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Toll-like receptors as therapeutic targets in cystic fibrosis.

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**Toll-like receptors as therapeutic targets in cystic fibrosis**

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**Background:** Toll-like receptors (TLRs) are pattern recognition receptors that act as a first-line of defence in the innate immune response by recognising and responding to conserved molecular patterns in microbial factors and endogenous danger signals. Cystic fibrosis (CF)-affected airways represent a milieu potentially rich in TLR agonists and the chronic inflammatory phenotype evident in CF airway epithelial cells is probably due in large part to activation of TLRs. **Objective/methods:** To examine the prospects of developing novel therapies for CF by targeting TLRs. We outline the expression and function of TLRs and explore the therapeutic potential of naturally-occurring and synthetic TLR inhibitors for CF. **Results/conclusion:** Modulation of TLRs has therapeutic potential for the inflammatory lung manifestations of CF. **Keywords:** cystic fibrosis, inflammation, innate immunity, therapeutics, toll-like receptors


1. **Introduction**

The innate immune system plays a key role in regulating responses to infection and inflammation in the pathophysiology of cystic fibrosis (CF). Toll-like receptors (TLR) are important components of innate immunity that are activated in response to both infective and inflammatory stimuli. Here we focus on the current paradigm regarding the function of TLRs. The important roles of each member of the TLR family in regulating pro-inflammatory gene expression in response to microbial and endogenous agonists in the CF lung is addressed. The major signalling pathways activated by TLRs are explained and methods to target either TLRs or their signalling pathways using microbial and naturally-occurring endogenous inhibitors or antiproteases are described. Current treatment regimens and how they may affect TLR function in the CF lung are also discussed.

2. **Infection and inflammation in the pathophysiology of CF**

CF is caused by mutations of the CF transmembrane conductance regulator (CFTR) gene. It is a lethal hereditary disorder that is relatively common in Europe and North America, accounting for 1 in 3000 live births. Amongst Caucasians, 1 in 20 is a heterozygotic carrier of a mutant CFTR allele. Other races including Hispanics, Blacks and Asians are also affected but in smaller numbers [1]. The clinical consequences of CF are protean with multiple organs potentially being involved. The liver, pancreas and intestinal tract can all be affected, however the major causes of morbidity and mortality are the lung disease [2]. This is characterised by chronic airway infections with *Pseudomonas, Staphylococcus, Haemophilus, Aspergillus* and *Burkholderia* species, overproduction of thick mucus and inflammation [2,3]. Other complications of CF affecting the airways can include haemoptysis, pneumothorax, pulmonary hypertension and *cor pulmonale*.

Although improvements in healthcare have enhanced survival the outlook for individuals with CF could be better.

The lung disease in CF is characterised by infection/colonization, inflammation and mucus overproduction [4]. Inflammation in CF is to a large extent compartmentalised to the epithelial surface and represents an interaction between bacteria, inflammatory cells, their secretions and epithelial cells and their receptors. It also represents an inability of the lung’s innate defences to clear the infective causes of inflammation. Historically the lung has been perceived as an organ primarily involved in gas exchange. However, due to its unique relationship with the environment, the lung must defend itself from infection by numerous inhaled microorganisms. Various innate defences protect the lung from infection, including the cough reflex, the mucociliary escalator, and the intrinsic antimicrobial properties of the mucosal surface. In addition, an extensive alveolar-capillary membrane containing immune and non-immune cells is exposed to microbial challenges. As a result, pulmonary tissues generate a brisk innate host response to both inhaled and haematogenous pathogens in order to clear the offending microorganism and preserve gaseous exchange. Innate immunity plays a key role in these events in the CF lung.

3. TLR expression and function

The TLR family represent a conserved and increasingly well-characterised group of pattern recognition receptors (PRRs). TLRs constitute the most significant component of pulmonary PRRs and can recognize and discriminate a diverse array of microbial antigens. The family comprises a selection of transmembrane proteins that constitute an important unit of the innate immune system. TLR expression is widespread and includes, but is not limited to, cells of myeloid and lymphoid origin, endothelial and epithelial cells.

First identified in the fruitfly *Drosophila melanogaster*, the *Drosophila* or dToll was initially characterised as a factor regulating embryonic--ventral axis formation. Later dToll was shown to act as a key receptor regulating antimicrobial defense in the adult fly [5]. In 1991 Gay and Keith reported the identification of structural and functional similarities between dToll and the mammalian Type I IL-1 receptor (IL-1RI) [6] an important receptor in innate immunity. This prompted a surge of research leading to the identification and partial characterisation of ten human TLRs sharing sequence similarity with the cytosolic signalling domain of IL-1RI.

TLRs are germ-line-encoded pattern recognition receptors. Each has a role in the innate immune response [7]. An extracellular leucine-rich repeat (LRR) ligand recognition domain and an intracellular signalling domain integrating the functional signature motif of TLRs, the so-called TIR (Toll/interleukin-1 receptor) domain, characterises all of the TLR type I transmembrane proteins. The conserved cytosolic TIR domain consists of up to 200 amino acids [8,9] essential for signalling whilst the external LRR motifs probably confer specificity to TLRs with respect to their pattern recognition properties [10]. TLR4, the mammalian lipopolysaccharide (LPS) receptor, was the first mammalian TLR to be identified. Its properties were elucidated from studies on the LPS hyporesponsive mouse strain C3H/HeJ [11] which have a dominant-negative Pro712His mutation in the TIR domain of their TLR4 and are resistant to challenges with lethal doses of LPS.

