Clinical Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Review

Lung inflammation in cystic fibrosis: Pathogenesis and novel therapies ☆

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ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 11 December 2013
Accepted 16 December 2013
Available online xxxx

Keywords:
Cystic fibrosis
CFTR
Inflammation
Cytokines
New therapies

ABSTRACT

Despite remarkable progress following the identification of the causing gene, the final outcome of cystic fibrosis (CF) remains determined mainly by the progressive reduction of lung function. Inflammation of the airways is one of the key elements of the pathogenesis of the disease: it is responsible for the destruction of lung architecture, resulting in progressive loss of respiratory function. Bronchial infection induces an intense inflammatory reaction characterized by a massive invasion of neutrophils, the properties of which seems altered in CF. Moreover, the inflammatory process is also marked by a profuse release of soluble pro-inflammatory mediators, such as interleukin (IL)-6, IL-1β and IL-8 cytokines. In contrast, release of the anti-inflammatory mediator IL-10 is reduced, thus reflecting a pro-/anti-inflammatory imbalance. The inflammation/infection pair seems hard to dissociate, and the origin of the baneful consequences of the persisting excessive inflammatory responses remains to be cleared up: does inflammation follow or rather precede infection?
Recent data suggest that uncontrolled inflammation is constitutive in CF. Countering it at early stages of the disease in order to prevent irretrievable damages in lungs remains a major priority in treating patients with CF. In this review, we discuss the usefulness and limitations of mouse models of CF to study the pathogenesis of human lung inflammatory disease, and the development of new potential strategies to reduce the inflammatory burden in the airways.

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Introduction

Morbidity and mortality observed in CF are principally related to lung alterations characterized by a vicious circle of obstruction, infection and chronic inflammation of the airways. The sequence of events leading to progressive destruction of the lung architecture and loss of respiratory function is not fully understood. It has been suggested that the initiating event is dehydration of the fluid layer lining the surface of the tracheobronchial tree [1]. Depletion of airway surface layer (ASL) volume develops, in large part, as a consequence of abnormal transepithelial ion transport related to loss of function of CF Transmembrane Conductance Regulator (CFTR) protein [1], and it also seems regulated by local levels of nucleotide and nucleoside, ATP and adenosine [2]. Preclinical studies on various experimental models of animal and human origins all agree on a key role of the CFTR in the homeostasis of fluids on the surface of epithelia. The protein mainly acts as a low conductance cAMP-dependent chloride channel, but it also regulates several other membrane transport proteins, most notably the epithelial sodium channel ENaC [3,4]. Transepithelial ion fluxes, mainly of chloride and sodium, are coupled to water movements that maintain adequate hydration of the epithelium surface. CFTR dysfunction impairs the water balance of

☆ Authors' contributions: BD and SN helped in drafting the manuscript; FH helped in editing the manuscript; and TL designed and coordinated the work and edited the manuscript. All authors read and approved the final version of the manuscript.

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0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2013.12.020

Please cite this article as: Dhooghe B, et al, Lung inflammation in cystic fibrosis: Pathogenesis and novel therapies, Clin Biochem (2014), http://dx.doi.org/10.1016/j.clinbiochem.2013.12.020

ASL, reducing the volume of its aqueous film and thickening its mucus film produced by submucosal glands with a minor contribution of goblet cells, endangering the mucociliary clearance [5–7]. The thick dehydrated sputum obstructs the airways and prevents elimination of dust, bacteria and other impurities from the lungs, thus making them more vulnerable to repeated bacterial infections and chronic inflammation.

In addition to typical transepithelial ion transport abnormalities, other epithelial cell dysfunctions, including decreased sialylation of cell membranes [8–10], defective bacterial internalization [11], imbalanced omega3/omega6 polyunsaturated fatty acid (PUFA) metabolism [12–22] and cell membrane accumulation of ceramides [23–26] have allowed to make the link between mutated CFTR protein and chronic inflammation in CF airways.

Decreased sialylation of CF cell membranes has been reported in CF epithelial cells; it is associated with increased concentrations of the gangliotetraosylceramide asialoganglioside M1 (asialo-GM1), which serves as a cell receptor for the two main pathogens for CF patients, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [8,9]. Binding of *P. aeruginosa* and *S. aureus*, but not of *Escherichia coli*, the latter not associated to significant pulmonary disease in CF, was reportedly increased in polarized CF bronchial and pancreatic epithelia [9]. However, studies on the role of asialo-GM1 as an epithelial cell receptor for *P. aeruginosa* have provided contradictory results. Indeed, no enhanced binding of several laboratory strains and fresh clinical isolates of *P. aeruginosa* to asialo-GM1-treated cells was observed [10]. It has been reported that in normal lungs, CFTR protein itself serves as a cellular receptor for binding, endocytosing, and clearing *P. aeruginosa* [11]. Internalization of *P. aeruginosa* was about a hundred times larger in epithelial cells expressing functional CFTR compared to cells lacking it or expressing the mutant F508del CFTR protein. The first extracellular domain of CFTR was suggested to be the specific ligand for the bacterium. If binding to functional CFTR is the initial step of the process clearing *P. aeruginosa* from the lungs, a direct link is established between mutations in CFTR and a higher vulnerability to trigger and perpetuate infections [11].

