Toll-Like Receptors in Cystic Fibrosis: Impact of Dysfunctional microRNA on Innate Immune Responses in the Cystic Fibrosis Lung

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Abstract
Toll-like receptors (TLRs) are a class of pattern recognition receptors that are particularly expressed in the sentinel and epithelial cells in the body, including the lung. They are central players in the innate immune system in response to microbial infection, and are the triggers of a complex pathway network that both promotes the inflammatory response and influences the adaptive immune response. These pathways are transiently and finely tuned by cellular factors, including a cell’s microRNA response program. MicroRNAs are small, non-coding RNAs that specifically regulate gene expression. In this article, we review the disease-specific microRNA regulatory network of cystic fibrosis, a debilitating and ultimately fatal disease and, specifically, its effect on TLR signalling.

Introduction
Innate immunity is a rapid, short-lived process that occurs in response to invading micro-organisms. Originally considered non-discriminatory, the innate immune system is actually highly specific. This property is due to pattern recognition receptors (PRRs), a collection of receptors with key roles in innate immunity. Conserved microbial structures are recognised by PRRs. Toll-like receptors (TLRs) are a major subclass of PRRs that can detect and discriminate specific molecular microbial patterns, induce intracellular signalling that leads to the activation of transcription factors that control innate immune responses and they also promote adaptive immunity. How, where and when TLRs are expressed can have a major impact on the effectiveness of an individual’s innate immune response. So mechanisms that control the expression of TLRs and their signalling intermediates fine-tune this process. MicroRNAs (miRNAs) are post-transcriptional regulators of protein expression. In this article, we review the disease-specific microRNA regulatory network of cystic fibrosis, a debilitating and ultimately fatal disease and, specifically, its effect on TLR signalling.

TLRs and TLR Signalling Pathways
TLRs are a family of germ-line encoded transmembrane pattern recognition receptors belonging to the TLR and interleukin (IL)-1/IL-18 receptor superfamily [1]. Unlike the IL-1/IL-18 receptors, which have immuno-
globulin-like domains located extracellularly, TLRs display leucine-rich repeats on the cell surface which are required for ligand recognition. The intracellular domains of this family of proteins are more highly conserved and all carry a critical TLR-IL-1R (TIR) domain, first described by Heguy et al. [2], which facilitates intracellular signal transduction via TIR-TIR interactions between TLRs and specific intracellular adaptor proteins. There are at least 11 TLRs encoded in the human genome, although hTLR11 is truncated due to a premature stop codon mutation and unlike its murine counterpart, mTLR11, cannot transduce signals induced by uropathogenic Escherichia coli [3]. On phagocytic cells such as dendritic cells and macrophages, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are located in the endosome.

All TLRs can recognise and discriminate a specific set of microbial-derived ligands, commonly referred to as pathogen-associated molecular patterns (PAMPs). Engagement of TLRs with these motifs leads to the activation of pro-inflammatory and innate immune gene expression via the activation of the transcription factors NFκB, AP1 and interferon-regulatory factors (IRFs). In addition, TLRs can also be activated by a set of endogenous host factors that act as signals that something is awry – these are the so-called damage- or danger-associated molecular patterns (DAMPs). Table 1 is a list of common PAMPs and DAMPs.

The immediate first step that occurs following activation of any TLR by its cognate ligand is the association of that TLR’s TIR domain with the TIR domain in the adaptor proteins MyD88, TIRAP (also called MAL), TRIF (or TICAM-1) or TRAM (or TICAM-2). Apart from TLR3, all TLRs can signal directly via MyD88. TLR3 can engage directly with TRIF, as can TLR4 indirectly via TRAM; TIRAP can be utilised by TLR2 and TLR4 to engage with MyD88. A fifth, related protein exists, called SARM. This is a TIR domain-containing protein that acts as a negative regulator of TRIF-dependent TLR signalling [4].

