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Current Concepts and Controversies in Innate Immunity of Cystic Fibrosis Lung Disease

Anjali Ralhan[a] Julie Laval[a] Felipe Lelis[a] Marlene Ballbach[a] Charlotte Grund[a] Andreas Hector[a] Dominik Hartl[a, b]

[a]Department of Pediatrics I, University of Tübingen, Tübingen, Germany; [b]Immunology, Inflammation and Infectious Diseases (I3) Discovery and Translational Area, Roche Pharma Research and Early Development (pRED), Roche Innovation Center Basel, Basel, Switzerland

Key Words

Cystic fibrosis · Lung disease · Host defense · Immune response · Neutrophils · Pattern recognition receptors · Toll-like receptor

Abstract

Cystic fibrosis (CF) lung disease is characterized by chronic infection and inflammation. The inflammatory response in CF is dominated by the activation of the innate immune system. Bacteria and fungi represent the key pathogens chronically colonizing the CF airways. In response, innate immune pattern recognition receptors, expressed by airway epithelial and myeloid cells, sense the microbial threat and release chemoattractants to recruit large numbers of neutrophils into CF airways. However, neutrophils fail to efficiently clear the invading pathogens, but instead release harmful proteases and oxidants and finally cause tissue injury. Here, we summarize and discuss current concepts and controversies in the field of innate immunity in CF lung disease, facing the ongoing questions of whether inflammation is good or bad in CF and how innate immune mechanisms could be harnessed therapeutically.

CF Lung Disease

Cystic fibrosis (CF) lung disease, the most common inherited lethal disease in Caucasians [1], is characterized by an early [2], nonresolving [3] and harmful [3, 4] activation of the innate immune system. CF is caused by mutations in the CF transmembrane conductance regulator *(CFTR)* gene, mainly expressed at the apical membrane of epithelial cells [5]. However, besides *CFTR*, other genes ('modifier genes') also appear to play a significant role in modulating lung disease severity and immune response [6–9], particularly genetic variants of transforming growth factor β_1 (TGF-β_1) [10–12], mannose-binding lectin (MBL2) [13] and interferon-related developmental regulator 1 (IFRD1) [14, 15]. Exome sequencing has revealed that the variants in dynactin protein, DCTN4, are linked with the chronic infections in CF [16]. A more recent meta-analysis [17] has identified 5 loci: *MUC4/MUC20*, *SLC9A3*, *HLA Class II* and *AGTR2/SLC6A14* to be associated with the lung function in CF. Labenski et al. [18] have reported 2 cytokine receptor genes, *INFGR1* and *IL1B*, and a transcription factor, STAT3, which is associated with the basic *CFTR* defect as candidate modi-

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Dr. A. Ralhan or Prof. Dr. D. Hartl
Department of Pediatrics I, University of Tübingen
Hoppe-Seyler-Strasse 1
DE–72076 Tübingen (Germany)
E-Mail Anjali.Ralhan@med.uni-tuebingen.de or Dominik.hartl@med.uni-tuebingen.de

fier genes in a study comparing F508del homozygous CF patient subsets. Some lesser-known genetic variations linked to CF lung disease are *EDNRA* [19], *IL-8* [20] and *SERPINA1* [9].