Abnormal essential fatty acid (EFA) metabolism could be related to excessive inflammatory responses in CF [12–14]. Pancreatic insufficient patients display a more disturbed lipid metabolism [15], but it would be too simple to ascribe EFA deficiency in CF to reduce intake or to alter gastrointestinal handling (digestion, absorption, transport) as the sole pathophysiological mechanism. Reduced EFA circulating values have been found as early as in the first weeks of life in infants with CF [16]; they were also present in well-nourished young CF patients who do not receive a low-fat diet and do not present with fat malabsorption [17,18]. The underlying mechanisms of altered EFA metabolism in CF seem indeed to be multifactorial [19]: increased lipid turnover in cell membranes, increased oxidation of fatty acids for energy needs, increased production of eicosanoids linked to an exacerbated inflammatory status and decreased desaturase activity. A characteristic imbalance between arachidonic acid (a representative derivative of omega6 PUFA) and docosahexaenoic acid (DHA, a representative derivative of omega3 PUFA) have been identified in patients with CF [13,15–17,20] and in animal models of the disease [21,22].

Ceramides, a class of sphingolipids, were reported to accumulate in an age-dependent manner in CF respiratory cells [23]. Accumulation of ceramides has been related to pulmonary inflammation, apoptosis and death of respiratory epithelial cells, deposits of DNA in bronchi and high susceptibility to severe *P. aeruginosa* infections [24–26].

Regardless of the cause, chronic inflammation progressively leads to suppurative pulmonary disease characteristic of CF. As inflammation and infection are extremely difficult to dissociate, this opens the debate on the origin of the inflammation: does it follow or rather precede the infection? Recent research has shed some new light on the issue of the origin of uncontrolled inflammatory overreactions in CF. The fact that muco-modulating agents, although effective in improving rheology and transportability of the hyperviscous mucus, fail to modulate inflammation in CF [27,28] could outline a direct link between inflammation and the genetic defect in the disease. Another argument in favor of this hypothesis is the detection of pulmonary inflammation in asymptomatic patients very early in life [29–33], even in newborns [29]. Clinical observations suggest that, in CF, inflammation precedes infection [29–33]; however, the cellular and soluble mediators as well as the intracellular signaling pathways involved in the intense inflammatory responses remain very poorly understood.

In this review, we describe the characteristics of airway inflammation and its relationship to progressive lung disease in CF patients. We discuss the usefulness and limitations of mouse models of CF to study the pathogenesis of human lung inflammatory disease, and the development of new potential strategies to reduce the inflammatory burden in the airways.

Characteristics of CF human inflammation

Chronic bacterial infection is assuredly the leading cause of progressive suppurative inflammation in CF lungs. However, several lines of evidence have suggested that altered pro-inflammatory responses in CF epithelia, described in the past as single barrier cells, may manifest independently of any detectable infection. The introduction of CF newborn screening has led to a better understanding of the early natural history of CF lung disease, as it has allowed assessing the evolution of pulmonary signs and clinical course of CF lungs shortly after birth [29–33]. In newborns with CF, lungs appear structurally normal and bacterial cultures of respiratory secretions often fail to yield specific pathogens. However, bronchoalveolar lavage (BAL), undertaken for culture and measurements of pro- and anti-inflammatory cytokines in infants with CF aged from 1.5 to 71 months, has shown neutrophilic-dominant lower airway inflammation with elevated concentrations of interleukin (IL)-8, the principal neutrophil chemoactractant in CF lungs, even in the absence of any detected pathogen [30].

