygen consumption was independent of mitochondrial respiration [38]. Following discovery of the respiratory burst, it was discovered that neutrophils from patients with chronic granulomatous disease (CGD) failed to mount a respiratory burst during phagocytosis and these individuals are characterised by an abnormality of neutrophil function and recurrent life-threatening infections [39, 40]. CGD provides the most definitive evidence for the physiological and clinical importance of the respiratory burst, or alternatively referred to as the NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase). Indeed, patients with CGD have played a vital role in understanding the structure and mechanism of the NADPH oxidase (Figure 2). The burst of oxygen consumption upon phagocytosis and the absence of this process in CGD leading to impaired bacterial killing was understood to indicate that the oxygen consumed was converted to antimicrobial oxidants. By exposing neutrophils to opsonised bacteria, Cohen and colleagues demonstrated that 99% of oxygen consumed was converted to superoxide (O_2^-) [41] and the requirement of O_2^- as a precursor to hydrogen peroxide (H_2O_2) was confirmed [42]. It is not known what concentration of H_2O_2 is attained within the vacuole, with measurement from $0.01\mu M$ [42] to 100mM estimated, depending on the amount of phagocytosis and the intracellular pH [43]. These oxidants produced by neutrophils may also contribute to lung tissue damage and the shield against oxidant-modulated injury is the extracellular anti-oxidant glutathione; but this is significantly decreased in CF epithelial lining fluid [44-47]. Of major relevance is that CFTR has been linked to extracellular glutathione

transport [48] and in paediatric reports of extremely high levels of protein oxidation have been detected in airway samples [49].
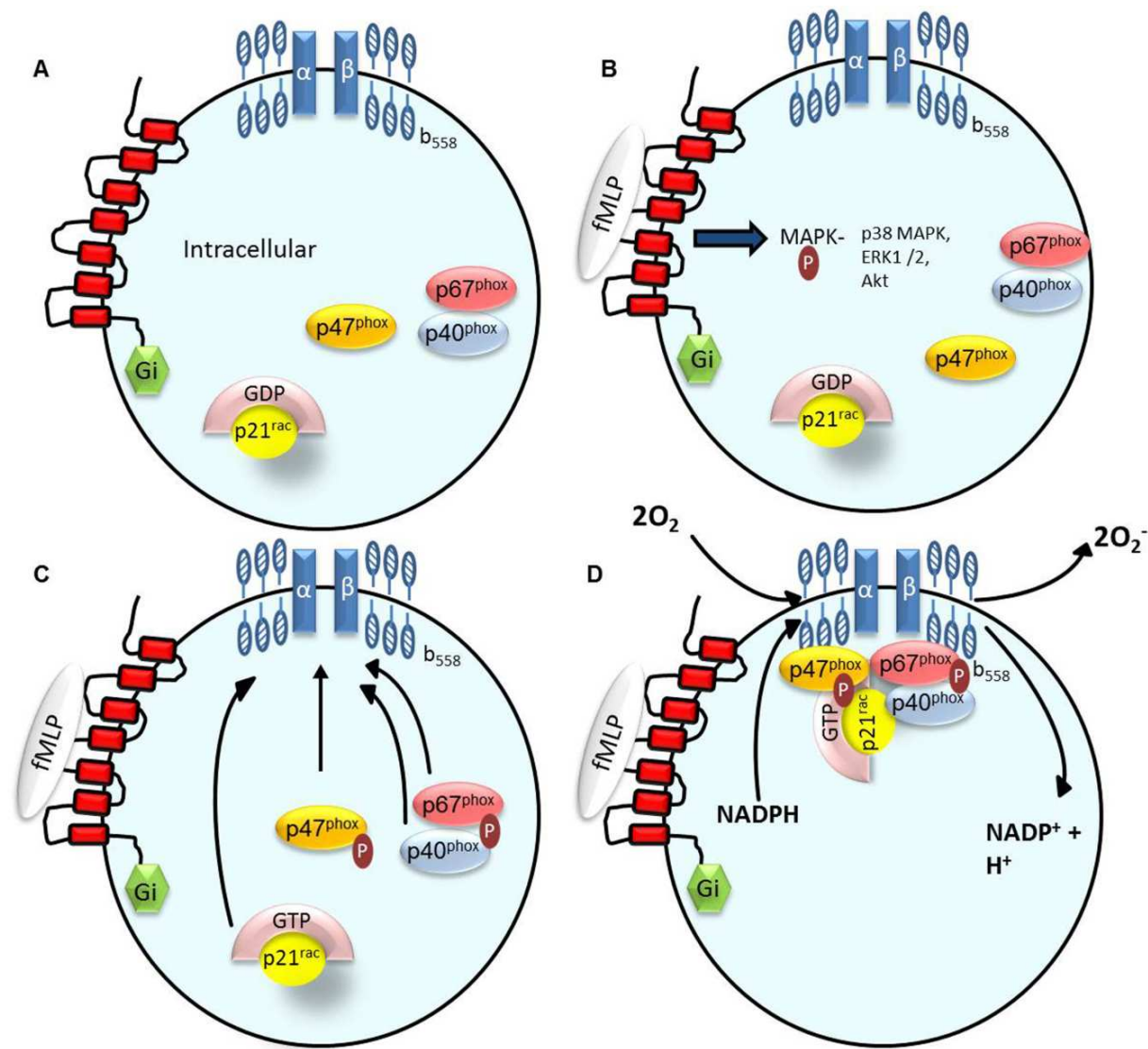
The neutrophil respiratory burst, or NADPH oxidase, generates superoxide (O_2^-) as the initial oxygen-derived reactive species in response to bacteria or a variety of soluble stimuli (fMLP). A) The enzyme complex is latent in quiescent unstimulated neutrophils and approximately 20% flavocytochrome b_{558} is found in the plasma membrane pool, and 80% in the specific granules. The active site of this enzyme is located in an integral membrane cytochrome, b_{558}, which consists of the two subunits gp91phox and p21phox (α and β subunits, respectively). B) Stimulation of the neutrophil by fMLP induces activation and phosphorylation (P) of a number of kinases including Akt. C) p21rac is converted into the active guanosine triphosphate GTP-bound form and the phosphorylation of the cytosolic components (p67phox, p47phox, and p40phox) occurs. D) These subunits then translocate to the plasma membrane where they interact with cytochrome b_{558} to initiate reactive oxygen species production.