Studies from regions with CF newborn screening indicate that the innate immune system, as reflected prototypically by neutrophil products present in CF airway fluids, is operative in infants with CF and predicts the later outcome of irreversible pulmonary disease [2]. Based on these and other studies, innate immune cells have come into the focus of understanding and treating CF lung disease [3]. Whilst there are several studies supporting the notion that unopposed neutrophil products, such as extracellular elastase, are detrimental for tissue integrity and innate immune cell receptors [3, 21] and can be used as noninvasive biomarkers for CF airway inflammation [22, 23], therapeutic approaches to dampen excessive neutrophilic inflammation in CF lung disease have remained largely unsuccessful [24]. Neutralizing neutrophil elastase (NE) by using antiproteases showed some effects in preclinical and clinical studies; however, the benefits for lung function are so far not convincing [25]. Interfering with neutrophil recruitment through CXCR2 antagonists was safe and showed anti-inflammatory potential, yet no beneficial effects on lung function were found [26]. As CF airways are chronically colonized with bacteria and fungi [27], completely abrogating neutrophil recruitment into the lung bears the inherent risk of unleashing bacterial and fungal infections. Collectively, innate immune pathways are activated early in CF and seem to cause more harm than good within the pulmonary microenvironment; however, the therapeutic implications of these insights remain a matter of debate. To dissect the innate immune response in CF and develop future pharmacotherapeutic strategies, we have composed this review, embedded in a thematic CF series in the *Journal of Innate Immunity*.

Current Controversies in Innate Immunity of CF Lung Disease

Innate immunity comprises both cellular and humoral factors. Here, we focus on the cellular components of innate immunity and their pathogenic, diagnostic and/or potentially therapeutic role in CF lung disease. However, before considering innate immune cells as pharmacotherapeutic targets, one must understand their activation and effector functionalities. Therefore, we start with summarizing and discussing the mechanisms by which innate immune cells sense and are activated by CF pathogens. Based on this, we focus on the role of neutrophils, probably the key type of innate immune cell in CF lung disease, including their distinct innate immune receptor profiles and phenotypes in the proinflammatory CF airway microenvironment. Overall, our review should stir a discussion of the following controversies in the field:

- Is inflammation good or bad in CF lung disease? The correlation between neutrophil activation and irreversible lung tissue remodeling (bronchiectasis) [2] suggests a harmful role, but without functional neutrophils (as exemplified in patients with the primary immunodeficiency chronic granulomatous disease), we cannot efficiently defend against bacteria and fungi. Consequently, dampening neutrophil activation would be reasonable, while completely abrogating neutrophil influx or function might be dangerous.
- How does harmful proinflammatory neutrophil activation in CF get dampened? Harmful unopposed neutrophil functions, such as unopposed protease release and neutrophil extracellular trap (NET) formation should be controlled, but how? Antiproteases show limited success so far, but studies are ongoing. NET formation still represents a controversial area [28]. On the one hand, NETs can entrap pathogens and may therefore act beneficially. On the other hand, abundant NETs, as found in CF airways, can obstruct the airway lumen and correlate with decreased lung function in CF patients [29]. Recombinant DNase (Dornase alfa) is clinically effective in CF patients by cleaving DNA strands and facilitating airway mucus clearance [30]. A recent study suggested that the majority of extracellular DNA in CF airways is derived from NETs [31]. Thus, the clinical effectiveness of recombinant DNase might support the concept that the prevalence of NETs causes more harm than good in CF lung disease. However, DNases cleave extracellular DNA and do not prevent de novo NET generation or release. Approaches how to target NET generation may involve interfering with reactive oxygen species (ROS) or MAPK, which have been found important for NET formation [32, 33]. Studies comparing the effect of inhibiting intracellular NET generation versus cleaving free extracellular DNA strands would shed more light on the kinetics and dynamics of NET-pathogen interactions in lung disease and beyond. Alternatively, specific neutrophil phenotypes, such as olfactomedin-4- or CD177-expressing neutrophil subsets, could be targeted [28]. Their functional role and CF disease relevance remains to be defined.

- When should inflammation be targeted? At first glance, the earlier, the better, in order to prevent inflammation-associated tissue damage and avoid irreversible pulmonary tissue remodeling as soon as possible in the course of disease. On the other hand, neutrophils could be essential in early host-pathogen interactions by restricting airway pathogen colonization in the first years of life, when the airways are intensively exposed to environmental microbes and vaccinations are performed. Further investigations into CF lung disease are required to define the time windows when inflammation could be targeted safely without significantly impairing the protective innate immune defenses.

