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### CITATION

Greene, Catherine M.; Branagan, Peter; McElvaney, Noel G. (2008): Toll-like receptors as therapeutic targets in cystic fibrosis.. figshare. Journal contribution. <https://hdl.handle.net/10779/rcsi.10783976.v1>

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# Expert Opinion

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Anti-inflammatory

## 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

*Expert Opin. Ther. Targets (2008) 12(12):1-15*

### 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*

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

60 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 lung 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 micro-  
organisms. 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

80 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.

90 First identified in the fruitfly *Drosophila melanogaster*, the  
*Drosophila* or dToll was initially characterised as a factor  
regulating embryogenic –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.

100 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 hypo-  
responsive mouse strain C3H/HeJ [11] which have a domi-  
nant-negative Pro712His mutation in the TIR domain of their  
TLR4 and are resistant to challenges with lethal doses of LPS.

115 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 pneumoniae*, Hsp60, flavolipin and murine retro-  
viruses 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].

120 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].

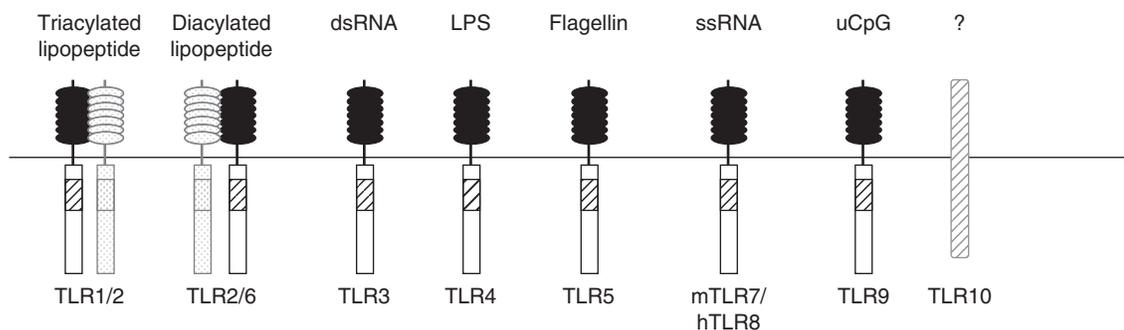
130 TLRs 3, 7, 8 and 9 have roles in the recognition of  
nucleic acids. TLR3 responds to double-stranded (ds)RNA [26,27]  
a potential by-product in virally infected cells. TLRs 7 and 8,  
although not well expressed by lung epithelium [28] are  
expressed by immune cells within the lung and have a 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 featuring unmethylated  
CpG (uCpG) dinucleotides motifs activate TLR9 [33]. These  
occur frequently in bacterial but not mammalian DNA.

140 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 bronchispetica* [35-38]. TLR10 is an orphan  
member of the human TLR family [39].

### 4. TLR signalling pathways

155 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.

160 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].



**Figure 1. Microbial Toll like receptor (TLR) agonists.**

dsRNA: Double-stranded RNA; hTLR: Human TLR; LPS: Lipopolysaccharide; mTLR: Murine TLR; ssRNA: Single-stranded RNA; uCpG: Unmethylated CpG dinucleotide motifs.

165 TIRs provide a scaffold for protein-protein interactions most notably those leading to the activation of NF- $\kappa$ B 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.

170 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 the cell membrane [44] and CD14, a glycosphatidyl inositol-anchored receptor which binds to LPS-LPS-binding protein complexes [45]. NF- $\kappa$ B 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- $\beta$  (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.

190 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- $\beta$ -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 I $\kappa$ B kinase (IKK) complex (IKK $\alpha$ , IKK $\beta$  and NF- $\kappa$ B essential modulator (NEMO)/IKK $\gamma$ ) [55], ensues culminating in phosphorylation, ubiquitylation and proteosomal degradation of I $\kappa$ B and nuclear translocation of NF $\kappa$ B.

