Toll-like receptors and chronic lung disease

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ABSTRACT

TLRs (Toll-like receptors) comprise a family of proteins whose function is principally to facilitate the detection of, and response to, pathogens. Protozoa, helminths, viruses, bacteria and fungi can all activate TLR signalling, and these signals have important roles in the activation of host defence. TLRs may also respond to products of tissue damage, providing them with roles in infective and sterile inflammation. Their role as detectors of pathogens and pathogen-associated molecules provides molecular mechanisms to underpin the observations leading to the hygiene hypothesis. Targeting of TLR signalling has implications in the control of infection, vaccine design, desensitization to allergens and down-regulation of inflammation. This review will explore TLR history, molecular signalling and the potential roles of TLRs in chronic lung disease.

INTRODUCTION

Inflammation underpins a majority of disease processes. Adaptive immunity has dominated research in lung disease, but a recent appreciation of the importance of innate immunity is leading to new areas of research and the development of novel therapeutic targets for the treatment of inflammatory diseases. This is perhaps best illustrated by the explosion of research into a recently identified family of proteins, the TLRs (Toll-like receptors). TLRs possess pivotal roles in innate and adaptive immunity, and targeting of their signalling provides substantial new opportunities to intervene in lung disease.

The principal role of TLRs appears to be induction of immune responses to a broad range of pathogens (including viruses, helminths, bacteria, protozoa and fungi). Additionally, there is evidence that antimicrobial molecules [ROS (reactive oxygen species)] and products of cell damage and death can activate TLRs. Their activation is crucial to innate immunity and for an effective presentation of antigens to the adaptive system. Thus TLRs are implicated in immunity, formation of immunological memory and polarization of antigen responses, and responses to tissue damage. TLRs are likely to play important roles in the aetiology of inflammatory lung diseases ranging from ARDS (acute respiratory distress syndrome) to asthma and COPD (chronic obstructive pulmonary disease), and new molecules targeting these receptors are already in trials. This review will outline our current knowledge of TLRs and signalling, their potential roles in the development of chronic lung diseases and their use as novel therapeutic targets. The information presented here complements many recent excellent reviews in TLR biology and disease, some of which are noted for the reader here [1–12]; of these, Basu and Fenton [3] also examine the roles of TLRs in lung disease.

TLRs: A BRIEF HISTORY

Work in Drosophila opened the door to our understanding of the roles of Toll in innate immunity. It is striking that crucial signalling systems activating innate immunity are preserved across evolution from species...
such as *Drosophila*; indeed, proteins containing leucine-rich repeats, the motifs found in the extracellular face of TLRs mediating their interactions with their agonists, are also found extensively in plants. Toll in *Drosophila* is involved in establishing the dorsal ventral axis in embryogenesis, and flies with conditional mutations in Toll (expressed only in the adult, allowing normal fly development) exhibit reduced survival against fungal infections [13]. A mammalian family of proteins with homology with Toll was identified (the TRLs; now comprising ten principal members) and, in 1997, it was shown that these putative receptors had the potential to activate the signalling pathways of adaptive immunity [14]. The crucial link to mammalian immunity came through the study of naturally occurring mouse strains that responded poorly to endotoxin, when painstaking genetic analysis revealed that these mice had mutations in TLR4 [15,16]. These studies identified TLR4 as the signalling protein for LPS (lipopolysaccharide), a key component of the Gram-negative bacterial cell wall, and placed this receptor family at the heart of innate immunity.

**PATHOGEN-ASSOCIATED TLR AGONISTS**

To date ten TLRs have been described (an eleventh appears to be functional in mice but not man). Numerous studies, often based on studies of TLR-knockout mice, have now identified a wide variety of diverse endogenous (i.e. originating from the host) and exogenous, pathogen-derived, products that serve as putative ligands for the TLR proteins. These studies are complicated by a relative paucity of pharmacological data confirming direct interactions between agonists and receptors [17]. Even where direct interactions between the TLR and the agonist have been demonstrated {e.g. for PGN (peptidoglycan) binding to TLR2 [18]}, it may be that these interactions are not responsible for signalling, since recent work suggests that TLR2 activation by PGN may be mediated not by the PGN, but by contaminating LTAs (lipoteichoic acids) [19]. Contamination of biological preparations has resulted in further confusion, as exemplified by studies of heat shock proteins (preparations contaminated with LPS may show artefactual signalling via TLR4 [20,21]) and LPS itself (contamination with lipopeptides accounts for apparent actions of most LPSs via TLR2 [22]). A broad summary of TLR agonists is shown in Figure 1.