CF-associated airway inflammation is characterized by a profuse influx of neutrophils into the lungs; however other types of leukocytes, including eosinophils [34], lymphocytes [35] and monocytes [36] could also play a role. The fact that several neutrophil cellular functions seem to be deregulated in CF [37,38] could solve the enduring paradox of an overwhelming infiltrate of inflammatory cells that fails to resolve infections. Neutrophil elastase (NE), a serine protease released from primary neutrophil granules, has been found to be elevated in CF airways very early in life [39]. NE has been claimed to be a very informative biomarker of disease progression, with higher sputum levels being associated with more rapid lung function decline and bronchiectasis [39]. The enzyme perpetuates the vicious cycle of inflammation: its broad substrate specificity is related to disruption of structural tissue components, such as elastin and fibronectin, and to activation of the proform of matrix metalloprotease (MMP)-9, contained in tertiary neutrophil granules [40]. MMP-9, a marker involved in matrix extracellular proteolysis, may also contribute to the increased transmigration capacity of neutrophils, observed in isolated CF cells [41]. Besides, higher concentrations of pro-inflammatory mediators such as IL-6, IL-8 and IL-1β have been found in the BAL of children with CF [29]. Similarly, increased levels of IL-17, also involved in lung neutrophil infiltrate, have been found to be increased in CF [35]. Conversely, reduced concentrations of IL-10 [42] and of lipoxins [43], which possess anti-inflammatory properties, were measured, highlighting an imbalance in inflammatory signals in CF. Moreover, airway neutrophils isolated from CF patients showed a blunted phagocytic capacity that could contribute to altered host defense functions and poor bacterial clearance [44].

On cell death, CF neutrophils release DNA, which increases mucous viscosity, and abundant oxidases, which contribute to the occurrence of oxidative stress. The abnormal flux of reactive oxygen species in CF lungs exacerbates pulmonary deterioration and favors progression of bronchiectasis [45].

Lungs of CF patients are often colonized or infected in infancy and early childhood with organisms such as *S. aureus* and *Haemophilus influenza*. In

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teenagers and young adults, these microorganisms are progressively replaced by *P. aeruginosa*. Chronic infection with *P. aeruginosa* adversely affects the prognosis of the disease and is the main proven perpetrator of lung function decline and ultimate mortality in CF patients. Other emerging bacterial pathogens, *Burkholderia cenocepacia*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*, have become more frequent [46]. The virulence of *P. aeruginosa* stems from a number of bacterial properties, including antibiotic resistance, release of microbial toxins, ability to utilize quorum-sensing signals to form biofilms, conversion to an exopolysaccharide alginate-overproducing mucoid phenotype, which renders the bacterium resistant to both the innate and acquired immune defenses of the host [47].

Co-infections with the two Gram-negative biofilm-forming bacteria, *P. aeruginosa* and *B. cenocepacia*, likely worsen the prognosis of CF lung disease. Accordingly, interspecies interplay has been evidenced by the demonstration that co-infection with *P. aeruginosa* and *B. cenocepacia* leads to increased biofilm formation and increased host inflammatory responses [48].

Infections with *P. aeruginosa* also seem to favor colonization of the airways with non-bacterial agents, such as *Aspergillus fumigatus*, and to favor the development of a hypersensitive *A. fumigatus*-related condition, known as allergic bronchopulmonary aspergillosis (ABPA) [49,50]. Although the pathophysiological mechanisms underlying the link between CF and ABPA are poorly understood, the contribution of chemokines, especially of the chemokine ligand 17 (CCL17), also known as thymus and activation-regulated chemokine (TARC), and its receptor CCR4, has been postulated. TARC has been used as a diagnostic serological biomarker of ABPA [51]; however, further studies are needed before the chemokine can be integrated routinely in diagnostic algorithms.

It has been well recognized that early referral to a specialist healthcare center favorably impacts on the prognosis of CF [52]. Indeed, the adherence to standardized principles of multidisciplinary therapy by CF centers has been lauded as an important factor responsible for increasing the median survival over time: for 2008–2012 year band, the median predicted survival in the United States was 37.8 years [53]. A more favorable respiratory outcome has been demonstrated with benefits both in terms of lung function and *P. aeruginosa* prevalence in CF children referred early to a specialized CF center [52]. Moreover, the possibility of an increased risk of cross-infection for patients followed up in the centers has to be taken into account.

Cell-based studies using immortalized human CF and non-CF epithelial cell lines have confirmed that CF airway epithelia exhibit inherent pro-inflammatory overresponses [54,55]. CFTR-deficient airway epithelial cells display signaling abnormalities and aberrant intracellular processes which lead to transcription of pro-inflammatory mediators. Transcription factors, such as nuclear factor-(NF)-κB and activator protein AP-1, seem to be activated in CF [54,55]. It can be postulated that cell stress triggered by accumulation of misfolded CFTR in the endoplasmic reticulum, as occurs in the presence of the most frequently found F508del-CFTR mutation, may provoke unbalanced pro-inflammatory responses in epithelial and in immune cells [40]. However, comparing studies using cell-based models of CF disease is difficult, owing to considerable variability in cell phenotypes, depending on a myriad of factors including the cell model system used, cell culture conditions and cell differentiation/dedifferentiation processes [56]. And this raises the question of the validity of cell-based rather than *in vivo* models to study the pathophysiology of CF lung inflammatory responses.