Figure 2. Schematic illustration of the NADPH oxidase of resting and fMLP activated cells.

H_2O_2 generated during the oxidative burst has limited bactericidal properties and the best-defined function of H_2O_2 in the antimicrobial activities of neutrophils comes from the function of H_2O_2 as a substrate for myeloperoxidase (MPO) in the presence of halides (chloride (Cl^-)). Neutrophil MPO was initially called verdoperoxidase, and later its name subsequently

changed to MPO. It is present in exceptionally high concentrations in neutrophils, with levels estimated to be no less than 5% of the dry weight of the cell. MPO is synthesized and packaged into azurophilic, also referred to as primary granules of neutrophils, during the promyelocyte stage of granulocyte development and is present in mature resting granulocytes.

Mature MPO is a 150 kDa tetramer composed of two glycosylated 59–64 kDa heavy subunits and two unglycosylated 14 kDa light subunits as a pair of protomers linked together by a disulphide bond. When the phagosome containing microorganisms fuses with cytoplasmic granules, MPO, along with the other components of the granules, is released into the vacuole. A role for MPO as a component of the antimicrobial armoury of neutrophils was proposed with the finding that MPO was strongly microbial when combined with H_2O_2 and a halide [50, 51]. MPO and H_2O_2 form an enzyme-substrate complex, which oxidises ions to the toxic agent hypohalous acid. Any of the halide ions (I^-, Br^-, Cl^-) can be oxidised with iodide and bromide being more effective than Cl^- on a molar basis [51]. It is more likely however that the neutrophil uses Cl^- because it is present in high concentration in biological fluids, resulting in the formation of hypochlorous acid (HOCl). There are three proposed means of Cl^- transport to the phagosome: extracellular Cl^- intake during phagocytosis of a pathogen, stored Cl^- within granules released into the phagosome upon vesicle fusion, and passive or active transport of Cl^- from the cytosol to the phagosome [52]. Only active or passive transport has been suggested to provide a constant supply of Cl^- (Figure 3) [53] and two Cl^- ion channels (ClCs), ClC-3and CFTR have been implicated in the transport of Cl^- within the neutrophil and the phagosome [33, 54]. The influx of protons to the phagosomal lumen by V-ATPase has been demonstrated to facilitate the transport of Cl^- ions into the phagosome by ClCs including the CFTR [55] (Figure 3).

It is generally believed that HOCl is the most bactericidal oxidant known to be produced by the neutrophil. Levels of HOCl produced are based on calculations made after phorbol ester stimulation, which results in the secretion of O_2^- across the membrane to the surrounding supernatant and levels achieved are estimated at 80μM.

Levels produced in the phagocytic vacuole have been estimated using chlorinated fluorescein as a specific marker for HOCl production and by use of this technique it was calculated that 11% of oxygen consumed was converted to HOCl, resulting in 28μM within the phagosome [56]. HOCl is an extremely strong non-radical oxidant and bacterial targets include adenosine triphosphate (ATP)-generating systems [57], disruption of basement membranes or cell membranes, and fragmentation of proteins [58]. Chloramines are generated indirectly through the reaction of HOCl with amines, but are less reactive than HOCl but much more stable, and are therefore called "long lived oxidants." Because of the high intracellular concentration of the β-amino acid taurine, *N*-chlorotaurine is the major compound of low molecular weight long lived oxidants produced by neutrophils [59, 60]. Of major relevance to bacterial killing in CF it was reported that CF neutrophils had impaired chlorination of internalised bacteria within the phagosome when compared to healthy control neutrophils [33]. The authors highlighted the role of CFTR in facilitating Cl^- flux into the phagosome of neutrophils which was impaired in CF cells. This CFTR dysfunction resulted in limited availability of phagosomal Cl^- required for the generation of HOCl, resulting in reduced intravacuolar killing of *Pseudo-*
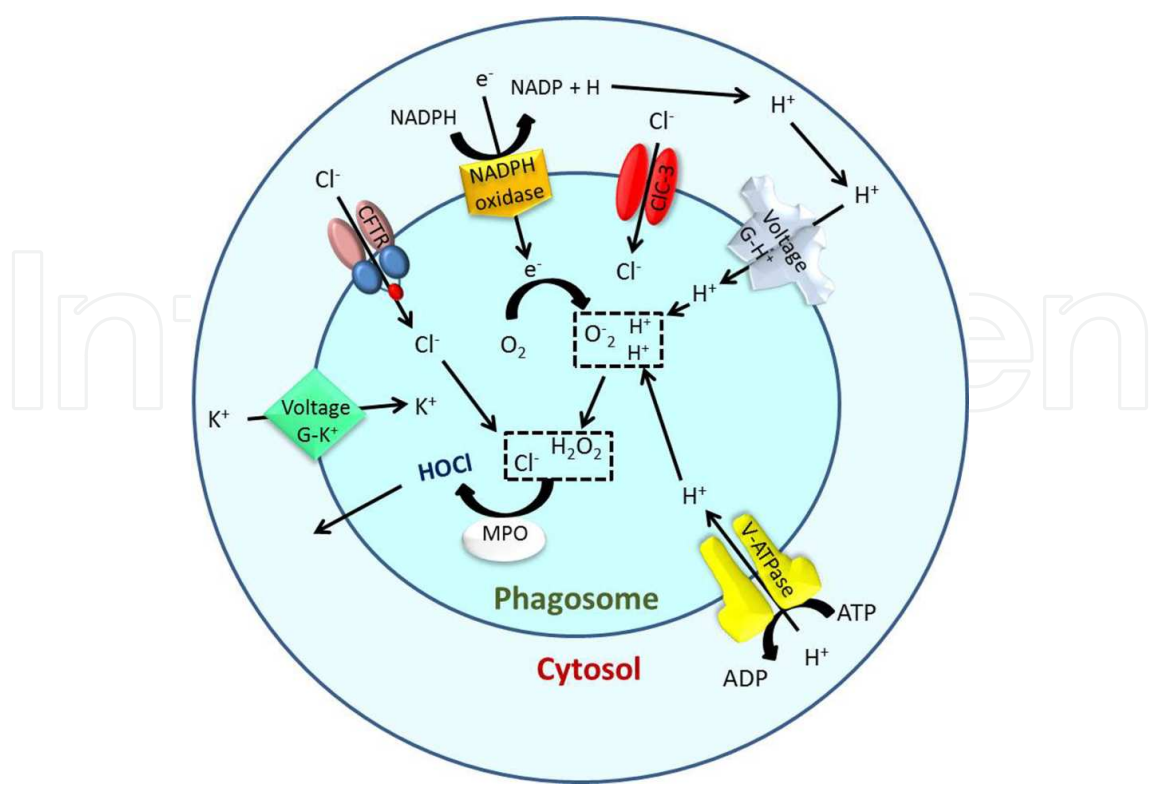
Upon activation of the NADPH oxidase, the cytochrome takes electrons from NADPH and passes them, via FAD and haem, to O_2 in the following reaction: $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$. NADPH oxidase generates an electron current which is compensated through voltage gated ion influx of protons (Voltage G-H^+) and potassium (Voltage G-K$^+$). Cl$^-$ transport is facilitated by CFTR and also other channels including ClC-3. The influx of H^+ to the phagosomal lumen by V-ATPase facilitates Cl$^-$ transport into the phagosome by ClCs including the CFTR. O_2^- is a mild oxidant and reductant with restricted biological activity. H_2O_2 is a slow acting oxidising agent and HOCl is a strong non-radical oxidant.

