

# Neutrophils in cystic fibrosis

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## ABSTRACT

Lung injury in cystic fibrosis is caused by recurrent airway infection and inflammation. Neutrophils are important in combating these infections but are also the predominate cells involved in the inflammatory process. This review of neutrophils in cystic fibrosis describes the cellular mechanisms involved in their migration into the airways and their role in bacterial phagocytosis. We discuss the inflammatory process and its resolution and ultimately how neutrophil function can be modulated.

Infection and inflammation damage the lungs in patients with cystic fibrosis (CF), with changes beginning early in life.<sup>1</sup> Recent CF data suggest infection initiates and sustains airway inflammation.<sup>2</sup> The resultant lung injury is the main cause of morbidity and mortality in CF. Neutrophils play a vital role in lung defence against bacteria and are a fundamental component of the innate immune response. The airways in CF are characterised by a neutrophil dominated inflammatory process predominately on the respiratory epithelial surface.<sup>3</sup> Indeed, neutrophils are considered responsible for the onset and promotion of the inflammatory response within the CF lung.<sup>4</sup>

This review describes aspects of neutrophil biology, including migration, activation, phagocytosis, apoptosis and modulation. There are few data on whether there is a fundamental difference in the initial mechanisms of neutrophil migration into the airways of patients with CF. Here, inference is derived mainly from non-CF *in vitro* and *in vivo* studies but specific CF differences are discussed where available. Subsequent sections primarily reference available CF data.

## NEUTROPHIL MIGRATION INTO THE LUNG

Recruitment of neutrophils into the lung occurs via the alveolar capillary bed and postcapillary venules. It involves complex interactions between cytokines, cell adhesion molecules and chemoattractants, leading to migration across the endothelium, the extracellular matrix and the alveolar epithelium due to a chemoattractant gradient. These processes are summarised in fig 1.

### Transendothelial migration

Transendothelial migration principally involves three stages: rolling adhesion, strong adhesion and migration. Rolling adhesion is mediated by carbohydrate binding molecules called selectins, which promote leucocyte rolling.<sup>5</sup> In the resting state, the endothelium expresses few adhesion molecules and is relatively non-adhesive. Adhesion begins by the rolling of neutrophils along the endothelium. This transient tethering of neutrophils is mediated by selectins and is initiated

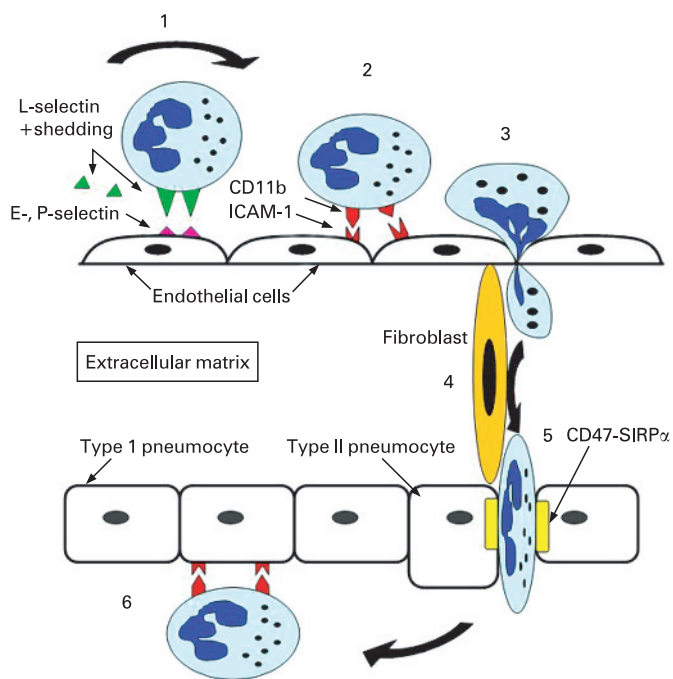
by expression of E- and P-selectins on the endothelial surface. It is recognised that patients with CF have raised levels of circulating E-selectin<sup>6</sup> and P-selectin<sup>7</sup> compared with healthy controls and may reflect a persistent inflammatory process. Leucocytes constitutively express L-selectin, which can be found on the tips of leucocyte microvilli that make contact with the endothelium first.<sup>8</sup> The binding capacity of L-selectin is rapidly and transiently increased following neutrophil activation, possibly via receptor oligomerisation.<sup>9</sup> However, the adhesion is only strong enough to induce rolling and not to stop the neutrophil completely.<sup>5</sup> L-selectin is rapidly shed from the surface of the leucocytes after their activation.<sup>10 11</sup> This can be mediated by interleukin 8 (IL8) and other chemoattractants, such as formyl-methionyl-leucyl-phenylalanine and platelet activating factor (PAF).<sup>11</sup> L-selectin shedding is required for the regulation of leucocyte rolling. Russell *et al* described a decrease in the shedding of L-selectin in stimulated neutrophils from patients with CF with an acute infective exacerbation.<sup>12</sup> Inhibition of L-selectin shedding decreases the leucocyte rolling velocity and increases the transit time of rolling leucocytes.<sup>13</sup> The transit time has been shown to be an important determinant of leucocyte recruitment *in vivo*.<sup>14</sup> The shedding of L-selectin may also increase neutrophil recruitment from the bone marrow.<sup>15</sup> During their time in the bone marrow, neutrophils increase their mobility, deformability and chemotactic responsiveness.<sup>16 17</sup> Normally only fully differentiated neutrophils enter the circulation, but stimulation of the bone marrow during an inflammatory reaction results in the release of more immature neutrophils into the circulation.<sup>18</sup> As immature neutrophils are larger and less deformable than mature ones, they preferentially sequester in lung microvessels and may mediate inappropriate lung injury.<sup>19 20</sup>