Characteristics of CF mouse inflammation: contribution of mouse models

Recent progress in genetics, with the identification of the *CFTR* gene [57,58] and the sorting out of the protein sequence and its highly conserved homology throughout the animal kingdom, have led to the development of CFTR transgenic mice. Several strains of mice have been generated, carrying different classes of mutations [59,60]. Among these, the mouse model homozygous for the F508del mutation developed by the Erasmus Medical Center of Rotterdam (F508del^{EUR}) [61] offers multiple advantages. First, the F508del mutation is by far the most widespread in European countries and world-wide: in Belgium, for example, about two-thirds of CF patients carry at least one copy of the mutation and half of them are homozygous for the mutation [62]; in the US, the figure reaches almost 90% [53]. Second, compared to the other mouse models of F508del [63,64], normal or near normal amounts of mRNA are expressed in a majority of target tissues in the F508del^{EUR} mouse and survival rates are much better (fewer than 5% of F508del animals generated by the Cambridge University [63], and 40% of those of Iowa University [64] reach maturity). These properties underline the clinical relevance of the F508del^{EUR} model, especially for the study of the pathogenesis of the disease and of new strategies with therapeutic aims.

Indeed, it has been shown that inflammation in the F508del^{EUR} mouse model of disease seems to be constitutive: in the absence of any detectable infection or induction of the inflammatory response, CF animals display an accumulation of inflammatory cells, among which neutrophils, in the bronchoalveolar lumen [65]. This seemingly spontaneous cellular infiltration combines with an increased release of pro-inflammatory cytokine MIP-2 (for Macrophage Inflammatory Protein-2), a mouse functional analog of human IL-8, and with an increased enzymatic activity in lung homogenates of lactate dehydrogenase, a marker of tissue injury [65]. Differences between wild-type and CF mice are still more conspicuous after induction of an acute inflammatory response; this is obtained by endotracheal instillation of lipopolysaccharide (LPS) extracted from the walls of *P. aeruginosa*. In such conditions, F508del^{EUR} mice react more rapidly and more strongly than do wild-type mice, by releasing pro-inflammatory mediators (MIP-2 and Tumor Necrosis Factor—(TNF)-α) and by favoring migration of neutrophils towards the bronchial lumen. These responses in F508del^{EUR} mice, associated with reduced amounts of IL-10, confirm the pro/anti-inflammatory imbalance [42,66] and validate this mouse model for *in vivo* studies of the inflammatory response in CF.

In addition to the increased surge of neutrophils, it has been demonstrated that macrophages make up an increased proportion of cells populating the bronchial lumen of F508del^{EUR} mice. Even in naive conditions, the number of macrophages in the fluid of BAL is roughly doubled relative to wild-type mice [66]. The larger proportion of macrophages seems to be related to increased concentrations of the macrophage chemoattractant cytokine CCL-2 (Chemokine C–C motif ligand-2): these are about 10 times larger in the bronchoalveolar space, but also in the peritoneal cavity of F508del^{EUR} mice. Studying cultured alveolar and peritoneal macrophages revealed that these cell populations are deregulated in CF: their differentiation pattern is altered towards a pro-inflammatory status (M1), deleterious for the lung tissue [66]. Taken all round, these results support a major role of macrophages in the pathogenesis of CF.

The F508del^{EUR} mouse allowed confirming that epithelial cells lining the airways also play an active role in the inflammatory response in CF and that their immune functions are altered in the disease. Although epithelial cells are known to be able to secrete IL-8 and other cyto/chemokines [67,68], the link between the CFTR protein and the control mechanism of secretion of mediators of the inflammatory response remains to be elucidated. It has been demonstrated that the expression of genes involved in the inflammatory response (MIP-2, TNF-α, IL-1β, NOS-2, CCL-2) is altered in cultured respiratory epithelial cells isolated and purified from the nasal mucosa and trachea of F508del^{EUR} mice [68].

In addition, morphological and functional responses and biology of lung and dermal fibroblasts are also altered in CF [69]. It has indeed been shown that the presence of the F508del-CFTR mutation confers to fibroblasts an increased sensitivity to express and release, in response to different stimuli, a variety of powerful pro-inflammatory and profibrogenic mediators associated to exaggerated fibrotic tissue transformation with collagen and reticulin accumulation [69].

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These experimental data help towards understanding that in CF, different cellular actors intervene in concert in the inflammatory response, although so far, their intra- and extracellular signaling pathways have not been fully clarified. It can be assumed that macrophages play a major role in orchestrating the responses: with a pro-inflammatory status (M1) in CF, they overexpress pro-inflammatory mediators (IL-1β, TNF-α, NOS-2) (Fig. 1). Activation of the IL-1β pathway, in which other pro-inflammatory mediators take part, attracts immune cells including macrophages and neutrophils. Airway epithelial cells secrete excess amounts of IL-8 favoring evolution of the inflammatory response in CF towards a chronic, dysfunctional and non-resolving state.