Innate Immune Activation in CF Lung Disease

Sensitive microbial detection mechanisms as well as tailored immune responses are required to efficiently protect the host from pathogens. Simultaneously, inflammation has to be tightly controlled and limited to avoid overshooting immune responses and collateral tissue injury. In 1989, Janeway [34] proposed the pattern recognition theory, stating that the microbial presence is sensed by the host innate immune system through the detection of distinct molecular structures called pathogen-associated molecular patterns (PAMPs) that are expressed by the pathogen but are absent in the host. To sense the presence of microorganism, the cells of the immune system possess germline-encoded pattern recognition receptors (PRRs) with 4 different families having been currently identified. These families include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as cytoplasmic proteins such as the retinoic acid-inducible gene *(RIG)*-I-like receptors (RLRs) and NOD-like receptors (NLRs). Apart from PAMPs, PRRs also recognize host-derived patterns/molecules, termed damage- or danger-associated molecular patterns (DAMPs).

CF lung disease is mainly characterized by bacterial and fungal colonization and infection. Therefore, in the sections below, we will focus on these 2 microbial entities and the corresponding innate immune responses.

Bacterial Recognition: TLRs

The main bacteria commonly identified in CF lungs in early disease/infancy are *Staphylococcus aureus* and *Haemophilus influenzae*, followed in adolescence and adulthood by the major CF pathogen *Pseudomonas aeruginosa*. However, beyond these 'classical' CF bacteria, microbiome studies indicate that a much broader variety of bacterial species, including anaerobes, colonize CF airways [35–37]. TLRs are the main innate immune receptors (PRRs) to sense bacteria. Ten and 12 TLRs have been identified in humans and mice, respectively, and TLR1–9 are conserved in both species [38]. The PRRs responsible for the recognition of *P. aeruginosa* in CF lung disease are TLRs, Asialo-GM1 receptors [39] and the NLRC4/IPAF inflammasome [40]. TLR2, TLR4, TLR5 and/or TLR9 have been reported to sense *P. aeruginosa* [41]. The bacteria-derived ligands known to bind TLR2 are lipoproteins, components of the extracellular capsule and secreted toxin, ExoS, with C-terminal-specific interaction [42–44]. Reports have shown a role for TLR2 in the recognition of mannuronic acid polymer, a major component of the alginate capsule and slime GLP, produced by mucoid and nonmucoid strains of *P. aeruginosa* [45, 46]. Lipopolysaccharide (LPS) is mainly sensed through TLR4 and, after recognition, the TLR4/LPS complex is rapidly endocytosed and trafficked for lysosomal degradation in order to terminate further inflammatory cascades [47]. The lipid A component of LPS ligates TLR4, inducing a potent immune response [48], with the hexacyclated form being a strong activator of TLR4-mediated signaling in humans [49]. Hexacylated lipid A is often produced by bacterial strains adapted to the chronic CF microenvironment [50, 51], leading to escape from the host antimicrobial peptides and increased recognition by human TLR4. In contrast to this structural peculiarity, a recent study by Di Lorenzo et al. [52] sheds new light on the activation mechanism of TLR4/MD2 complex by penta-acylated lipid A produced by the CF isolates of *Burkholderia cenocepacia.* TLR5 specifically binds to flagellin, a primary constituent of flagella important for microbial motility [53]. However, the correlation between bacterial motility and immune evasion by *P. aeruginosa* remains controversial [54]. An in vivo study highlighted the proinflammatory role of flagellin-mediated TLR5 activation [55]. Descamps et al. [56] reported that TLR5, rather than TLR4, is essential for bacterial phagocytosis and killing by murine alveolar macrophages (AMs) in vitro and in vivo. The authors also demonstrated that nonflagellated *P. aeruginosa* or mutants defective in TLR5 activation are resistant to AM clearing, which is dependent on TLR5 signaling and IL-1β production. The intracellular function of TLR9 is characterized by detection of unmethylated CpG motifs in bacterial DNA [57, 58]. Synergistic effects of TLR2, TLR6 and TLR9 have been reported using in vivo studies

[59]. Further studies report a resistant phenotype of TLR9$^{-/-}$ mice to *P. aeruginosa* infection compared to wild-type mice [60]. These unexpected findings are attributed to increased airways cytokine production leading to effective bacterial clearance in the lungs of the TLR9$^{-/-}$ mice.