Strong adhesion of neutrophils involves  $\beta 2$  integrins. They are glycoproteins that have a common  $\beta$ -chain (CD18) and different  $\alpha$ -chains, including CD11a, CD11b, CD11c and CD11d. Integrins are present on the neutrophil surface but in a low avidity state and unable to bind with ligands on the endothelial surface until the neutrophils are activated. It has been shown that neutrophil expression of CD11b is reduced following intravenous antibiotics for pulmonary exacerbations of CF.<sup>21</sup> Inflamed endothelium produces chemoattractants such as PAF, leukotriene B4 and various chemokines, including the most potent neutrophil chemokine in CF, IL8. IL8 binds to the luminal surface of activated endothelium where it is able to activate neutrophils.<sup>22</sup> Bacterial cell products such as formylated peptides and lipopolysaccharide also activate neutrophils. These cell

products and other cytokines such as tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) also stimulate endothelial cells to synthesise IL8 and E-selectin.<sup>23</sup> Also, CD18 is activated during E-selectin mediated neutrophil adherence to endothelium.<sup>24</sup> The activation of CD11/CD18 on the neutrophil surface causes a change in its conformational state to a form that recognises the endothelial ligand. The important ligands for CD11/CD18 on the endothelial surface are intercellular adhesion molecule 1 (ICAM-1) and ICAM-2, which are members of the immunoglobulin superfamily. ICAM-2 is constitutively expressed whereas ICAM-1 expression is increased on inflamed endothelium by proinflammatory cytokines (eg, TNF $\alpha$ ).<sup>25</sup> Serum levels of soluble ICAM-1 are raised in patients with CF, even at times of clinical stability, compared with healthy controls,<sup>6</sup> suggesting an ongoing inflammatory process. This interaction between integrins and their ligands promotes strong adhesion and stops the neutrophil rolling.

Neutrophil migration occurs predominately at the borders of endothelial cells where modifications of cell junctions allow this. The cell adhesion molecules, platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and junctional adhesion molecule<sup>26-28</sup> are involved in neutrophil transmigration. Migration occurs via PECAM-1/PECAM-1 interaction while maintaining the permeability barrier of the endothelial cell monolayer.<sup>27</sup> The endothelial surface density of ICAM-1 is important in regulating this migration.<sup>29</sup>

Neutrophil adhesion to pulmonary endothelial cells and migration into the lung may occur by CD11/CD18 dependent or CD11/CD18 independent mechanisms.<sup>30</sup> Different stimuli



**Figure 1** (1) Transient tethering of the neutrophil to the endothelial surface is mediated by selectins (green and pink) and results in rolling adhesion. (2) Strong adhesion to the vessel wall is mediated by activated  $\beta$ 2 integrins (red). (3) Migration between the endothelial cells is facilitated by chemokines and chemoattractants. (4) Migration through the extracellular matrix occurs along fibroblasts. (5) Migration through the alveolar epithelial cells uses CD47-signal regulatory protein  $\alpha$  (SIRP $\alpha$ ) (yellow). (6) Tethering to the apical surface of the alveolar epithelial cells via  $\beta$ 2 integrins and ICAM-1 (red). ICAM-1, intercellular adhesion molecule 1.

