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Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease

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Abstract

The pathological hallmark of cystic fibrosis (CF) chronic inflammatory response is the massive neutrophil influx into the airways. This dysregulated neutrophil emigration may be caused by the abnormal secretion of chemoattractants by respiratory epithelial cells and polarised lymphocyte T-helper response. Neutrophils from CF patients have a different response to inflammatory mediators than neutrophils from normal subjects, indicating that they are primed in vivo before entering the CF airways. CF neutrophils secrete more myeloperoxidase and elastase, mobilise less opsonin receptors and release less L-selectin than non-CF neutrophils. Moreover, they show altered cytokine production and a dysregulated chemotaxis response. Laboratory studies now suggest that CFTR is involved in regulating some neutrophil functions and indicate that altered properties of CF neutrophils may depend on genetic factors. Current gene therapy approaches are targeted to the respiratory epithelium, but many hurdles oppose an efficient and efficacious CFTR gene transfer. The possibility of CFTR gene therapy-based approach targeting CF neutrophils at the hematopoietic stem cell level is discussed.

Keywords: Cystic fibrosis; Hematopoietic stem cell; Inflammation; Respiratory epithelial cells; Neutrophils; *Pseudomonas aeruginosa*; Gene therapy

1. Introduction

One of the clinical hallmark of cystic fibrosis (CF) is chronic inflammatory lung disease dominated by polymorphonuclear neutrophil influx in the airways. However, these neutrophils are not able to clear bacterial infections, especially in the case of *Pseudomonas aeruginosa* colonisation. Repeated bacterial infections lead to a vicious cycle of endobronchial and endobronchiolar infection and inflammation, leading to further airway and lung damage. Early events in the development of CF lung disease could be a decreased mucociliary clearance due to mucus dehydration, a breach in innate immunity at the airway surface and an altered regulation of inflammatory responses by respiratory epithelial cells [1].

In this review, we will focus on the crosstalk between airway epithelial cells and neutrophils, and its involvement in the pathophysiology of CF lung disease. Moreover, we will discuss therapeutic implications.

2. CF lung disease is a prolonged, frustated acute inflammatory response to infections

The neutrophil-dominated inflammatory response of CF lung disease is unusual for a chronic-type inflammation and is typically characterised by the mononuclear infiltrates and granulomatous tissue. The neutrophil is a predominant cell type infiltrating the CF lung, suggesting that CF represents a prolonged primary inflammatory response like that seen in acute infection. This is consistent with the hypothesis that inflammation in CF airways is primarily driven by products of the local environment (macrophages and bronchial epithelial cells) rather than by T-cell-derived-lymphokines generated as part of the systemic immune response [2]. Alternatively, one could assume that CF is driven by a T lymphocytic response. T helper (Th) cell responses

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can be either Th1 (distinguished by IFN-γ production, low antibody response and macrophage activation) or Th2 (characterised by IL-4, IL-5 or IL-10 production and high antibody response) predominant [3,4]. The outcome of chronic infections has been assigned to the type of Th-cell response. Thus, high antibody levels [5] and an abundant number of neutrophils in the lumen of the infected bronchi/bronchioli might indicate that CF lung disease is a Th2-dominated response. Indeed, it has been shown that CF patients are characterised by an unbalanced Th1/Th2 lymphocytic response [6]. The reason for this predominant Th2 response is unclear. Many factors may influence the differentiation of $CD4^+$ Th cells: the cytokine profile and balance of cytokines evoked by the antigen; antigen dose; antigen-presenting cells and the cytokines they produce; host genetic background and the activity of co-stimulatory molecules; and hormones present in the local environment [7]. There is growing evidence that chronic inflammatory diseases in the gut mucosa, such as inflammatory bowel disease, and in the lung, such as allergic asthma, are due to dysregulation of the mucosal immune system and pathological T-cell responses in a genetically susceptible host (see [4] and references therein). Investigation of the correlation between CF genetics and Th cell differentiation will be useful for understanding the immunopathology of CF lung disease.

