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Role of CFTR, *Pseudomonas aeruginosa* and Toll-like receptors in cystic fibrosis lung inflammation

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Abstract

CF (cystic fibrosis) is a severe autosomal recessive disease most common in Northwest European populations. Underlying mutations in the CFTR (CF transmembrane conductance regulator) gene cause deregulation of ion transport and subsequent dehydration of the airway surface liquid, producing a viscous mucus layer on the airway surface of CF patients. This layer is readily colonized by bacteria such as *Pseudomonas aeruginosa*. Owing to the resulting environment and treatment strategies, the bacteria acquire genetic modifications such as antibiotic resistance, biofilm formation, antimicrobial peptide resistance and pro-inflammatory lipid A structures. Lipid A is a component of the lipopolysaccharide cell wall present on bacteria and is recognized by TLR4 (Toll-like receptor 4). Its detection elicits a pro-inflammatory response that is heightened over time due to the addition of fatty acids to the lipid A structure. Eradication of bacteria from the lungs of CF patients becomes increasingly difficult and eventually leads to mortality. In the present review, we describe the role of lipid A as a virulent factor of *Ps. aeruginosa*; however, it appears that further work is needed to investigate the role of CFTR in the innate immune response and in modifying the pathogen–host interaction.

Introduction

CF (cystic fibrosis; OMIM no. 219700) is a lethal autosomal recessive disease found worldwide, but most commonly in Northwest European populations, where the prevalence is approx. 1 in 2500 newborns [1]. Originally described in 1938 [2], its biochemical basis was later linked to a mutation in the CFTR (CF transmembrane conductance regulator) gene in 1989 [3]. CFTR is a unique protein belonging to the ABC transporter (ATP-binding-cassette transporter) superfamily [3]. Principally, CFTR is expressed apically in exocrine epithelial cells, and functions primarily as a chloride ion (Cl[−]) channel [3]. As epithelial cells are found ubiquitously throughout the body, multiple organs can be affected by disease, such as the pancreas [4,5], sweat glands [6], vas deferens [3], adult epididymis [3], gall bladder and the bile duct [7]. However, the main cause of morbidity and mortality in CF is characteristically associated with respiratory disease, which is a result of persistent pulmonary infection and chronic inflammation [8,9].

CFTR

The underlying molecular basis for the inflammatory lung disease seen in CF patients stems from mutations in the CFTR gene. The gene itself is located on chromosome 7q31.2 and produces a transcript that is 6.5 kb in size [3,10]. This transcript codes for the CFTR protein, which comprises 1480 amino acids and has a molecular mass of 1.7 kDa. The crystal structure showed that the overall protein is composed of five domains: two MSDs (membrane-spanning domains) each consisting of six α -helices, two NBDs (nucleotide-binding domains; capable of ATP hydrolysis) and a regulatory domain (target of phosphorylation by protein kinase A) [3,11]. To date, throughout the CFTR gene approx. 1600 mutations have been sequenced (<http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html>); however, the mutation that accounts for approx. 70% of CF chromosomes is $\Delta F508$ (F508del) [13]. The mutation results in a 3-bp deletion, producing a protein lacking a phenylalanine at position 508 located in the NBD1 [3]. Owing to the loss of this residue the protein formed after translation fails to mature properly and is subsequently tagged for ubiquitination and degradation, resulting in a loss of CFTR at the apical membrane [14].

CFTR mutations

Owing to the large number of mutations and the variation in translation and Cl[−] channel function, CFTR mutations have been split into five different classes [15,16]. Class I mutations are generally deletions, nonsense or frame shifts that result in

Key words: cystic fibrosis, inflammation, lipid A, lipopolysaccharide, *Pseudomonas aeruginosa*, Toll-like receptor (TLR).

Abbreviations used: ASL, airway surface liquid; CAMP, cationic antimicrobial peptide; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; ENaC, epithelial Na⁺ channel; IL, interleukin; IL-1R, IL-1 receptor; IL-1RI, type I IL-1R; IRAK-4, IL-1R-associated kinase 4; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; NBD, nucleotide-binding domain; NF- κ B, nuclear factor κ B; PAMP, pathogen-associated molecular pattern; PCL, periciliary liquid layer; TIR, Toll/IL-1R; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF-6, TNF-receptor-associated factor-6.