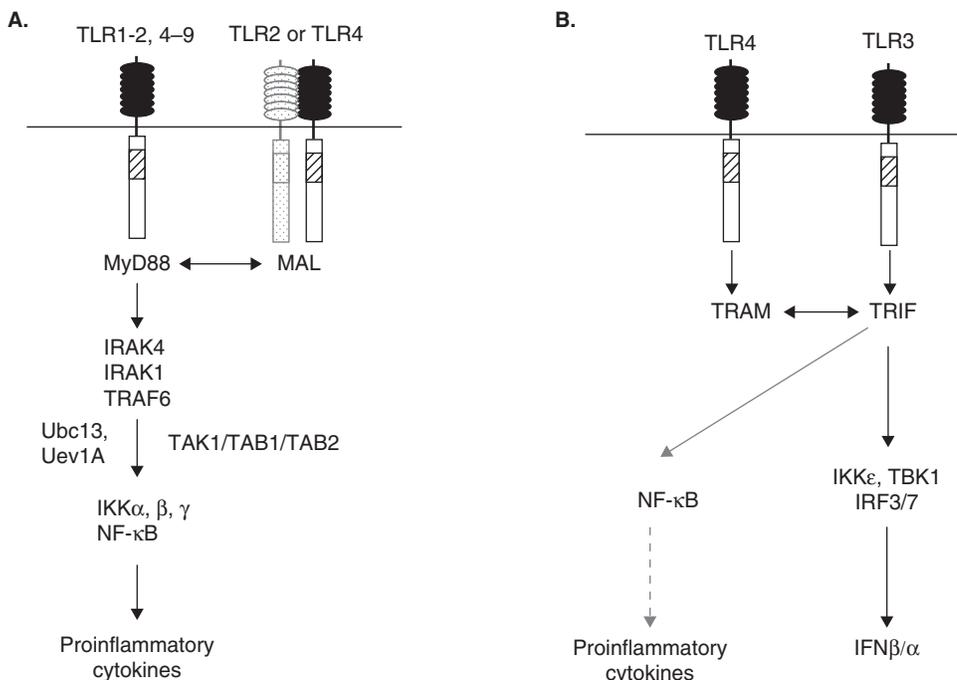
200 Signalling from TLR3 or TLR4 via TRIF and TRAM can also trigger a signalling pathway leading to expression of the type I interferons. This pathway involves the non-canonical

205 IKKs, TANK-binding kinase 1 (TBK1) and IKK $\epsilon$ /IKK $\iota$  and culminates in activation of the transcription factors IRF 3 and 7 (Figure 2B) [50,56,57] which regulate IFN- $\beta$  and IFN- $\alpha$  expression, respectively.

210 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- $\alpha$  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].

225 The human  $\beta$ -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].

235 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].



**Figure 2. Toll like receptor (TLR) signalling cascades leading to NF- $\kappa$ B 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- $\beta$ -activated kinase-1 (TAK1), which associates with TAK1-binding protein (TAB)1/TAB2 leading to phosphorylation and activation of the I $\kappa$ B kinase (IKK) complex, culminating in activation NF $\kappa$ B 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 IKK $\epsilon$  and TBK1 via TIR domain-containing adaptor inducing interferon- $\beta$  (TRIF)/TRIF-related adaptor molecule (TRAM) leading to interferon regulatory factor (IRF)3 and IRF7 activation and production of interferon- $\beta$  and - $\alpha$ . TLR3 can also activate the IKK complex via TRIF, leading to classical NF- $\kappa$ B activation.

TBK1: TANK-binding kinase 1.

245 **5. TLR agonists in the CF lung**

245 The CF lung represents a milieu that is potentially rich in a  
 250 variety of microbial (Figure 1) and endogenous TLR agonists.  
 It is widely accepted that there are higher than normal levels  
 255 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*  
 260 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  
 265 express agonists for TLRs 3, 7 and 8, and viral infections in  
 CF have the potential to modulate TLR activity.

265 **5.1 The role of viral infection in CF**

Increasing interest is focusing on the role of viral infections  
 270 in CF in predisposing to bacterial superinfection and as

independent pulmonary pathogens. Up to 40% of pulmonary 266  
 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* 270  
 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].

275 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 280  
 influenza and adenovirus have cytotoxic 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 285  
 human alveolar macrophages showed an impairment of 286

287 cytokine responses to bacterial LPS and LTA [88] thus a  
 290 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  
 295 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  
 300 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.  
 305 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 adamantanes,  
 amantadine and rimantadine, are not generally used, and  
 resistance rates with these for influenza A of up to 92%  
 310 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  
 315 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

320 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, pharma-  
 325 cological 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

330 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  
 335 here are A20, toll interacting protein (Tollip), single  
 immunoglobulin IL-1-related receptor (SIGIRR), MyD88s,  
 IRAK-M, suppressors of cytokine synthesis (SOCS), sterile  
 $\alpha$  and HEAT-Armadillo motifs protein (SARM), Src  
 homolog protein tyrosine phosphatase 1 (Shp-1), protein  
 340 tyrosine phosphatase, non-receptor type 1 (PTP1B), mucin 1  
 341 (MUC1) and peroxisome proliferator activated receptor

gamma (PPARgamma) (Table 1). This diverse range of 342  
 molecules includes transmembrane proteins and nuclear  
 receptors, ubiquitin-modifying and adaptor proteins, kinases  
 and phosphatases. Although each of these proteins can 345  
 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]. 350  
 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 355  
 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 360  
 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 365  
 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 370  
 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 375  
 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 380  
 NF- $\kappa$ B 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 385  
 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 390  
 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 troglitazone 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, 4 395  
 and 7 [108]. 396

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

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

IRAK: Interleukin-1 receptor associated kinase; MyD88: Myeloid differentiation factor 88; MUC1: Mucin 1; PPAR $\gamma$ : 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- $\beta$ .