Whatever the mechanism that leads to neutrophil-dominated lung inflammation, neutrophils are not able to clear bacterial infections. This is due to the switching of *P. aeruginosa* from a non-mucoid to a mucoid strain [8,9]. The latter strain synthesises an exopolysaccharide, alginate, which protects the prokaryotic cells from neutrophil phagocytosis. In this sense, it is possible to speak about 'frustrated' phagocytosis. Here we review the cellular events underlying neutrophil recruitment and activation in the CF lung. For an in-depth review of the pathological consequences of neutrophil influx in the CF lung, we refer readers to other recent sources [2,10].

3. Recruitment of neutrophils and lymphocytes to the CF lung

Among host and bacterial chemoattractants (bacterial products, C5a and $C5a_{desArg}$, LTB_4), interleukin (IL)-8 is the major neutrophil chemoattractant in the CF lung. The synthesis of IL-8 by various pulmonary cell types including alveolar macrophages, bronchial epithelial cells and fibroblasts has been documented in vitro and in vivo. Alveolar macrophages may play a central role in the recruitment of neutrophils to the lung since they produce IL-8 in response to either an exogenous stimulus, i.e. bacterial-derived cell wall lipopolysaccharide (LPS) or autocrine stimuli such as TNF-α and IL-1β. Although the exact source of IL-8 in the airways of CF patients is still unclear, some studies suggest that respiratory epithelial cells play a key role in this respect. Indeed, CF airway tissues and epithelial cells produce significantly higher levels of IL-8 than non-CF cells. Bronchial epithelial cells from patients with CF produced detectable levels of IL-8 and IL-6, whereas cells obtained from healthy controls secreted little or no IL-8 and IL-6 [11]. Immunohistochemistry revealed that CF bronchial submucosal glands in patients homozygous for the ΔF508 deletion express elevated levels of IL-8 compared with non-CF bronchial glands [12]. This up-regulation was selective because the pro-inflammatory cytokines IL-1β and IL-6 were not differently expressed. Basal protein and mRNA expression of IL-8 were up-regulated in the cultured ΔF508 human bronchial gland cells [12,13]. Human tracheal gland serous cells from CF patients showed a much higher basal secretion of IL-6 and IL-8 than normal cells [14]. A tracheal CF epithelial cell line was shown to produce more IL-8, IL-6 and GM-CSF in response to *P. aeruginosa* than the control line, and these differences increased over time [15].

The chemokine RANTES (regulated upon activation, normal T cells expressed and secreted) is an inducer of chemotaxis of eosinophils, monocytes and memory T cells and it was found at the lower levels in CF than in the asthmatic patients [16]. TNF-α/IL-1β stimulation of RANTES was significantly greater in normal immortalised bronchial cells than in the CF counterpart [15]. Pyocianin, a secreted product of *P. aeruginosa*, reduced the release of RANTES by respiratory cells in the same conditions under which it increased IL-8 release [17]. Taken together, these findings indicate that CF airway epithelial cells have excessive IL-8 production either under basal conditions or in response to the bacterial stimulation. However, the lack of RANTES induction may determine a dysregulated chemotaxis of lymphocytes into the CF lung.

Fig. 1 summarises the complex interplay which may occur among macrophages, airway epithelial cells, neutrophils and lymphocytes in the context of the CF lung.

4. CF neutrophils show peculiar functional features (Table 1)

4.1. Metabolism, surface receptors and proteases

Not only there is an increased neutrophil burden in the lung of CF patients, but CF neutrophils differ from normal neutrophils. Neutrophils isolated from various CF patients show an increase in oxidative burst (induced by PMA or zymosan) in 30% of cases [18]. However, *P. aeruginosa* colonisation and IL-6 serum levels correlate significantly with an oxidative burst enhancement of resting neutrophils. The production of leukotrienes (LTB_4 and its metabolites) is significantly increased in CF neutrophils as compared to neutrophils isolated from

