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Abstract: Chronic lung disease determines the morbidity and mortality of cystic fibrosis (CF) patients. The pulmonary immune response in CF is characterized by an early and non-resolving activation of the innate immune system, which is dysregulated at several levels. Here we provide a comprehensive overview of innate immunity in CF lung disease, involving (i) epithelial dysfunction, (ii) pathogen sensing, (iii) leukocyte recruitment, (iv) phagocyte impairment, (v) mechanisms linking innate and adaptive immunity and (iv) the potential clinical relevance. Dissecting the complex network of innate immune regulation and associated pro-inflammatory cascades in CF lung disease may pave the way for novel immune-targeted therapies in CF and other chronic infective lung diseases.

Innate immunity in cystic fibrosis lung disease

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ABSTRACT

Chronic lung disease determines the morbidity and mortality of cystic fibrosis (CF) patients. The pulmonary immune response in CF is characterized by an early and non-resolving activation of the innate immune system, which is dysregulated at several levels. Here we provide a comprehensive overview of innate immunity in CF lung disease, involving (i) epithelial dysfunction, (ii) pathogen sensing, (iii) leukocyte recruitment, (iv) phagocyte impairment, (v) mechanisms linking innate and adaptive immunity and (iv) the potential clinical relevance. Dissecting the complex network of innate immune regulation and associated pro-inflammatory cascades in CF lung disease may pave the way for novel immune-targeted therapies in CF and other chronic infective lung diseases.

i. Epithelial dysfunction drives airway inflammation in CF

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause a critical impairment of innate host defense systems in the lungs of cystic fibrosis (CF) patients. This results in an early and severe form of chronic airways disease, featuring mucus obstruction, neutrophil-dominated airway inflammation and bacterial infection, finally leading to progressive pulmonary damage with bronchiectasis and emphysema[1-4].

The airways are lined by an epithelial layer that integrates several functions important for effective defense against inhaled pathogens and other noxious agents, ranging from a barrier function, proper surface hydration by regulated transport of ions and fluid, mucus secretion, and mucociliary clearance[5,6] to expression of antimicrobial peptides[7], receptors that recognize pathogens associated molecular patterns (PAMPs) and host-derived danger signals (DAMPs) and the secretion of chemokines and cytokines that orchestrate the recruitment of leucocytes[8] as well as the local immune responses in the airway lumen[9]. Given this broad functionality of the airway epithelium in innate lung defense, and the fact that the CFTR protein is expressed in the apical membrane of the surface epithelium and in submucosal glands[10,11], the link between epithelial CFTR dysfunction and the pathogenesis of CF airways disease has long been in the focus of CF research.

In healthy airways, CFTR acts as a cAMP-dependent Cl^- channel[12,13] that works in concert with the Ca^{2+}-activated Cl^- channel (CaCC)[14] to secrete Cl^- and fluid required for hydration of mucins and other secreted molecules that form airway mucus. In the surface epithelium, CFTR also functions as regulator of the amiloride-sensitive epithelial Na^+ channel (ENaC) that constitutes the limiting pathway for Na^+ and fluid absorption[15]. Given this dual functionality in vectorial ion transport, CFTR plays a key role in coordinate regulation of secretion and absorption of NaCl and fluid, and hence airway surface liquid (ASL) homeostasis[6,16]. Accordingly, in CF airways, epithelial CFTR dysfunction results in an imbalance between Cl^- secretion and ENaC-mediated Na^+ absorption rendering the airways

surfaces vulnerable to ASL volume depletion. In a series of studies using highly-differentiated primary airway cultures it was shown that airway surface dehydration impaired ciliary beating and mucus transport, and promoted mucus stasis and adhesion in CF airway epithelia[17,18]. Since mucociliary clearance acts as an important integral part of the innate pulmonary defense system, these results indicated that mucociliary dysfunction due to airway surface dehydration constitutes a disease-causing mechanism that links the basic CF defect to impaired airways defense and CF lung disease. The *in vivo* evaluation of the "dehydration hypothesis" was hampered for a long time by the fact that *Cftr*-deficient mice did neither show abnormal Cl^- or Na^+ transport in their lower airways, nor develop CF-like lung disease[19-23]. This limitation was overcome in a mouse model with airway-specific overexpression of the β subunit of ENaC (βENaC) developed to mimic increased Na^+ absorption characteristic of CF airways[19,24]. In this model, an imbalance between Cl^- secretion and Na^+ absorption caused ASL depletion and reduced mucus clearance *in vivo*, and resulted in a lung disease that shares many features of early lung disease in CF patients, including airway mucus obstruction, reduced bacterial clearance, chronic neutrophilic inflammation and emphysema, supporting the notion that epithelial impairment initiates and sustains inflammation[19,24,25]. Further, studies in βENaC-overexpressing mice demonstrated that preventive pharmacological targeting of ASL depletion by the ENaC inhibitor amiloride has substantial therapeutic effects on CF-like mucus obstruction and airway inflammation[26]. These results validated impaired ASL homeostasis resulting from a basic epithelial ion transport defect in CF airways as a disease-initiating mechanism in the *in vivo* pathogenesis and as a therapeutic target of CF lung disease. Hence, the βENaC-overexpressing mouse will also provide opportunities for further *in vivo* elucidation of the molecular mechanisms underlying the initiation and perpetuation of dehydration-induced airway inflammation, and its contribution to impaired host defense and lung damage that will be reviewed in the sections below.

In addition to the well established impairment of epithelial ion transport, other epithelial dysfunctions have been implicated in chronic inflammation and infection of CF airways, including increased apoptosis due to accumulation of ceramide, a promising approach for drug targeting[27,28], intrinsic pro-inflammatory properties, exaggerated inflammatory responses to viral and bacterial infections, and reduced clearance of *Pseudomonas aeruginosa* (*P. aeruginosa*)[29-31,32,33]. For studies indicating a link between CFTR and glutathione or Ca^{2+} channels we refer to a recent review by Ratner et al[34]. We expect that the recent development of small-molecule CFTR modulators designed to improve impaired CFTR Cl^- channel function in CF[35,36], together with sensitive assays for quantitative assessment of mutant CFTR function in native tissues[37,38], will help to dissect the relative roles of the CF ion transport defect and other epithelial dysfunctions in chronic inflammation and impaired host defense in the CF lung.

ii. CF pathogens are sensed through distinct pattern recognition receptors

Infections with *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa* are mainly characteristic for CF patients. Other emerging bacterial pathogens, such as *Burkholderia cepacia* and *Stenotrophomonas maltophilia*, or fungi like *Aspergillus fumigatus*[39], *Candida albicans*[40] or *Scedosporium apiospermum*[41] are increasingly appreciated to play a role in CF lung disease[3,42]. Whereas *Staphylococcus aureus* and *Haemophilus influenzae* infections manifest in younger CF patients, *P. aeruginosa* is the predominant pathogen in later infancy, teenage-age and adult CF patients. Colonization with *Aspergillus* and/or *Candida spp.* is mainly found in older CF patients, but their pathophysiological relevance is poorly understood so far[39,43,44]. Infections with *P. aeruginosa* seem to favor colonization with *Aspergillus fumigatus* and associated allergic bronchopulmonary aspergillosis (ABPA)[39,43], potentially due to a *P. aeruginosa*-mediated Th2 shift[45].

Given the long-term and intimate contact of pathogens with the lining CF airways, the mechanisms by which these pathogens are recognized by the host are of key relevance for the understanding of innate immunity in CF lung disease. In general, the innate immune system - senses conserved molecular patterns using pattern recognition receptors (PRR), with Toll-like receptors (TLRs) representing the prototypic PRRs[46,47]. Other non-TLR PRRs include complement receptors, Fc receptors, nucleotide-binding oligomerization domain family (NOD)-like receptors, scavenger receptors, RIG-like helicases, nucleic acid receptors and others[48]. The majority of TLRs recognize bacterial patterns (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9, TLR10, TLR11) with TLR4 sensing lipopolysaccharide (LPS), TLR5 flagellin, TLR9 CpG oligonucleotides and TLR11 uropathogenic bacteria and/or profilin from protozoa. Viral PAMPs are recognized through TLR3 (double-stranded RNA, dsRNA), TLR7 and TLR8 (single-stranded RNA, ssRNA). Beyond microbial PAMPs (recently also termed for microbes in general independent of their pathogenity as microbe-associated molecular pattern, MAMPs), TLRs sense endogenous ligands (DAMPs), including heat shock protein 70 (HSP-70), proinflammatory extra domain A of fibrinogen, extracellular CXCR1 moieties, heparan sulfate fragments, elastase, hyaluronic acid fragments (TLR4) and others. TLRs are expressed by a variety of immune and structural cells, including monocytes/macrophages, dendritic cells, lymphocytes, endothelial cells, myocytes, epithelial cells, neutrophils and others. Here we will focus on TLRs expressed by epithelial cells, since they represent the first cellular line of pattern recognition in the CF airway microenvironment.