The NLRC4 and NLRP3 Inflammasomes

NLRs are cytosolic proteins that respond to a variety of ligands, from bacterial and viral components to particulate matter and crystals. The mammalian NLR family comprises >20 members, containing a C-terminal leucine-rich repeat domain, a central nucleotide-binding NACHT domain and an N-terminal protein-protein interaction domain composed of a caspase activation and recruitment domain (CARD) or Pyrin domain [61–63]. The transmembrane secretion systems of intracellular pathogens or bacteria serve as cytosolic microbe-associated molecular patterns (MAMPs) that may interact with NLRs [64–66]. Regarding human pulmonary pathogens, NLRC4 and NLRP3 are the 2 most widely studied NLRs that orchestrate immune responses [67–69]. In addition to TLR5, bacterial flagellin is sensed by NLRC4 [70, 71]. Sutterwala et al. [40] have further described that NLRC4 triggers the activation of the inflammasome upon infection with *P. aeruginosa*, resulting in macrophage cell death and the secretion of the proinflammatory cytokines, IL-1β and IL-18. This activation cascade was shown to be IPAF-dependent, but flagellin-independent. Moreover, in vivo studies revealed an increased susceptibility of NLRC4-deficient mice against *P. aeruginosa* infection [72]. In addition to *Pseudomonas*, other Gram-negative bacteria, such as *Salmonella*, *Legionella* and *Shigella*, have also been found to activate the NLRC4 inflammasome [73–75]. In a recent study, the role of NLRP3 inflammasome activation in the CF lung has been described in association with elevated levels of ceramide [76]. The authors demonstrated an upregulation and recruitment of the adapter protein apoptosis-associated speck-like protein (ASC) and caspase-1 in the lungs of CF mice. The activation of NLRP3 is characterized by a canonical two-step deubiquitination mechanism that is initiated by priming through TLR signaling (e.g. TLR4), inducing NF-κB-dependent NLRP3 protein synthesis, followed by a second signal leading to full NLRP3 inflammasome assembly [77]. In CF airway epithelial cells, *P. aeruginosa* infection has been shown to trigger mitochondrial dysfunction and enhance mitochondrial Ca^{2+} uptake, leading to NLRP3 inflammasome activation [78, 79].

Fungal Recognition

With constant inhalation of fungal spores, the human airway immune system has evolved a plethora of fine-tuned defense mechanisms for effective fungal clearance, involving, mainly, AM, neutrophils and antimicrobial peptides [80–85]. With ageing and more intensified antibiotic treatments, prevalence rates of fungal colonization increase in CF lung disease, traditionally known to be mainly colonized by a bacterial community [86–88]. The reported emerging rate of filamentous fungal species, such as *Aspergillus fumigatus*, in CF, is found to be most frequent; however, other important filamentous fungi including *Scedosporium* sp. and *Exophiala dermatitidis* have also been identified [89, 90]. The sensitization of CF patients to the airway microenvironment presents a wide range of unresolved questions. However, previous reports have proposed a crucial role for dendritic cells and Th2-associated chemokines, like CCL17 [91]. Phagocytic cells play an essential role in protection against the fungal infections, and abrogation of these cells leads to increase susceptibility towards pathogens [92]. The receptors involved in these processes include secreted factors such as pentraxin-3 (PTX3), C-type lectins, complement system and membrane PRRs such as TLRs [93]. Previous reports have shown that *A. fumigatus* conidia are recognized by TLRs [94, 95] and β-glucan receptor dectin-1 on dendritic cells, AM and lung epithelial cells [96, 97]. TLRs, in particular TLR2 [98, 99], TLR4 [100, 101] or an interplay between TLR2, 4 and 9 via an MyD88-dependent pathway [96], are described as playing an important role in the host immune response to *A. fumigatus*. The endocytic PRR dectin-1 is crucial in the recognition and internalization of specific morphotypes of *A. fumigatus* in AM [102, 103], and a novel mechanism of dectin-1 induction in human bronchial epithelial cells and its consequences for innate immune responses against *A. fumigatus* have been described by Sun et al. [97]. Secreted receptor pentraxin PTX3 also plays an important role in the clearance of fungal burden in vivo after *A. fumigatus* pulmonary infection. PTX3 levels in a CF patient's respiratory secretions and sputum samples were found to have decreased [104]; this could be one of the explanations for recurrent lung infections in CF lungs. Another study showed that a serum opsonin, H-ficolin, modulates host immune response by binding to *A. fumigatus* [105]. The authors further showed that following pathogen recognition, there is an enhanced activation of the lectin complement pathway and fungal association with lung epithelial cells.