The 2 major intracellular pathways that are activated by TLRs are referred to as the MyD88-dependent and TRIF-dependent pathways (fig. 1; reviewed by Greene [1]). Engagement of a TLR with MyD88 leads to the activation of mitogen-activated protein kinases (MAPKs) and the transcription factor NFκB, via an intracellular signalling cascade involving the sequential activation of IL-1 receptor-associated kinase 4 (IRAK4), IRAK1 and IRAK2, and the E3 ubiquitin ligase TRAF6. TRAF6 and

| TLR | PAMP | DAMP |
|-----|------|------|
| TLR1 | triacyl lipopeptides, lipoproteins | | |
| TLR2 | lipoproteins, lipoteichoic acid, peptidoglycan, GPI anchors, lipourabinomannan, phenol-soluble modulin, zymosan, glycolipids | eosinophil-derived neurotoxin, HMGB1, HSP60, HSP70, versican, hyaluronan, heparan sulphate |
| TLR3 | dsRNA (virus) | dsRNA (necrotic cells) |
| TLR4 | LPS, envelope proteins, mannans, protozoan glycoprophospholipid inositol, pneumolysin, Chlamydia pneumoniae Hsp60, flavolipin, murine retroviruses and RSV fusion protein, taxol, paclitaxel | HSP60, HSP70, fibronectin, hyaluronan, heparan sulphate, fibrinogen, S100 protein |
| TLR5 | flagellin | | |
| TLR6 | diacetylated lipopeptides | | |
| TLR7 and TLR8 | viral ssRNA, imidazolquinolines | | |
| TLR9 | CpG motifs, dsDNA, hemozoin | Self-nucleic acids complexed to auto-antibodies HMGB1 or LL-37 |
| TLR10 | orphan receptor | | |
| mTLR11 | surface-exposed factor on uropathogenic E. coli, protozoan profilin | | |
IRAK1 undergo lysine63-linked ubiquitination via the ubiquitin-conjugating enzymes Ubc13 and Uev1. Next, the regulatory proteins TAB2 and TAB3 bind leading to activation of TAK1, the kinase that phosphorylates and activates the β subunit of the IKK complex. Nuclear localisation sequences on NFκB become exposed as a result of the phosphorylation, ubiquitination and degradation of IκB proteins by the proteasome. Translocation of the NFκB dimer into the nucleus facilitates the binding of this transcription factor to its consensus sequences in gene promoters followed by their transactivation. The MAPKs ERK1, ERK2, p38 and JNK, which can induce AP1 activation and influence translation, are phosphorylated by TAK1. Finally, IRFs 5 and 7 can be activated by TRAF6 leading to the induction of proinflammatory cytokines and type I interferons, respectively.

The TRIF pathway (also frequently referred to as the MyD88-independent signalling pathway) commonly leads to activation of the IRF transcription factors and upregulation of type I interferons. TRAF3 is activated by TRIF and the signal is transduced via IKKi and TBK1, culminating in the phosphorylation and activation of IRF3. TRIF can also activate NFκB either via TRADD, Pellino-1 and RIP1 or via TRAF6, leading to NFκB and MAPK activation as described above.
**TLRs in the CF Lung**

The first reports describing TLR expression in primary and transformed CF airway epithelial cells provided evidence that TLRs 1–10 and MyD88 are expressed and functional in CF airway epithelia [5–7]. These findings complemented previous work performed in non-CF bronchial epithelial cells, which showed that miRNAs for all TLRs are expressed by tracheal, bronchial and alveolar type II airway epithelial cells and that the receptors are exposed on the mucosal surface of the airway, presumably in order to facilitate activation by exposure to PAMPs. TLR2 is the predominant TLR expressed on the surface of bronchial epithelial cells in vivo, whereas TLR3, TLR4 and TLR5 reside mainly intracellularly or display only low-level surface expression [7–9]. Interestingly, whilst TLR9 resides in endosomes in phagocytic cells, it has been shown to be expressed on the cell surface of primary and transformed differentiated airway epithelial cells. Of note, stimulation of airway epithelial cells with microbial factors such as respiratory syncytial virus, flagellin and *Pseudomonas aeruginosa* can enhance the expression of TLR2, TLR4 and TLR5 on the epithelial cell surface, respectively [9–11]; this has relevance for the CF lung when considering the molecular effects of viral and bacterial infective exacerbations. People with CF are frequently colonised with fungal species such as *Aspergillus*. Whether conidial dsRNA can induce interferon-β via activation of TLRs in CF airway epithelial cells, as has been demonstrated in non-CF cells, is not known [12]. Alternatively, the mechanism involved may be similar to what occurs in murine macrophages that display chitin-induced activation of TLR2 [13].