TLR2 and TLR4 are the principal receptors involved in the recognition of various bacterial cell wall components. TLR4 is crucial for effective responses to Gram-negative LPS [22]. Delivery of LPS to TLR4 requires the accessory proteins LBP (LPS-binding protein; found in serum), CD14 and MD-2 (the latter two proteins can exist in soluble form, or bound to the membrane or TLR itself). Additional cell-surface molecules, such as the integrin CD11b/CD18, may also facilitate responses to LPS [23].

TLR4 has also been linked to responses to pneumolysin, a major virulence factor of *Streptococcus pneumoniae* [24], and proteins of respiratory syncytial virus [25].

TLR2 mediates responses to lipoproteins and LTA from Gram-positive bacteria (e.g. *S. pneumoniae*) and mycobacteria [26,27], and to some rare LPS species such as that from *Porphyromonas gingivalis* [28]. TLR2 has also been shown to have a role in the immune response to lipoproteins from a wide range of organisms, including *Borrelia burgdorferi*, *Treponema pallidum*, *Aspergillus fumigatus* and *Mycoplasma fermentes*. The range of agonists to which TLR2 responds appears to be broadened by heterodimerization, so that TLR1/2 heterodimers respond to a subtly different panel of lipoproteins to TLR2/6 heterodimers.

TLR2 and TLR4 are both involved in recognition of helminth-derived molecules, and it is interesting that the helminths may also suppress signalling via TLRs [29,30], presumably with some survival advantage for the parasite.

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**Figure 1** The potential of TLRs to influence respiratory disease

TLRs have a principal role in the detection of pathogens. Their activation influences processes of adaptive and innate immunity, regulating immediate response and development of immunological memory. TLRs have profound influences on activation of cells of the innate immune system, such as monocytes, macrophages and neutrophils; additionally, they are expressed on epithelia and endothelia. Furthermore, some TLRs may mediate responses to tissue damage in the absence of infection. Thus TLRs occupy a central position in inflammatory responses, with the potential to contribute to a diverse range of respiratory diseases. Reprinted from Encyclopaedia of Respiratory Medicine (Laurent, G. and Shapiro, S., eds), Sabroe, I., Dower, S. K. and Whyte, M. K. B., Toll-like receptors, in the press, © (2005), with permission from Elsevier.
TLR9 is also of critical importance to respiratory disease. It mediates responses to bacterial DNA, through the recognition of cytosine–guanine pairs in the bacterial DNA (‘CpG’ motifs) [31]. TLR9 is also activated by Herpes viruses [32,33] and Aspergillus [34]. Its importance in respiratory disease lies in the immense power of TLR9 to alter Th1/Th2 responses to antigens (see below). TLRs 3, 7, and 8 appear to play important roles in responses to viruses. TLR3 responds to double-stranded viral RNA [35], and TLRs 7 and 8 mediate responses to single-stranded RNAs [36,37], and potent immunomodulatory nucleoside or nucleoside-like drugs, such as the imidazoquinolines and loxoribine [38,39].

ENDOGENOUS TLR AGONISTS

In addition to their abilities to detect pathogens, TLRs (principally TLR4 but also where noted TLR2) are also apparently able to mediate responses to host molecules, including antimicrobial molecules (defensins [40], ROS [41]: TLR2 response), proteins released from dead or dying cells (HMGB1 [high-mobility group box 1] [42]: TLR2 and TLR4 response), surfactant protein A [43], fibrinogen [44] and breakdown products of tissue matrix, such as fragments of fibronectin [45] and hyaluronic acid oligosaccharides [46]. It is not clear how one receptor manages to facilitate responses to so many agonists. Heterodimerization may help to broaden the range of agonists recognized by TLRs [47,48], but, particularly in the case of TLR2 and TLR4, agonists causing activation of the same receptor often share very little structural similarity. A number of mechanisms by which this can occur have been postulated. One possibility is that accessory molecules/receptors, such as MD-2, CD14 and potentially other unidentified proteins, play a role by recognizing these ligands and facilitating activation of the TLR, and TLRs certainly assemble in large activation complexes in the membrane in close association with a number of other proteins [12,17,49]. It may even be that TLRs act as integrators of signalling, activated in signalling complexes without direct interaction with a ligand [17]. However, some caution is needed, given the potential for small amounts of contaminants such as LPS to cause an artefact when studying specific effects of putative agonists. Thus the final lists of molecules that truly require TLRs to signal, and the mechanisms of pharmacological interaction with the TLR, remain unknown.