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premature truncated proteins. Class II mutants such as $\Delta F508$ result in a protein that is defective in protein processing and trafficking. Class III mutants code for a protein that is capable of reaching the apical membrane, but cannot be activated with ATP or cAMP. Both are considered to be the mechanism for channel activation. The result is a protein with little or no ion channel activity. Class IV mutants result in a CFTR that has defective chloride ion channel transport and, finally, class V mutants are affected by mutations in promoter and splicing regions. Thus, as a result, a fully functional CFTR protein is produced but its presence on the membrane is reduced. Genotype–phenotype studies have shown that mutations within classes I, II and III are associated with increased severity of pulmonary disease, due to the almost total lack of functional CFTR on the apical cell membrane [15,16]. Further studies have highlighted class I mutations as the most dominant cause of morbidity and mortality [17,18].

ASL (airway surface liquid)

It is apparent that mutations in the CFTR cause little or no Cl^- channel transport, but how does this affect the lungs of CF patients and bring about the characteristic pro-inflammatory lung disease? Normally the airway surface is covered with a thin liquid film called the ASL [19], which consists of the PCL (periciliary liquid layer), which is in direct contact with epithelial cells and the upper mucus layer. Together they play a critical role in effective mucociliary and cough clearance of the airway [19]. The PCL extends and maintains the beating of the cilia, as well as providing a low viscous environment to lubricate and separate the mucus layer from the epithelial cell surface. This prevents mucus adhesion and the formation of mucus plugs [19–22]. Finally, the mucus layer on top of the PCL is involved in trapping and removing inhaled pathogens without triggering a substantial innate immune response.

In order for the ASL to effectively assist removal of bacteria by mucociliary clearance, it is important that it remains hydrated and retains a height of approx. $7\ \mu\text{m}$ [21]. The ASL height and composition are maintained by two opposing active ion transport channels, namely CFTR and ENaC (epithelial Na^+ channel) [23]. CFTR is involved in the secretion of Cl^- into the ASL and the inhibition of ENaC to prevent Na^+ absorption into epithelial cells [19]. As epithelial cells are highly water-permeable, water is drawn out of the cells via osmosis on to the iso-osmotic ASL [24] (Figure 1A). CFTR mutations result in reduced levels of CFTR on the epithelial membrane, Cl^- can no longer be secreted on to the airways and regulation of Na^+ absorption through ENaC is diminished. As a consequence of this, increased amounts of Na^+ are absorbed into the cell, with Cl^- following through the paracellular pathway. Subsequently water is drawn from the ASL into the cells (Figure 1B). This depletion of water from the ASL results in (i) increased mucus concentration, (ii) flattening of the cilia, and (iii) adhesion of the mucus layer to the airway surface [21]. The environment produced as a consequence is rich in thick sticky mucus and provides the perfect milieu for colonization and

Figure 1 | Role of CFTR in regulating the ASL in healthy individuals (upper panel) and CF patients (lower panel)

propagation of bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

TLRs (Toll-like receptors)

The adhesion of mucus to the epithelium surface brings epithelial cells into the first line of host defence against colonizing bacteria [25]. Invading bacteria display PAMPs (pathogen-associated molecular patterns), which are recognized by TLRs on the airway epithelium and their activation results in a pro-inflammatory response [26,27]. In 1991 TLRs were initially identified in the fruitfly *Drosophila melanogaster* as a key receptor in fungal defence [28]. Later that year, it was found that the receptor shared structural homology with an important receptor in mammalian innate immunity, the IL-1RI [type I IL (interleukin)-1 receptor] [29]. To date, 12 mammalian TLRs have been identified, and all of them are postulated or have proved to play a significant role in innate immune responses [30–32]. TLRs are type I transmembrane glycoproteins, which possess a characteristic horseshoe-like extracellular domain composed of LRR (leucine-rich repeat) motifs [30]. Its cytoplasmic signalling domain is composed of 150–200 conserved residues that share homology with IL-1RI; hence it is named the TIR (Toll/IL-1R) domain [33]. The TIR domain plays a key role in recruiting those adaptor proteins containing a TIR domain, which subsequently leads to activation of downstream signalling cascades such as NF- κ B (nuclear factor κ B) and production of pro-inflammatory cytokines and chemokines that are abundantly found in the airways of CF patients.