There have been other reports on TLR expression and function in CF airway epithelium. For example, John et al. [14, 15] reported that TLR4 is displayed at very low levels on the apical surface of CF airway epithelial cells in vivo; unsurprisingly, the cells fail to respond strongly to LPS stimulation and consequently have weaker than normal MyD88- and TRIF-dependent signalling. TLR5 mediates inflammatory responses to flagellin-expressing *Burkholderia cepacia*, *B. cenocepacia* and *P. aeruginosa*. In CF airway epithelial cells, inhibition of TLR5 abolishes *P. aeruginosa*-induced IL-6 production, and CF adults with a dysfunctional TLR5 1174C>T SNP have an improved body mass index [16, 17].

Due to microbial infection, the CF lung is a PAMP-rich milieu. Ongoing inflammation and protease-mediated damage generates DAMPs. In addition to these classical TLR agonists, the CF lung also contains a selection of factors that can indirectly activate TLRs. For example, heme, which may be present in the lumen of the CF lung due to cleavage of hemoglobin in microbleeds by neutrophil elastase and *Pseudomonas*-derived proteases, can activate TLR signalling and pro-inflammatory gene expression via EGFR [18]. Neutrophil elastase itself can also indirectly activate TLR4 via a mechanism involving meprin and/or TACE, TGFα and EGFR. This has been shown to lead to exaggerated IL-8 and mucin gene expression [19, 20].

**miRNAs and TLR Signalling in the Lung**

Several miRNAs target key points along the TLR signalling pathways, effecting a large degree of epigenetic control. These relationships between particular miRNAs and TLR pathway members have been characterised in resident white blood cells, in pulmonary tissue/cell lines, or both. The TLR pathways themselves also transcriptionally regulate miRNAs, in some instances forming feedback loops. The synergistic action between TLR signalling and miRNA regulation has previously been extensively reviewed [21, 22]. It is thought that miRNAs control the TLR pathways in response to inflammatory stimuli, regulating their strength and timing during the inflammatory response. Furthermore, miRNAs can control the cellular location of the pathway elements and enable cross talk with other physiological pathways [21, 22]. Most miRNAs are upregulated by the activation of TLR signalling, suggesting that those that form feedback loops can protect against hyperstimulation of TLRs and aid inflammatory resolution [21, 22]. However, the role here of miRNAs is selective and timed, as targeting by TLR pathway-miRNAs can have both pro- and anti-inflammatory properties.

miRNAs have many roles in the development and maintenance of the lung and its immune system. Several of these also have been linked to TLR signalling pathways. miR-21 is strongly upregulated in mouse lung upon exposure to *E. coli* LPS and in allergic airway inflammation [23, 24]. This has also been found in other tissues and cell lines exposed to various inflammatory stimuli, and it was discovered that miR-21 can be induced through TLR4/MyD88 activation of NFκB or AP1 while also negatively regulating TLR signalling by inhibiting MyD88 and IRAK1 [25–27]. Although not yet specifically characterised as such, it is likely that these feedback loops between miR-21 and TLR signalling pathways also exist in airway leukocytes and epithelial cells.
miR-155, the most commonly described miRNA in the innate immune system, is upregulated in mouse lungs and lung epithelial cells upon inflammatory and mechanical insult [28–31]. Although not fully explored in the lungs, miR-155 is likely induced by NFκB and inhibited by STAT3 transcription factors through several TLRs (TLR2, TLR3, TLR4 and TLR9) and cytokines (notably inhibited by anti-inflammatory IL-10) [22, 32, 33]. Generally considered a pro-inflammatory miRNA, miR-155 can directly target the negative regulators of TLR4 signalling, SHIP1 and SOCS1, an observation also documented in the lung and bronchial epithelium [29, 31]. However, miR-155 can also suppress TLR signalling at key points of convergence along the pathways, indicating opposing functions. These include the targeting of the pro-inflammatory signal transducers IKK, IκBα, MyD88 and TAB2 [34–37].