SIGNALLING OF TLRs

TLR signalling has been extensively reviewed elsewhere [1,7,11,12]. Like Drosophila Toll, TLRs consist of an extracellular domain comprising a series of leucine-rich repeats (thought to be involved in ligand recognition, although as noted above there is still little pharmacological data defining these interactions), and a conserved region of approx. 200 amino acids in their cytoplasmic portion. Within the cytoplasmic component, a well-characterized region shares sequence and functional homology with the IL-1R (IL (interleukin)-1 receptor), providing regions crucial to signalling, and is thus named the TIR (Toll/IL-1R) domain [50] (Figure 2). It is in this crucial domain that the point mutant of the C3H/HeJ mouse that prevents TLR4 signalling is found; and it is on this sequence mutation that many
dominant-negative TLR signalling inhibitors are based, providing important research tools for unravelling the complexity of TLR signalling [51]. The TIR domain serves as a scaffold for a series of protein–protein interactions in which association with adapter proteins results in a complex cascade of interactions, and ultimate activation of downstream signalling pathways, including MAPKs (mitogen-activated protein kinases), PI3K (phosphoinositide 3-kinase) and NF-κB (nuclear factor κB) [1]. A family of five adapters couples variously to the TLRs, providing opportunities for tailored responses to specific pathogens [1]. The biggest divergence in signalling between TLRs is exemplified by those receptors that activate IRF3 (IFN (interferon) regulatory factor 3) and the induction of IFN-β generation, followed by a further phase of IFN-dependent gene expression; and those receptors that do not activate this pathway. This generation of type 1 IFNs is a feature of TLR3 and TLR4, for example, but not TLR2 [52], and is largely driven by the use of the adapter TRIF (TIR domain-containing adaptor inducing IFN-β) by TLR3 and TLR4 [1,17]. To complicate things further, activation of antiviral genes that are normally dependent upon the generation of IFNs can be independent of type 1 IFN generation in the neutrophil [53]. Additionally, adapter use may be cell-type dependent, and may vary between species, as recent studies have shown that dominant-negative adapters do not always produce the anticipated loss of signalling in primary human macrophages [54]. Selective signalling in response to specific pathogens is also likely to be affected by usage of coreceptors (CD14, CD11b/CD18 and dectin-1 [12,17,23,55,56]) and the pharmacological interactions of agonist and receptor, although many of these mechanisms remain to be elucidated.

**TLRs AND THE LUNG**

Respiratory epithelial cells lie at the interface between host and environment and represent the initial site of bacterial colonization in the respiratory tract. Airway and alveolar type II epithelial cells express TLRs [57–61], and airway epithelial cells can up-regulate TLR expression to their apical surface under conditions of infection [62]. Engagement of TLRs on these cells results in induction of antimicrobial responses [59]. Surfactant proteins are also heavily implicated in host defence, and SP-A (surfactant protein A) can modulate signalling via TLRs 2 and 4 [43,63]. The alveolar macrophages also provide a crucial early warning and response system for infections and, as would be expected given their roles, express functional TLRs [26,64–66]. These macrophages serve critical roles in the recognition of inhaled LPS and initiation of an inflammatory reaction [67]. Monocyte-dependent TLR-driven responses can result in the activation of airway smooth muscle [68,69] and neutrophils [70–72]; infiltrating neutrophils express TLRs and mediate antimicrobial signalling through their activation [71–75]. TLRs are expressed by endothelium [76] and fibroblasts, vascular smooth muscle [77], memory T-cells [78], regulatory T-cells [79], mast cells [80] and dendritic cells [81]. Thus TLRs are placed, by nature of their potent pro-inflammatory signalling and wide expression, to play central roles in pulmonary inflammatory disease. Interestingly, the role of macrophages may dominate in response to inhaled endotoxin [67], whereas tissue (probably endothelial) TLR4 may be more important than leucocyte TLR4 when endotoxin is presented systemically [76].