Each TLR has the ability to recognize and discriminate a specific PAMP present on invading pathogens. Together the TLR family is able to respond to a diverse array of species including bacteria, viruses, mycoplasma, yeasts and protozoa. For example, TLRs 3, 7, 8 and 9 recognize pathogen nucleic acids such as viral dsRNA (double-stranded RNA), ssRNA

Figure 2 | General signalling pathway of TLR4 resulting in the release of pro-inflammatory mediators

(single-stranded RNA) and bacterial unmethylated CpG dinucleotide motifs respectively [30–35]. However, the most studied TLRs in CF are TLRs 2, 4 and 5. Heterodimerization of TLR2 with TLR1 or TLR6 results in the recognition of microbial components from the cell wall of Gram-positive bacteria, such as lipoproteins, peptidoglycan and lipoteichoic acid [36], whereas TLR4 is involved in the detection of LPS (lipopolysaccharide) and TLR5 with flagellin, which are both found in Gram-negative bacteria. LPS, which is found on *Ps. aeruginosa*, plays a major role in producing the chronic infection seen in the CF airways.

The binding of LPS results in TLR4 homodimerization, which subsequently causes recruitment of TIR-domain-containing adaptor proteins such as MyD88 (myeloid differentiation factor 88). MyD88 then interacts with downstream signalling protein kinases through its death domain, allowing activation and phosphorylation of IRAK-4 (IL-1R-associated kinase 4), which in turn activates TRAF-6 [TNF (tumour necrosis factor)-receptor-associated factor-6]. Ubiquitination of TRAF-6 and TAK-1 [TGF (transforming growth factor)- β -activated kinase] then occurs followed by the activation of IKK [$\text{I}\kappa\text{B}$ (inhibitor of NF- κB) kinase] complex. This reverses its inhibiting function on NF- κB and allows its subsequent translocation into the nucleus. NF- κB is one of the key regulating transcription factors of genes that are involved in inflammation and immunity

[32] (Figure 2). Activation of any TLR results in a Th1 innate immune response, which is characterized by NF- κB activation and the release of pro-inflammatory mediators. Cytokines and chemokines such as IL-8, TNF- α and IL-6 alert the immune system to the presence of infection [37], up-regulate antimicrobial peptides and increase phagocytosis of bacteria.

Bacterial infection

Infections by Gram-positive and Gram-negative bacteria aggravate the underlying mutations in the CFTR by exaggerating pro-inflammatory gene expression of cytokines such as IL-8, a potent chemoattractant of neutrophils. This recruitment of additional neutrophils to the airways results in further release of pro-inflammatory cytokines, constructing a cycle of inflammation. Initially throughout infancy and early childhood in CF patients, *S. aureus* and *Haemophilus influenzae* are the main infecting bacteria. It is thought that damage to the epithelial surface occurs as a result of *S. aureus* infection, helping to prime and increase attachment of *Ps. aeruginosa* [38,39]. However, more recent research suggests that continuous antistaphylococcal prophylaxis increases the rate of *Ps. aeruginosa* growth when compared with patients who receive no *S. aureus* antibiotic treatment [40]. Despite this, it is evident that by adulthood 80% of CF patients are chronically infected by *Ps. aeruginosa*, and this species correlates with a high mortality rate [41,42]. Current treatment of pulmonary infection in CF with antibiotic chemotherapy and chemoprophylaxis has increased patient life expectancy. However, this also provides a selective pressure causing bacteria such as *Ps. aeruginosa* to undergo genetic modification. This allows the bacteria to gain traits such as antibiotic resistance [43] and biofilm formation. These biofilms are intricate bacterial communities, which are surrounded by a protective bacterial extracellular matrix, which reduces the rate of bacterial phagocytosis by immune cells [43]. Propagating *Ps. aeruginosa* within the biofilm is then released causing further inflammation and colonization.