within the lung can determine whether CD18 is required for neutrophil migration into the lung. It is felt that stimuli from gram negative bacteria require CD18, as 75–80% of neutrophil migration is inhibited by CD18 antibodies.<sup>31 32</sup> Animal studies indicate that neutrophils migrate to the lung via the CD18 dependent pathway in acute *Pseudomonas aeruginosa* infection, but the migration pathway shifts to the CD18 independent route after chronic exposure.<sup>33</sup> Mackarel *et al* have demonstrated the preferential use of the CD18 independent migratory mechanism by both control and CF neutrophils,<sup>34</sup> suggesting that blockade of the CD18 independent pathway may be a method of decreasing neutrophil influx into the CF airways.

### Migration through the extracellular matrix

Following transendothelial migration, neutrophils must pass through the interstitium before entry into the lung. The interstitium consists of two types of fibroblast, one that is arranged parallel to the epithelium and the other perpendicular. Burns *et al* demonstrated that neutrophils had increased adherence (which is partially CD18 dependent) and motility (which is totally CD18 dependent) on canine lung fibroblasts when stimulated with PAF and IL8, respectively.<sup>35</sup> Neutrophil adhesion to cultured lung fibroblasts is also partially dependent on fibroblast ICAM-1.<sup>35</sup> It has also been noted that neutrophilic expression of  $\beta$ 1 integrin is significantly increased after transendothelial migration,<sup>36</sup> and ligation of  $\beta$ 2 integrin provides a signal for  $\beta$ 1 integrin upregulation.<sup>37</sup> Several extracellular matrix proteins, including fibronectin, vitronectin, collagen and laminin have been shown to function as ligands for integrins.<sup>38</sup> It has recently been demonstrated that mindin, an extracellular matrix protein, functions as a novel ligand for integrins and plays a critical role in neutrophil recruitment.<sup>39</sup>

### Migration across the alveolar epithelium

Neutrophils enter the alveolar lumen at epithelial tricellular corners where the border of two type I pneumocytes meet the border of a type II pneumocyte.<sup>40 41</sup> It is possible that the positioning of fibroblasts in the interstitium direct them to this site.<sup>42</sup> The processes regulating neutrophil migration across the alveolar epithelium are not well understood. Transepithelial migration is in a basal to apical direction and, unlike transendothelial migration, the selectins and PECAM do not appear to be involved.<sup>43</sup> Much of the understanding of the processes of transepithelial migration are based on studies of intestinal epithelial monolayers. CD11b/CD18 plays an important role in neutrophil migration but its ligand on the basal wall of alveolar cells to facilitate this has not been totally elucidated. ICAM-1 is not expressed in this region<sup>44</sup> but is expressed on the apical region of cell boundaries<sup>45</sup> and is strongly induced by viral infection of airway epithelial cells.<sup>46</sup> Thus expression of ICAM-1 on the apical epithelial surface provides adhesive sites for neutrophils at the site of infection that assist in antibacterial activity.<sup>47</sup> This has been investigated by blocking ICAM-1 receptors on CF bronchial epithelial cells; this inhibited the adherence of neutrophils by 64%.<sup>48</sup> Furthermore, lung tissue collected at transplantation showed that neutrophils preferentially accumulated in the CF surface epithelium which over-expressed ICAM-1.<sup>7</sup> Therefore, following neutrophil migration, ICAM-1 can provide a mechanism for retention of neutrophils at sites where they are required.

CD47, an immunoglobulin superfamily transmembrane glycoprotein, is expressed on epithelial cells and neutrophils (where it is stored in secondary specific granules). This

glycoprotein is involved in neutrophil transmigration of intestinal epithelia following  $\beta 2$ -integrin dependent adhesion.<sup>49</sup> Its role in neutrophil migration of the alveolar epithelium has not been described. However, Rosseau *et al* demonstrated that monocyte migration across cultured alveolar epithelial cells depended on both CD11b/CD18 and CD47.<sup>50</sup> Signal regulatory protein (SIRP $\alpha$ ) is a transmembrane glycoprotein and is a cellular ligand for CD47. Interactions between CD47 and SIRP $\alpha$  have been shown to regulate neutrophil transmigration.<sup>51</sup> The interactions are complex and may involve SIRP on neutrophils and tissue expressed CD47 (trans interactions) or cis interactions between SIRP $\alpha$  and CD47 within the neutrophil membrane.<sup>52</sup>

Further studies are required to explore these interactions and enhance our understanding of neutrophil migration across the CF alveolar epithelium to the site of infection.