**TLRs, ALLERGEN SENSITIZATION AND AIRWAY RESPONSIVENESS**

Strands of evidence from epidemiological studies, in vitro biology and mouse models of allergic airways inflammation have come together to place TLRs at the heart of the processes controlling sensitization. Polymorphisms in TLR2 appear to influence risk of developing asthma in cohorts with high endotoxin exposure [82], although there is less evidence at the genetic level for a role of TLR4 in regulating asthma risk [82,83]. A polymorphism in TLR10 appears to show association with asthma risk, although the agonists (endogenous or pathogen-related) for this receptor are unknown, so it is harder to speculate on the mechanism in this case [84]. The dendritic cell plays a crucial role in regulating the Th1/Th2 bias of the subsequent T-cell response [85], and it is here that TLR agonists have a dramatic effect. Presentation of allergen to DCs (dendritic cells), and thence to T-cells, together with agonists of TLR9, drives a potent Th1-type cytokine response [86], and the strong establishment of Th1-type memory T-cells. Activation of plasmacytoid DCs by TLR9 agonists also strongly induces production of regulatory T-cells [87]; TLR9 also activates DC-independent pathways resulting in the local production of IDO (indoleamine 2,3-dioxygenase) in the lung, suppressing T-cell activity [88]. Thus administration of allergen complexed to TLR9 agonists may provide a potent mechanism of desensitizing established atopic responses, and administration of immunostimulatory CpG-containing motifs may, on their own, directly inhibit allergic responses [89].

Likewise, other TLR agonists have profound actions as adjuvants and immunomodulators. TLR4 activation by LPS can favour both Th1- and Th2-type responses to allergen, depending upon the dose of LPS [90–94]. More surprisingly, signalling via TLR2, which given its role in bacterial and mycobacterial infections might be predicted to activate Th1 type immunity, seems to favour Th2 responses [90,91,95]. TLR2 itself is also expressed on memory T-cells, and its activation enhances their production of IFN-γ [78].
TLR agonists are by their nature pro-inflammatory, and there has been concern that the use of these to modulate allergic inflammation may be associated with aggravation of disease. In keeping with this possibility, immunostimulatory DNA can cause airway inflammation [96], levels of exposure to endotoxin in early life can correlate with risk of wheezing [97] (although endotoxin exposure in other studies of older children is protective compared with atopy [98]), asthma severity may be more dependent upon environmental levels of LPS than levels of allergen [99], and TLR4 activation may play a role in ozone-induced airway hyper-reactivity [100]. Furthermore, activation of DCs by TLR agonists results in release of IL-6 which can overcome endogenous suppression of T-cell proliferation by regulatory T-cell populations [101]. These data suggest caution may need to be employed before the use of therapeutics targeting these axes in man; however, on the other hand, most recent data point to the fact that TLR agonists can probably be used safely [89,102], and new therapeutic TLR agonists are being developed that may lack some of the potentially unwanted pro-inflammatory responses [2,103].

**TLRs AND COPD**

Exposure to ozone is associated with a TLR4-dependent induction of airways hyper-reactivity and TLR4 may be a susceptibility locus for this process [100,104]; however, we found no evidence for an association between a common TLR4 polymorphism and severity of COPD [105]. Nonetheless, the extensive expression of TLRs in many cells in the lung, including infiltrating monocytes and neutrophils that are thought to play pivotal roles in disease pathology, suggests that TLR activation is likely to contribute to the development of COPD. Activation of monocytes and macrophages by LPS stimulates release of matrix metalloproteinases that probably play major roles in release of IL-6 which can overcome endogenous suppression of T-cell proliferation by regulatory T-cell populations [101]. These data suggest caution may need to be employed before the use of therapeutics targeting these axes in man; however, on the other hand, most recent data point to the fact that TLR agonists can probably be used safely [89,102], and new therapeutic TLR agonists are being developed that may lack some of the potentially unwanted pro-inflammatory responses [2,103].