Lipid A modification

Several genetic modifications such as antibiotic resistance and biofilm formation have been well researched; however, recently other less noticeable changes have been brought to the forefront. These types of changes are not regularly characterized in CF patients and, as a result, treatment efficiency may be reduced. One such adaptation is *Ps. aeruginosa*'s ability to modify its LPS structure. LPS consists of three distinct structural elements: O-antigen, core and lipid A. The O-antigen and the core are both composed of polysaccharides, whereas lipid A is composed of two central glucosamine dimers to which fatty acid chains, phosphates and amino sugars are attached [44]. It is a result of addition and/or extension of the fatty acid chains present in the lipid A component of LPS that helps *Ps. aeruginosa* convey resistance to CAMPs (cationic antimicrobial peptides) and produce a higher pro-inflammatory response [45,46]. By

Figure 3 | The lipid A structure of *Ps. aeruginosa* isolates from the environment (A), a CF patient with mild lung disease (B) and a CF patient with severe lung disease (C)

changing its isoelectric properties lipid A becomes less negatively charged, reducing the attractive forces to CAMPs. Initially *Ps. aeruginosa* lipid A contains two amide-linked fatty acids and two ester-linked fatty acids, producing a tetra-acylated structure [44–47]. The fatty acids attached in this structure vary in length from ten to twelve carbons and are hydroxylated at the 3-position. This base structure is modified further by the addition of two secondary fatty acids C₁₂ and 2-OH C₁₂. Analyses of LPS from *Ps. aeruginosa* isolates taken from CF patient airways have shown differential expression of fatty acid chains compared with isolates from acute infections or patients with broncheolitis. The general trend observed is the addition and lengthening of fatty acid chains over the duration of disease. For example, the lipid A structure (Figure 3A) of *Ps. aeruginosa* LPS isolated from the environment shows a hexa-acylated structure (six fatty acid chains) with all but one having a carbon chain length of 12. Scrutinization of lipid A structure of *Ps. aeruginosa* LPS isolated from teenage CF patients with mild disease shows a hexa-acylated chain (six fatty acid chains, Figure 3B) with the new additional chain reaching a length of 16 carbons [48]. Finally, analysis of the lipid A structure of *Ps. aeruginosa* LPS isolates from a subset of older CF patients with severe pulmonary disease produces a hepta-acylated structure (seven fatty acid chains, Figure 3C), which is thought to occur by loss of PagL deacylation activity [48]. Approx. 48% of isolates from CF patients with severe disease have a hepta-acylated structure and this is associated with an increased resistance to specific classes of antibiotics [49]. This intriguing association between disease severity and lipid A structure led us to our current investigations of the pro-inflammatory effect of these *Ps. aeruginosa* LPS isolates using CF and non-CF tracheal epithelial cells and IL-8 as a biomarker of inflammation. First results show that only CF epithelial cells respond with significant inflammation to some LPS isolates. The non-responsiveness of non-CF cells suggests a modified pathogen–host interaction as a significant virulent mechanism in pulmonary CF inflammation [46].

Conclusion

While the defects and modifications mentioned above contribute to the chronic infection and inflammation, which in turn results in mortality in CF patients, it is only a small part of the whole CF picture. Other cell types, for example macrophages and neutrophils, play critical roles in the cycle of inflammation. Often these cells produce more innately pro-inflammatory responses than epithelial cells. As well as the pro-inflammatory phenotype of the host, there is clear evidence that *Ps. aeruginosa* has developed mechanisms to avoid host defence and once established in CF lungs contributes significantly to the severity of disease. *Ps. aeruginosa* has shown to be resistant to numerous antibiotic treatments, which often encourage natural selection of modifications in *Ps. aeruginosa*. In this review, we have described the role of lipid A as a virulent factor of *Ps. aeruginosa*; however, it appears that further work is still needed to investigate the role of CFTR in the innate immune response and in modifying the pathogen–host interaction (e.g. TLRs). Moreover, counteracting genetic adaptation of *Ps. aeruginosa* would be beneficial to CF patients. Treatments that help remove additional fatty acid chains and aminoarbinose sugars, or prevent biofilm or mucoid phenotypes may help reduce inflammation and clear *Ps. aeruginosa* infection.

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