Airway epithelial cells including tracheal, bronchial and alveolar type II cells express a broad repertoire of TLRs[9,49-52]. Most anti-bacterial TLRs are appropriately exposed on the mucosal surface of the airway and can be readily activated by superficial exposure to microbial factors. The predominant TLR expressed on the surface of bronchial epithelial cells *in vivo* appears to be TLR2, with other TLRs (TLR3, TLR4, TLR5) residing mainly intracellularly yet displaying low level surface expression. Whilst in macrophages and

dendritic cells[53] TLR7, TLR8 and TLR9 reside endosomes, in both immortalised and differentiated primary airway epithelial cells[54,55], TLR9 has also been detected on the cell surface expression using flow cytometry. Some TLRs can be mobilised to the airway epithelial cell surface following stimulation with microbial factors. For example cell surface localisation of TLR4 and TLR5 is promoted by Respiratory Syncytial Virus (RSV) infection and flagellin, respectively[56,57], whereas infection with *P. aeruginosa* enhances TLR2 localisation within lipid rafts on the apical surface of airway epithelial cells[58].

Three early reports described TLR expression in primary and transformed CF airway epithelial cells[55,59,60]. These studies provided the first evidence that TLRs 1-10, the TLR4 accessory protein MD2 and the TLR adaptor protein myeloid differentiation primary response gene 88 (MyD88) are expressed and functional in CF airway epithelium. As in non-CF airway epithelial cells, bacterial stimulation of CF cells caused up regulation of TLR2, albeit reduced. Similarly, TLR4 is displayed poorly on the apical surface in CF cells and fails to respond well to stimulation with LPS. John *et al.* investigated the functional consequences of this, and independently confirmed that TLR4 expression is decreased in CF bronchial epithelium resulting in weaker than normal MyD88 and TRIF-dependent signalling[61,62], possibly leading to impaired adaptive immune responses. For further detailed discussion of the role of TLRs in CF airway epithelial cell responses we also refer to an recently published review[63].

TLR5 is a key TLR expressed by airway epithelial cells and can mediate inflammatory responses to flagellin-expressing but not *fliC* (flagellin-deficient) mutants of *P. aeruginosa* and *Burkholderia cepacia (B. cepacia)*[64]; it represents also the predominant TLR responsible for lung epithelial inflammatory responses to *B. cenocepacia*[65]. In CF airway epithelial cells inhibition of TLR5 can abolish IL-6 production normally generated following exposure to *P. aeruginosa*, identifying TLR5 as a potential therapeutic target for CF lung inflammation[66]. Interestingly CF adults with a TLR5 1174C>T SNP, which encodes a premature stop codon in TLR5 that is associated with decreased flagellin-responsiveness, appear to have improved

nutritional status as measured by higher body mass index[67]. Thus TLR5 could also represent a modifier gene for CF. However antagonism of TLR5 alone may not be sufficient to decrease CF airway epithelial cell responses to *P. aeruginosa*. Reduced glutathione levels sensitize these cells to the action of reactive oxygen species generated by NADPH oxidase leading to enhanced CXCL8/IL-8 expression[68]. Therefore, targeting both oxidants and TLRs, in addition to other recently reported experimental anti-inflammatory strategies, is likely to have greater therapeutic benefit. CF patients are commonly co-colonised with *Aspergillus* species[69]. Conidial dsRNA can induce interferon-β via activation of TLRs in non-CF airway epithelial cells[70], however it remains to be shown whether CF airway epithelial cells respond in a similar way or whether their response to *Aspergillus* occurs as a result of chitin-induced activation of TLR2 as seen in murine macrophages[71].

The CF lung is a MAMP/PAMP- and DAMP-rich milieu and, in addition to classical TLR agonists, contains a selection of factors that can indirectly activate TLRs. Neutrophil elastase activates TLR4 via a mechanism involving meprin and/or Tumor necrosis factor-alpha-converting enzyme / a disintegrin and metalloproteinase domain 17 (TACE/ADAM17), TGFα and EGFR[72-75], leading to exaggerated IL-8 and mucin gene expression whilst hemoglobin, which can be present in the fragile CF lung due to micro-bleeds, liberates free heme as a result of cleavage by elastase and/or *P. aeruginosa*-derived proteases that can similarly activate TLR signaling and proinflammatory gene expression via EGFR-TLR signaling[76]. Although TLR hyper-responsiveness is largely accepted to contribute to CF lung pathology there are a number of contexts where inadequate bronchial epithelial TLR–mediated responses can occur and are likely to impact negatively on pulmonary inflammation. In CF females high circulating estrogen levels can impair TLR-induced NFκB activation and cytokine expression via estrogen-receptor-β-mediated up regulation of secretory leucoprotease inhibitor (SLPI)[77]: SLPI is an antiprotease with anti-inflammatory and immunomodulatory properties that can prevent NFκB activation by interfering with

proteasomal degradation of IκB[78,79] and by blocking p65 binding to NFκB consensus sequences in chromosomal DNA[80]. This, along with other estrogen-induced effects, likely contributes to the gender gap in CF whereby CF females have poorer lung function, worse exacerbations and convert to mucoid strains of *P. aeruginosa* prematurely compared to males.

Altered microRNA expression in CF bronchial epithelium *in vivo* can also impact on TLR signalling potentially leading to TLR hypo-responsiveness[81]. Decreased miR-126 controls reciprocal increases in TOM1 which can interfere with TLR2 and TLR4 responses and may also contribute to CF-specific decreased cell surface expression of TLR4. These subtle regulatory mechanisms underscore the importance of carefully considering the context and timing in which future TLR-directed therapies should be prescribed.

iii. Chemokines and extracellular matrix break-down products orchestrate the recruitment of leukocytes into CF airways

As consequence of epithelial dysfunction and pathogen sensing, a pro-inflammatory cascade is initiated in CF airways that leads to the recruitment of leukocytes from the bone-marrow niche via the circulation into the airways. While professional phagocytes, such as macrophages and microphages (neutrophils), are essential in clearing the lungs from bacterial and fungal pathogens, their uncontrolled action in the absence of microbial threat causes tissue damage and tissue remodelling. Therefore, the in-depth understanding of mechanisms driving leukocyte migration into CF airways under infectious and non-infectious conditions is pivotal for future therapeutic approaches targeting specific, host-damaging recruitment pathways, while preserving antimicrobial host defense capacities.

Our current view of leukocyte migration in chronic neutrophilic inflammation becomes increasingly complex and has overcome the traditional concept that single disease-associated chemoattractants direct leukocyte migration into tissues. Leukocytes transmigration into the extravascular space represents a multi-step process, involving rolling,

activation, adhesion and diapedesis, requiring the concerted action of integrins, selectins and chemokines[82-84]. There is limited evidence for CF-associated dysregulations in the context of neutrophil transmigration. The relevant pathomechanisms seem to be complex, since CFTR neutrophil-intrinsic, airway epithelial cell-dependent and transmigratory lung epithelium feed-back factors have to be considered[85,86,87]. Related to transmigration mechanisms, dysregulations in L-selectin shedding[88], complement receptor (CD11b/CD18) expression[89] and ICAM-dependent airway epithelial cell-neutrophil interactions[90] have been described.

Tissue-released chemokines (major source: epithelial cells and macrophages) attract leukocytes via chemokine gradients to the site of inflammation, putting chemokines and their G-protein coupled receptors in the spotlight of targeted anti-inflammatory strategies[91]. However, the previous years have substantially extented the notion that chemokines are predominantely responsible for attracting leukocytes to diseased organs by demonstrating that products liberated from necrotic cells (alarmins) and tissue extracellular matrix break-down products, as discussed below in detail, are capable of initiating and sustaining leukocyte recruitment[92,93].

Cytokines and chemokines

Cytokines, as small molecular weight proteins, influence a plethora of biological processes including cellular activation, recruitment, cell death, and repair and play a critical role in the regulation of innate immunity in a variety of disorders including CF[94]. Due to the chronic bacterial colonization of the CF airways, cytokines are continuously produced at the pulmonary site of inflammation and exert their influence in a paracrine manner at the interface of bacteria and airway epithelial cells[95]. This ongoing activation of innate immune receptors by bacteria and bacteria-associated PAMPs/MAMPs leads in the CF microenvironment to the dysregulated production of cytokines; elevated levels of pro-inflammatory cytokines (such as TNF-α, IL-6, IL-1β) and reduced levels of anti-inflammatory cytokines (such as IL-10) have been reported in the bronchoalveolar lavage (BAL) and

sputum of patients with CF lung disease[96]. Despite general agreement about the imbalance of these cytokines in the CF lung, it remains a challenge to assess how these cytokines regulate biological and cellular functionalities in the CF lung.