miR-146a is a highly documented anti-inflammatory miRNA with several critical targets in TLR signalling pathways [38]. It is highly expressed in the lung and is further induced in lung macrophages, epithelial cells and basal tissues upon their exposure to inflammmagens such as LPS and pro-inflammatory cytokines, but also injury [39–44]. miR-146a induction can be mediated through TLR signalling (specifically through TLR2, TLR3, TLR4 and TLR5) and is directly induced by NFκB [21, 22, 45]. In the lung, miR-146a has been found to ameliorate the inflammatory response by directly targeting critical pro-inflammatory members of TLR signalling, including IRAK1 and TRAF6 [40, 44, 45]. The TLR signalling targets of miR-146a validated in other tissues and cell lines include TLR4 and IRAK2, making them likely additional targets in the lung [46].

miR-21, miR-155 and miR-146a are the best-characterised miRNAs involved in TLR signalling; however, in recent years, many more miRNAs have been linked with these pathways. Table 2 contains a list of miRNAs discovered to be involved in TLR signalling in the lung.

### miRNAs and CF

Since our publication showing a role for miRNAs in CF for the first time, several miRNAs have been found to be pathologically dysregulated in the CF lung compared to non-CF lungs [47]. miRNA dysregulation in CF has recently been extensively reviewed by McKiernan and Greene [48] and Sonneville et al. [49]. Numerous deregulated miRNAs have now been identified to be specifically deregulated in the lungs of people with CF, with predict-

| miRNA | Target molecules | Reference(s) |
|-------|-----------------|--------------|
| miR-155 | SHIP1 SOCS1 | [29, 58] [31] |
| miR-21 | IL-12 PDCD4 | [23, 24] [59] |
| miR-146a | IRAK1 TRAF6 IRF5 | [40, 44, 45] [40, 44, 45, 60] [60] |
| miR-132 | ACHE FOXO3 | [61] [33] |
| miR-19a | STAT3 SOCS1 | [62] [63] |
| miR-203 | TAK1 | [64] |
| miR-212 | ACHE | [65] |
| miR-29c | TNFAIP3 | [66] |
| miR-199a-5p | CAV1 | [55] |
| miR-17 | IL-8 STAT3 | [51] [68] |
| miR-20a | STAT3 | [68] |
| miR-106b | STAT3 | [68] |
| miR-221 | TNFAIP3 | [69] |
| miR-365 | IL-6 | [70] |
| miR-106a | IL-10 | [71] |
| miR-15a | TLR1 | [72] |
| miR-16 | TLR1 | [72] |
| miR-124 | TLR6 MyD88 TRAF6 TNFα | [73] [73] [73] |
| miR-302 | IRAK4 | [74] |
| miR-7 | PIK3R3 | [75] |
| miR-126 | TOM1 | [47] |
| miR-31 | IRF-1 | [53] |
| miR-93 | IL-8 | [52] |

CF-specific miRNA-target interactions and related studies are in bold type. Instead of inhibition, miR-29c protects TNFAIP3 from degradation.
A number of dysregulated miRNAs functionally affect the innate immune system in the CF lung. Only a few publications address miRNA regulation of, or by, TLR signalling pathways in CF specifically. Nonetheless, recent discoveries have begun to address the complex role of miRNAs in the innate immune system of the CF lung. The first discovery of pathologial miRNA dysregulation in CF involved the targeting of TOM1, which was upregulated in CF bronchial brushings, by miR-126, which was downregulated [47]. TOM1 is a negative regulator of TLR2, TLR4 and IL-1R1 signalling, and its chronic upregulation in the CF lung is suggested to be a compensatory measure to limit runaway inflammation. In separate studies, miR-17 and miR-93, both of which are downregulated in CF bronchial epithelia, have been found to target IL-8 [51, 52]. IL-8, the major neutrophil chemotactic factor, is the most important pro-inflammatory chemokine in the CF lung and is highly upregulated in the CF lung environment. In both studies, miR-17 and miR-93 were found to be downregulated by *Pseudomonas*-induced infection in bronchial epithelial cell lines, contributing to IL-8 overexpression. This mechanism could potentially be expanded to include a wider gamut of chronic infectious diseases.