Limits of CF mouse models and knowledge brought forth by the CF pig model

As discussed above, F508del-CF mice display overexpression, at protein and mRNA levels, of multiple inflammatory mediators and immune cells in lung tissue and in BAL fluid [65,66,69,70]. The mouse model also displays increased mortality following challenge with LPS [65] or with bleomycin [69]. However, CF mice do not develop a major spontaneous lung phenotype as observed in human CF. More recently, other animal models to study the pathophysiology of CF have been generated. A critical role for imbalanced ion transport, with increased sodium efflux, in the development of CF lung disease has been supported thanks to the development of a mouse model genetically modified to overexpress the β subunit of ENaC [71]. In the β-ENaC overexpressing mouse model, ASL is depleted, mucus clearance is reduced, and the resulting lung disease shares typical features of early lung disease in patients with CF, including airway mucus obstruction, reduced bacterial clearance and chronic neutrophil-dominated inflammation and emphysema. Interestingly, early intervention in β-ENaC mice, *i.e.*, from the first day of life, with intranasal administration of amiloride, an ENaC blocker, significantly reduced pulmonary mortality, airway mucus obstruction, epithelial necrosis, goblet cell metaplasia, and airway inflammation [72]. In contrast, consistent with previous clinical trials in patients with CF [73], amiloride administration did not bring in benefits if treatment was started after the development of CF-like lung disease in ENaC mice [72]. These results suggest that treatment with amiloride, or with second generation amiloride analogs showing a longer duration of effect and a larger potency [74], may be an effective preventive therapy for patients with CF if initiated early in life before the onset of lung disease.

Methods for generating pig and ferret CF models have been developed in the last decade [75–77]. Pigs knocked out for the CFTR protein or homozygous for F508del were then generated with the claimed advantage that the anatomy and the structure of their respiratory tract are more similar to that of humans than that of mice. As observed in CF mice, CFTR-deficient piglets develop a meconium ileus syndrome that, if not adequately treated, is lethal in 100% of CFTR-knockout animals [78]. Encouraging to the CF field was the report that CFTR knockout and F508del piglets developed a spontaneous CF-like lung phenotype. At birth, their lungs lack infection and inflammation but have reduced ability to eradicate bacteria. Within months after birth, they spontaneously develop airway inflammation, infection, tissue remodeling, mucus accumulation and airway obstruction [79]. The porcine CF model has also been valuable to study CF sinus disease. Indeed, chronic sinusitis is nearly universal in patients with CF and is accompanied by sinus hypoplasia, the origin of which remains unknown: is the impaired sinus development a primary feature of loss of CFTR or a secondary consequence of chronic infection? Sinuses of newborn CF pigs were not infected and showed no evidence of inflammation, yet they were hypoplastic at birth [80]. Older CF pigs spontaneously developed sinus disease similar to that seen in humans with CF [80].

It is however surprising that the respiratory epithelium of CF pigs does not show any abnormal sodium transport across the nasal and tracheal/bronchial epithelia or depleted ASL volume [81], recognized as the initial change in the pathogenesis of CF [1]. Furthermore, both practical and financial conditions required by pig breeding limit its applicability on a larger scale.