The prototypic pro-inflammatory cytokine TNF-α is mainly released from monocytes and macrophages and has pleiotropic effects central to innate immunity, including inflammatory cell recruitment and induction of additional cytokines[97]. TNF-α has been traditionally regarded as an early release cytokine and, therefore, its persistence in the CF airways strongly suggests ongoing production from both resident alveolar macrophages and recruited monocytes[98]. Although TNF-α is elevated in the CF lung, it has not shown a strong correlation with progression of disease[99,100]. In addition, there have been variable results in the regulation of TNF-α production during pulmonary exacerbation of CF lung disease[101,102]. Besides TNF-α, IL-1 (both IL-1α and IL-1β) have been studied in the context of CF. Similar to TNF-α, IL-1β is released from macrophages/monocytes and is actively involved in inflammatory cell recruitment and the amplification of the pro-inflammatory cytokine response[103]. Both isoforms have been found to be elevated in the blood and airway samples (BAL and sputum) from CF patients[100,104-106]. IL-1β levels have been shown to be increased both in the sputum and BAL of CF patients who were chronically colonized with *P. aeruginosa* compared to those individuals who were not colonized[107]. In parallel, eradication of *P. aeruginosa* leads to decreased IL-1β levels in the BAL of CF children[108]. Despite these findings, the correlation of IL-1β with progression of disease remains poorly linked. IL-10 is a critical anti-inflammatory protein capable of reducing the production of multiple pro-inflammatory proteins. The levels of IL-10 have been found to be decreased in the CF lung of older patients[107]; this may in part be due to differences in secretion of IL-10 from airway epithelial cells[109]. Interestingly, younger children do not seem to exhibit differences in IL-10 levels between CF and non-CF individuals[110]. Due to potential differences observed of IL-10 in older patients, there is significant interest in directed gene therapy to restore IL-10 balance

in CF lung disease and preclinical studies are underway[111]. The potential role of these CF-associated cytokines as disease biomarkers is reviewed in detail elsewhere[112]. Therapeutically, anti-cytokine strategies (biologicals), which are already successfully implemented in the therapy of rheumatoid arthritis, are reasonable to consider in progressive CF lung disease. However, the increased infection susceptibility and related side effects[113] have to be taken into account, in particular in older CF patients suffering from chronic bacterial infections.

A distinct subset of chemotactic cytokines, termed chemokines, orchestrates the recruitment and activation of inflammatory cells[114,115]. Perhaps the best studied chemokine in CF lung disease is CXCL8 (IL-8). CXCL8 acts on both CXCR1 (IL-8RA, CD181) and CXCR2 (IL-8RB, CD182) surface receptors; this chemokine exhibits pleotropic effects on neutrophils, acting as a potent chemoattactant and inducing degranulation and superoxide production[116]. Many groups have reported elevated levels of this chemokine in the BAL and sputum of both CF adults and children[107,112,117-119]. Furthers cellular studies demonstrated that CXCR1, but not CXCR2, is involved in antibacterial effector functionalities by neutrophils and that CXCR1 is proteolytically cleaved in CF airways, thereby disabling CXCL8-mediated enhancement of anti-bacterial effector functions[117]. There are also reports of CXCL8 levels correlating with patients' clinical status, including Schwachmann scores, bacterial colonization with *P. aeruginosa*, and lung function[120-122]. Despite these findings, studies targeting this molecule in chronic obstructive pulmonary disease (COPD), featuring a similar chronic neutrophilic lung disease, have not shown clinical benefits so far[123], strongly suggesting additional, redundant chemotactic and activation pathways being important in COPD and probably CF disease pathogenesis. Other chemokines that have been studied in CF include CCL2[124], CCL3, CCL4, and CCL20[125] as well as a deficiency of the Th1- and human Immunodeficiency Virus (HIV)-associated chemokine CCL5 (RANTES)[126,127]. In ABPA, the Th2-associated CC chemokines CCL17 and CCL22 have been proposed as potential biomarkers[43,128-132].

Proline-Glycine-Proline and high mobility group box protein-1

Beyond the canonical pathways of preformed cytokines/chemokines regulating inflammatory response in CF, recent evidence suggests a significant noncanonical pathway responsible of neutrophil influx. This pathway involves the coordinated cleavage of extracellular matrix (ECM) to small fragments, capable of acting on chemokine receptors on inflammatory cells. Although hypothesized in human disease for over 50 years, the identification of these fragments and mechanisms related to their action was poorly understood for a long time[133]. Recently, however, a fragment of collagen proline-glycine-proline (PGP) has been identified in the sputum and serum of CF patients[134]. This peptide acts as a ligand mimetic for CXCR1 and CXCR2 due to similarities in the structure of ELR+ CXC chemokines. PGP (and its N-terminal acetylated form, Ac-PGP) have been shown to induce chemotaxis, produce superoxide, and induce release of proteases from neutrophils[135]. The liberation of PGP from collagen is a multiprotease pathway, with the initial cleavage of collagen mediated by matrix metalloprotease (MMP)-8 and MMP-9 and subsequent cleavage conducted by prolyl endopeptidase (PE), a serine protease first described in this work as playing a role in pulmonary inflammation[136]. Recently, a pathway regulating PGP degradation and inactivation has been described through a novel aminopeptidase activity of the enzyme leukotrieneA4 hydrolase (LTA4H)[137]. Persistence of PGP in chronic neutrophilic lung disorders has been well-documented and PGP peptides were found to be elevated in the sputum of individuals with CF lung disease compared to non-lung disease controls[136]. At the beginning of inpatient exacerbation, PGP peptides were increased and these peptides decreased at the end of therapy, consistent with improvement in lung function. However, the end-of-exacerbation PGP levels were still significantly higher than non-lung disease controls, suggesting ongoing inflammatory response in the lung despite resolution of exacerbation. PGP peptides have also been detected in the serum of CF patients, suggesting a possible systemic role for this peptide in human disease; ongoing research is focusing on the variety of systemic effects of this

peptide in CF disease. Figure 1 illustrates the proposed mechanisms by which PGP drives and perpetuates chronic neutrophilic airway inflammation in CF.

A further host-derived inflammatory protein involved in CF lung is high mobility group box protein-1 (HMGB1). This chromatin protein is released from inflammatory cells (such as monocytes) or from necrotic cells[138] and acts intracellularly to enhance nucleoprotein interactions[139]. Once released, this protein can act as a potent proinflammatory cytokine by its binding to RAGE, TLR and CXCR4 receptors[140,141]. Recently, Rowe et al. have described the increased presence of extracellular HMGB1 levels in the secretions of CF patients and in the βENaC mouse model[134]. In addition, this elevation of HMGB1 closely correlated with the presence of the chemotactic collagen fragment PGP and offers the consideration if these pathways of inflammation are linked[142].

iv. Recruited phagocytes are impaired in the CF microenvironment

Bone marrow-derived immune cells are continuously recruited to the infective pulmonary microenvironment in CF lung disease in order to clear the bronchoalveolar pathogens. Neutrophils and macrophages are the major antibacterial effector cells, are therefore termed as ‚professional phagocytes' and are here discussed in detail, while dendritic cells (DCs) phagocytose but act mainly as antigen presentators and T cell instructors to bridge innate and adaptive immunity and are only summarized briefly at the end of this parapraph, particularly regarding the paucity of data concerning DCs in CF.

Neutrophils

In healthy adults, 10^{11} neutrophils daily transit through the circulation[143]. Upon infection, these neutrophils rapidly transmigrate to the site of inflammation, where they sense PAMPs and/or DAMPs and combat pathogens. Airway fluids (sputum, BALF) of CF patients contain millions of neutrophils, which led to the well-established notion that CF lung disease is dominated by a neutrophilic airway inflammation[8,117]. In general, the pathophysiological role

of airway neutrophils in CF and other chronic neutrophilic lung diseases is two-faced: On the one hand they are required for antibacterial and antifungal host defense, on the other hand they can cause significant parenchymal lung tissue damage when they accumulate over longer time periods and liberate their toxic granule contents, mainly serine and metalloproteases as well as oxidants, in an uncontrolled fashion[144]. Based on this scenario, the targeting of distinct harmful subtypes of airway neutrophils, while preserving other beneficical subtypes, may provide the possibility to specifically modulate the neutrophilic activity in CF lung diseases.

Regarding the role of CFTR in neutrophil homeostasis and function, studies suggest a functional relevance of CFTR in regulating anti-bacterial neutrophil activities (see discussed in detail below)[145-147]. Isolated neutrophils from CF patients were found to release more elastase from primary granules than control cells[148] and showed enhanced migratory capacities[149], but a blunted phagocytic capacity[150]. On the other hand, studies indicated that neutrophils from CF patients showed no difference in terms of phagocytosis (in newborns)[151] or ROS generation[152], suggesting that local/pulmonary factors play also a role in modulating neutrophil effector functionalitis *in vivo*. This notion is supported by the finding that CF sputum stimulates CD18-independent neutrophil migration across endothelial cells[153]. Studies analyzing cell death pathways in peripheral blood-isolated cells added another angle by demonstrating that circulating neutrophils from CF patients feature a slower apoptosis rate[154,155 156,157]. When viewing these studies in combination, both systemic/CFTR-associated, cell death-related as well as pulmonary factors probably contribute in the regulation of neutrophil functionality in CF airways.