**TLRs AND PULMONARY INFECTION**

Although beyond the scope of this review, it is appropriate to note that TLR signalling is likely to play an important part in host defence to acute infections with prokaryotes, fungi and viruses. Humans with genetic mutations in TLR2 and TLR4 probably show increased susceptibility to infection [108–110]. Interestingly, deletion of an individual TLR does not necessarily increase mortality in pneumonia models, as seen in a recent study of the role of TLR2 in pneumococcal pneumonia [26]. These data probably reflect activation of other multiple antimicrobial response systems by bacterial infection and suggest that therapeutic antagonism of TLRs in the future may not be associated with an excessively severe immune paresis.

TLR2 and TLR4 have been shown to have distinct roles in the immune response to tuberculosis (reviewed in [6]). Human polymorphisms in TLR2 increase susceptibility to leprosy, and possibly tuberculosis [111]. TLR2- and TLR4-knockout mice can show impaired clearance of tuberculosis and increased mortality [112,113], and deficiency in a key TLR and IL-1R signalling adapter, MyD88, is associated with a profound defect in the innate immune response to tuberculosis [114]. These findings are not ubiquitous; studies of knockout mice exposed to BCG have both supported roles for TLRs 2 and 4 in control of infection [115,116] and refuted roles for TLRs 2, 4 and 6 [117].

Strikingly, the TB (tuberculosis) bacillus may use TLR2 to actually further its own survival. A 19 kDa M. tuberculosis protein impairs macrophage IFN-γ signalling and antigen presentation [118,119]; however, this protein also induces macrophage apoptosis (cell death), which appears to reduce intracellular survival of M. tuberculosis, suggesting it may also activate host defence pathways [120]. Overall, a role for TLRs in lung protection from TB seems to be the prime principal. TLR agonists are showing promise as immunomodulators, providing non-specific enhancement of immunity [2], and thus may boost anti-TB immune responses. However, TB infection often co-exists with HIV, and TLR agonists can drive HIV replication [121–123]. Thus mechanisms also exist for TB infection to speed progression of HIV infection and, potentially, therapeutic TLR agonists could enhance this process, so caution is still needed.

**TLRs AND CHRONIC LUNG DISEASE:** IN CONCLUSION

The broad potential for TLRs to be implicated in infective and non-infective inflammatory responses in the lung suggests that the study of other pulmonary disease processes will also implicate TLRs in their pathology. How diverse will such involvements be? Genetic evidence
suggests that TLRs may play roles in risks of rejection of lung transplants [124]. Roles in interstitial lung disease, cystic fibrosis and bronchiectasis are all plausible. Vasculitis is associated with activation of neutrophils and endothelia and the formation of granulomata; triggering of exacerbations of Wegener’s granulomatosis by Staphylococcus aureus infections and the intense involvement of the innate immune system suggest a role for TLRs in these diseases as well. Furthermore, the use of TLR agonists as effective adjuvants to boost the efficacy of cancer vaccines may lead to a role for these molecules in the treatment of lung cancer.

What form might drugs take that act on TLRs? Agonists of these signalling pathways are likely to be clinically useful in desensitization, by rebiasing the immune system towards Th1 responses. Their adjuvant effects may improve vaccination efficacy in infectious diseases and anticancer strategies. Non-specific up-regulation of innate immune responses may allow these drugs to provide protection against a range of infections; specific up-regulation of TLR signalling may aid clearing of chronic infections such as TB. Disadvantages of these drugs may include chronic unwanted driving of inflammation, leading to further disease and induction of apoptosis in bystander cells; reassuringly, fears of induction of lupus-like syndromes or inflammatory disease have not been supported in experimental work to date. TLR antagonists may down-regulate overexuberant inflammation, favouring healing and resolution in asthma, COPD, ARDS and many other inflammatory pathologies. Genetic defects in TLR signalling are associated with reduced risks of atherosclerosis [108], for instance, and parallel examples in pulmonary disease are likely. Unwanted immunosuppression may not be as great as with drugs that affect both the innate and adaptive immune system (e.g. steroids), and some animal models are reassuring that immunosuppression may not be as great as with drugs such as azathioprine [29], accompanied by global failure of the immune system.

Overall, TLRs provide a fascinating and rapidly expanding area of research; it is likely that therapies that target these receptors and their signalling will be of use in human disease.

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