TLRs facilitate the recognition and discrimination of invading microbes and induce an appropriate immune response. They are activated by specific microbial agonists including ones derived from bacteria, viruses, mycoplasma, yeasts and protozoa (Figure 1). TLR4, the principal receptor for Gram-negative LPS can also recognise other microbial agonists including respiratory syncytial virus (RSV) [12], *Chlamydia pneumonia*, Hsp60, flavolipin and murine retroviruses F protein [13-16]. Interestingly a number of endogenous signals can also activate TLR4 and other TLRs including hyaluronan and neutrophil elastase (see below) [17,18].

TLR2 appears to recognize a broad repertoire of agonists and is a functionally important PRR in the airways, which can respond to lipoteichoic acid, peptidoglycan and *M. pneumoniae* [19-21]. As a dimer with other TLRs, TLR2 confers responsiveness to a selection of agonists [22]. For example, with TLR1 it recognizes triacylated lipopeptides, Gram-positive lipoteichoic acid and Streptococcus pneumoniae [23], whereas with TLR6 it recognises diacylated lipopeptides [24].

There has been a single report of TLR2’s involvement in the response to flagellin [25].

TLRs 3, 7, 8 and 9 have roles in the recognition of nucleic acids. TLR3 responds to double-stranded (ds)RNA [26,27] a potential by-product in virally infected cells. TLRs 7 and 8, although not well expressed by lung epithelium [28] are expressed by immune cells within the lung and have a known role in the antiviral response [29,30]. Their major agonists are guanosine- and uridine-rich single-stranded (ss)RNA found in many viruses [31,32]. Microbial DNA containing unmethylated CpG (uCpG) dinucleotides motifs activate TLR9 [3]. These occur frequently in bacterial but not mammalian DNA.

Flagellin is a protein subunit of bacterial flagellae expressed by Gram-negative bacteria. It can induce TLR5-dependent signalling [34]. Airway epithelial cells utilise TLR5 in their responses to *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Bordetella bronchiseptica* [35-38]. TLR10 is an orphan member of the human TLR family [39].

4. TLR signalling pathways

An important feature of TLR signal transduction is that highly conserved pathways can be activated by the different TLRs. Thus, both TLRs and their intracellular signalling molecules represent key inhibitory targets for therapeutic drug design.

Activation of TLRs can lead to downstream signalling cascades resulting in the activation of pro-inflammatory gene transcription. These TLR-mediated changes in gene expression are critically dependent on the cytosolic TIR domain [7,40].
TIRs provide a scaffold for protein-protein interactions most notably those leading to the activation of NF-κB and the interferon regulatory factors (IRFs) [41,42]. Activation of AP1 and the MAPKs jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK)1/2 [43] are other classical signals regulated by TLR signalling.

Agonist-induced homo- or heterodimerisation of TLRs represents the first step in all TLR signalling cascades. TLR4 uniquely requires two accessory proteins for full responsiveness to its agonists; myeloid differentiation protein-2 (MD-20, a soluble glycoprotein on the outer surface of cell membrane [44] and CD14, a glycosylphosphatidylinositol-anchored receptor which binds to LPS–LPS-binding protein complexes [45]. NF-κB activation by TLRs then occurs in response to recruitment of TIR-domain-containing adaptor proteins that interact with the TIR domains of TLRs. Four TIR adaptor proteins MyD88 [46], MyD88-adpater like (Mal)/TIRAP [47,48], TIR domain containing adaptor inducing IFN-β (TRIF) [42,49] and TRIF-related adapter molecule (TRAM) [50-52] integrate TLR activation with downstream signalling. MyD88 transduces signals for all TLRs with the exception of TLR3, which utilises TRIF instead, whilst TRAM and Mal are involved in TLR3 and TLR2/4 signalling, respectively.

Following its recruitment MyD88 associates with IL-1 receptor-associated kinase-4 (IRAK-4) (Figure 2A) [53]. A series of protein-protein interactions then assemble involving IRAK-1, TNF receptor-associated factor 6 (TRAF6), TGF-β-activated kinase-1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2. Ubiquitin conjugating enzyme (Ubc)13 and ubiquitin-conjugating enzyme E2 variant 1A (Uev1A), next catalyse the synthesis of a polyubiquitin chain on TRAF6 [54] triggering activation of TAK1 by phosphorylation. Activation of the 1kB kinase (IKK) complex (IKKα, IKKβ and NF-κB essential modulator (NEMO)/IKKγ) [55], ensues culminating in phosphorylation, ubiquitylation and proteosomal degradation of 1kB and nuclear translocation of NFκB.

Signalling from TLR3 or TLR4 via TRIF and TRAM can also trigger a signalling pathway leading to expression of the type I interferons. This pathway involves the non-canonical IKKs, TANK-binding kinase 1 (TBK1) and IKKe/IKKi and culminates in activation of the transcription factors IRF 3 and 7 (Figure 2B) [50,56,57] which regulate IFN-β and IFN-α expression, respectively.