Figure 3. Suggested mechanism of Cl$^-$ and ion transport within the neutrophil phagosome.

monas aeruginosa, the archetype infecting organism in CF [33, 34, 53, 61]. Moreover, new data has shown that mutant CFTR fails to target to the neutrophil vacuole resulting in impaired intraphagosomal HOCl production and neutrophil microbial killing [62].

Having described the importance of MPO mediated halogenation for adequate microbial killing, there is also a need to mention that MPO deficiency occurs with a high prevalence and patients are not clinically afflicted by serious bacterial infections [63], with infections by *Candida* species being the main difficulty. Although more slowly than healthy cells, neutrophils deficient in MPO can kill bacteria *in vitro* [64], a result incompatible with the concept that HOCl is the major mediator of neutrophil bactericidal function in man. MPO deficient cells illustrate a prolonged respiratory burst resulting in increased levels of H_2O_2 and this coupled with non-oxidative methods of bacterial killing has been proposed to compensate for MPO deficiency. Furthermore, despite the fact that neutrophils of CGD patients do not produce O_2^- and H_2O_2, studies have shown that neutrophils from CGD patients are capable of killing significant numbers of phagocytosed bacteria and yeast [65, 66] and the presence of oxygen-independent microbial mechanisms in neutrophils is demonstrated by the ability of these cells to kill bacteria in the absence of oxygen [67].

3.2. Oxygen independent microbial killing

The presence of an oxygen independent microbial mechanism in neutrophils is demonstrated by the ability of these cells to kill bacteria under anaerobic conditions. Hirsch and colleagues reported that neutrophil lysates killed bacteria and that this effect was due to a substance they called phagocytin [68]. The component responsible for bacterial killing was localized to the cytoplasmic granules which are released into the phagocytic vacuole [69]. Neutrophil-derived microbial molecules are packaged within four distinct subgroups of granules (Figure 4) and are released either into the phagocytic vacuole or to the outside of the cell upon activation. Granule biogenesis follows the granulocyte differentiation pathway. The azurophilic (also referred to as primary) granules first emerge at the stage of the promyelocytes [70] and contain MPO, the serine proteases neutrophil elastase (NE), cathepsin G and proteinase 3, defensins and bacterial permeability-increasing protein [71], and are considered as the true microbial compartment mobilized upon phagocytosis. Later in differentiation, at the metamyelocyte stage, specific granules containing lactoferrin [72], 18 kDa human cathelicidin antimicrobial protein (hCAP-18), and lysozyme emerge [73], followed by a third population termed the gelatinase granules which predominantly contain gelatinase (matric metalloprotease (MMP)-9 and MMP-2), lysozyme, and leukolysin [74, 75]. A forth type of granule, called the secretory vesicles, appears at the stage of the mature neutrophil. Movement of the cytoplasmic granules following ingestion of bacteria was first observed by Robineaux and Frederic [76], and by use of chicken leukocytes with their large dense granules, Hirsch observed by phase contrast microscopy, degranulation and release of the granule contents directly into the phagocytic vacuole, by fusion of the granule membrane with the invaginated cell membrane [77]. As for any form of intracellular vesicle transport, degranulation is a tightly regulated process. Small GTPases of the Ras superfamily are known key regulators of cellular events including vesicle transport, cell division, control of cytoskeletal rearrangements, and nuclear assembly. Secondary and tertiary granules are tethered through a small GTPase Rab27a and its effector protein Munc13-4 [78] which interacts with the soluble N-ethylmaleimide association protein receptor (SNARE), a protein complex composed of vesicle-associated membrane proteins (VAMPs) on the vesicle surface and SNAP23 and STX4 on the plasma membrane [79-81]. Once the granule is docked the granule membrane fuses with the plasma membrane enabling release of granule contents. The small GTPase Rac2 has been shown necessary for the release of primary granules [82] but of interest, a sub-population of Rab27a positive primary granules was also found [83] with different Rab27a effectors Slp1 and Munc13-4 reported necessary for primary and tertiary granules release, respectively [84].