Regarding the usefulness of neutrophils or neutrophil-derived proteins, a large number of studies investigated the relationship between neutrophil numbers, phenotypes and neutrophilic mediators in CF patients and correlated them with disease outcome parameters, such as pulmonary function [32,45,112,118,130,131,158-165]. These studies provided evidence that both the extent of neutrophilic airway inflammation as well as neutrophil proteins, prototypically

neutrophil elastase, correlate with pulmonary obstruction (FEV1) in different CF patient cohorts. A large US multicenter study compared different CF sputum markers as predictors for FEV1 decline and found that neutrophil elastase showed the highest correlation with longitudinal FEV1[118], suggesting sputum elastase as promising biomarker for CF airway inflammation. Other studies analyzing BALF found increased elastolytic activity in a subset of CF infants very early in the course of CF lung disease[1], supporting, in line with murine βENaC studies[19,24], the notion that neutrophilic-proteolytic inflammation starts early in the pathogenesis of CF lung disease. Beyond elastase, other studies found evidence for neutrophil-derived proteins as potential biomarkers in CF lung disease, in particular calprotectin[166,167], a S-100 protein located in specific granules, MMP9[159,158,168] stored in tertiary granules, myeloperoxidase (MPO)[169] and proteinase 3[170,171] stored in primary granules, the chitinase-like protein YKL-40[160] and others. In a study comparing gene expression in peripheral blood neutrophils from CF patients and healthy controls, 1050 genes were found to be upregulated[172], with the majority not being studied so far in the context of CF lung disease, indicating that the field of neutrophilic inflammation in CF is complex and requires future studies to gain mechanistic insights.

Neutrophils release their granules sequentially, starting with secretory vesicles that shuttle between cytoplasmic compartments and the plasma membrane, which are pivotal for recycling and mobilization of complement- , Fc- and other receptors. Tertiary granules contain MMP-9, a marker protein involved in transmigration of neutrophils and the initiation of proteolytic extracellular matrix degradation. Upon intensified and prolonged stimulation, neutrophils liberate their secondary granules that contain anti-microbial proteins, such as lactoferrin and cathelicidine, liberated to cope with encountering pathogens. Upon direct pathogen contact, neutrophils engage their strongest granule weapon, the primary or azurophilic granules. This event liberates serine proteases that target both engulfed pathogens in the phagolysosome or, when liberated in an uncontrolled or pathogen-independent fashion,

extracellular matrix and leukocyte surface receptors, such as the CXCL8 / IL-8 receptor CXCR1 (CD181), T cell or complement receptors. These proteolytic effects are dependent on the protease/anti-protease balance in the CF airway microenvironment, a comprehensive topic by itself[173,174]. Of note, ASL without proteolytic activity has been shown to have no major effect on neutrophil activity *in vitro*[175]. Given the stepwise granule release mechanism, granule proteins detectable in CF airway fluids may represent distinct stages of neutrophil activation. For a more in-depth review on neutrophil granules we refer to Hager et al.[176]. Consequently, studies investigating proteins characteristic for all four intracellular neutrophil granule/vesicle compartments in peripheral and airway neutrophils from CF patients and controls are warranted to understand the differential activation state of CF neutrophils comprehensively. Furthermore, longitudinal studies analyzing neutrophil markers in CF airway fluids are required to understand the kinetics of neutrophilic activation in CF lung disease.

Beyond these studies, desmosin, an urinary marker of elastin was found to be associated with disease outcome and inflammation in CF patients[177] [29]. Recently, the neutrophil-derived alpha-1 antitrypsin and CD16 protein heterocomplex (AAT:CD16) has been reported as potential biomarker for exacerbations in CF[178]. Intriguingly, Voglis et al. showed that human neutrophil peptides impaired the phagocytic capacity of neutrophils in an auto- and/or paracrine manner in both CF and non-CF bronchiectatic conditions[179].

In the airway microenvironment of chronic *P. aeruginosa* infections, infiltrated neutrophils are faced with *Quorum sensing*-induced bacterial biofilms[180] and this interaction substantially modulates the phagocyte's behaviour. *P. aeruginosa* can expoit the neutrophil-derived host DNA to form biofilms[181]. In turn, CF airway fluid biofilm-associated neutrophils have been described to be distinct in terms of impaired migration with preserved phagocytosis[182], modulated respiratory burst activity[183] and ongoing oxygen consumption[184]. Further interaction mechanisms between *P. aeruginosa* and neutrophils implicate TLRs, with

TLR5 seeming to play a major role[185], pyocyanin, a *P. aeruginosa*-derived exotoxin that is capable of inducing neutrophil apoptosis[186], neutrophil extracellular trap (NET) formation (NETosis)[187] (as discussed below in detail) and type III secretion-dependent oncosis[188]. Further abnormalities reported for CF neutrophils include catalase- and MPO-dependent actions[189], oxidant release[190,191,192,193], neutrophil apoptosis[157], Coronin-1-associated neutrophil survival[156] and others not discussed here in detail[194, 195,87,196].

The phenotypical characterization of neutrophils is CF airways is hampered by several facts: (i) heterogeneitity among CF patients (extent of neutrophilic inflammation, infections), ability to sample sputum (impeding sputum studies in CF infants younger than about 5/6 years of age), (iii) pre-analytical processing of complex airway fluids material (sputum, see[197] for details) and cleavage of surface markers essential for neutrophil subtype characterization. A few studies used advanced optimized flow cytometric approaches to dissect airway neutrophil subpopulations[198,199,8,185,200]. These studies provided evidence that CF airway neutrophils contain different subphenotypes, which feature non-canonical surface receptor expression characteristics, in particular CXCR4, CD39, RAGE, CD114 and CCR5. The functional and pathophysiological relevance of these CF airway-site specific neutrophil phenotypes remains to be defined[201,198,195].

Neutrophils at the pulmonary site of inflammation are instrumentalized with three main anti-bacterial weapons: phagocytosis, granules release and NET formation[202], which are employed depending on the duration and severity of host-pathogen interactions (Figure 2). There is evidence for impaired phagocytosis[150] and release of protease-rich primary granules[148] in CF neutrophils as discussed above and summarized in Table 1. When neutrophils at the pulmonary site of host-pathogen interaction are unable to cope with the encountered pathogen by phagocytosis or granule attack, they employ their final armamentarium which is their own DNA. Upon prolonged stimulation, neutrophils release NETs, that are DNA fibres that entangle, immobilize and kill mainly bacterial and fungal

pathogens. The mechanisms by which NETs kill the captured and immobilized bacteria are still not fully understood, but histones and granule proteins, such as proteases and calprotectin, could play a role[203,204]. CF airway fluids feature abundant extracellular DNA[205] and the amount of free extracellular DNA shows an association with pulmonary function in several studies[206-209], supporting the concept that recombinant DNases acts beneficial in CF lung disease by cleaving the NET-DNA meshwork and thereby facilitating mucociliary clearance of cleaved DNA strands. NETs in the CF airway context are mainly formed upon contact with *P. aeruginosa* and a recent study nicely demonstrates that NETs are capable of killing *P. aeruginosa* bacteria[210]. The *CFTR* gene defect does not seem to affect NET-otitc killing, since no difference between CF and non-CF neutrophils were found[210]. Intriguingly, isogenic clinical *P. aeruginosa* isolates from CF patients exhibited an acquired resistance towards NET-mediated killing, which correlated with the mucoid phenotype, but was not a direct result of excess alginate production[210]. NET formation seems to be critically involved in the host defense against other CF-associated pathogens, such as *Aspergillus fumigatus*[211] and this interaction may be essential for the common *Aspergillus fumigatus* colonization in CF patients.

Traditionally, neutrophils have been traditionally described to be devoid of CFTR expression. However, recent studies indicate that neutrophils express CFTR in lysophagosomes[145-147]. These studies demonstrate that CF neutrophils are defective in chlorination of phagocytosed bacteria and further suggest that CFTR as chloride channel is involved in the MPO-hydrogen peroxide-chloride-mediated microbicidal function, relevant for killing of *P. aeruginosa*[145,146]. These studies using primary human neutrophils were extended by showing that iRNA-mediated knock-down of *CFTR* in neutrophil-like immortalized HL-60 cells impairs microbicidal effector functionalities[212] and by demonstrating that Zebrafish with reduced *cftr* expression featured impaired respiratory burst

and neutrophil migration[213]. The potential role of CFTR in neutrophils has been further supported by studies in mice. Upon pharmacologic or genetic inhibition/deletion of CFTR in neutrophils, pro-inflammatory pathways (NF-κB, MIP-2 and TNF-α) were enhanced and reconstituion of wild-type mice with *cftr* F508del neutrophils showed increased pulmonary inflammation after LPS challenge[214]. What is currently completely unknown is the turnover/survival of neutrophils in the CF airway microenvironment. Recent studies challenged the paradigm that neutrophils are short-lived cells and showed, using sophisticated *in vivo* neutrophil labelling methodology, that neutrophils live for 5.4 days in healthy individuals[215]. Future studies are required to assess the neutrophil turnover / life-span in CF airways to evaulate the effects of currently used anti-inflammatory drugs, such as corticosteroids and ibuprofen, in CF patients.