age-matched controls [19]. Circulating neutrophils isolated from uninfected CF homozygotes show an increased ability to generate myeloperoxidase (MPO)-derived oxidants as compared with neutrophils isolated from control subjects, both under basal conditions and upon stimulation [20]. Moreover, MPO and the long-lived oxidants chloramines are released at higher levels by CF than from control neutrophils. Since this functional disturbance is greatest after stimulation with complement and immunoglobulin-opsonised zymosan (a particle that can be phagocytosed), a link to opsonin receptor–mediated activation was suggested. Thus, opsonin receptor CR1 (CD35) and CR3 (CD11b) function and phenotypic expression were studied in whole blood leukocytes of different patient categories. Study of circulating and platelet-activating factor (PAF)-primed phagocyte luminol luminescence responses showed that neutrophils from CF children presented decreased mobilization of opsonin receptors in response to PAF exposure as compared to controls [21]. In this case, phenotypic expression correlated with functional capacity. Taken together, these data indicate a loss in opsonin receptor reserve in CF, consistent with in vivo immune activation (i.e. inflammation) (Table 1).

Neutrophils obtained from CF patients were shown to have an increased propensity to release their granule proteins, including eosinophil cationic protein and MPO, as compared to neutrophils from asthmatic patients and control subjects [22]. Again this suggests that CF neutrophils have been primed in vivo, possibly by cytokines.

Table 1
Dysregulated functions of neutrophils observed in CF [19–21,23,29,34,37]

1	Increased production of LTB_4
2	Oxidative burst
	(a) Increased MPO activity
	(b) Increased MPO-derived oxidants
3	Surface receptor function and expression
	(a) Decreased mobilisation of opsonin receptors
	(b) Decreased FcγRIII levels
	(c) Decreased shedding of L-selectin
	(d) Decreased IL-8 receptors
4	Increased production of elastase
5	Increased IL-8 and decreased IL-1ra production
6	Altered chemotaxis

Neutrohils from normal subjects and individuals with CF contained similar amounts of elastase (NE). However, after pre-incubation with CF bronchoalveolar lavage (BAL) fluid, significantly more NE was released by CF neutrophils [23]. Elastase release was reduced after neutralisation or immunoprecipitation of IL-8 and TNF-α in CF BAL fluid. Serum IL-8 and TNF-α levels