TLRs expressed by immune and epithelial cells in the CF lung contribute to the pulmonary immune response by regulating the production and secretion of chemokines, cytokines and antimicrobial peptides and by enhancing cell surface adhesion molecules expression. Surface expression of intercellular adhesion molecule 1 (ICAM-1) can be increased on airway epithelial cells in response to triacylated lipopeptide, LPS, uCpG DNA, dsRNA and Influenza virus A [58,59]. Multiple pro-inflammatory cytokines can be regulated by TLR activation in these cells. TLR2, TLR4 and TLR9 agonists upregulate IL-8, TNF-α and IL-6 amongst others [58,60-62]. IL-8 is a potent neutrophil chemoattractant and a key factor regulating the neutrophil-dominated airway inflammation characteristic of CF. The expression of other chemokines by airway epithelial cells however is also regulated by TLR agonists that activate TLRs 2-5. Macrophage inflammatory protein 3 (MIP-3) expression, for example, is increased in response to zymosan, dsRNA, LPS and flagellin [63].

The human β-defensins (HBD) are antimicrobial peptides produced by epithelial cells. TLR2 activation by bacterial lipoprotein enhances HBD2 expression in tracheobronchial epithelium [64] whilst lipoteichoic acid and peptidoglycan are also known to induce TLR2-mediated increases in HBD2 expression in a variety of airway epithelial cells [61,65]. TLR4 agonists, including neutrophil elastase, similarly regulate HBD2 expression in both immortalized and primary airway epithelial cells [66].

In addition to microbial agonists, a number of host-derived factors with TLR-activation properties have been described. Endogenous agonists with activity against TLR2 or TLR4 include neutrophil elastase, hyaluronan, heat-shock proteins, oxidants and fibronectin amongst others [17,18,67-72]. Stimulation of TLRs by these danger-associated molecular patterns (DAMPs) points to the existence of mechanisms whereby TLRs can recognise host molecular patterns that represent a danger signal due to chronic inflammation [73].
Toll-like receptors as therapeutic targets in cystic fibrosis

The CF lung represents a milieu that is potentially rich in a variety of microbial (Figure 1) and endogenous TLR agonists. It is widely accepted that there are higher than normal levels of neutrophil elastase in CF bronchoalveolar lavage fluid whilst the presence of both bacterial and yeast-derived factors has also been demonstrated [74]. For example Pseudomonas DNA has been detected in CF sputum and bronchoalveolar lavage fluid [58,75] flagellin expressed by planktonic Pseudomonas may act as a TLR5 agonist in early colonisation and LPS is also likely to be present at high levels in the CF lung. TLR2 agonists such as microbial lipopeptides or lipoteichoic acids (LTA) derived from Pseudomonas and/or Staphylococcus species or fungal-derived factors are other potential candidates. Viruses express agonists for TLRs 3, 7 and 8, and viral infections in CF have the potential to modulate TLR activity.

5. TLR agonists in the CF lung

The CF lung represents a milieu that is potentially rich in a variety of microbial (Figure 1) and endogenous TLR agonists. It is widely accepted that there are higher than normal levels of neutrophil elastase in CF bronchoalveolar lavage fluid whilst the presence of both bacterial and yeast-derived factors has also been demonstrated [74]. For example Pseudomonas DNA has been detected in CF sputum and bronchoalveolar lavage fluid [58,75] flagellin expressed by planktonic Pseudomonas may act as a TLR5 agonist in early colonisation and LPS is also likely to be present at high levels in the CF lung. TLR2 agonists such as microbial lipopeptides or lipoteichoic acids (LTA) derived from Pseudomonas and/or Staphylococcus species or fungal-derived factors are other potential candidates. Viruses express agonists for TLRs 3, 7 and 8, and viral infections in CF have the potential to modulate TLR activity.

5.1 The role of viral infection in CF

Increasing interest is focusing on the role of viral infections in CF in predisposing to bacterial superinfection and as independent pulmonary pathogens. Up to 40% of pulmonary exacerbations are attributed to viruses in individuals with CF [76-78] and are associated with a persistent decrease in lung function [76,78-80], prolonged hospital admission and increased likelihood of earlier acquisition of Pseudomonas aeruginosa colonization [80,81]. A distinction between upper and lower respiratory tract viral infections exists and in CF, increased incidences of lower respiratory tract viral infections are associated with poorer outcomes [79].

Viral infection can increase expression of IL-8 thus exaggerating the inflammatory cascade [82]. The co-existence of bacterial pathogens can also further predispose to epithelial cell damage as part of the inflammatory process, with resultant increased transepithelial permeability [83]. Influenza A infection has been associated with neutrophil infiltration [84] and both influenza and adenovirus have cytopathic effects on airway epithelial cells [85]. In vitro studies suggest that viruses promote increased bacterial adherence to the airways [86,87]. On a molecular level it would appear that viral infection interferes with TLR responses. In response to rhinovirus human alveolar macrophages showed an impairment of

Figure 2. Toll like receptor (TLR) signalling cascades leading to NF-κB or interferon regulatory factor IRF activation.

A. Following triggering of TLRs 1, 2 and 4-9 the Toll/IL-1R (TIR) domains of TLRs and myeloid differentiation factor 88 (MyD88) interact. The signal is transduced via interleukin-1 receptor associated kinase (IRAK)-4, IRAK-1 and TNF receptor-associated factor 6 (TRAF-6). Next TRAF6 is ubiquitinated by the E2 ligases ubiquitin conjugating enzyme 13 (Ubc13) and ubiquitin-conjugating enzyme E2 variant 1A (Uev1A). This activates transforming growth factor-β-activated kinase-1 (TAK1), which associates with TAK1-binding protein (TAB)1/TAB2 leading to phosphorylation and activation of the IkB kinase (IKK) complex, culminating in activation NFκB which can then translocate to the nucleus to regulate gene expression. TLR2 or TLR4 can also activate the IKK complex indirectly via MyD88 adaptor-like (Mal)/MyD88.