In summary, the neutrophil is quantitatively the predominant cell in the airways of CF lung disease and contributes to disease progression through release of oxidants, proteases and other proteins. A major challenge for future studies in the field will be the in-depth characterization of neutrophil turnover and subset functionality in CF lung disease in order to identify specific neutrophil subtypes relevant for disease monitoring and therapeutic targeting.

Macrophages and dendritic cells

Alveolar macrophages (MΦs), together with the epithelium, are part of the lung's innate cellular surveillance system. MΦs efficiently phagocytize bacteria, dead cells and debris and MΦ' activation by external triggers (viruses, bacteria, bacterial products, etc.) leads to a cascade of events that contribute to the migration of neutrophils into the alveolar space and, eventually, to the activation of dendritic cells and T cells, initiating the adaptive arm of immune response.

The number of alveolar MΦs in young, non infected CF patients has been reported to be elevated compared to non-CF individual controls[125], suggesting a constitutive/intrinsic and

early mononuclear inflammation in CF. The high number of MΦs in CF BAL correlated with an increased concentration of the monocyte chemoattractant chemokine (MCP-1) CCL2[124,125]. A similar phenotype is observed in the BAL of several CF mouse models[216,217]. During an inflammatory response, MΦs are differentiated toward distinct subpopulations, which have different functionalities[218]. The so-termed "classically" or "M1" MΦs represent inflammatory cells that produce high amounts of pro-inflammatory cytokines and have accentuated anti-microbial activity. M1 MΦs are generally polarized by exposure to pathogen products (e.g. LPS) and interferon-gamma (INF-γ). The "alternatively" (M2) activated MΦs instead have an immunomodulating function, and promote tissue remodeling and repair after an inflammatory stress response. However, under pathological condition, M2 cells can also mediate chronic inflammation and tissue fibrosis. M2 MΦs are polarized by cytokines that are characterized by a T helper (Th) 2 response, such as IL-4 IL-13 or IL-10. Adequate activation of an inflammatory response and the subsequent resolution requires the balanced and coordinated activity of both MΦs subpopulations. There has been some attempt to study MΦs polarization in CF patients, however, results are inconclusive so far. Circulating CF monocytes[219] and macrophages isolated from BAL fluid from *P. aeruginosa* infected CF patients[220] exhibit a M2 phenotype compared to controls, while MΦs isolated from CF nasal polyp explants showed a M1 profile[221]. Studies on murine CF alveolar MΦs suggest that these cells, in absence of any inflammatory triggers, express an M1 polarization profile, and therefore remain in a pro-inflammatory state. Murine CF alveolar MΦs show an exacerbated expression of M1 activation markers when polarized with LPS/INF-γ; the M2 activation also tends to be elevated in CF MΦs[222]. In a subsequent study, it has been reported that, in comparison to healthy controls, the analysis of MΦs isolated from induced sputum of CF patients revealed a pronounced expansion of a unique small $CD14^{+}$ DR^{+} $CD68^{dim}$ population characterized by reduced expression of the scavenger receptors macrophage receptor with collagenous structure (MARCO) and CD206 (mannose receptor). The authors speculate thet

MΦs with altered plasma membrane receptor profile may lead to impaired clearance of inhaled particles and apoptotic cell, and increased inflammation and damage in CF lungs[124]. Thus, MΦs from CF lungs display differences in number and phenotype, which can be the result of exposure to the CF lung environment and/or of cell-intrinsic/autonomous CF MΦs defects.

Based on these observations of skewed macrophage phenotypes in CF, the questions arises whether these changes are just reflecting inflammation and infection in CF or whether macrophages directly contribute to the pathogenesis of CF lung disease? The creation of stable bone marrow (BM) chimeras, in which WT and CF mice were total body irradiated, transplanted with either WT BM or CF BM and, three months later challenged with LPS, demonstrated that the enhanced secretion of pro-inflammatory cytokines (most of which are abundantly secreted by macrophages) depended on the lack of functional CFTR in immune rather than resident epithelial cells. In contrast, the exuberant migration of neutrophils in the airspace derived from the absence of CFTR in both epithelial cells and immune cells[217]. These data were elegantly corroborated on a myeloid specific *Cftr* -/- mouse model generated by the Cre-recombinase LoxP system in which *Cftr* exon 10 was floxed under the control of the myeloid specific promoter lysozyme. After ten days of exposure with *P. aeruginosa* intra-tracheally, the myeloid specific deletion of *Cftr* featured an impaired ability to resolve infection and inflammation with an increased number of BAL cells, increased concentration of pro-inflammatory cytokines, reduced survival, greater weight loss, and more severe clinical and lung pathology scores compared to controls[223]. The contribution of CF MΦs to the *P. aeruginosa*-related lung pathology was milder in a CF mouse model ($G551D^{-}/G551D^{-}$) in which the tissue specific expression of functional Cftr was achieved by gene complementation[224]. When viewed in combination, these studies provide experimental evidence supporting the notion that immune cells actively contribute to CF-related lung disease.

The question arises which dysfunctions characterize CF MΦs? In CF pigs[225], a model that is greatly helping to increase our knowledge of disease progression, a bacterial clearance defect establishes from the time of birth, suggesting that non-functional CFTR gives rise to a primary host response defect, despite total cells, numbers of neutrophils and CXCL8 levels did not differ between CF and control pigs. In-depth immunological studies will be essential to dissect the various components of the innate immune system and their potential impairment in CF pigs.

Although changes in the CF lung environment, such as decreased secretion of anti-bactericidal molecules by submucosal gland and epithelial cells; abnormal ASL ion composition and pH, may contribute to this defect, there is evidence that absence of functional CFTR leads to impaired bacterial killing in murine [226-229]and human[230] MΦs, suggesting that reduced MΦ bactericidal activity may impair the maintenance of CF lung sterility. Macrophages also play a crucial role in the production of cytokines during the innate immune response. Human[231,232] and murine [233,234,222,217] CF MΦs were found to be hyper-responsive when exposed to the bacterial endotoxin LPS, with enhanced signal transduction through the NF-κB and MAPK pathways[231,234,232], increased secretion of pro-inflammatory cytokines [233,217,232] and alteration of LPS-induced metabolic pathways (e.g. PPAR/LXR)[234]. Interestingly, secretion of pro-inflammatory cytokines was correlated with the amount of functional CFTR, as MΦs isolated from obligated carriers[231] or from mice heterozygous for the *Cftr*-null allele[217] had a concentration of inflammatory cytokines between non-affected and CF-affected cells. In contrast, murine CF MΦs exposed to *P. aeruginosa* for two hours did not differ in chemokine secretion[28]. These studies indicate that the CF MΦ's hyper-responsiveness to bacterial products may contribute to the exuberant migration of other immune cells to the lungs and, therefore, to the resulting lung damage.

Based on these studies, it remains to be considered whether MΦs express functional CFTR and whether the observed MΦ phenotypes in CF are cell-intrinsic. The expression of

CFTR in non-epithelial tissues has been described[235], however, the impact of non-functional CFTR in non-epithelial cells is becoming better understood with the recent development of a mouse model in which the expression of CFTR can be abolished in a tissue-specific manner. At low levels, the CFTR protein is detectable in both murine MΦs[227,229] and human MΦs[236] and the CFTR-like Cl^- conductance has been recorded in monocytes/MΦs[230,236,228,229,232], suggesting that in these cells CFTR functions as a cAMP-dependent chloride channel. In addition, inhibition of functional CFTR in wild-type MΦs led to a phenotype that resembles CF MΦs[227,230,232]. Thus, CF MΦs express functional CFTR, suggesting that CF MΦs dysfunctions are cell-autonomous rather than a consequence of MΦs exposure to the CF lung surrounding micromilieu.