Innate Immune Cells

Airway epithelial cells form the first line of defense against microbial infections and serve as a central player in the mucociliary clearance of the lung. The key innate immune functions of the epithelium include (1) secretion of a variety of antimicrobial substances, (2) release of chemokines, cytokines and growth factors that mediate leukocyte recruitment, (3) modulation of adaptive immunity and (4) tissue repair and remodeling [3, 106, 107]. Direct interaction between the CFTR protein and pathogens has been previously suggested, where CFTR serves as a receptor for *Salmonella typhi* [108] and *P. aeruginosa* [109, 110] when expressed on intestinal or airway epithelial cells, respectively. Moreover, *A. fumigatus* spores are readily ingested by airway epithelial cells and the uptake and killing of conidia are both impaired in epithelial cells lacking CFTR [111]. The bronchial epithelium has been previously shown to modulate its sensitivity towards microbial recognition by regulating receptor expression levels [112]. Upon pathogen recognition by specific PRRs, the activation of intracellular signaling cascades initiates proinflammatory and antimicrobial responses. Bacterial infection in CF can exacerbate lung inflammation by exaggerating proinflammatory gene expression via TLR activation in airway epithelial cells [43]. In vitro as well as in vivo studies have shown that excessive cytokine release upon *P. aeruginosa* exposure to CF airway epithelial cells is mainly mediated by TLR5/flagellin or TLR4/LPS interactions [113, 114]. In particular, intracellular TLR4 trafficking seems to be dysregulated and attenuated in human CF airway epithelial cells compared to non-CF cells [115–117]. Hyperresponsiveness of primary airway epithelial cells to LPS, despite expressing normal levels of TLR4, has been attributed to the reduced surface expression of coreceptor CD14 and lower levels of the costimulatory molecule MD2 [118]. Conflicting studies have been reported regarding the localization of TLR5 on airway epithelial cells, with apical dominance on human and murine cells [119–122] and basolateral expression on polarized human nasal and bronchial epithelium [123–125]. Specific cell source, modulation of culture conditions and/or specific stimuli might explain these discrepancies. A strong synergism between TLR2/PGN- and TLR4/LPS-mediated IL-8 production and IL17A was found in human bronchial epithelial cell lines [126]. Recently, genotyping of TLR polymorphisms revealed that CF airway epithelial cells are homozygous for TLR1 SNP 1602S and possess a diminished innate immune response towards *Mycobacterium abscessus* infection. [127]. In a separate study, TLR SNPs were associated with CF lung function decline [128]. A recent study [129] demonstrated that *S. aureus* filtrates inhibit *P. aeruginosa* filtrate-mediated IL-8 production.