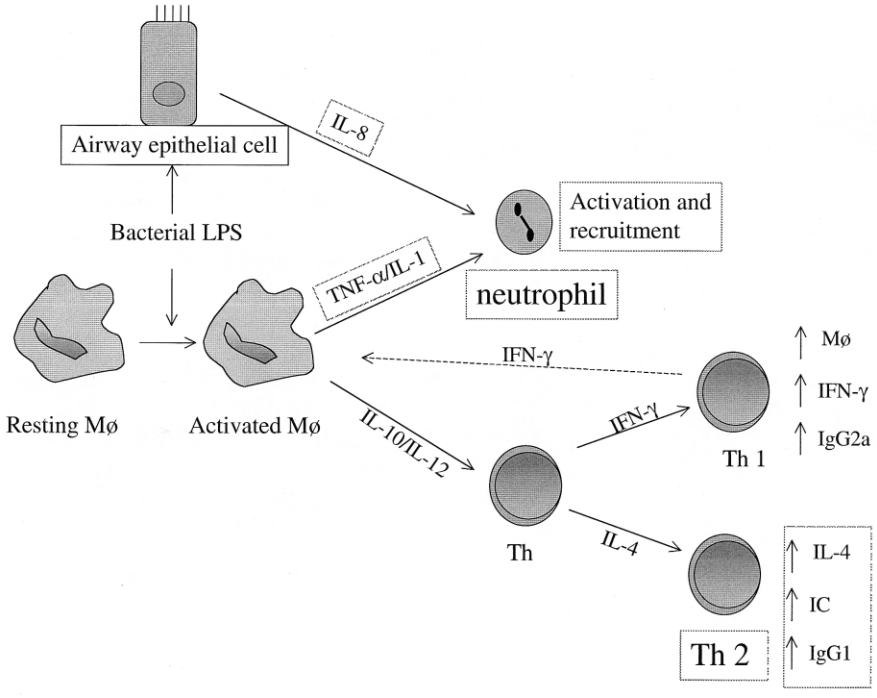

Fig. 1. Involvement of innate and adaptive immunity in chronic CF lung disease. Bacterial LPS can activate both macrophages (Mø) and airway epithelial cells, which in turn produce IL-8, TNF-α and IL-1β. These primary pro-inflammatory mediators are responsible for neutrophil activation and recruitment into the airways. Finally, cytokines secreted by activated Mø favour the development of either a Th1 or a Th2 response. The exaggerated activation of airway epithelial cells, neutrophils and Th2 response in CF is denoted by boxes. It is hypothesised that IFN-γ secretion by Th1 cells and activation of bactericidal activity of Mø cells is somehow impaired in CF (---). IC: immune complexes.

are elevated in CF patients [24], and this might be due to their leakage from the lung environment into the systemic blood circulation. Indeed, it was found that direct incubation of neutrophils with both cytokines released more NE in CF than in non-CF neutrophils [23].

In neutrophils, many functional responses, including triggering of secretion in azurophil granules (containing NE and MPO), oxidant production and microbe killing, are pH dependent. Coakley and associates found that intracellular pH after phorbol ester activation is more acidic in CF than in normal neutrophils [25], indicating that pH regulation in CF neutrophils is intrinsically abnormal, contributing to the alterations in CF neutrophil functionality. These data are in keeping with a recent report describing hyperacidifcation of trans-Golgi network in CF lung epithelial cells [26], indicating that the CF defect may be directly responsible for these pH abnormalities.

Taken together, these findings suggest that neutrophils are already primed for activation before they enter the lungs of CF individuals and that their activation is likely due to CFTR mutation.

4.2. Chemotaxis

The in vitro chemotactic response of peripheral neutrophils from patients with CF was shown to be normal for C5a [27] and decreased for LTB_4 [28]. Subsequently, Dai et al. [29] reported that CF neutrophils displayed a migration similar to that of non-CF cells at optimum IL-8 concentrations (10^{-8} M), but a reduced responsiveness to sub-optimal concentrations ($1–6\times10^{-9}$ M). Moreover, they detected lower numbers of receptors on CF neutrophils (22 000 per cell) than from control neutrophils (75 000 per cell).

Dean et al. [30] have shown that IL-8 concentration in sputum and BAL fluid of CF children is 3000–6600 pM, while serum concentration is 490 pM. While the accuracy and sensitivity of gradient concentration is reduced when receptor density is reduced, detection by neutrophils of steep concentration gradients is independent of the number of receptors. In view of the steep IL-8 concentration gradient across the lung of CF patients, it is unlikely that receptor down-regulation by systemic IL-8 will limit neutrophil recruitment in the lung.

Sener and associates [31] reported that neutrophil chemotaxis was significantly lower in acutely infected patients than in the clinically stable and healthy control groups. In a recently published report, neutrophil chemotaxis to IL-8 was studied in clinically stable patients. The migratory response of neutrophils from children with CF was significantly higher than that of non-CF children, and particularly so at 10^{-8} M IL-8 [32]. Interestingly, no difference in neutrophil migration to the bacterial derived peptide formyl-methionyl-leucyl-phenylalanine (fMLP) was seen between CF and non-CF neutrophils [29,32], indicating a selective abnormal chemotactic responses in CF.

Taken together, these results indicate that during acute episodes of infection neutrophil chemotaxis may be decreased due to the bacterial exoproducts, which have been shown to inihibit neutrophil chemotaxis [33]. This phenomenon may contribute to the persistence and pathogenesis of chronic bacterial infections in CF.