Given these studies supporting the notion that MΦ express CFTR, it remains to be discussed by which mechanisms non-functional CFTR leads to MΦ' dysfunctions? It has been proposed that CF MΦs fail to kill bacteria due to defective phagolysosome acidification as a result of a Cl^--related electrogenic imbalance[227,228]. However, this finding was challenged by other investigators showing that phagolysosomal acidification in MΦ was not dependent on CFTR channel activity[237]. In a more complex scenario, the alteration of the pH in cellular vesicles led to the malfunction of a pH-sensitive enzyme (acid sphingomyelinase) involved in ceramide metabolism. The release of ceramide during *P. aeruginosa* infection in alveolar MΦs favors the formation of ceramide-enriched membrane platforms, which mediates the assembly and activation of the NADPH oxidase[28]. This complex triggers the production of reactive oxygen species, which, in turn, favor the killing of *P. aeruginosa*. In CF MΦs, ceramide has been shown to accumulate in vesicles, impairing the formation of ceramide-reach membrane platforms and activation of the NADPH complex. However, the role of CFTR in controlling vesicle pH remains controversially discussed[238,239]; discrepancies that are likely due to differences in experimental settings. An additional observation is that the phagosome Cl^- concentration can modulate the behavior of intracellular bacteria by altering

bacterial protein activity or host factors. For instance, the CFTR-mediated Cl^- flux in MΦs contributes to the *L. monocytogenes* phagosomal escape to the host cytosol by favoring its hemolytic activity[229]. In addition, the defective CFTR-mediated Cl^- flux in CF MΦs was found to impair autophagy and autophagosome formation during *B. cepacia* infection. CF mouse MΦs infected with *B. cepacia* downregulated the expression of autophagy-related genes compared to WT cells, impairing the autophagosomes-lysosomes fusion and thus reducing bacterial clearance. Prolonged *B. cepacia* survival in CF MΦs has been associated with increased secretion of the pro-inflammatory cytokine IL-1ß[226]. CF MΦs are also characterized by defective endosomes to late-endosomes/lysosomes maturation during LPS challenge. This defect was shown to lead to dysregulated TLR4 trafficking from the plasma membrane to the lysosomes, where the activated TLR4 is degraded. As a result, CF MΦs were shown to feature a more robust TLR4 signaling with an increased secretion of pro-inflammatory cytokines compared to WT cells[232]. Studies analyzing human monocytes in CF patients and control subjects found that the receptor *Triggering receptor expressed on myeloid cells 1* (TREM1) was downregulated on circulating CF monocytes and that this was associated with circulating PAMPs and an unresponsiveness / tolerance state toward LPS in CF[240,241]. This finding is in contrast to studies using murine CFTR-/- or human CF patient macrophages. The underlying reasons are not fully understood, but based on the notion that monocytes upregulate a large array of genes upon entering the airways and differentiation to alveolar macrophages[242], it is conceivable that this differentiation process modulates the CFTR-related responsiveness towards PAMPs and DAMPs. Nevertheless, these discrepant observations necessitate future studies in the field of monocytes/macrophages in CF, utilizing both murine and human experimental systems.

Macrophages represent the main scavengers of apoptotic neutrophils, a process termed efferocytosis[243]. Efferocytosis is essential in compartments where large numbers of neutrophils accumulate and undergo ageing and apoptosis, such as the CF airways where

large numbers of particularly neutrophils undergo several forms of apoptiosis and cell death[244]. Successful efferocytosis prevents secondary necrosis and pro-inflammatory cascades and thereby acts anti-inflammatory. Notably, previous studies found that clearance of apoptotic cells is impaired in the CF microenvironment due to elastase-mediated cleavage of phosphatidylserine receptors or other CFTR-related mechanisms in epithelial cells[245-247]. These findings underpin the complexity of the initiation, maintainance, resolution and perpetuation of neutrophilic inflammation in CF. Figure 3 summarizes the complex role and paracrine interaction partners of macrophages in CF lung disease.

CF-DCs challenged with *P. aeruginosa* were found to exhibit an abnormal expression of genes involved in the maintenance of membrane structure and lipid-metabolism; in addition, CF DCs displayed a delay in the early phase of differentiation[248,249]. On the other hand, neutrophil elastase in CF airway fluids was found to inhibit DC maturation and functionality by shedding CD86 and impairing antigen presentation capabilities[250]. In combination, these data suggests that in CF, the cross-talk between the innate and the adaptive immune response, orchestrated by DCs, may also be compromised, caused by CFTR-dependent and CFTR-independent proteolytic mechanisms. More studies are needed to dissect the role of this arm of the immune system in the development of CF lung disease.

Taken together, these observations suggest that CF MΦs display several cell-autonomous/intrinsic dysfunctionalities that alter their ability to properly control their response to inflammatory triggers and to kill bacteria in the context of CF lung disease. In the early stages of CF lung disease, the inappropriate MΦ behavior may contribute to conditions that favor bacterial lung adaptation, harmful chronic infection, and lung damage.

v. Th17 cells link innate and adaptive immunity

While neutrophils are the dominant cell population in the broncho-alveolar compartment (bronchial > alveolar), recent studies indicate that T cells accumulate within the subepithelial bronchial tissue but are almost devoid od the bronchial space[251], supporting the notion that the pulmonary immune system is compartmentalized and the role of T cells has probably been under-appreciated in CF lung disease due to their paucity in sputum or BALF[252-254].

In recent years, there have been significant advances in our understanding of T cell subsets beyond the canonical Th1/Th2 paradigm. It is now widely accepted that a normal immune system harbours a regulatory T cell population specialized for immune suppression, as well as a Th17 cell subset that mediates inflammatory processes and autoimmunity[255]. Th17 cells are $CD4^+$ T cells that produce IL-17-like cytokines named IL-17-A/F, besides IL-22 and other effector cytokines, and regulate both granulopoesis and recruitment of neutrophils into sites of inflammation, thereby linking innate and adoptive immunity[256]. IL-17 signals through the IL-17 receptor (IL-17R), a type I transmembrane protein which is ubiquitously expressed in tissues. IL-17 can stimulate a number of different cell types including macrophages, dendritic cells, endothelial cells or fibroblasts to release effector molecules leading to pathology[257]. Th17 cells are activated by IL-23, mainly derived from dendritic cells and macrophages, as well as by IL-6 and TGF-β[258].

There is a rapidly growing body of evidence that IL-17-related pathways are involved in the immunopathogenesis of a broad spectrum of inflammatory human disease conditions, including multiple sclerosis, rheumatic diseases, inflammatory bowel disease, asthma, and atopic dermatitis[259]. In the lungs, IL-17 has been shown to be crucial for maintaining control of host defence against extracellular pathogens. It was demonstrated that IL-17, and its driving factor IL-23, are important for maintaining mucosal host defence against *Klebsiella pneumoniae* infection in human bronchial epithelial cells. In mice, IL-17 and IL-23 are involved in host responses to infection by gram-negative bacteria, in particular *Klebsiella, P. aeruginosa, Escherichia coli, Salmonella and Bordetella species*[256,260,261]. The potential role

of IL-17-related pathways in CF lung disease is now greatly supported by a considerable number of recent studies[162,262,263]. Of particular interest for CF lung disease may be the observations that IL-23 and IL-17 mediate inflammatory response to mucoid *P. aeruginosa* infection and are critical for neutrophil inflammation[264]. The IL-23-driven recruitment of neutrophil inflammation is, however, not uniquely dependent on IL-17: newer studies suggest that in addition to IL-17 production by γδT cells after IL-23 stimulation, neutrophil recruitment is promoted by IL-23 independently of IL-17 in an early inflammation phase[265]. In human studies, IL-17 and IL-23 were markedly elevated in BAL fluid or sputum of CF patients with chronic *P. aeruginosa* colonization undergoing pulmonary exacerbation as well as in explanted CF lungs[162,266,267]. A more recent study likewise found substantially increased levels of IL-17 protein and mRNA, and IL-23 in the sputum of stable CF patients[268]. This observation was especcially pronounced in patients chronically infected with *P. aeruginosa*, further pointing to an important role of IL-17 in the neutrophil inflammation of CF lung disease.

Though Th17 cells are regarded as the major producer of IL-17, other cellular sources have been associated with production of IL-17 depending on the underlying pathology, especially γδT cells, natural killer T cells and $CD8^+$ T cells[256]. Beyond these cell types, IL-17 protein was detected also in eosinophils, mastcells, neutrophils and human blood monocytes[266,267]. In CF, the complexity of IL-17-producing cells has recently been nicely demonstrated by Tan and colleagues analysing endobronchial biopsies. They showed that IL-17 cell counts were significantly increased in lung tissue of young children with established CF compared to healthy controls[269]. Whereas Th17 cells were the predominant IL-17-producing cell type in the airway walls of newly diagnosed as well as established CF and non-CF bronchiectasis, other important sources of IL-17 were NKT cells (in end stage lung disease) and ~~gamma~~ γδT cells. IL-17-producing cell counts depended on severity, as young children showed intermediate counts compared to end stage lung disease. ~~Similar observation~~

~~was made for IL-17 levels in BAL. However, not only lymphocytes seems to contribute to IL-17-related immunity CF.~~ Immunhistochemistry staining of explanted CF lungs suggested that IL-17 is increased in lower airways compared to non-CF lung tissue, and that IL-17 production localizes to neutrophiles[266,267]. The observation that elevated numbers of IL-17-producing cells are already found in early lung disease in very young CF patients and the correlation of disease severity and *P. aeruginosa* colonisation to IL-17^{+} cells and IL-17 protein levels in BAL fluid render IL-17 and associated cytokines potentially interesting biomarkers for monitoring CF lung inflammation. In addition to their potential value as biomarkers, IL-17-related cytokines such as anti-IL-17 or anti-IL-23 antibodies may be therapeutic targets to modify pulmonary inflammation in CF.