### GENES, CFTR AND THE NEUTROPHIL

Gene expression has been compared in blood neutrophils from patients with CF and healthy controls. A macroarray of 1050 genes revealed upregulation of 62 genes (including those coding for some chemokines and IL8) and downregulation of 27 genes in CF neutrophils.<sup>53</sup> None of the genes coding for adhesion molecules were modulated (eg, ICAM-1 and ICAM-2). CF sputum and blood neutrophils were also compared; this demonstrated upregulation of two genes in sputum neutrophils.<sup>53</sup> This included amphiregulin which is an epidermal growth factor receptor ligand that contributes to TNF induced IL8 release from airway epithelial cells, thus suggesting that amphiregulin is a new marker of lung inflammation in CF.

The most common genetic defect in CF results in defective transmembrane regulator protein (CFTR) processing so that the CFTR protein does not reach the apical surface of the epithelial cell. There is no direct link between the CF genetic defect and the process of neutrophilic migration across the airway epithelium.<sup>54</sup> CFTR mutations do not lead to aberrant synthesis of IL8. However, when CF neutrophils are cocultured with CFTR deficient bronchial epithelial cells, there is increased adherence and a threefold increase in IL8 levels.<sup>48</sup> This interaction may contribute to the sustained inflammatory response seen in CF. It has also been demonstrated that CFTR is expressed in neutrophils at the mRNA and protein levels<sup>55</sup> but it is unclear whether this specifically alters CF neutrophil function.

### NEUTROPHIL FUNCTIONS

When neutrophils arrive in the CF airway, they are primed, activated and engage in bactericidal phagocytosis releasing oxidants and proteases. These functions are described below.

#### Neutrophil priming and activation

Circulating neutrophils need to be primed to express their full bactericidal capacity. Neutrophils can also cause extensive endothelial cell injury after priming.<sup>56</sup> There is evidence that TNF $\alpha$  and IL8 in bronchoalveolar lavage from patients with CF play a significant role in the priming and activation of CF neutrophils.<sup>57</sup> When TNF $\alpha$  and IL8 are used as activating stimuli, CF neutrophils release significantly greater amounts of neutrophil elastase compared with neutrophils from control subjects and bronchiectatic patients.<sup>57</sup> Recent data have shown that airway neutrophils from patients with CF are primed and resistant to anti-inflammatory signals delivered by IL10.<sup>58</sup>

### Neutrophil phagocytosis

Following transport to the site of infection, bacterial phagocytosis by neutrophils can take place. This involves two different receptor classes found on the neutrophil surface: Fc $\gamma$  receptors, which include Fc $\gamma$ RIIA (CD32) and Fc $\gamma$ RIIIB (CD16), and complement receptors which include CR1 (CD35) and CR3 (CD11b/CD18 integrin).<sup>59</sup> Fc $\gamma$ RIIIB and CD11b/CD18 are the functional phagocytic receptors. Fc $\gamma$  receptor ligation initiates the vigorous extension of pseudopods that surround and ultimately entrap the bacteria.<sup>60</sup> Changes in the level of cytosolic calcium are required for granule secretion<sup>61</sup> and for granular fusion with neutrophilic phagosomes.<sup>62</sup>

Both intracellular and extracellular environments are important in the regulation of neutrophil function. It is recognised that pH can modulate neutrophil function. Lack of a CFTR dependent apical epithelial bicarbonate conductance has been suggested to cause increased acidification of the airway surface liquid. In this normally acidic milieu, bacterial ingestion may induce neutrophil necrosis, rather than apoptosis, and thus promote lung parenchymal degradation.<sup>63</sup>

Furthermore, with the overwhelming bacterial load and mucus characteristics of CF, the efficiency of neutrophil phagocytosis is reduced. Chronic *P aeruginosa* infection results in the secretion of quorum sensing compounds which play an important role in the formation of bacterial biofilms.<sup>64</sup> Neutrophils that settle onto biofilms appear to be unable to migrate away from the point of contact even though they are still capable of phagocytosis.<sup>65</sup> Neutrophil accumulation within biofilms may result in self-injury of the neutrophil by released oxidants which in turn compromises host defense mechanisms<sup>66, 67</sup> and necrotic neutrophils can also serve as a biological matrix to facilitate *P aeruginosa* biofilm formation.<sup>68</sup> Morris *et al* demonstrated that neutrophils from patients with CF had a lower phagocytic capacity than circulating neutrophils from the same patients or from normal control subjects.<sup>69</sup> The authors postulated that a failure of neutrophil phagocytic priming during migration into the lung was the cause.