B. TLR3 and TLR4 activate IKKe and TBK1 via TIR domain-containing adaptor inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM) leading to interferon regulatory factor (IRF)3 and IRF7 activation and production of interferon-β and -α. TLR3 can also activate the IKK complex via TRIF, leading to classical NF-κB activation.

TBK1: TANK-binding kinase 1.

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cytokine responses to bacterial LPS and LTA [88] thus a more thorough investigation of the dynamics of innate immunity in CF in the context of both bacterial and viral infection is warranted.

Prevention and therapeutic strategies for viral infection have proven controversial. A Cochrane Review from 2000 showed no increased protection with influenza vaccine in individuals with CF [89]; conversely a recent trial has shown this may not be the case [90]. Despite this the vaccine has been shown to be safe and is currently recommended for individuals with CF. Palivizumab a monoclonal antibody to RSV, has been developed for passive immunoprophylaxis and has shown some promise. However, there is currently no RSV vaccine.

The focus of research has recently swung towards antiviral therapy to limit the inflammatory response, with limited results to date. Ribavirin, a nucleotide analogue, is licensed for RSV infection but not in CF, and results have been variable.

The neuraminidase inhibitors oseltamivir and zanamavir, if used early, may be effective for the treatment and prophylaxis of influenza infection, and are safe to use. The adamantan, amantadine and rimantadine, are not generally used, and resistance rates with these for influenza A of up to 92% have been reported [91,92].

Developing newer strategies for therapy necessitates evaluation of more defined viral signalling pathways and should possibly focus on TLR involvement. Viruses detectable in CF secretions during exacerbations act mainly through TLR3 (influenza A, influenza B, rhinovirus). Signalling pathways for parainfluenza virus via TLRs are undetermined whereas RSV has been shown to act through TLR4 [14].

6. Targeting TLRs in CF

There are a variety of potential strategies available to interfere with TLR function in the CF lung. These include traditional approaches such as the use of inhibitory peptides, pharmacological methods to promote the expression of endogenous anti-TLR molecules or administration of purified versions of these molecules. Exploring the therapeutic properties of naturally-occurring TLR antagonists is an area that is also likely to have merit.

6.1 Endogenous inhibitors

Like the autocrine mechanisms that exist to control regulation of TNF and IL-1 signalling, a number of endogenous TLR inhibitors have been identified that can negatively regulate TLR signalling processes. The factors that we will consider here are A20, toll interacting protein (Tollip), single immunoglobulin IL-1-related receptor (SIGIRR), MyD88s, IRAK-M, suppressors of cytokine synthesis (SOCS), sterile α and HEAT-Armadillo motifs protein (SARM), Src homolog protein tyrosine phosphatase 1 (SHP-1), protein tyrosine phosphatase, non-receptor type 1 (PTP1B), mucin 1 (MUC1) and peroxisome proliferator activated receptor gamma (PPARgamma) (Table 1). This diverse range of molecules includes transmembrane proteins and nuclear receptors, ubiquitin-modifying and adaptor proteins, kinases and phosphatases. Although each of these proteins can function to control TLR signalling, their mode of action differs; nonetheless, they can commonly be grouped as TLR inhibitors.

A20 is a ubiquitin-modifying enzyme and zinc-finger protein that was first reported to regulate TLR4 signalling [93,94].

It is now known that A20 can suppress both TLR2- and TLR4-induced IL-8 expression in airway epithelial cells and can be upregulated by measles P virus protein [95]. Tollip is an inhibitor of both IL-1 and LPS signalling [96].

Inhibition by Tollip is mediated through its ability to block the activity of IRAK after TLR activation [97]. Like Tollip, the TIR family member TIR8, also known as SIGIRR, is a negative regulator of IL-1 and TLR signalling [98]. An alternatively spliced form of MyD88 exists, termed MyD88s. This variant is unable to recruit IRAK-4 and thus transcriptionally controls negative regulation of innate immune responses to MyD88-dependent TLRs [99] IRAK-M is a novel member of the IRAK family [100] and a negative regulator of TLR signalling [101].

It has an important role in endotoxin tolerance and its expression is restricted to monocytes and macrophages. The SOCS proteins are induced by stimulation of TLRs and both SOCS1 and 3 have been shown to have the ability to suppress TLR signalling. Rothlin et al. [102] uncovered the complex negative feedback mechanism limiting TLR signalling involving the Tyro3/Axl/Mer (TAM) family of receptor tyrosine kinases, which induce expression of the inhibitory proteins SOCS1 and SOCS3.

MyD88, Mal, TRIF and TRAM are the four TIR adaptors associated with TLR-activation functions. The fifth known TIR adaptor, SARM, acts as a negative regulator of TRIF-dependent TLR signalling and thus can impair TLR3 and TLR4 responses [103]. Recently SHP-1 [104] and PTP1B [105] have been identified as phosphatases that can negatively regulate TLR-mediated production of pro-inflammatory cytokines by inhibiting activation of the transcription factors NF-κB and MAPK (and IRFs for PTP1B). Interestingly, SHP-1 concomitantly increases the production of type I interferon mediated by TLRs by directly binding to and inhibiting activation of IRAK1. Thus, SHP-1 appears to have an important role in skewing the balance between expression of pro-inflammatory cytokines and type I interferons in the innate immune response [104].