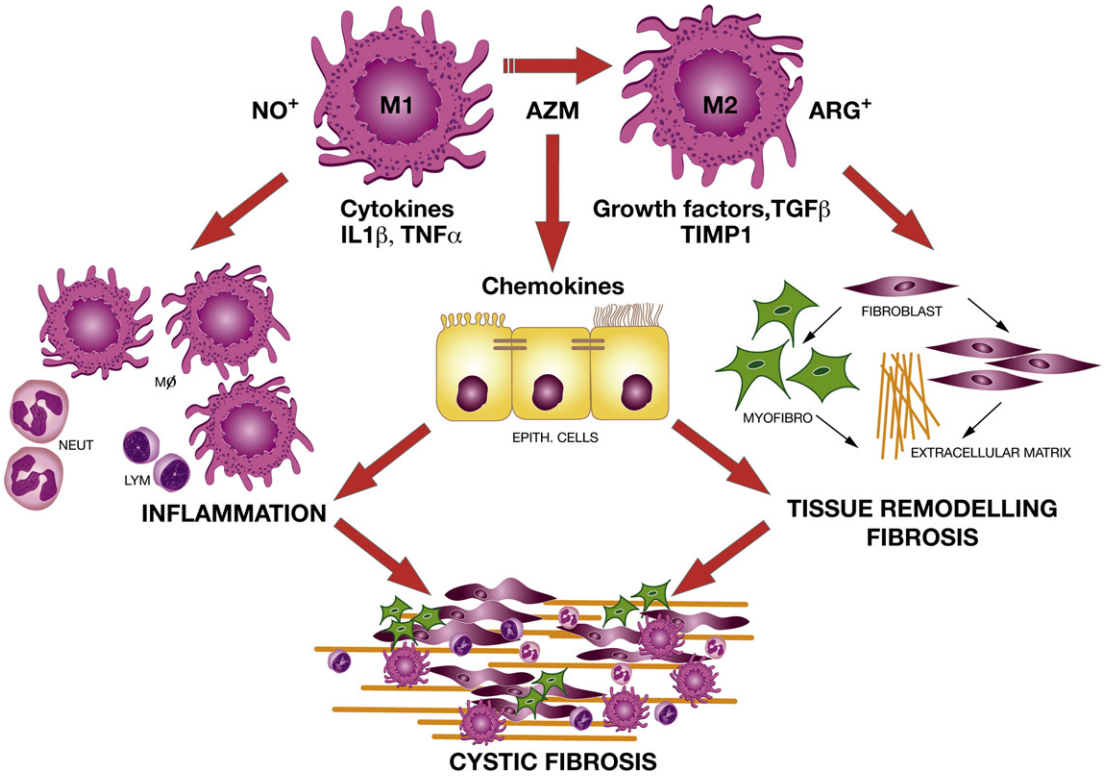

Fig. 1. Multiple interactions among cells and cytokines involved in the pathogenesis of CF in lungs. Effect of azithromycin (AZM) on M1/M2 macrophage cell differentiation.

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From deeper knowledge to better treatment: future perspectives

Recent progress in the understanding of the pathogenesis of the disease, together with a multidisciplinary and multifaceted management organized by reference centers of the disease [52], has led to a progressive and significant increase in the life expectancy of patients, to such an extent that in developed countries, around 50% or more of them reach adulthood. Pulmonary inflammation has been increasingly viewed as a therapeutic target in CF patients.

For a rational treatment of inflammation, like for all other manifestations of CF, understanding underlying mechanisms involved in the observed pathophysiological responses is essential. Counteracting pulmonary inflammation at early stages of the disease in order to prevent irretrievable lung damage remains a top priority of the treatment [40]. An anti-inflammatory therapy, often combined with more common tools such as antibiotics, is a headstone of the management of the respiratory syndrome. More precisely targeting, controlling the excess accumulation of inflammatory cells (neutrophils, eosinophils, lymphocytes, macrophages and fibroblasts), and trying to act upon their cellular differentiation process and to modulate their functions could all be beneficial to patients, irrespective of their classes of mutation.

In the clinical field, multiple therapeutic strategies applied to CF are being developed by building up of a vast network of collaborations of scientists, academics and pharmaceutical industry of different horizons [82]. Of note in the particular context of the contribution of depleted ASL in the pathogenesis of CF lung disease, muco-modulating agents have been developed aiming at increasing the fluidity of secretions by inhalation of hypertonic salt solutions or osmotic agents such as mannitol (Bronchitol, Pharmaxis). In a long term clinical trial, inhalation of hypertonic salt solution has demonstrated beneficial effects on the respiratory function and on the occurrence of bronchial exacerbations; however, tolerance to the treatment, associated to bronchospasms, is a source of difficulties [83]. Bronchitol, recently approved as a symptomatic treatment of CF, has been ingeniously developed as hollow microspheres for dry powder inhalation therapy ensuring a better deposition of mannitol in the smaller airways [84]. A very attractive muco-modulating strategy consists in reducing mucus viscosity by hydrolyzing DNA from bacteria and neutrophils massively present in airway secretions. Therapeutic trials using recombinant human DNAse 1 (dornase alfa, Pulmozyme, Genentech) [85] have shown an improvement of the respiratory function [86].

Several studies have reported beneficial effects of systemic corticosteroids on the progression of lung disease in CF, particularly in children with mild lung disease. Effectiveness and adverse events of long-term use (over 30 days of treatment) of oral corticosteroids have been recently revisited in a Cochrane database systematic review [87]. Even though oral corticosteroids at prednisolone-equivalent dose of 1 to 2 mg/kg alternate days appear to modestly and/or transiently slow progression of lung disease in CF, benefit should be weighed against occurrence of adverse effects including glucose intolerance, diabetes, and growth retardation that persists for years after treatment withdrawal. Given that the risk–benefit ratio is unfavorable, systemic corticosteroids are logically not widely applied in patients with CF. Inhaled corticosteroids are frequently prescribed, but evidence from randomized or even quasi-randomized clinical trials is insufficient to establish that they are certainly beneficial in CF. It has not been firmly established that long-term use is able to reduce lung inflammation. Moreover, there is some evidence that they may be harmful in terms of growth [88].