Although the neutrophil possesses an armoury of anti-microbial proteins and peptides, individual components have been shown to exert microbial effects. For example, NE has long been regarded as the major antibacterial protein and mice made homozygous for a disrupted NE gene have demonstrated impaired resistance to *Klebsiella pneumonia* and *Escherichia coli* sepsis [85]. A target for NE is the bacterial outer-membrane protein OmpA [86]. NE degradation of OmpA results in cell death as a result of loss of bacterial integrity by localized weakening of the cell wall followed by osmotic lysis [86]. The amino acid composition of cathepsin G shares 37% sequence homology with NE and plays a role in neutrophil responses against a
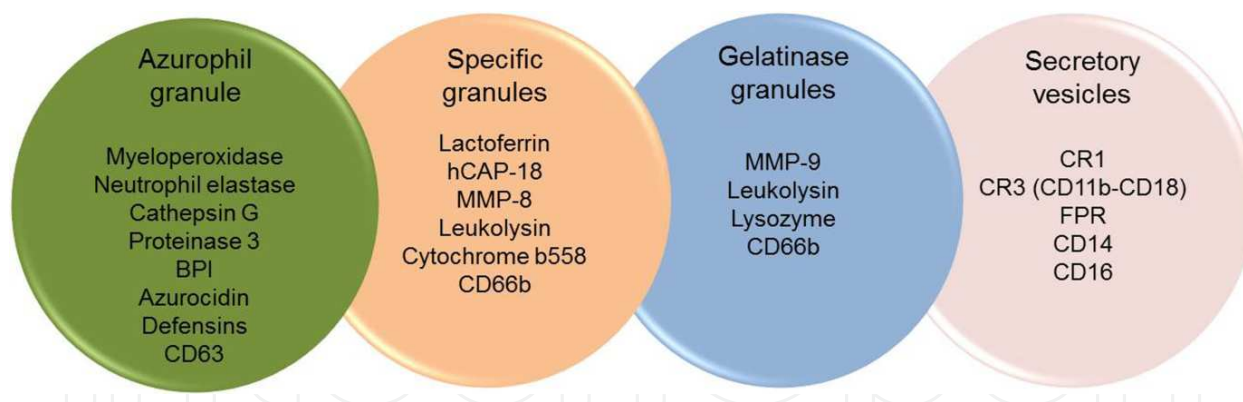
Figure 4. Granule and vesicle contents of the neutrophil. The most abundant organelles within the cytoplasm are the granules, which are membrane-bound organelles containing an array of antimicrobial proteins. Three major types have been identified, azurophilic, specific, and gelatinase-containing granules. A forth type of granule, called the secretory vesicles, are endocytic in nature and act as an internal reservoir of membrane/cytokine receptors.

variety of bacteria. Purified cathepsin G has been shown to inhibit the growth of several organisms including *Staphylococcus aureus*, *E. coli*, *P. aeruginosa*, and *Neisseria gonorrhoea* [87, 88]. Moreover, Tkalcevic and colleagues performed *in vivo* studies of mice deficient in cathepsin G, NE, or both and demonstrated the importance of cathepsin G in the successful clearance of *Aspergillus fumigatus*. Wild type mice almost completely cleared the fungal pathogen, while the single mutants showed an intermediate phenotype between the wild type and double mutants, thus establishing a critical role for both elastase and cathepsin G in the control of fungal infection *in vivo* [89].

Examples of proteins stored in specific (secondary) and gelatinase (tertiary) granules include human lactoferrin and MMPs. Lactoferrin is a major component of the specific granules and is active against a variety of pathogens [90]. This protein binds to bacteria through its highly positively charged *N*-terminus and displays antimicrobial properties against Gram-positive and Gram-negative bacteria by limiting the availability of environmental iron. However, since iron-saturated lactoferrin is also capable of killing certain bacteria, mechanisms other than iron depletion are involved. Further studies have indicated that peptides obtained after enzymatic hydrolysis of lactoferrin are much more effective in killing bacteria than is the intact protein. It is likely that the *N*-terminal cationic domain of human lactoferrin plays an essential role in the bactericidal activity and has been shown to be highly effective against infections with antibiotic-resistant *S. aureus* [91]. MMP-9 is stored in an inactive preform that requires activation by a serine protease such as NE. Its main function is the degradation of type V collagen in the extracellular matrix to aid migration to the site of infection [92]. The importance of MMP-9 in host defence was illustrated by a higher frequency of peritoneal sepsis in MMP-9 knockout mice due to impaired migration of neutrophils to the site of infection [93].

In spite of their original role in host defence, NE and proteinase 3 have been strongly implicated in the pulmonary pathology of CF. Indeed it has been shown that NE is the main mediator of proteolysis (Figure 1) but can also cause up-regulation of expression of other proteases including MMPs and cathepsins and as a result it has been proposed that neutralisation of NE activity is central to reducing the overall protease burden [94]. In line with this thought, NE