MUC1 is a transmembrane glycoprotein expressed by airway and other epithelial cells. Recently, it has been shown to have an anti-inflammatory role particularly with respect to Pseudomonas aeruginosa or its flagellin but also against TLR2, 3, 4, 7 and 9 agonists [106,107] 15deoxy-prostaglandin-J2 and treoglizatone are natural and synthetic PPARgamma agonists. Their activation of PPAR-gamma can result in reduced stimulation of DCs via the ligands for TLR 2, 3, 4 and 7 [108].
Toll-like receptors as therapeutic targets in cystic fibrosis

Table 1. Endogenous Toll like receptor (TLR) inhibitors.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Signalling target</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20</td>
<td>TLR2, TLR4</td>
<td>[21,93-95]</td>
</tr>
<tr>
<td>Tollip</td>
<td>TLR4 (and IL-1R) at IRAK1</td>
<td>[96,97]</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>TLR4 (and IL-1R)</td>
<td>[98]</td>
</tr>
<tr>
<td>MyD88s</td>
<td>TLR4 (and IL-1R) Myd88-dependent signalling</td>
<td>[99]</td>
</tr>
<tr>
<td>IRAK-M</td>
<td>TLR2, TLR4</td>
<td>[100,101]</td>
</tr>
<tr>
<td>SOCS1/3</td>
<td>TLRs</td>
<td>[102]</td>
</tr>
<tr>
<td>SARM</td>
<td>TLR3, TLR4, TRIF-dependent signalling</td>
<td>[103]</td>
</tr>
<tr>
<td>Shp-1</td>
<td>TLR3, TLR4 at IRAK1</td>
<td>[104]</td>
</tr>
<tr>
<td>PTP1B</td>
<td>TLR5, MyD88- and TRIF-dependent signalling</td>
<td>[105]</td>
</tr>
<tr>
<td>MUC1</td>
<td>TLR2, TLR3, TLR4, TLR5, TLR7, TLR9</td>
<td>[106,107]</td>
</tr>
<tr>
<td>PPARγ</td>
<td>TLR2, TLR3, TLR4, TLR7</td>
<td>[108]</td>
</tr>
</tbody>
</table>

IRAK: Interleukin-1 receptor associated kinase; MyD88: Myeloid differentiation factor 88; MUC1: Mucin 1; PPARγ: Peroxisome proliferator activated receptor gamma; PTP1B: Protein tyrosine phosphatase; non-receptor type 1; SARM: Sterile alpha and HEAT-Armadillo motifs; Shp-1: Src homolog protein tyrosine phosphatase 1; SIGIRR: Single immunoglobulin IL-1-related receptor; SOCS: Suppressors of cytokine synthesis; Tollip: Toll interacting protein; TRIF: TIR domain-containing adaptor inducing interferon-β.

397 6.2 Microbial TLR antagonists

In addition to naturally-occurring endogenous TLR inhibitors, there exist in nature a number of microbial TLR antagonists that may have therapeutic potential for CF (Table 2). To date a selection of such microbial proteins have been discovered and partially characterised. These include the viral proteins A46R, A52R and N1L from *Vaccinia* virus and RSV G/soluble G protein [109-112] and the TIR-domain-containing-proteins typhimurium large plasmid A (TlpA) from *Salmonella*, *Brucella* TIR-containing protein 1 (Btp1)/TIR domain containing-protein (TcpB) from *Brucella* spp. and TcpC from *Escherichia coli* [113-115].

A46R and A52R share amino acid sequence similarity with the mammalian TIR domain and can interfere specifically with IL-1 signal transduction [109]. Ectopic expression of A46R can inhibit intracellular signalling by a range of TLRs. It targets all four host TIR adaptors MyD88, Mal, TRIF and TRAM, and interferes with downstream activation of NFκB and MAPKs. A46R also disrupts TRIF-induced IRF3 activation and induction of regulated upon activation, normally T-expressed, and presumably secreted (RANTES) [110]. A46R is functionally distinct from the other described *Vaccinia* virus TLR inhibitor, A52R which can potently block both IL-1- and TLR4-mediated NFκB activation. A52R mimics the dominant-negative effect of a truncated version of MyD88 on IL-1, TLR4 and IL-18 signalling but has no effect on MyD88-independent signalling pathways.

Together A46R and A52R are used by *Vaccinia* virus to 424 suppress TIR domain-dependent intracellular signalling. Of 425 particular interest here is the demonstration that either A46R or A52R, like dominant negative versions of MyD88 and Mal can interfere with the intracellular mechanisms by which neutrophil elastase upregulates inflammatory gene expression in both primary and transformed bronchial 430 epithelial cells [60].

Bioinformatics has identified a third *Vaccinia* virus protein, N1L, which shares significant similarity with A52R [111]. N1L protein strongly affects *Vaccinia* virulence in vivo by suppressing NF-kB and IRF3 activation following engagement of TLRs. N1L also disrupts signalling to NF-kB by the TNF superfamily of receptors by targeting the IKK complex for inhibition.

RSV infection of monocyte-derived dendritic cells (mDCs) leads to activation of the IFN-inducing pathway leading to 440 type I IFN induction. Having shown that RSV initial attachment to cells can block polyIC-mediated IFN-beta induction Shingai et al. [112] further demonstrated that polyIC- or LPS-mediated IFN-beta production were inhibited by RSV G or soluble G (sG) proteins. These anti-TLR3/4 effects 445 were mediated via the TRIF pathway and suppressed the production of IFN-beta. The exact molecular mechanism by which this inhibition occurs remains to be determined.