Brinkmann *et al* have demonstrated that neutrophils generate extracellular fibres, or neutrophil extracellular traps. They are composed of granule (eg, neutrophil elastase and myeloperoxidase (MPO)) and nuclear constituents that disarm and kill bacteria extracellularly.<sup>70</sup> Interestingly, when neutrophil extracellular traps were dismantled with recombinant human deoxyribonuclease, an enzyme which selectively cleaves DNA, the killing of bacteria was negligible.<sup>70</sup>

### Oxidative burst

During phagocytosis of bacteria, neutrophils increase their oxygen consumption through the activity of NADPH-oxidase and superoxide is produced ( $O_2^-$ ). The  $O_2^-$  then rapidly dismutates to form hydrogen peroxide ( $H_2O_2$ ) catalysed by superoxide dismutase, and MPO is released from neutrophil primary granules.<sup>71</sup> This oxidative burst is crucial for bacterial killing but it has also been implicated in inflammatory damage to the CF airways. MPO is capable of enhancing oxidative induced injury to epithelial cells, most likely because of the formation of the cytotoxic oxidant HOCl.<sup>72-74</sup> It has been shown that stimulated neutrophils from individuals with CF release significantly more oxidants.<sup>75</sup> However, a recent study demonstrated that CF neutrophils exhibit normal extracellular production of HOCl but have a defect in their ability to chlorinate bacterial proteins from *P aeruginosa*, unveiling defective intraphagolysosomal HOCl production.<sup>55</sup> This potentially results in reduced neutrophil phagocytic efficacy against

this important CF organism. Van Der Vliet *et al* have demonstrated the formation of MPO derived oxidising and possibly nitrating species within the respiratory tract of patients with CF, which collectively may contribute to lung damage.<sup>76</sup> MPO levels have been found to correlate with decreases in pulmonary function and disease severity<sup>77 78</sup> with the MPO polymorphism -463G associated with more aggressive pulmonary disease in CF.<sup>79</sup>

### Proteases

Neutrophils also release proteases which are an important component of the phagocytic process. They can also degrade extracellular matrix and are therefore implicated in CF lung damage. The most important are elastase and the matrix metalloproteinases (MMPs).

A central role in the pathophysiology of CF has been attributed to neutrophil elastase. Neutrophil elastase is stored within the primary (azurophilic) granules and released following surface activation, phagocytosis and cell death. It has been demonstrated that isolated peripheral blood neutrophils from patients with CF spontaneously release more elastase than control neutrophils.<sup>80</sup> Importantly, this elastase production was not significantly altered following treatment with intravenous antibiotics, suggesting continuing elastase activity despite clinical improvement. Although its physiological role is to degrade phagocytosed proteins, it causes significant damage to the CF airway by degrading nearly all the structural proteins of the lung, including elastin, collagen type I-IV, fibronectin and proteoglycans.<sup>81</sup> Urinary excretion of desmosine, a cross linking amino acid specific to elastin, is a reflection of elastin degradation. We have recently demonstrated that this and neutrophil elastase are raised in patients that ultimately have a poor outcome.<sup>82</sup> Elastase can also cause prolongation of the inflammatory process by degrading complement and releasing C5a, a potent chemoattractant for neutrophils.<sup>83</sup> Neutrophil recruitment may be further augmented by the effect of elastase on the epithelium to synthesise and secrete IL8.<sup>84</sup> It can cause a reduced ciliary beat frequency of the respiratory epithelium and directly damage the epithelial cells.<sup>85</sup> As it is a potent stimulator of airway gland serous cells,<sup>86</sup> bacterial colonisation can be facilitated by excessive mucus production. Elastase may inactivate several components of the immune system (eg, immunoglobulins, immune complexes, complement components<sup>87</sup> and neutrophil cell surface receptors),<sup>88</sup> thus interfering with the ability of neutrophils to opsonise and eliminate bacterial pathogens. Recently, Hartl *et al* have demonstrated that IL8 promotes bacterial killing by neutrophils through its chemokine receptor CXCR1 (IL8RA) and that elastase activity in bronchoalveolar lavage fluid from patients with CF cleaves CXCR1 on neutrophils and disables their bactericidal capacity.<sup>89</sup>