has the ability to degrade structural proteins in the lung including elastin, collagen, and fibronectin and to promote IL-8 production by bronchial epithelial cells [95], to degrade antimicrobial peptides [96], and to degrade antiproteases including alpha-1 antitrypsin, SLPI [97], and elafin [98] leading to a protease/antiprotease imbalance [99]. Moreover, it has been shown that the process of primary granule release by CF neutrophils appears altered, as greater levels of NE [100] and MPO [101] were recorded in the extracellular fluids post stimulation with either CF airway samples, TNF-alpha and IL-8, or serum-opsonised particles. Of importance, altered cytosolic pH regulation in CF neutrophils has been demonstrated [102, 103], which could in turn influence the process of degranulation. In further support of increased primary granule release by CF neutrophils, MPO and NE levels have been reported to be present at significantly increased levels in airway samples from patients with CF compared to controls [49, 104]. Moreover, levels of NE degranulation were not significantly altered following intravenous antibiotic treatment of patients with CF, indicating continued dysregulation of neutrophil activity even with clinical improvement [105]. While the mechanism for excessive primary granule release by CF neutrophils has not been fully investigated [104, 106], new research on the cause of reduced secondary and tertiary granule release has recently been revealed [35]. Contrary to increased release of secondary and tertiary granules by neutrophils of individuals with airway disease linked to alpha-1 antitrypsin deficiency (AATD) [107], evidence was presented indicating that abnormal CFTR function contributes to impaired neutrophil killing in CF due to inadequate Rab27a activation, which regulates the release of antimicrobial proteins from secondary and tertiary granules. In this study reduced degranulation of lactoferrin of secondary granules and MMP-9 of tertiary granules from patients with CF compared to healthy control cells was observed, an effect mirrored in healthy control cells post CFTR inhibition. Collectively results revealed that CFTR inhibition or dysfunction reduces cytosolic Mg^{2+} levels resulting in impaired Rab27a activity, ultimately reducing the CF neutrophils ability to kill bacterial pathogens [35].

4. The involvement of CFTR in neutrophil adherence leading to migration

Before describing the process leading to neutrophil adhesion, it is important to stress the complexity of studying neutrophil adhesion and migration. Blood neutrophils isolated from patients with CF are chronically exposed to pro-inflammatory cytokines, including LTB_4 and IL-8, and pathogenic particles including fMLP (formyl-methionyl-leucyl-phenylalanine) and LPS, resulting in these cells being in a constant primed state. Therefore, independent of the expression of CFTR protein, neutrophils in CF may illustrate enhanced cell adherence and migration. To circumvent this dilemma and to eliminate the potential for bias towards inflammatory versus altered neutrophil adhesion due to a lack of CFTR function, an approach taken by researchers is to include neutrophils isolated from inflammatory control patients. For example, in 1998, Russell and colleagues demonstrated that L-selectin shedding is altered in patients with CF resulting in increased neutrophil adhesion in response to IL-8 and fMLP [108]. This paper reported that non-CF bronchiectasis patients did not possess alterations in L-selectin shedding, suggesting that the defect in L-selectin shedding is CF specific and could be

a result of defective CFTR rather than the inflammatory status of the individual [108]. Indeed, CFTR expression in human neutrophils has provoked the idea that altered neutrophil migration and adhesion in CF could be caused by an intrinsic defect. Counteracting this concept however, Pohl *et al.* (2014) did not demonstrate impaired neutrophil migration in healthy control cells exposed to the CFTR inhibitor, CFTR(inh)-172, suggesting that altered neutrophil migration in CF is not inherent.

Circulating neutrophils generally adhere and migrate in response to pro-inflammatory mediators including TNF-alpha and pathogenic components including N-formyl peptides produced by bacteria. Lipid mediators involved in neutrophil adhesion and chemotaxis include LTB_4 [109, 110], with significantly increased levels quantified in sputum of patients with CF [111]. The chemokine IL-8 is the main neutrophil chemo-attractant involved in CF lung neutrophil infiltration [112], and increased levels of IL-8 have been detected in bronchial lavage fluid and sputum of patients with CF [113]. IL-8 is produced by a number of cells, including fibroblasts [114], epithelial cells [115], and by neutrophils themselves [116]. Interestingly, neutrophils isolated from children with CF demonstrate increased migration to IL-8 [117], and also release significantly increased levels of the chemokine when compared to the blood neutrophils of the same donor, suggesting that the environment that the cell is found triggers disproportionate release of IL-8 [116].

Neutrophils migrate in a multistep process consisting of rolling, tight binding, diapedesis, and migration. Initially, E-selectin and P-selectin are upregulated on the epithelium cell surface, and reversibly bind to L-selectin found on the neutrophil cell surface. In turn, L-selectin is shed from the neutrophil membrane resulting in up-regulation of integrins LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) which can both bind to ICAM-1 on the epithelium. Research studies have demonstrated that activation of integrins is in part mediated by IL-8 [118], and this is particularly relevant in CF as studies involving infants and children have demonstrated increased expression of IL-8 and ICAM-1, possibly indicating intrinsic inflammatory changes at a very early stage in disease progression supporting cell adhesion [119, 120]. This latter study is reinforced by data indicating that CF neutrophils show higher migratory responsiveness to IL-8 [117] supporting elevated numbers of neutrophils migrating to the airways. Moreover, neutrophil activation results in increased cytosolic Ca^{2+} levels triggering activation of calpain, a calcium dependent protease. Calpain has been demonstrated to liberate adhesion molecules CD11b and CD18, facilitating cell adhesion through the tight binding of integrins to epithelium cell surfaces [121]. Of major importance, studies have shown that CF neutrophils possess increased calpain activity, affecting cleavage of the cholesterol transporter caveolin-1, thereby modulating cholesterol trafficking to the plasma membrane [122]. Interestingly, and of major relevance to CF, Solomkin *et al.* (2007) demonstrated that cholesterol depletion in human neutrophils results in increased cell adhesion [123].

## 5. The involvement of CFTR in neutrophil apoptosis

Timely and effective neutrophil programmed cell death is essential for the resolution of inflammation [124, 125] and abnormal neutrophil apoptosis is associated with decreased

antimicrobial defences [126], incomplete microbial clearance, and sustained inflammation [127, 128]. Three different pathways are involved in the regulation of apoptosis and particular attention will be given to signalling pathways that are relevant to the neutrophil as it has been recognised that neutrophils have unique mechanisms of cell death due to their short half-life and phagocytic activity (Figure 5). Essential to the regulation and execution of apoptosis are the caspases, a family of cytosolic proteases or cysteine-dependent aspartate-directed proteases, involved in all three pathways. The first pathway described is the mitochondrial or intrinsic pathway. This pathway responds to physical and chemical stress signals, including growth factor withdrawal, DNA damage, and endoplasmic stress, and is transduced by members of the Bcl-2 family, which ultimately trigger the mitochondrial outer membrane permeabilisation (MOMP). As a result, several pro-apoptotic mitochondrial proteins, the most important being cytochrome C, are released into the cytosol. Subsequently cytochrome C, apoptotic protease activating factor-1 (Apaf-1) and caspase-9 form the apoptosome [129-131], this results in the activation of caspase-9 which thereafter activates caspase-3 and initiates the execution of apoptosis. In addition, caspase-8 can cleave Bid into truncated Bid (TBid), which ultimately triggers MOMP, thus providing a link from the intrinsic pathway to the extrinsic pathway.