4.3. Cytokine production

The dysregulation of the inflammatory response in the CF airways may be due to an abnormal release of inflammatory mediators not only by epithelial cells but also by neutrophils. A recent report by Corvol and associates [34] compared the capacity of blood and lung CF neutrophils to release IL-8 and the anti-inflammatory cytokine IL-1ra. Blood neutrophils from CF patients constitutively secreted higher IL-8 and lower IL-1ra amounts than those from control subjects, suggesting either a sustained in vivo exposure of CF cells to various inflammatory mediators or a genetic component in altered cytokine production by neutrophils in CF. The spontaneous release of IL-8 and Il-1ra by airway neutrophils was significantly higher than that from blood neutrophils, indicating that the local environment may modify the functional properties of CF neutrophils. Interestingly, the spontaneous release of IL-8 was significantly lower in airway neutrophils from children with dyskinetic cilia than that from CF airway neutrophils, providing support for a role of genetic component in the altered neutrophil function in CF.

In summary, the ability of CF epithelial cells to produce large amounts of pro-inflammatory cytokines in conjunction with the hyperactive secretory response of CF neutrophils can both initiate and propagate a severe cycle of inflammation both at local (respiratory) and systemic level.

5. Transmigration of CF neutrophils

Migration of neutrophils from the blood-stream to sites of tissue inflammation involves adherence of the neutrophil to activated endothelial cells, squeezing through the endothelium, crossing the subepithelial matrix and finally migration through the epithelium. The adherence of neutrophils to the endothelium involves shedding of L-selectin from the surface and release of β-integrin Mac-1 (CD11b/CD18), contained in the neutrophil-specific granules, to the surface. In the recent years, it has become clear that neutrophil adhesion to pulmonary endothelial cells and migration into the distal air spaces of the lungs occur through one pathway that requires CD11/CD18 and one that does not [35]. Neutrophil emigration in response to *Escherichia coli*, *E.*

coli LPS, *P. aeruginosa*, immunoglobulin (Ig)G immune complexes, IL-1 and phorbol myristate acetate occurs through adhesion pathways that require CD11/CD18. In contrast, *Streptococcus pneumoniae*, Group B Streptococcus, *Staphylococcus aureus*, hyperoxia and hydrochloric acid elicit neutrophil emigration independent of CD11/CD18. Whether there is a similar pattern for the bronchial circulation is not known. However, in CF patients neutrophil extravasation at the level of bronchi, bronchioli and alveoli has been found [36]. The role of neutrophils that emigrate in the alveolar spaces is still unclear.

Upon stimulation with IL-8 or fMLP, neutrophils from both CF and non-CF subjects showed similar up-regulation of CD11b, while CF neutrophils shed significantly less L-selectin than control subjects [37]. This diminished L-selectin responsiveness was not observed in non-CF bronchiectasis patients. *P. aeruginosa*-induced pneumonia shows a dual behaviour in respect to the CD11/CD18 dependency. Neutrophils migrate to the lung via the CD18-dependent pathway in acute *P. aeruginosa* infection whereas, after chronic exposure the migration pathway shifts to the CD18-independent route, and is accompanied by a decrease in the number of neutrophil migrating to the lung [38,39]. Thus, it has been suggested that the reduced L-selectin shedding observed in CF patients [37] may reflect the maintenance of a heightened 'acute-type' response to *P. aeruginosa* (see Section 2).

Neutrophils release massive amounts of active proteases, including elastase, the major mediator of the observed lung damage. However, to cross the subepithelial matrix neutrophils need to synthesize and secrete specific proteases. One of these is urokinase-type plasminogen activator (u-PA), which binds on the leukocyte surface to its glycosylphosphatidylinositol-linked high affinity receptor, the urokinase receptor (u-PAR, CD87). The u-PA/u-PAR system is pivotal for leukocyte, smooth muscle cell and cancer cell migration [40–44]. There are no specific studies on u-PA/u-PAR involvement in neutrophil migration in the context of CF lung disease. However, it has been recently reported that mice deficient in u-PAR have profoundly diminished recruitment in response to *P. aeruginosa* pneumonia and impaired bacterial clearance compared to wild-type mice [45]. Further studies are necessary to evaluate the exact role of the u-PA/u-PAR system (or other protease systems) in allowing neutrophil migration through the sub-epithelial matrix in the lung.