Twenty-three different MMPs have been cloned to date, with additional members continuing to be identified.<sup>90</sup> Matrilysin (MMP-7) is induced in response to airway injury and is markedly upregulated in CF and its catalytic activity is essential for the repair of epithelial wounds.<sup>91</sup> MMP-7 is much more efficient than other metalloproteinases in the proteolytic inactivation of  $\alpha_1$ -antitrypsin (AAT).<sup>92</sup> Macrophage metallo-elastase (MMP-12) is the most elastolytic enzyme of the MMP family.<sup>93</sup> Liu *et al* demonstrated that neutrophil derived MMP-9 provides a shield for neutrophil elastase activity.<sup>94</sup> A recent study of children with CF showed that induced sputum MMP-9 had a significant correlation with neutrophils, IL8 and neutrophil elastase and an inverse relationship with forced expiratory volume in 1 s (FEV<sub>1</sub>).<sup>95</sup>

In the normal lung, the airways are protected from the damaging effects of proteases mainly by AAT and secretory leucoprotease inhibitor. Secretory leucoprotease inhibitor is produced by the respiratory epithelium mainly in the larger airways. Only 33% is functionally active in the epithelial lung fluid and it is therefore unlikely that it plays a significant role in lung protection.<sup>96</sup>

AAT is the main elastase inhibitor in CF sputum.<sup>97</sup> In the absence of inflammation in the normal lung the antiprotease activity of these molecules outweighs the protease burden, preventing elastase damage to the airways and local host defences. However, elevated levels of bronchoalveolar lavage neutrophil elastase have been found in patients with cystic fibrosis younger than 6 months.<sup>98</sup> Therefore, AAT is overwhelmed due to a relative imbalance of protease and antiprotease. It can also be inactivated by the proteases themselves.<sup>99</sup> Thus neutrophil elastase induced lung injury may potentially occur early in life.

### RESOLUTION OF INFLAMMATION

Apoptosis plays a critical role in the host immune response and contributes to the regulation of inflammation. It is the major mechanism for removal of neutrophils from the sites of lung inflammation.<sup>100</sup> It involves a coordinated series of morphological and biochemical steps in the cell causing its removal by scavenger phagocytes.<sup>101</sup> Early changes on the neutrophil cell surface are particularly important as they signal macrophages to phagocytose rapidly moribund cells before toxic breakdown products or contents can injure surrounding tissue.<sup>102</sup> It also prevents macrophages from releasing proinflammatory mediators such as chemokines, granule enzymes and thromboxane<sup>103</sup> thus limiting the potential damage to the lung.

Apoptosis is a mechanism essential to the regulation of neutrophil haemostasis and inflammation. Therefore, alteration of neutrophil apoptosis in CF would have significant effects on the inflammatory response and resolution of infection.

The Fas (CD95)/Fas ligand (FasL) system is an important cellular pathway regulating the induction of apoptosis. Fas is a type 1 integral membrane protein and is a member of the TNF receptor (TNFr) family that mediates apoptosis following interaction with FasL. FasL is a type II protein member of the TNF family that includes TNF $\alpha$ . Fas is constitutively expressed on neutrophils, monocytes and eosinophils, whereas FasL expression is restricted to neutrophils. Therefore, co-expression of Fas and FasL on neutrophils could provide a mechanism for the spontaneous apoptosis seen in neutrophils,<sup>104 105</sup> or adjacent cells could initiate apoptosis if one expressed Fas and the other FasL.<sup>106</sup> However, a recent study has investigated Fas expression on neutrophils following intravenous antibiotics for pulmonary exacerbations of CF. Its expression on sputum neutrophils did not alter with treatment but its expression on blood neutrophils decreased following antibiotics.<sup>21</sup> Soluble FasL, an inducer of apoptosis, also decreased following treatment. This at first seems counter intuitive as it would be expected that neutrophil apoptosis should increase to aid resolution of infection and inflammation. Therefore, other apoptotic pathways may be involved to assist in this.