Like viruses, pathogenic bacteria have evolved sophisticated molecular strategies to subvert host defenses. Btp1 is a 450 aa protein expressed by *Brucella abortus* that contains a C-terminal 130-amino acid domain with significant sequence similarity to the TIR domains of TLR2, TLR4, SIGIRR and MyD88. This similarity exists both in box 1, the signature sequence of the TLR family, and in box 2 which is important for signalling [114]. Btp1 shares homology with TlpA, a *Salmonella* effector that interferes with TLR signalling in vitro [115]. Cirl et al. have also identified a mechanism by which virulent bacteria can interfere directly with TLR function by secreting proteins that act as inhibitory homologs of the 460 mammalian TIR domain [115]. In addition to the TIR domain containing-protein (Tcp) TcpB from *B. melitensis* this group also identified a gene encoding a Tcp in *Escherichia coli* CFT073 (TcpC). *In silico* analysis predicts significant tertiary structure homology between these proteins and the TIR 465 domain of human TLR1. Tcps can directly bind to MyD88, impede TLR signalling and suppress innate immunity.

6.3 Inhibitory peptides

Peptide mimetics that can inhibit agonist binding or signal transduction are appealing therapeutic agents for CF. In addition to their well-defined specificity, peptides can be engineered to gain access to intracellular compartments and thus directly interfere with signalling pathways. As a class, these low-molecular-weight compounds have significant anti-inflammatory potential. To date just a few peptide-blocking studies targeting TLRs have been published. Bartfai et al. [116] 477
SLPI is an 11.7 kDa protein, secreted by airway epithelial cells [119]. Along with A1AT, it comprises the body’s major antineutrophil elastase defence [120]. As an antiprotease, it has been shown to protect tissue locally by inhibiting other proteases such as trypsin, chymotrypsin, chymase, trypase and cathepsin G [121]. In addition to its antiprotease actions, SLPI has been shown to have anti-inflammatory and anti-infective properties [122]. It displays antimicrobial action against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* [123,124]. Other studies have also demonstrated its anti HIV properties [125].

With respect to targeting TLRs, SLPI has been shown to inhibit TLR- induced cytokine expression, impairing TLR2- and TLR4-mediated responses in monocytic cells. In the setting of CF, bacterial colonization and persistent infection, particularly with *Pseudomonas aeruginosa* is one of the main determinants of morbidity and mortality in patients, thus inhibition of TLR2- and TLR4-mediated responses by SLPI underlines its therapeutic potential in this area [126]. Interestingly LPS is known to upregulate SLPI production in macrophages suggesting the existence of a novel endogenous negative feedback loop [127].

The mechanisms by which SLPI interferes with LPS effects are dependent on its antiprotease activity and involve impairing degradation of IkB without affecting phosphorylation or ubiquitination [128] and by binding directly to p65 NFκB binding sites in a site-specific manner [129].

SLPI as an anti fungal is also of potential importance in the CF population. *Aspergillus* colonization is associated with accelerated lung function decline and poorer overall outcomes. Recombinant SLPI (rSLPI) has been shown to dose-dependently kill hydrated but not airborne conidia [130]. The same group found a partial inhibition of fungal protease activity (*A. fumigatus* virulence factor) by rSLPI, and this also inhibited the induction of the pro-inflammatory cytokine response in airway epithelial cell lines [131].

Excessive mucus production as a result of an exacerbation of CF is a huge symptomatic problem for the patient, particularly when difficult to expectorate, and leads to increased patient anxiety, as well as detrimental effects on lung function and worsening respiratory failure. SLPI’s potential as an antimucin agent in CF has also been highlighted [132] in a study that demonstrated its inhibitory capacity against TLR2, TLR4 and TLR9 agonist-induced MUC2 and 5AC expression via a mechanism primarily dependent on the inhibition of TGF-α release. SLPI may therefore have a potential role as an antimucin agent in CF.

### 6.4.1 SLPI

SLPI is an 11.7 kDa protein, secreted by airway epithelial cells [119]. Along with A1AT, it comprises the body’s major antineutrophil elastase defence [120]. As an antiprotease, it has been shown to protect tissue locally by inhibiting other proteases such as trypsin, chymotrypsin, chymase, trypase and cathepsin G [121]. In addition to its antiprotease actions, SLPI has been shown to have anti-infective and anti-inflammatory properties [122]. It displays antimicrobial action against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* [123,124]. Other studies have also demonstrated its anti HIV properties [125].

With respect to targeting TLRs, SLPI has been shown to inhibit TLR- induced cytokine expression, impairing TLR2- and TLR4-mediated responses in monocytic cells. In the setting of CF, bacterial colonization and persistent infection, particularly with *Pseudomonas aeruginosa* is one of the main determinants of morbidity and mortality in patients, thus inhibition of TLR2- and TLR4-mediated responses by SLPI underlines its therapeutic potential in this area [126]. Interestingly LPS is known to upregulate SLPI production in macrophages suggesting the existence of a novel endogenous negative feedback loop [127].

The mechanisms by which SLPI interferes with LPS effects are dependent on its antiprotease activity and involve impairing degradation of IkB without affecting phosphorylation or ubiquitination [128] and by binding directly to p65 NFκB binding sites in a site-specific manner [129].

SLPI as an anti fungal is also of potential importance in the CF population. *Aspergillus* colonization is associated with accelerated lung function decline and poorer overall outcomes. Recombinant SLPI (rSLPI) has been shown to dose-dependently kill hydrated but not airborne conidia [130]. The same group found a partial inhibition of fungal protease activity (*A. fumigatus* virulence factor) by rSLPI, and this also inhibited the induction of the pro-inflammatory cytokine response in airway epithelial cell lines [131].