However, great therapeutic caution needs to be applied in an environment of enhanced infection susceptibility, as it is the CF lung. Suppressing of one particular inflammatory pathway may result in uncontrolled bacterial growth and severe pulmonary exacerbation. For these reasons, a deep understanding of the IL-17-related pathways in the context of CF lung disease is critical to assess the potential implications of an anti-IL-17 therapy, or interventions targeting it`s cellular source. Beyond Th17, previous studies provided evidence that lymphocytes from CF patients exhibit a cytokine dysregulation with an intrinsic Th2 bias[270,271] and increased Nuclear Factor of Activated T Cell (NFAT) translocation due to the cellular CFTR defect, a topic related to adaptive immunity that is reviewed in greater detail in a recent review[34].

vi. Innate immunity in CF – any relevance?

Innate immunity in CF is a growing field of interest in both basic and translational research. Within the previous years, it turned out that the interplay between cellular and soluble, CFTR-dependent and –independent and pathogen-associated and sterile pathomechanisms are of higher complexity than previously expected. While the link between the basic CF defect and

innate immune inflammation is increasingly appreciated[272], key questions on the survival, subsets and functionalities of phagocytes in CF airways remain controversial.

Single nucleotide polymorphisms (SNPs) in genes involved in innate immunity, such as mannose binding lectin 2 (MBL2)[273-278], Transforming growth factor β (TGF-β)[279], CD14[280], Interferon-related developmental regulator 1 (IFRD1)[281] and others, as well as recent genome-wide association studies add another layer of complexity[279,282,283]. Considering the manifold components and mechanisms of innate immunity in CF lung disease, the question remains which diagnostic or therapeutic relevance remains for the clinician. Diagnostically, several immune proteins, proteases or proteolytically-generated peptides, such as CXCL8/IL-8, IL-6, TNF-α, YKL-40, TGF-β, PGP, HMGB1, AAT:CD16, elastase or MMP-9 have been proposed as biomarkers for CF lung disease, but their clinical usefulness requires further cross-sectional multi-center and longitudinal studies to understand their kinetics, treatment responses and comparing their reliability, validity and cost-effectiveness. Therapeutic consequences seem to be farer on the horizon and have to be implemented with great caution[2,284,285,286], since interfering with innate immune recruitment or effector mechanisms harbours the risk of disabling innate host defense mechanisms and favoring bacterial and fungal infections. Therefore, future studies dissecting the kinetics and dynamics of the innate immune system in cells, mice, ferrets, pigs and humans will be essential to identify specific cell populations or released proteins that are worth targeting in a time- and inflammation-specific manner in CF lung disease.

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TABLES

TABLE 1

Innate immune cell	Functional role	Impairment in CF lung disease
Neutrophils	• Anti-bacterial and-fungal host defense • Cytokine / chemokine release	• Impaired chlorination / intracellular killing of phagocytosed bacteria • Delayed apoptosis • Impaired phagocytosis* • Proteolytic and oxidative tissue damage • Cleavage of leukocyte surface receptors • DNA release (necrosis / NETosis) • Increased elastase release • Enhanced chemotaxis
Macrophages / Monocytes	• Anti-bacterial and-fungal host defense • Removal of apoptotic cells (efferocytosis) • Antigen-presentation • Cytokine/ chemokine release • Immune regulation	• Increased secretion of pro-inflammatory cytokines • Impaired intracellular killing of bacteria* • Defective phagolysosomal acidification* • Disturbed ceramide metabolism • Dysregulated TLR4 trafficking • Downregulated autophagy

		• *Macrophages:* Hyper-responsiveness towards LPS • *Monocytes:* Downregulation of TREM / Hyporesponsiveness to LPS • M1 / M2 dysregulation* • Reduced expression of scavenger receptors • Cleavage of phosphatidylserine receptors / impaired efferocytosis
T cells	• Anti-bacterial, -fungal and –viral host defense • Antibody production • Neutrophil recrutiment (Th17) • Allergic responses (ABPA, Th2) • Immune regulation (Tregs)	• CFTR-mediated Th2 shift • *Pseudomonas aeruginosa*-mediated Th2 shift • Th17 activation • *Pseudomonas aeruginosa*-mediated impairment of T cell proliferation

* controversial studies

FIGURE LEGENDS

Figure 1. Mechanisms initiating neutrophil influx into the CF Lung

Panel A represents the healthy airway; Panel B represents the CF airway. Airway surface liquid (ASL) is reduced in the CF airway, leading to decreased mucociliary transport and bacterial colonization. In response to bacteria (blue ovals), epithelial cells in CF patients release CXCL8, resulting in neutrophil influx. Degranulation of activated neutrophils releases MMP-9 and PE (red circles) as well as LTA4H (green triangles). In healthy individuals, LTA4H is able to degrade PGP generated by MMP-9 and PE-mediated cleavage of collagen, while unopposed proteolytic cascades in CF patients lead to PGP generation, triggering neutrophil influx and thereby perpetuating inflammation.

Figure 2. Neutrophil flexibility in CF

When neutrophils in CF airways are exposed to PAMPs and/or DAMPs, distinct effector functions are activated depending on the duration and the severity of the PAMP/DAMP contact. Neutrophils rapidly phagocytose bacteria. As CFTR is localized in the phagolysosome, CF neutrophils feature an intrinsic phagocytotic dysregulation depending on phagolysosomal chlorination. Extracellular pathogens and tissue structures are attacked by granule release. CF neutrophils feature an increased release of primary granules (1 G), while characteristics of secondary (2 G) and tertiary (3 G) granules in CF versus healthy neutrophils have not been studied so far. After prolonged stimulation, NETs are generated to entangle extracellular bacteria and fungi. NET formation capacity does not differ between CF and control peripheral blood neutrophils.

Figure 3. Macrophage flexibility in CF

The figure illustrates the complex involvement of macrophages in host-pathogen and cell-cell interactions in CF lung disease. CF macrophages are hyper-responsive when exposed to bacterial (such as *P. aeruginosa* derived) PAMPs, with enhanced signal transduction and increased secretion of pro-inflammatory cytokines. Through secretion of cytokines, alveolar macrophages communicate with neutrophils, lymphocytes, dendritic cells and epithelial cells, thereby orchestrating the outcome of the pulmonary immune response in CF. In addition, CF macrophages have been described to show defective intracellular bacterial killing (controversial finding) and apoptotic cell removal (efferocytosis) activities. In summary, these dysfunctions may led to altered paracrine signaling within the CF lung environment.

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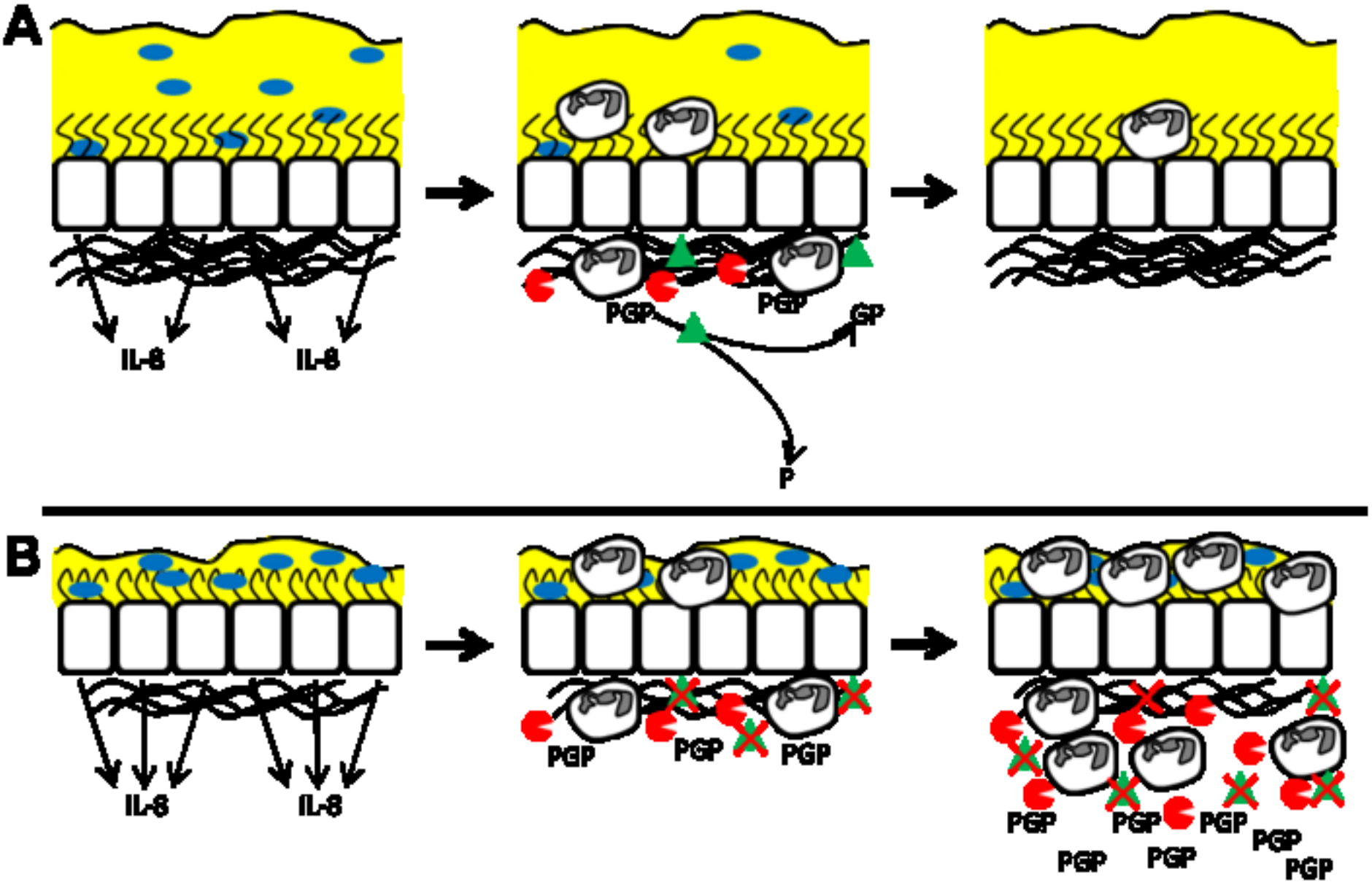