## 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 NIL 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 (Tcp)B 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 NF $\kappa$ B 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 NF- $\kappa$ B 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 suppress TIR domain-dependent intracellular signalling. Of 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 epithelial cells [60].

Bioinformatics has identified a third *Vaccinia* virus protein, NIL, which shares significant similarity with A52R [111]. NIL protein strongly affects *Vaccinia* virulence *in vivo* by suppressing NF- $\kappa$ B and IRF3 activation following engagement TLRs. NIL also disrupts signalling to NF- $\kappa$ B 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 type I IFN induction. Having shown that RSV initial attachment to cells can block polyI:C-mediated IFN-beta induction Shingai *et al.* [112] further demonstrated that polyI:C- or LPS-mediated IFN-beta production were inhibited by RSV G or soluble G (sG) proteins. These anti-TLR3/4 effects 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 250 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* [113]. 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 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 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.*

**Table 2. Microbial Toll like receptor (TLR) antagonists.**

Protein	Organism	Target	Ref.
A46R	Vaccinia virus	TLR3, TLR4 at TRIF, TRAM, MyD88, Mal	[109,110]
A52R	Vaccinia virus	TLR4 (IL-1, IL-18)	[109,110]
N1L	Vaccinia virus	TLR2, TLR3, TLR4 at IKK complex	[111]
RSV sG	RSV	TLR3, TLR4	[112]
TlpA	<i>Salmonella enterica</i> serovar Enteritidis	TLR4 (IL-1) at MyD88	[113]
Btp1	<i>Brucella abortus</i>	TLR2	[114]
TcpP	<i>Brucella melitensis</i>	TLRs at MyD88	[115]
TcpC	<i>Escherichia coli</i>	TLRs at MyD88	[115]

Btp1: *Brucella* TIR-containing protein 1; IKK: IκB kinase; Mal: MyD88 adaptor-like; MyD88: Myeloid differentiation factor 88; RSV: Respiratory syncytial virus; TlpA: Typhimurium large plasmid A; Tcp: TIR domain containing-protein; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor inducing interferon-β.

478 designed and synthesized a low molecular weight MyD88  
 480 mimic, hydrocinnamoyl-L-valyl pyrrolidine, modeled on a  
 tripeptide sequence of the BB-loop [(F/Y)-(V/L/I)-(P/G)] of  
 the TIR domain. Examination of its pharmacological effects  
 focussed on its ability to inhibit IL-1- rather than TLR-mediated  
 events but did indicate a concentration-dependent inhibitory  
 capacity against IL-1-induced phospho-rylation of p38 MAPK  
 485 in murine lymphocytes. *In vivo*, this cell-penetrating TIR  
 domain mimic also significantly attenuated IL-1-induced  
 fever. Davies *et al.* [117] also tested TIR mimetics based on  
 MyD88, whilst Toshchakov and Vogel [118] performed a  
 comprehensive study using a set of blocking peptides comprised  
 490 of the 14 aa corresponding to the sequences of the BB loops of  
 MyD88, Mal, TRIF and TRAM linked to the cell-penetrating  
 segment of the *Antennapedia* homeodomain. TLR4-mediated  
 changes in gene expression, as well as MAPK and transcription  
 factor activation associated with both MyD88-dependent and  
 495 -independent signalling pathways, were disrupted. All four  
 peptides success-fully disrupted TLR4-mediated responses whilst  
 TLR2 responses were less well inhibited with the exception  
 of a modest effect of the MyD88 peptide on triacylated  
 lipopeptide-induced IL-1β mRNA. The data suggest that the  
 500 adapter BB loops of different MyD88 adaptor proteins may  
 serve distinct roles in TLR4 and TLR2 signalosome assembly.  
 This specificity is likely to determine the effectiveness of  
 developing TIR-based peptides as anti-inflammatory drugs.