Excessive mucus production as a result of an exacerbation of CF is a huge symptomatic problem for the patient, particularly when difficult to expectorate, and leads to increased patient anxiety, as well as detrimental effects on lung function and worsening respiratory failure. SLPI’s potential as an antimucin agent in CF has also been highlighted [132] in a study that demonstrated its inhibitory capacity against TLR2, TLR4 and TLR9 agonist-induced MUC2 and 5AC expression via a mechanism primarily dependent on the inhibition of TGF-α release. SLPI may therefore have a potential role as an antimucin agent in CF.

### 6.4.2 Elafin

Elafin is a 6 kDa serine antiprotease expressed in mucosal surfaces [133]. Pre-elafin, also known as trappin 2, is its precursor which undergoes proteolytic cleavage to release...
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mature elafin [134]. There is increasing interest in this antiprotease as an immunomodulatory molecule. Cytokine-mediated increases in elafin production by epithelial cells are greater than the increase in SLPI production [135], and it has been suggested that elafin is of greater significance during an inflammatory challenge to the lungs. Antimicrobial properties have been described for elafin against Pseudomonas aeruginosa and Staphylococcus aureus [123].

As an anti-inflammatory agent elafin can inhibit LPS-induced production of monocyte chemotactic protein 1 (MCP-1) in monocytes. This effect is mediated via impairment of both AP-1 and NF-κB activation, via an effect on the ubiquitin-proteosome pathway [136]. Elafin also has antifungal properties [137]. Thus, like SLPI, elafin may hold therapeutic promise in the treatment of inflammation and infection in CF.

6.4.3 A1at
A1AT is the body’s most abundant serine antiprotease. Increasing evidence exists to suggest that A1AT possesses properties other than its antiprotease function including both anti-inflammatory and immunomodulatory effects. For example, native, polymerised or oxidised A1AT can inhibit LPS-stimulated synthesis of TNF-α and IL-1β whilst enhancing the release of the anti-inflammatory cytokine IL-10 [138]. Whether A1AT has direct anti-TLR4 activity remains to be shown, however, C-36 peptide, a degradation product of A1AT, also has potential LPS-modulatory activity against human monocytes [139].

7. Delivery of therapeutics to the CF lung
A major hurdle in targeting therapeutics to treat the pulmonary inflammatory manifestations of CF is the effective delivery of an agent to the lung. The presence of biofilm, excessive mucus and parenchymal damage in the CF lung can negatively affect drug distribution and bioavailability. Whilst aerosol delivery ensures direct administration to the site of action and less systemic toxicity, its drawbacks include problems in sampling of epithelial lining fluid to quantify anti-inflammatory effects and determine pharmacokinetics. An ideal aerosolised therapeutic agent for CF should be sterile, nonpyrogenic, chemically stable and have a particle size ranging from 1 to 5 µm for reproducible drug delivery to the airways [140,141]. The drug should have a wide margin of dosage safety to allow for the varying conditions in the lungs of CF patients and should be easily nebulised.

8. Current therapeutics and their effect on TLRs
Continuous cycles of infection lead to increased morbidity and mortality in the setting of CF. Prompt antibiotic therapy based on appropriate sensitivities is associated with reduced decline in lung function and improved patient outcomes. Symptomatically, inhaled/nebulised broncho-dilator therapy provides relief from breathlessness, and mucolytic therapy can be very useful in enabling patients to expectorate sputum. There is increasing focus on therapy targeting the underlying pathogenesis, and in particular underlying inflammation.

8.1 Anti-inflammatory therapies
The underlying inflammatory state is central to the disease process, and many therapies have been initiated in an effort to counter this, including NSAID’s, steroids, and, more recently azithromycin. Initial studies looking at diffuse pan bronchiolitis in Japan showed improvement with macrolide therapy [142]. Studies in CF have shown similar improvements, with improved forced expiratory volume in one second (FEV1), reduced number of hospital admissions, increased weight, and improved quality of life [143-145]. Azithromycin is a macrolide antibiotic, and in addition to its antibacterial properties which may include effects on biofilm formation, may have anti-inflammatory [146] and immunomodulatory effects.

For example, azithromycin has been shown to reduce TNF-α levels in CF airway epithelial cells [147] and to suppress activation of NF-κB and pro-inflammatory cytokine expression in tracheal aspirate cells from premature infants [148]. Some evidence exists for TLR4- and TLR5-specific properties of azithromycin. An experimental model has shown that azithromycin attenuates the effects of LPS administration in mice [149]. Azithromycin has been shown to reduce expression of Pseudomonas aeruginosa flagellin [150,151]. A more recent model, with Salmonella typhimurium, has shown that azithromycin inhibits the formation of flagellar filaments without suppressing flagellin synthesis [152].

Glucocorticoids are well documented as anti-inflammatory agents but chronic use is associated with long-term sequelae including osteoporosis, peptic ulcer disease and cataracts. Their mechanism of action is associated with upregulation of TLRs although they can impair the differentiation and antigen presenting ability of dendritic cells [153].

8.2 Mucolytic therapies
Nebulised hypertonic saline has been shown to be useful for sputum induction in the setting of CF, and short-term studies have shown improved pulmonary function following treatment [154,155]. Recently it has been shown in a mouse model post thermal injury that hypertonic saline enhances host defense to bacterial challenge by augmenting TLRs [156]. Nebulised hypertonic saline, although a different mode of delivery and for a different purpose, may in fact work in a similar way.