The second pathway, the extrinsic or external death receptor pathway, is activated in response to extracellular signals such as FasL and TNF-alpha and is mediated by the binding of these members of the tumour necrosis factor family to death receptors on the cell surface (e.g., Fas, TNFR). The binding of the FasL and TNF-alpha to its cognate receptor results in the multi-merisation of the death receptor and the formation of the death inducing signal complex (DISC), containing multiple adaptor molecules such as the Fas associated death domain (FADD) or TNF-R1-associated death domain (FADD) or TNF-R1-associtaed death domain (TRADD). The respective adaptor death domain then interacts with caspase-8 [132, 133], which in turn leads to the autolytic activation from pro-caspase-8 to caspase-8. After activation and release of its active subunit (p18), caspase-8 then activates caspase-3, which finally executes apoptosis by releasing caspase-activated DNase (CAD) from its inhibitor (ICAD) with DNA fragmentation as a consequence [134]. The third pathway is the ER stress pathway. This pathway is thought to involve the activation of caspases, increase in cytosolic calcium and pro-apoptotic transcription factors in response to stress signals such as hypoxia, accumulation of unfolded protein, and alteration in calcium homeostasis within the ER [135, 136].

In the neutrophil the enzymatic activity of active caspase-3, -7, and -9 can be selectively inhibited by X-linked inhibitor of apoptosis (XIAP), a member of the conserved inhibitor of apoptosis (IAP) family of proteins [137]. Moreover, there are a number of key distinguishing features of neutrophil apoptosis. Firstly, the prominent role of the Bcl-2 homologue, Mcl-1, as a survival protein is central to the neutrophil's ability to undergo rapid apoptosis and may therefore limit the neutrophil's lifespan [138].

Mcl-1 is an unusual bcl-2 protein and can be rapidly turned over in the proteasome giving it a short half-life (2h) in the cell and is in contrast to the essential pro-apoptotic bcl-2 homologues, which are known to persist in the cell beyond 12 h. A critical excess of pro- over anti-apoptotic homologues decides the fate of the neutrophil and this eventually leads to loss of mitochondrial membrane potential and the progression of apoptosis [139]. Secondly, the involvement of
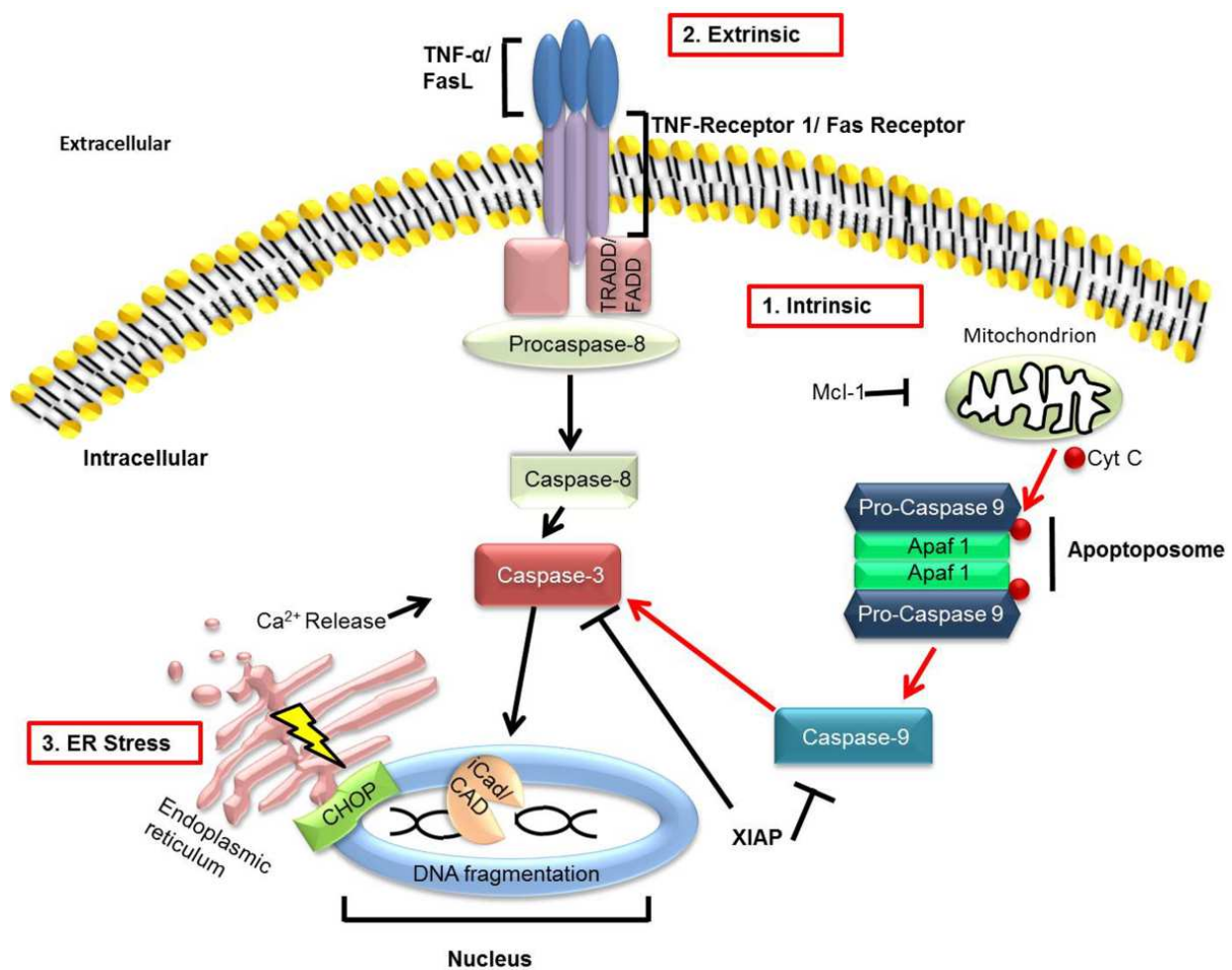
Figure 5. Summary schematic of apoptosis pathways in the neutrophil. [1] Intrinsic mitochondrial pathway (red): mitochondria release cytochrome c (Cyt C) in response to cellular stress. Together with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, Cyt C will form the apoptosome complex. This results in the proteolytic activation of the procaspase. Mature caspase-9 can then proteolytically activate caspase-3. [2] Extrinsic/ligand death-receptor pathway (black): death factors such as tumour necrosis factor alpha (TNF-alpha) and Fas ligand (FasL) trigger apoptosis by binding on death receptors such as tumour necrosis factor receptor 1 (TNFR1) and Fas. The death receptors recruit procaspase-8 by means of an adaptor protein, TNF-R1-associated death domain (TRADD), or Fas associated death domain protein (FADD). After cleavage the mature caspase-8 can then directly activate caspase-3. [3] Endoplasmic reticulum (ER) stress pathway (yellow bolt): the ER can also induce apoptosis as a reaction to ER stress by releasing intracytosolic calcium (Ca^{2+}) thereby activating effector caspases or through the expression of pro-apoptotic transcription factors such as CHOP. These different initiation pathways converge further downstream into activation of caspase-3. The effector caspase-3 cleaves ICAD (inhibitor of CAD) and releases it from CAD (caspase-activated DNase). CAD translocates from the cytoplasm to the nucleus and can now act as active endonuclease and fragment DNA. Inhibitors of apoptosis such as myeloid leukaemia cell differentiation protein (Mcl-1) and X-linked inhibitor of apoptosis (XIAP) act upstream against pro-apoptotic Bcl-2 family members at the mitochondria and downstream, directly inhibit caspases, respectively.