A four-year trial using high-dose ibuprofen, an oral non-steroidal anti-inflammatory medication, showed a slower annual rate in pulmonary function and maintained body weight without change in the frequency of hospitalizations [89]. The effects were more pronounced in children aged less than 13 years and presenting with mild lung disease. Ibuprofen is currently being used by approximately 1000 people with CF [82]. Despite its apparent benefit, high-dose treatment with ibuprofen may be associated with gastrointestinal and renal toxicities. Moreover, treatment with low-dose ibuprofen has been associated with a parodoxical pro-inflammatory effect, increasing neutrophil migration into the lungs [67].

Numerous review studies and clinical trials have been dedicated to the efficacy of azithromycin as a potential anti-inflammatory therapy for CF lung disease. Azithromycin is an azalide, a subclass of macrolide antibiotics derived from the natural compound erythromycin; it has attracted attention of CF therapists for at least fifteen years [90]. Several clinical investigations have revealed that an azithromycin treatment improves respiratory function and reduces the occurrence of pulmonary exacerbations and the frequency of courses of antibiotic therapies [91–93]. These observations have been confirmed by more recent studies in children and teenagers not infected by *P. aeruginosa* [94–96]. It has been demonstrated that the amounts of neutrophils and of some plasma inflammatory markers are significantly reduced by 28 days of azithromycin treatment; these changes are associated with the improvement of respiratory function [95,96]. Within the context of F508del^{EUR} mice, we have demonstrated anti-inflammatory properties of azithromycin, both *in vivo* and *ex vivo*. Treating F508del^{EUR} mice for four weeks reduces neutrophil and macrophage infiltration and blunts release of pro-inflammatory cytokines TNF-α and MIP-2 [65].

Working on murine models of CF, it has been demonstrated that both macrophages and epithelial cells are targets of the anti-inflammatory effects of azithromycin [66]. The drug modulates the pro-inflammatory status of alveolar but not of peritoneal macrophages from F508del^{EUR} mice by shifting their differentiation towards a sub-population with a more anti-inflammatory profile (M2) [66]. The effects of azithromycin on epithelial cells look more complex: it promotes expression not only of neutrophil-attracting cytokines, but also of the anti-inflammatory IL-10 [68].

To address the continuing need for development of new treatments for CF lung disease, basic scientific investigations are identifying novel targets for the development of new therapeutic approaches of CF lung inflammation. Using cell electrophysiology methods, the F508del^{EUR} mouse model is useful to study the efficacy of compounds with a therapeutic potentiality to correct the typical abnormal transepithelial ion transport in CF. Along this line, it has been demonstrated that inhibitors of cGMP-specific phosphodiesterase type 5, which provoke accumulation of the intracellular second messenger cyclic GMP, are able to correct the transport of chloride ions across the nasal mucosa of F508del^{EUR} mice [97,98]. Sildenafil, vardenafil and tadalafil, which have been approved in the treatment of erectile dysfunction and pulmonary arterial hypertension, could prove useful in the treatment of CF. Furthermore, we have demonstrated that vardenafil also has anti-inflammatory properties by downregulating the expression of several mediators such as CCL-2 and IL-1-β [70]. More recently, we have shown that micromolar doses of vardenafil reduce the extensive upregulation of pro-inflammatory and fibrogenic functions of CF fibroblasts [69]. In view of the deleterious effect of inflammation and fibrosis in CF, such compounds that can simultaneously correct the abnormal ion transport of the CFTR protein and downregulate inflammation and fibrogenic processes clearly bring up a multiple advantage. Phase 2 clinical trials are under way in patients with CF who are 12 years of age or more, whatever their mutation type, to test the efficacy of sildenafil on inflammatory parameters (NCT00659529). Positive preliminary results were obtained using oral sildenafil (20 or 40 mg TID, for 6 weeks): the treatment was safe and significantly reduced sputum NE [99].

In view of the omega3/omega6 imbalance, formulations containing widely variable DHA doses have been administered to patients with cystic fibrosis and anti-inflammatory markers were included as outcome parameters [100,101]. Supplementations with DHA have decreased serum concentrations of inflammatory markers such as IgG and α-1 antitrypsin [100] and decreased the LTB4/LTB5 ratio generated by stimulation of neutrophils from CF patients [101]. Randomized controlled

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trials are still needed in order to draw firm conclusions on the therapeutic effect of omega3 fatty acid supplementation in cystic fibrosis.