Dornase alfa, a DNase, has been shown to reduce viscosity of sputum, allowing easier expectoration. Improvements in lung function have also been documented [157,158]. Viscosity is decreased by enzymatic degradation of host and bacterial DNA, with the added advantage that TLR9-mediated signalling in response to uCpG DNA is likely to be abrogated also.
8.3 Delivery of agents to the CF lung

The mucus lining the airways of the CF lungs is a complex biological environment posing significant barriers to efficient therapeutic drug delivery. CF mucus, which acts as the primary extracellular barrier in the CF lung contains mucin fibres that form a dense matrix intertwined with other macromolecules such as actin and DNA that decrease its permeability. The viscoelasticity of CF sputum greatly reduces the diffusion rates of colloidal particles, limiting the effectiveness of drug delivery to underlying lung cells. According to Dawson et al. [159], nanoparticles dispersed in CF sputum are transported primarily through lower viscosity pores within a highly elastic matrix. Neutral particles with a diameter of less than 200 nm undergo more rapid transport in CF sputum than charged or larger particles. Whilst DNase can reduce the macro-viscoelastic properties of CF sputum by up to 50% by hydrolyzing chromosomal DNA released from dead neutrophils, surprisingly, it does not significantly alter the average particle diffusion rate. This is most probably due an increased microviscosity [160].

Polyethylene glycol (PEG)ylated poly-L-lysine nanoparticles have been used to efficiently transfect lung epithelium following intrapulmonary administration [161]. This technology could assist any inhaled therapeutic in penetrating the airways.

9. Concluding remarks

As a major portal of entry for bacteria and microbes, the lung represents a key component of the innate immune system. Airborne pathogens encounter a number of effective defense mechanisms designed to rapidly counteract potential damage, inhibit colonization and protect against invasion by pathogens. The existence of TLRs equips the lung with an exquisitely designed mechanism for controlling microbial infection. Unfortunately CF is a disease in which the lung is so badly affected that innate immunity and TLR activity are dysfunctional. Thus, modulation of TLR function has obvious important implications. Enhancing TLR responses using targeted approaches directed at TLR3 could accelerate antiviral responses. Conversely suppression of other TLR responses, by the use of appropriate inhibitors could reduce the chronic inflammation characteristic of this disease. Thus, new therapeutics designed to selectively activate or inhibit TLR function specifically and reversibly represent powerful tools for the prevention and treatment of the pulmonary inflammatory manifestations of CF.

10. Expert opinion

TLR-targeted approaches to the management of inflammation and infection in CF differ with respect to bacterial and viral factors. In chronic bacterial colonization, as seen in CF, the problem is partly one of an overexuberant inflammatory response, which may need to be dampened down or be made more effective in clearing bacteria. With respect to viral infection the focus is on enhancing rather than inhibiting type I interferon production in an attempt to eliminate the virus as soon as possible. Thus, the challenge is to design appropriate TLR-directed therapeutics with selective properties. In this regard designing drugs that activate or interfere with components of the signalling pathways rather than the TLRs themselves would appear to be the more targeted strategy. Progress in this area will be guided by the emerging knowledge regarding the mechanisms by which endogenous and microbial antagonists of TLR signalling exert their effects at a molecular level.

Manipulation of TLR signalling has inherent risks that must be carefully considered. Immunosuppression in the face of constant bacterial and fungal challenge may allow spread of infection and involvement of previously undamaged lung tissue. Therefore, until such time as we know the level of inflammation required to confine infection we must be conservative in our immunosuppressive approaches and any new trials must include careful monitoring of sputum organisms, inflammatory mediators, lung function and structural changes. Currently we are at a point where we are faced with determining how best to identify and minimize adverse events. By studying patients with known deficiencies in TLR signalling (e.g IRAK-4 [162]), we may be able to identify pathway-specific events that represent hallmarks of adverse suppressive effects. The corollary to this exists when attempting to enhance TLR function. Immunostimulatory therapies can create an undesirable 'hypercytokine' milieu. Data from animal studies will help to decipher such events. Indeed impairing antiviral immunity may actually confer an unexpected benefit as per the recent demonstration of an unexpected survival advantage in influenza-virus-infected mice deficient in TLR3, despite higher viral production in the lungs [163]. Thus, on balance, given that CF airways have aberrantly high cytokine levels even in the absence of detectable microbial infection, TLRs antagonists would appear to be the way forward.

With respect to existing conventional therapies there is evidence that many of these approaches directly or indirectly affect TLR-mediated responses and that their efficacy is mediated by this. We can learn a lot from current conventional therapies. We have seen that many of these, for example azithromycin or hypertonic saline exert their effects at least partly via TLRs. By understanding in more detail how these modulate the activity of TLRs to good effect we can design TLR-directed interventions that selectively inhibit the inflammatory part of the cascade whilst retaining the antimicrobial component. As with all therapeutic approaches for CF our enthusiasm for success must be tempered with a degree of caution. Important considerations for effective therapies, for example the use of A1AT augmentation therapy, or indeed any antiprotease therapy, designed to treat the pulmonary manifestations of CF include effective deposition in poorly aerated areas and protection against degradation and inactivation by serine and cysteinyl cathepsins present.
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in the CF airways. These challenges must remain at the forefront of our scientific and clinical thinking.

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Declaration of interest

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This paper presents a comprehensive approach to inhibit TLR signalling using inhibitory peptides based on the structure of the TIR domain.


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