reactive oxygen species (ROS) is very specific to neutrophil apoptosis. The observation that neutrophils isolated from CGD patients which are known to be NADPH oxidase defective display a significant delayed spontaneous cell death relative to that of neutrophils from healthy donors is important [140]. This suggests that activation of the NADPH oxidase, with consequent production of ROS, is involved in spontaneous apoptosis and in regulating the programmed cell death of neutrophils during phagocytosis.

In contrast to the reported accelerated apoptosis in neutrophils from patients with airways disease associated with AATD [141], prolonged neutrophil survival has been reported in people with CF independent of infectious state and mutation type [142]. In support of this concept, Moriceau *et al.* (2010) illustrated that neutrophils isolated from heterozygous asymptomatic parents of people with CF exhibited delayed apoptosis [143, 144]. Moreover, the expression of the apoptosis-inducing membrane receptor Fas and its ligand have been reported reduced on CF neutrophils [145] while in addition, NE can degrade the phosphatidylserine receptor on macrophages, further delaying the removal of apoptotic neutrophils in CF [146].

6. The effect of CFTR mutation targeted therapy on neutrophil function

There can be no doubt that our grasp and understanding of the complex physiology of CFTR protein function has progressed majorly and permitted the development of exciting new treatments designed to target the basic defects of CF. Along with this, continuing improvements in clinical care have allowed improved outcomes for patients with CF including reduced morbidity and preserved lung function. As CFTR has been shown to be an integral membrane channel on neutrophils, the influence of new CFTR therapeutics on neutrophil function is an area of intense interest.

Class I mutations are thought to affect 5% of the CF population in Western society and in this class of *CFTR* mutations, there is complete absence of stable CFTR protein (due to non-sense mutations from premature stop codons) and thus replacement of the defective *CFTR* gene or changes in how the protein is made is required. However, the focus relating to the research surrounding gene addition therapy is its potential use in all class mutations in CF, with the ultimate aim of functional gene insertion and normalized *CFTR* expression. Studies on gene addition therapy have been developed to replace the mutant *CFTR* gene, and in this respect a phase II trial with non-viral lipid vector for DNA instillation has commenced. The study investigators have recommended monthly inhaled therapy for one-year duration (NCT 01621867). Further developments involve a lentiviral vector for gene therapy in this patient population [147] and the collective results of gene therapy on neutrophil function will be an exciting area of research in the future.

A second area of immense interest is the therapeutic use of aminoglycosides (e.g., gentamycin) and ataluren to cause read through of premature stop codons thereby allowing translation to continue to the end of transcription [148, 149]. Ataluren (PTC 124) is under investigation for its use and role in targeting premature stop codons. This compound has the potential to allow processing of premature stop codons, resulting in the production of normal length and functional CFTR, with insertion at the cell surface. Its beneficial effects have been proven with analysis of nasal chloride transport. The phase III trial in 238 patients with CF failed to achieve its primary end point (improvement in FEV_1) at 48 weeks, except in a small subgroup of patients not on concomitant nebulised aminoglycoside treatment [150].