Weldon et al. [53] found a significant increase of cathepsin S in the bronchoaveolar lavage fluid of CF patients over that from non-CF lungs. Together with neutrophil elastase, this potent protease and pro-inflammatory mediator is thought to be an important exacerbator of lung tissue destruction and inflammation. They also found that miR-31, which is decreased in CF bronchial brushings, inhibits the transcription of cathepsin S by targeting its transcription factor IRF1. IRF1 is frequently part of TLR signalling as it can be activated directly downstream from TLR2 [54]. However, in this study, cathepsin S transcription was not found to be dependent on *P. aeruginosa* infection [53]. Although not further investigated in this study, TLR2 activation and subsequent cathepsin S transcription by IRF1 could still potentially exist through infection by *Staphylococcus aureus*, a potent trigger of TLR2 signalling and a common resident pathogen in CF lungs [54].

Zhang et al. [55] recently discovered the aberrant underexpression of caveolin 1/CAV1 by miR-199a-5p overexpression in CF macrophages and lung tissue. CAV1 is a negative regulator of TLR4 signalling and is normally upregulated upon LPS-induced activation of TLR4, which is facilitated through the concurrent downregulation of miR-199a-5p. In CF macrophages, a failure of miR-199a-5p to downregulate in response to LPS stimulation results in the persistent underexpression of CAV1 and consequent TLR4 hyperstimulation. The authors found that this is caused by aberrant PI3K-AKT signalling unique to CFTR-deficient macrophages, and possibly lung tissue. They further postulated that the lack of signalling functions by CFTR, otherwise present in non-CF macrophages, contribute to this. They have also shown that dysfunctional regulation of miRNA expression, directly linked to mutant CFTR, can contribute to the symptomatic manifestation of this disease, opening up additional routes for therapy. Likewise, the CFTR mutation can upset other TLR-related, self-regulatory effector miRNAs. Bhattacharyya et al. [56] have shown that the crucial feedback effector of TLR signalling, miR-155, is elevated in CFTR-mutant bronchial epithelial cells compared to in CFTR-corrected cells. They provide evidence that the unique underexpression of the ARE-mRNA destabilising protein, TTP, in CF cells, results in the chronic stabilisation and thus overexpression of pro-inflammatory miR-155.

These are a few important examples of miRNA regulation of TLR signalling in CF. The miRNA-target relationships that form part of the TLR pathways in CF are highlighted in table 2 (in bold type) and in fig. 1.

The number of miRNAs implicated with TLR signalling in CF is increasing. This opens up avenues for targeted therapies, possibly in the form of miRNA modulators such as mimics or miRNA inhibitors. Upon delivery to the lung, these miRNA modulators could suppress or activate the desired elements of the TLR pathways. However, due to the dual nature of many miRNAs as both pro-inflammatory and anti-inflammatory agonists, the risk of
unfavorable pathway activation should be taken into account. Nonetheless, miRNA modulation of TLR signaling may be an attractive anti-inflammatory option, and the first attempts at this have already been made [57].

**Concluding Remarks**

TLR signaling is a critical component of host defence, but its dysregulation can be harmful in chronic inflammatory diseases such as CF. The concerted effort to elucidate the TLR network has been given a new dimension with the discovery of the miRNA regulation of TLR signaling. Both advantageous and disadvantageous effects of miRNAs on TLR pathways in CF have been explored only recently. Their further investigation will provide new insights into TLR signaling as well as the potential for developing novel diagnostics and therapeutics.

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