Other important factors to consider are TNF receptors 55 and 75 (p55 TNFr-I and p75 TNFr-II). TNF $\alpha$  acting through these receptors has a unique ability, unlike other neutrophil priming and activating agents, to induce apoptosis. Murray *et al* have shown this action to be bimodal (ie, prolonged incubation of human neutrophils with TNF $\alpha$  can reduce apoptosis).<sup>107</sup> Receptor p75 has a relatively short cytoplasmic domain with

no death domain sequence, unlike p55.<sup>108</sup> Therefore, p75 TNFr appears to function as a facilitator of the death signal primarily initiated via p55 TNFr. It is suggested that occupancy of p55 by TNF $\alpha$  is a prerequisite for TNF $\alpha$  induced neutrophil apoptosis and that TNF $\alpha$  binding to p75 TNFr is not critical for this process.<sup>109</sup> A recent study has shown a reduction in soluble p55 TNFr in sputum following treatment of pulmonary exacerbations of CF.<sup>21</sup>

Pyocyanin, the major phenazine exotoxin, produced by *P aeruginosa*, has also been shown to induce apoptosis.<sup>110</sup> The authors suggested that inappropriate induction of apoptosis could deplete neutrophil numbers and function and in turn impair host defence. However, it has been shown that the percentage of apoptotic neutrophils in CF sputum did not vary with different types of bacterial infection.<sup>111</sup> Defective airway clearance of apoptotic cells in CF may be due to elastase mediated cleavage of phosphatidylserine receptors on phagocytes and therefore may contribute to ongoing airway inflammation.<sup>112</sup>

It is clear that further work is required to define the role of neutrophil apoptosis in CF and its place in the resolution of the inflammatory process.

## NEUTROPHIL MODULATION

Most of the major clinical manifestations, morbidity and mortality of CF are related to the progressive damage to the airways. Therefore, modulation of neutrophil function may attempt to redress this. The effects of pharmacological treatments on neutrophil function are discussed below.

### Non-steroidal anti-inflammatory drugs

As CF involves infection and chronic inflammation, studies have examined the role of non-steroidal anti-inflammatory agents such as ibuprofen.<sup>113</sup> A study by Konstan *et al* revealed that ibuprofen led to a slower decline in pulmonary function and improved body weight. However, there were concerns over side effects, and plasma concentrations must be tightly controlled. Paradoxically, neutrophil activation is increased by treatment with ibuprofen at doses lower than the therapeutic range. More recently, Konstan *et al* demonstrated a 31% reduction of neutrophils in CF oral mucosa if peak plasma ibuprofen concentration was >50  $\mu\text{g/ml}$ .<sup>114</sup> However, Fennell *et al* have investigated the use of high dose ibuprofen in a paediatric CF centre and discovered that nearly half of the patients discontinued therapy due to adverse events.<sup>115</sup> The authors commented that neither the use of ibuprofen nor its cessation resulted in a significant change in the rate of decline in pulmonary function or influenced hospitalisation rates.

### Corticosteroids

An *in vitro* study has demonstrated that prednisolone can reduce neutrophil migration across cultured human endothelial and bronchial epithelial cells.<sup>116</sup> Oral corticosteroids have also reduced the rate of decline of CF lung disease but side effects have limited the use of this as therapy.<sup>117</sup> Despite frequent usage, the role of inhaled corticosteroids is unclear at present but a double blind placebo controlled trial of inhaled corticosteroid therapy showed no benefit from inhaled beclomethasone.<sup>118</sup> A recent multicentre randomised controlled trial in CF demonstrated no change in lung function or usage of rescue bronchodilators when inhaled corticosteroids were withdrawn.<sup>119</sup>

## Macrolide antibiotics

Several recent studies have reported important clinical benefits of azithromycin in patients with CF,<sup>120–122</sup> including improved lung function and quality of life, reduced hospitalisation and reduced systemic markers of inflammation. Macrolides may act through antimicrobial action or through immunomodulatory properties. Recent years has seen the use of macrolides, especially azithromycin, as an anti-inflammatory agent. The mode of action is not entirely clear but is thought to act by suppressing proinflammatory cytokines and altering neutrophil function.<sup>123 124</sup> Azithromycin in a CF mouse model attenuated neutrophil recruitment and inhibited cytokine (TNF $\alpha$  and macrophage inflammatory protein 2) release in lipopolysaccharide induced inflammation.<sup>125</sup> Macrolides can inhibit superoxide generation by activated neutrophils *in vitro*<sup>126</sup> and accelerate apoptosis.<sup>127</sup> A recent study of healthy human subjects administered azithromycin observed initial neutrophil degranulation.<sup>128</sup> Immediate oxidative responses to particulate stimuli were enhanced after azithromycin exposure, but there were reductions in IL8 and IL6 concentrations and delayed and prolonged reduction in the oxidative burst which persisted for up to 28 days after administration of azithromycin. The authors hypothesised that acute neutrophilic degranulation and oxidative burst may contribute to an antimicrobial effect and delayed inflammatory responses may contribute to an anti-inflammatory effect of macrolides. Inhibition of neutrophil elastase can also be induced by the 14 membered macrolides erythromycin and flurithromycin.<sup>129</sup>