Class II (e.g., *ΔF508*) and III *CFTR* mutations (e.g., *G551D*) have seen the advent of CFTR corrector and potentiator therapies. Corrector agents facilitate appropriate protein folding and

targeting to the cell membrane. The correctors lumacaftor (VX 809) or VX 661 are designed to promote increased quantities of ΔF508 CFTR at the cell membrane surface. VX 809 has shown good results *in vitro* but it is likely that adequate correction of ΔF508 CFTR will need multi-compound drug therapy [147, 151]. The potentiator ivacaftor (VX 770) was originally developed to augment the activity and efficiency of the abnormal CFTR protein (Figure 6).
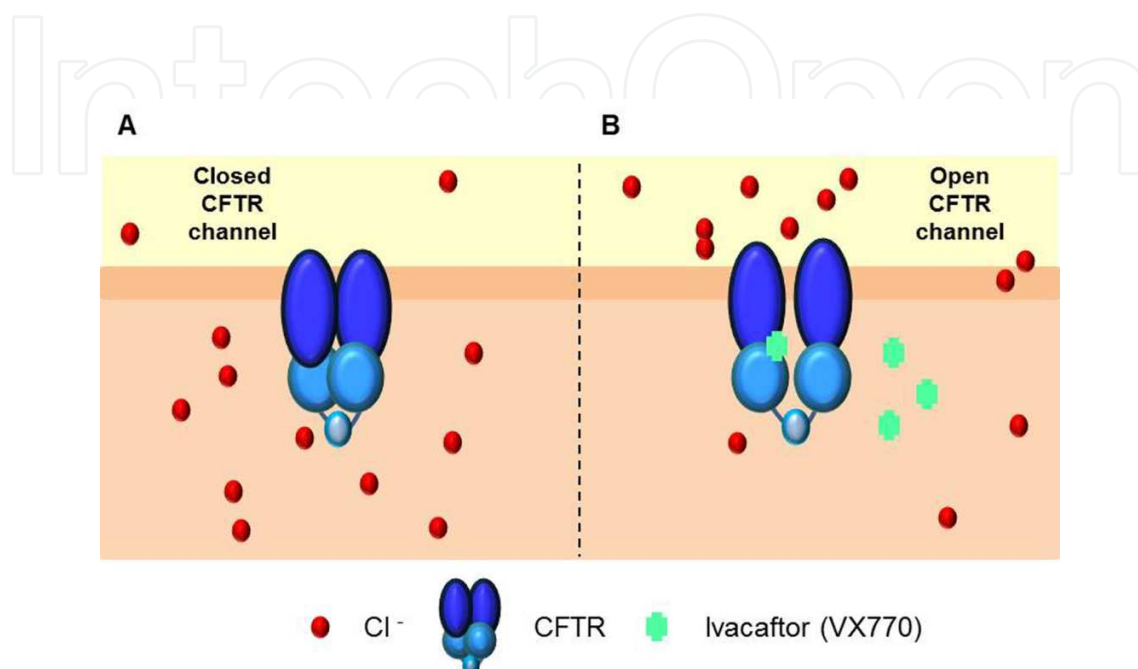
Figure 6. CFTR function in human epithelia. Panel (A) illustrates defective CFTR Cl⁻ transport due to the *G551D* mutation. Image (B) illustrates corrected function post VX-770 (ivacaftor) treatment.

Use of this therapy involved a well-designed randomized, double blind, placebo-controlled trial. The study subjects had at least one *G551D CFTR* mutation and were randomly assigned to ivacaftor 150 mg twice daily or placebo for 48 weeks. The treatment group showed a sustained improvement from baseline in FEV1 by 10% compared with the placebo group. They were also 55% less likely to suffer a pulmonary exacerbation compared with their placebo counter parts, had higher health scores, gained weight and normalization of their sweat chloride levels. These benefits were sustained for the duration of the trial and the frequency of adverse events in the two groups was equivocal [152]. A shortcoming of the use of ivacaftor is that only 2–3% of individuals with CF have the *G551D* mutation and research is currently underway to ascertain whether ivacaftor may be employed for other mutations. A multi-centre, phase II trial on the use of the CFTR corrector lumacaftor together with the CFTR potentiator ivacaftor for the treatment of patients with CF with the *ΔF508 CFTR* mutation was performed. The primary outcome was change in sweat chloride concentration with combination therapy in the *ΔF508* subjects, however a minimal effect on sweat chloride level was observed [153]. It is postulated that the reason behind this was that the potentiator rendered the CFTR protein less stable and increased its removal from the cell membrane. However,

currently there is a very motivated area of research into double or triple combination treatment for synergistic ΔF508 CFTR correction [154].

Of relevance to the circulating neutrophil and as *CFTR* mRNA transcripts have been reported in neutrophils at an expression level similar to those found in monocytes and alveolar macrophages [24, 33], the effect of CFTR corrector and potentiator therapies on neutrophil function is an anticipated area of research. In a study by Pohl *et al.* (2014), the mechanism leading to impaired degranulation by CF neutrophils was shown to involve altered ion homeostasis caused by defective CFTR function and significantly decreased levels of GTP-bound Rab27a. Of major importance, treatment of *G551D* patients with ivacaftor, normalized neutrophil cytosolic ion levels, and activation of Rab27a thereby leading to increased degranulation and pseudomonal killing. These results confirm that intrinsic alterations of circulating neutrophils from patients with CF are corrected by ivacaftor thus illustrating additional clinical benefits for CFTR modulator therapy. In line with this concept, neutrophils of ivacaftor-treated subjects demonstrated decreased cell surface CD63, a marker of primary granule release [155].

7. Conclusion

A plethora of studies on neutrophil function in CF have been performed and demonstrate alterations in cellular activities including impaired microbial uptake [156, 157], defective intracellular kinase activation [116], cellular inactivation [158], and increased oxidant formation [106, 159]. Furthermore, an additional area of intense research has focused on persistent mammalian target of rapamycin (mTOR) and cyclic AMP response element binding protein (CREB) pathway activation in CF airway neutrophils [160], with more recent data suggesting that neutrophils express augmented cell surface nutrient transporter expression and glucose uptake, consistent with metabolic adaptation [161]. As the CF neutrophil may shape the inflammatory response and influence patient outcome, further research investigating the CF neutrophil is required. In addition, a promising development in the treatment of airway inflammation involves the correction of CFTR dysfunction. If CFTR dysfunction is corrected at a very early age, it is possible that neutrophil induced inflammation involving impaired trafficking, delayed apoptosis, impaired degranulation, and bacterial killing may be significantly curtailed.

Acknowledgements

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