Consistent with the assumption that ceramides regulate airway inflammation and infection in mice and patients with CF, inhibition of acid sphingomyelinase cleavage of sphingomyelin to ceramide could represent a new therapeutic strategy for CF. The acid sphingomyelinase blocker, amitriptyline, has been identified as a candidate drug able to normalize pulmonary ceramide and to prevent susceptibility to infection [23]. Miglustat, an inhibitor of the synthesis of glycosphingolipids able to rescue a mature and functional F508del-CFTR in the intestinal crypts of ileal mucosa [102,103] and to correct sodium and chloride transport abnormalities in the nasal mucosa of F508del-CF mice [104], seems to modulate immune responses induced by challenge with LPS or infection with a laboratorial strain of *P. aeruginosa*. Miglustat strongly reduced recruitment of neutrophils into the lungs and the expression of several pro-inflammatory cytokines and chemokines [105].

S-nitrosoglutathione (GSNO) is an abundant endogenous nitrosothiol NO-donor based on naturally-occurring glutathione: it exhibits NO-like biological activities, is thought to function as an intracellular signaling molecule during oxidative stress and is able to induce apoptotic cell death. Glutathione, a major component of cellular antioxidant defenses, was found to be depleted in neutrophils and in ASL of patients with CF. Interestingly, it has been shown that GSNO is able to increase expression, trafficking and function of mutant and wild-type CFTR [106]. N30 Pharmaceuticals Inc. has developed GSNO reductase inhibitors and is conducting clinical trials in patients homozygous for F508del-CFTR to test the effect of the injectable inhibitor N6022. The purpose of the study is to investigate the safety, tolerability and pharmacokinetics of N6022, and to obtain descriptive information on the effect of N6022 on biomarkers of CFTR function and inflammation (NCT01746784). GSNO reductase inhibition increases levels of GSNO that have also been found reduced in people with CF.

Pharmacological inhibition of GSNO reductase may represent an attractive approach for the treatment of CF: this class of drugs also exerts anti-inflammatory and bronchodilator activities. Indeed, treatment of F508del-CFTR mice with an oral GSNO reductase inhibitor led to an increase of cAMP-dependent chloride transport across the intestinal tissue [107]. GSNO reductase inhibition provided potent bronchodilator effects of excised rat tracheal rings with attenuation of methacholin-induced contraction [108]. Using F508del-CFTR expressing cells, the GSNO reductase inhibitor showed CFTR modulator properties assessed by yellow fluorescent protein-based iodide influx [109].

Alpha-1 antitrypsin, a potent serine protease inhibitor used for many years as augmentation therapy in patients with alpha-1 antitrypsin deficiency, is currently under investigation in CF [82]. Recruitment of a randomized, double-blind, placebo-controlled, dose escalation Phase 2 clinical trial to assess the safety and tolerability of 100 mg and 200 mg of aerosolized alpha-1 antitrypsin in patients with CF aged 18 years or more has been completed (NCT01684410). The treatment is given once a day for three weeks. The rationale for a wider therapeutic spectrum of the serine protease inhibitor has followed the demonstration of its diverse anti-inflammatory, immune-modulatory and tissue-protective actions. Its indication, beyond an augmentation therapy in patients with the enzyme deficiency, has been recently broadened to the treatment of unrelated conditions, such as type 1 diabetes, cell/organ rejection, viral infection, cystic fibrosis, bronchiectasis and chronic obstructive pulmonary disease, heart failure, Crohn's disease and connective tissue diseases [110]. However, the therapeutic usefulness of alpha-1 antitrypsin in these conditions remains to be established.

KB001A is a humanized monoclonal Fab fragment that targets a *P aeruginosa* virulence factor (Type III secretion system). As KB001A reduces bacterial load, but not bacterial killing, the drug is being tested as an anti-inflammatory therapy for patients in CF aged 12–50 with chronic *P. aeruginosa* lung infection [82]. During a Phase 2 multi-center randomized, double-blind, placebo-controlled trial, the safety and effectiveness of KB001A to increase time-to-need for antibiotic treatment for worsening respiratory tract signs and symptoms are compared to a placebo (NCT01695343).

Conclusions

The causing gene of CF was identified more than 20 years ago; but in spite of major progress that followed, progressive loss of lung function still mainly determines the final prognosis and the outcome of the disease. Understanding the mechanisms of the inflammatory overresponse can help adjusting the treatment and developing novel strategies, with as a final aim to improve the quality and expectancy of life of patients.

Acknowledgments

Supported by grants of the French CF Association, *Vaincre la Mucoviscidose*; the Fonds de la Recherche Scientifique Médicale (FRSM); the FSR, and the Foundation St Luc (St Luc University Hospital, UCL). SN is a postdoctoral fellow with the Fonds Spéciaux de Recherche (FSR; Université catholique de Louvain, UCL) and Marie Curie Actions of the European Commission. FH is a research associate with the Fonds National de la Recherche Scientifique (FNRS). TL is a research associate with the Institut de Recherche Expérimentale et Clinique (IREC, UCL). Authors are indebted to Professor Jean Lebacq for the critical reviewing and editing.

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