The CF airways are characterized by a neutrophil-rich environment. Neutrophils have been mainly implicated in controlling bacterial and fungal infections, but can also lead to airway damage upon activation through the release of enzymes (proteases) and oxidants [28]. Neutrophils are the first cell type recruited to the CF airway compartment. The recruitment of blood neutrophils into the airway compartment is mainly regulated through chemokines, such as IL-8, and lipid-mediators, such as LTB_4. The efficient antibacterial function of neutrophils in the CF airway micromilieu is impeded due to several mechanisms, such as proteolytic damage of airway neutrophils, neutrophil cell death and bacterial/fungal biofilm formation that prevents phagocytosis [3]. At the site of infection, neutrophils sense PAMPs or DAMPs via PRRs. Expression and functionality of TLRs in neutrophils have been studied in the context of CF lung disease. Collectively, TLR2, TLR4 and TLR5 are suggested to be most essential for neutrophil-*P. aeruginosa* interactions. CF airway neutrophils express remarkably high levels of TLR5, which correlates with lung function in CF patients [130, 131]. In a separate study, TLR surface expression was investigated on circulating and induced sputum neutrophils in CF patients. Compared to healthy controls, decreased expression of TLR2 was detected on circulating neutrophils in CF patients [132]. Furthermore, an inverse relationship between TNF-α serum levels and TLR2 surface expression on circulating neutrophils has been described [130]. DAMPs such as proline-glycine-proline and high-mobility group box protein-1 (HMGB1) have been implicated in CF lung disease. A high concentration of these mediators is found in CF airways and they serve as neutrophil chemoattractants to drive lung inflammation [133]. S100A12, a member of the S100/calgranulin family and a neutrophil-derived DAMP, was found in abundance in CF airway fluids leading to activation of downstream metabolic and stress pathways following neutrophil entry into CF airways [134].

Novel Therapeutic Concepts

Despite a plethora of proinflammatory innate immune pathways having been studied and determined as playing a significant role in CF lung disease, therapeutic exploitation of these pathomechanisms remains scarce. For a broader and more in-depth discussion of this aspect, we