## Antiproteases

In order to protect the lung from damage mediated by neutrophil elastase, the use of AAT has been investigated. The neutrophil elastase burden in adults has been shown to be suppressed with aerosolised plasma purified AAT.<sup>130</sup>

A double blinded, randomised, placebo controlled, parallel group trial in 39 patients with CF was carried out using nebulised transgenic AAT.<sup>131</sup> The safety and tolerability of AAT was demonstrated. Although MPO levels were generally lower on AAT, sputum free neutrophil elastase activity remained unchanged. However, in a study of 52 patients with CF by Griese *et al*, elastase activity and neutrophil numbers were reduced following inhalation of AAT.<sup>132</sup> Unfortunately, these studies did not show an improvement in lung function following treatment. As the study drug was only administered for 4 weeks, it is possible that more prolonged periods of AAT usage may be required before a clinical effect is seen. However, 4 weeks of inhaled AAT by subjects with CF improved the bacterial killing capacity of airway neutrophils and as a consequence the number of colony forming units of *P aeruginosa* in CF sputum decreased.<sup>89</sup>

## Other treatments

Several other treatments have been recently investigated as potential anti-inflammatory therapies. Deoxyribonuclease is an established treatment modality in CF which is thought to have its major action as a mucolytic agent. It has also been demonstrated that it can stabilise bronchoalveolar lavage neutrophil numbers, elastase activity and IL8 concentration over time.<sup>133</sup> It can also decrease bronchoalveolar lavage MMP levels.<sup>134</sup> A recent study has investigated the use of high dose oral N-acetylcysteine, a glutathione prodrug, in 18 stable patients with CF.<sup>135</sup> It revealed a reduction in airway neutrophil burden and sputum elastase activity. Furthermore, as cysteinyl

leukotrienes have been found in the sputum of patients with CF, a recent study investigated the effects of montelukast.<sup>136</sup> In this 20 week, randomised, double blind, placebo controlled, crossover trial in 26 patients with CF, the authors discovered that montelukast treatment increased FEV<sub>1</sub> and decreased sputum levels of IL8 and MPO. Additional multicentre studies are needed to evaluate the potential anti-inflammatory roles of these therapies in patients with CF.

Research into currently available drugs have investigated their anti-inflammatory properties and therefore may provide future therapies in CF. Pioglitazone, a peroxisome proliferator activated receptor gamma ligand, can through its action on nuclear factor κB attenuate lung ischaemia–reperfusion injury in rats.<sup>137</sup> These protective effects involve inhibition of the production of proinflammatory cytokines and reduce lung neutrophil accumulation. The administration of simvastatin, a lipid lowering drug, to a murine inflammatory model of acute lung injury demonstrated reduced bronchoalveolar lavage myeloperoxidase activity and total neutrophil counts.<sup>138</sup>

Anticytokines may also play an important role. Anti-TNF is an anti-inflammatory treatment for rheumatoid arthritis but TNF also plays an important role in host defense and tumour growth control. A recent meta-analysis of patients with rheumatoid arthritis treated with anti-TNF therapy revealed an increased risk of serious infections and a dose dependent increased risk of malignancies,<sup>139</sup> suggesting that the anti-inflammatory effects can potentially have unexpected and deleterious effects on patients with chronic inflammatory conditions.

## CONCLUSIONS

In this review, we have followed the neutrophil in its path to the airway lumen and described its functions and modulation. Host and bacterial derived chemoattractants play a key role in the migration of neutrophils into the lung but this is a complex area and not fully understood. Acknowledging the limitations of non-CF neutrophil experimental data and its interpretation in this complex inflammatory milieu, it is recognised that there is an increased burden of neutrophils in the CF airways and that there are functional differences in these neutrophils. Importantly, neutrophils are not only important effectors of bacterial phagocytosis but are also at the centre of the inflammatory process in CF. This duality is important to understand and to investigate as insights into CF specific neutrophil function may lead to novel therapies.

**Competing interests:** None.

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