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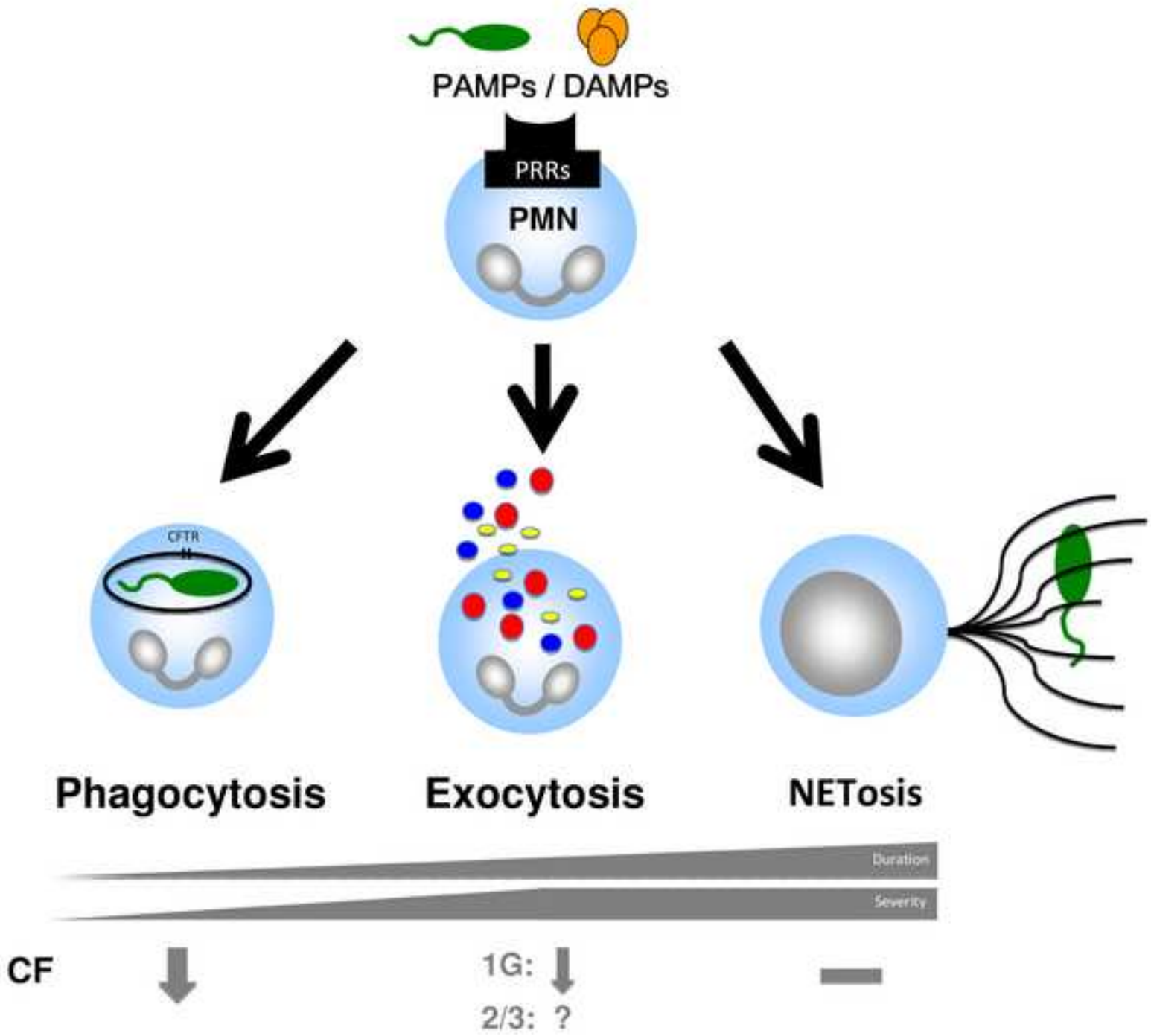
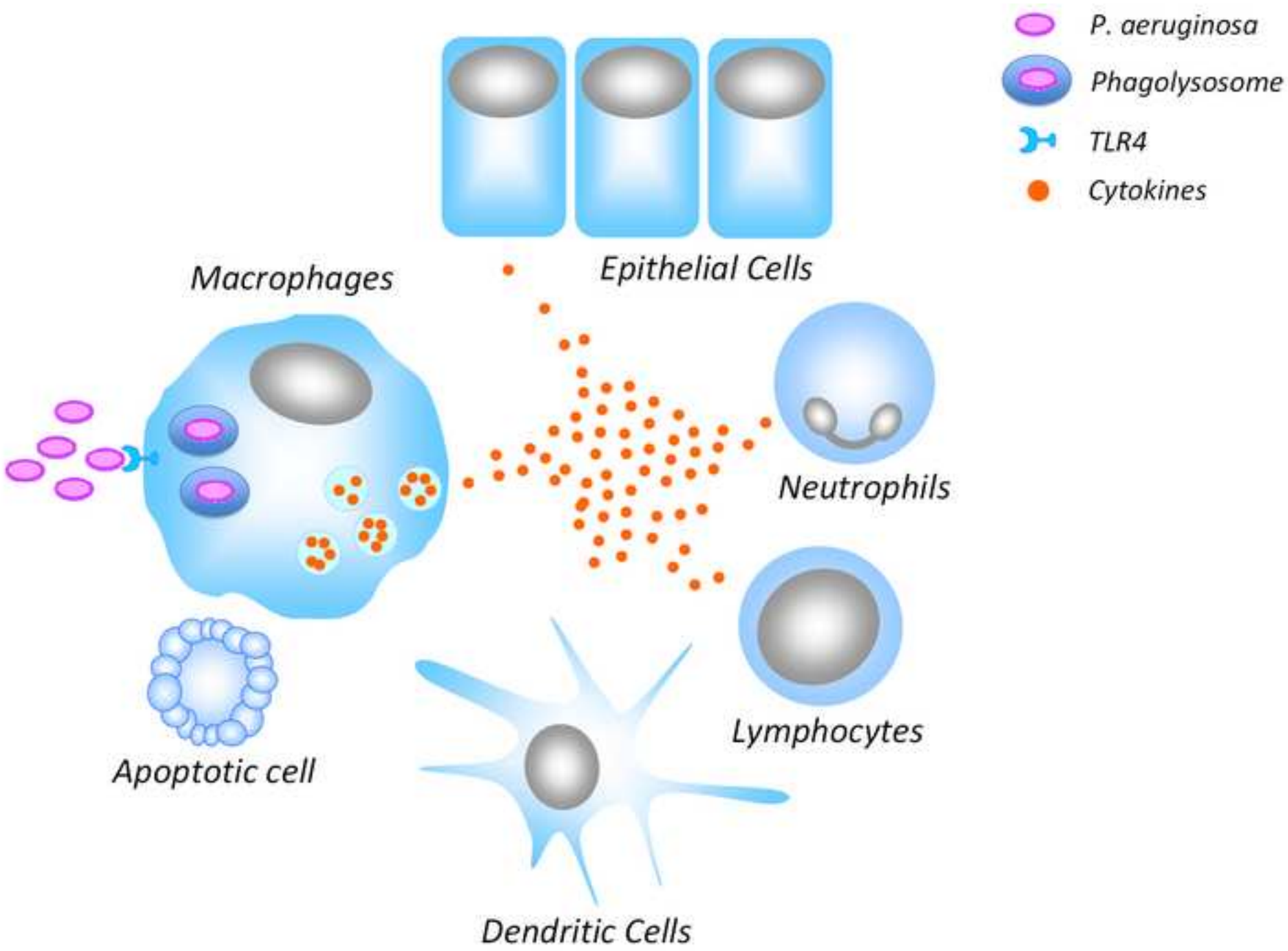
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