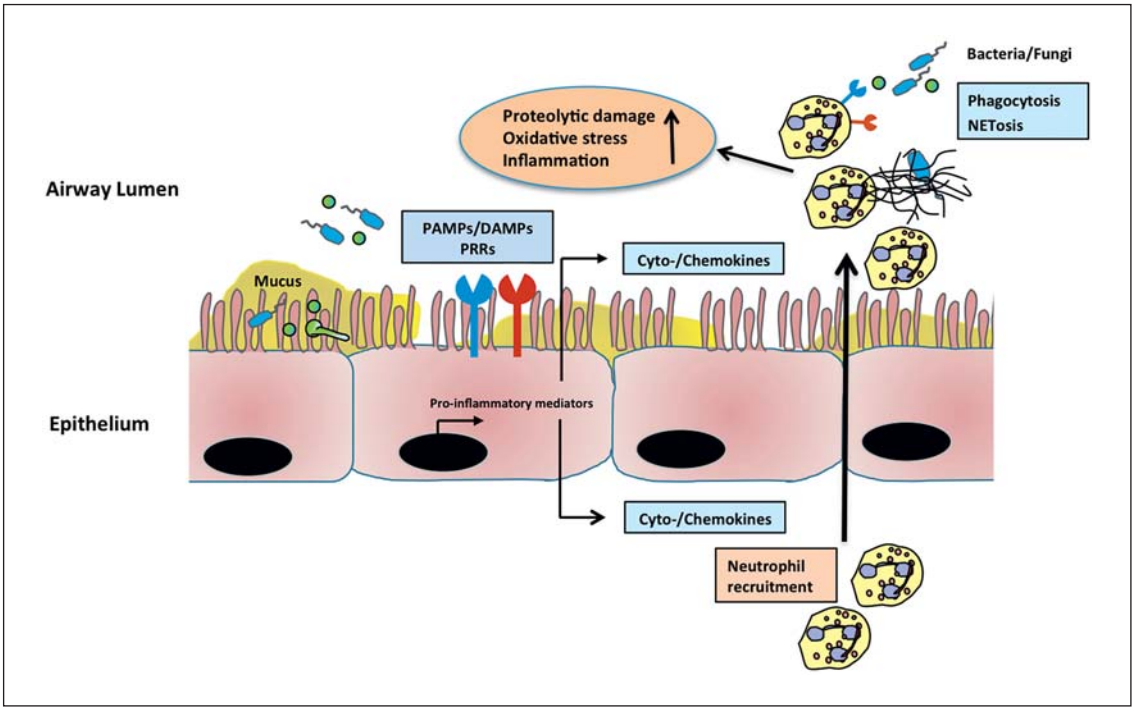

Fig. 1. Innate immune activation in CF airways. Due to continuous production of cytokines and chemokines, especially IL-8, neutrophils are recruited into the CF airways. Bacterial and fungal PAMPs and host-derived DAMPs further activate downstream signaling pathways through the activation of PRRs, and lead to enhanced cytokine and chemokine production. Infiltrated neutrophils release proteases and oxidants, resulting in perpetuated inflammation and tissue injury.

refer to thematic review articles [24, 135]. Ibuprofen represents a clinically available anti-inflammatory drug that has been shown to slow lung function decline in pediatric/adolescent CF lung disease [136–139], but its broad clinical usage outside the USA is restricted by drug-monitoring requirements. Correlations between lung function and inflammatory markers in CF airway fluids (neutrophil counts, IL-8 and NE) have been demonstrated in multicenter CF patient cohorts [22], suggesting that targeting neutrophil-related products may be beneficial in CF lung disease. However, clinical studies aiming to neutralize free NE activity in CF airways by the delivery of antiproteases, such as α-1 antitrypsin, showed modulated airway inflammation but failed to show convincing effects on lung function [25]. In contrast, the use of the oral antioxidant *N*-acetylcysteine, as a strategy to rebalance antioxidant deficiencies in CF, shows beneficial effects on lung function, but has no effect on neutrophilic inflammation [140]. Future studies are required to reconcile these findings and to further assess the therapeutic potential of antiprotease or antioxidant approaches in CF lung disease [24, 141]. The antibiotic azithromycin is known to have anti-inflammatory effects. A clinical trial [142] showed that azithromycin treatment reduced circulating neutrophil counts and systemic blood biomarkers, including C-reactive protein, serum amyloid A and calprotectin, and was correlated with the improvement in lung function and weight gain. Other anti-inflammatory therapeutic approaches include sildenafil (phosphodiesterase inhibitor) [143], CXCR2 inhibition [26] and others less-advanced ones that are not discussed here. Collectively, therapeutic interventions to dampen inflammation in CF remain an appealing yet challenging approach.

Conclusions and Outlook

There is broad consensus about the concept that the innate immune system is activated early and strongly in CF lung disease, leading to the continuous recruitment of neutrophils into CF airways [3]. These neutrophils release proteases that cause harm to the host by degradation

of the pulmonary tissue and the immune receptors (fig. 1). However, controversy exists as to whether the targeting of innate immune pathways, by neutrophil recruitment and/or activation, represents a promising strategy in CF lung disease. On the one hand, there are clear relationships between neutrophil products, prototypical NE and decreased lung function [22] as well as bronchiectasis [2]. On the other hand, targeting excessive proteolytic activities in CF has clinically not been successful so far. Interfering with neutrophil recruitment through CXCR2 inhibition represents a causative anti-inflammatory approach [26], but has also not shown any clinical benefits for lung function. Novel strategies to dampen innate immunity in CF in the future could involve anti-inflammatory pro-resolution lipid mediator pathways, such as resolvins [144], and the endocannabinoid system [145]. However, most of these pathways have mainly been assessed in acute lung inflammation models and not in chronic CF lung disease. Both preclinical and clinical studies are warranted to evaluate these and other anti-inflammatory mechanisms in the context of CF lung disease.

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Disclosure Statement

The authors have declared that no conflict of interests exists.

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