Neutrophils migrate across the epithelium via a paracellular pathway resulting in disruption of epithelial tight junctions. Transepithelial migration is dependent on the neutrophil β2 integrin CD11b/CD18 and appears to involve adhesive interactions with the membrane glycoprotein termed CD47 (reviewed in Ref. [46]). However, it has been recently observed that neutrophil transepithelial migration was reduced by pre-incubation of epithelial cells with a F $(ab')^2$ anti-ICAM-1, or by pre-incubation of neutrophils with anti-CD18, anti-CD11a, anti-CD11b or anti-CD11c [47].

While CF neutrophils have not been investigated in this context, Pizurki and associates [48] have found that CFTR-driven adenovirus expression in CF monolayers did not lead to a difference in neutrophil migration across CF airway epithelial cells. Moreover, adherent *P. aeruginosa* promoted no difference in neutrophil migration across monolayers rescued or not with CFTR, indicating that the combined presence of a mutated CFTR and of *P. aeruginosa* is not enough to explain the excessive number of neutrophils in CF airways colonised by these bacteria.

6. Therapeutic implications

Given the high neutrophil burden and inflammatory response in the CF lung, anti-inflammatory therapy has been envisioned for CF lung disease. Both corticosteroids and non-steroidal anti-inflammatory drugs have been used in this context with mixed results [49,50].

Gene therapy could be the resolutive treatment for CF. The respiratory epithelium has been identified as the main target of CF gene therapy. Although transfer of the CFTR gene into human airway epithelial cells by viral and non-viral vectors has been achieved in animal models and in humans [51], many hurdles must be overcome before CFTR gene transfer can be considered efficient [52]. We and others have recently shown that mucus and surfactant may be considered barriers to viral and non-viral gene transfer vectors [53–55].

The finding of abnormalities in CF neutrophils leads to the possibility of hematopoietic stem cells (HSCs) as a new target for CF gene therapy. This should be more feasible than the airway epithelial cell approach, as HSCs can be easily purified from blood or marrow, ex-vivo transduced and reinfused in the same patient. Although the expression of CFTR in cell types of non-epithelial origin and in blood cells such as lymphocytes has been described, the presence of CFTR mRNA in mature neutrophils has been reported only once [56]. There is no evidence of the presence of the CFTR protein in neutrophils. B and T lymphocytes express a functional CFTR and in CF patients, CFTR-regulated chloride channel function is impaired as seen in epithelial cells [57–59]. Therefore, in neutrophils a functional CFTR protein might be expressed. Numerous experimental evidence suggests a genetic component in altered neutrophil function in CF [20,23,25,34,37]. For example, MPO-dependent oxygenation activity is significantly higher not only in CF homozygotes but also in heterozygotes parents of CF patients [20]. Moreover, some studies suggest that MPO-dependent oxidant generation,

intracellular pH regulation and glutathione concentration might be attributable to CFTR mutation in CF [60].

HSC-based cell therapy is currently limited by a number of hurdles. In chronic granulomatous disease, phase I clinical trials based on retroviral-mediated gene transfer of HSC are ongoing [61]. The frequency of corrected neutrophils ranged from 0.06–0.2%, whereas it has been established that complete correction of at least 10% of circulating neutrophils is necessary to observe a clinically relevant outcome. Morever, it is likely that more efficient gene transfer vectors into human long-term repopulating HSCs are needed. Much work is now focused on the human immunodeficiency lentivirus, which has the advantage of transducing relatively quiescent HSCs as compared to conventional oncoretrovirus vectors [62].

In conclusion, current gene therapy approaches to CF should be limited to the respiratory epithelium until CFTR expression in neutrophils is demonstrated and strong evidence for CFTR-derived dysfunctions in CF neutrophils is presented.

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