#### 505 6.4 Anti-TLR properties of pulmonary antiprotease

Although classically regarded as antiproteases, secretory  
 leukoprotease inhibitor (SLPI), elafin, and alpha-1 antitrypsin  
 (A1AT) also function as antimicrobial agents. Each has  
 509 potential anti-TLR activity.

#### 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, trypsinase  
 515 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  
 520 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  
 525 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  
 530 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 IκBα without affecting phospho-  
 535 rylation 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  
 540 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  
 545 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  
 550 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  
 555 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  
 563

564 mature elafin [134]. There is increasing interest in this anti-  
 565 protease 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  
 570 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 chemoattractant protein 1 (MCP-1)  
 in monocytes. This effect is mediated via impairment of  
 575 both AP-1 and NF- $\kappa$ B activation, via an effect on the  
 ubiquitin-proteasome pathway [136]. Elafin also has anti-  
 fungal properties [137]. Thus, like SLPI, elafin may hold  
 therapeutic promise in the treatment of inflammation and  
 infection in CF.

#### 580 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-  
 585 inflammatory and immunomodulatory effects. For example  
 native, polymerised or oxidised A1AT can inhibit LPS-  
 stimulated synthesis of TNF- $\alpha$  and IL-1 $\beta$  whilst enhancing  
 the release of the anti-inflammatory cytokine IL-10 [138].  
 Whether A1AT has direct anti-TLR4 activity remains to  
 590 be shown, however, C-36 peptide, a degradation product of  
 A1AT, also has potential LPS-modulatory activity against  
 human monocytes [139].

### 595 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  
 600 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  
 605 therapeutic agent for CF should be sterile, nonpyrogenic,  
 chemically stable and have a particle size ranging from 1 to  
 5  $\mu$ m 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  
 610 and should be easily nebulised.

### 615 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.  
 618 Symptomatically, inhaled/nebulised broncho-dilator therapy

provides relief from breathlessness, and mucolytic therapy 619  
 can be very useful in enabling patients to expectorate sputum. 620  
 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 625  
 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 630  
 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 635  
 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- $\alpha$  levels in CF airway epithelial cells [147] and to suppress  
 activation of NF- $\kappa$ B and pro-inflammatory cytokine expression 640  
 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 645  
*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 650  
 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]. 655

#### 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 660  
 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 665  
 similar way.

Dornase alfa, a DNase, has been shown to reduce  
 viscosity of sputum, allowing easier expectoration. Improve-  
 ments in lung function have also been documented [157,158].  
 Viscosity is decreased by enzymatic degradation of host and 670  
 bacterial DNA, with the added advantage that TLR9-  
 mediated signalling in response to uCpG DNA is likely to  
 be abrogated also. 673

### 674 8.3 Delivery of agents to the CF lung

675 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  
 680 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  
 685 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  
 690 hydrolyzing chromosomal DNA released from dead neutro-  
 phils, surprisingly, it does not significantly alter the  
 average particle diffusion rate. This is most probably due an  
 increased microviscosity [160].

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

## 700 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  
 705 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  
 710 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,  
 715 by the use of appropriate inhibitors could reduce the chronic  
 inflammation characteristic of this disease. Thus, new thera-  
 peutics designed to selectively activate or inhibit TLR function  
 specifically and reversibly represent powerful tools for the  
 prevention and treatment of the pulmonary inflammatory  
 720 manifestations of CF.

## 725 10. Expert opinion

TLR-targeted approaches to the management of inflammation  
 and infection in CF differ with respect to bacterial and viral  
 730 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

729 more effective in clearing bacteria. With respect to viral  
 730 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  
 735 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. 740

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  
 745 of inflammation required to confine infection we must be  
 conservative in our immunosuppressive approaches and any  
 new trials must include careful monitoring of sputum organ-  
 isms, inflammatory mediators, lung function and structural  
 changes. Currently we are at a point where we are faced with  
 750 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  
 755 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  
 760 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. 765

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 conven-  
 770 tional 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  
 775 inflammatory part of the cascade whilst retaining the anti-  
 microbial 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  
 780 pulmonary manifestations of CF include effective deposition  
 in poorly aerated areas and protection against degradation  
 and inactivation by serine and cysteinyl cathepsins present 783

784 in the CF airways. These challenges must remain at the  
785 forefront of our scientific and clinical thinking.

Health Research Board, the CF Hope Source, the CF 791  
Association of Ireland and Molecular Medicine Ireland.

## Acknowledgements

790 Cystic fibrosis research in this laboratory is generously funded  
791 by the Programme for Research in Third level Institutes, the

## Declaration of interest

The authors declare no conflicts of interest and have received  
no payment for the production of this manuscript.

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