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Review

Toll-like receptors in the host defense against *Pseudomonas aeruginosa* respiratory infection and cystic fibrosis

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ABSTRACT

TLRs function in innate immunity by detecting conserved structures present in bacteria, viruses, and fungi. Although TLRs do not necessarily distinguish pathogenic organisms from commensals, in the context of compromised innate immunity and combined with pathogens' effector molecules, TLRs drive the host response to the organism. This review will discuss the evidence and role(s) of TLRs in the response to the opportunistic bacterial pathogen, *Pseudomonas aeruginosa*, as it relates to respiratory infection and CF, in which innate immune mechanisms are indeed compromised. Outer membrane lipoproteins, LPS, flagellin, and nucleic acids all serve as ligands for TLR2, -4, -5, and -9, respectively. These TLRs and their respective downstream effector molecules have proven critical to the host response to *P. aeruginosa*, although the protective effects of TLRs may be impaired and in some cases, enhanced in the CF patient, contributing to the particular susceptibility of individuals with this disease to *P. aeruginosa* infection. *J. Leukoc. Biol.* 92: 977–985; 2012.

Introduction

PRRs play an important role in innate immune defense by binding conserved molecular patterns on microorganisms and initiating appropriate responses. TLRs represent a highly conserved class of PRRs [1]. These are glycoprotein receptors consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular signaling portion [2]. A total of 13 TLRs has been identified in mice and humans, and TLR1–9 are conserved in both species [3]. Whereas most TLRs are thought to act as monomers, TLR2 also functions as a heterodimer, with TLR1 or -6 broadening the ligand specificity to include peptidoglycan and lipoproteins from the cell wall of gram-positive bacteria. TLR3 is a sensor of dsRNA and is thus able to respond to various viruses. TLR4 and -5 play key roles in the recognition of bacterial pathogens. TLR4 is specific for LPS, present on the outer membrane of gram-negative organisms. TLR5 binds to flagellin, a component of flagella. TLR7 and -8 ligands include guanosine- and uridine-rich ssRNA, characteristic of certain viruses. Nucleic acid also serves as a ligand for TLR9, which binds unmethylated CpG motifs, typically enriched in bacterial DNA. TLR11 binds to a profilin-like molecule produced by the parasite *Toxoplasma gondii* and responds to uropathogenic *Escherichia coli* [4, 5]. The ligands of the remaining TLR10, -12, and -13 have not been determined, although TLR10 is hypothesized to bind to bacterial lipoproteins [6, 7].

P. aeruginosa is a motile, gram-negative organism and opportunistic pathogen. Although *P. aeruginosa* is readily cleared in healthy individuals, it often causes chronic pulmonary infection and is a leading cause of morbidity in CF and immunosuppressed patients [8]. Stimulation through TLRs may be suitable for enhancing the CF lung defenses, but *P. aeruginosa* has proven to be an adaptable adversary that ensures it persists in the host. This review will consider the evidence for specific TLR recognition of *P. aeruginosa* at the host/pathogen interface in the respiratory tract, key second-messenger signaling molecules, and the modifications of these responses in CF patients.

P. aeruginosa AS AN OPPORTUNISTIC PATHOGEN

A leading cause of nosocomial infection, *P. aeruginosa* infects the respiratory, urinary, and gastrointestinal tracts and the eye, ears, skin, and joints in susceptible patients. Pathogenic mechanisms include LPS, pili, flagella, types III and VI secretion systems, and secreted virulence factors [9, 10]. The bacteria are also highly adaptable and with a hypermutable genome, genetic changes see it evolve from a virulent plantonic state to a mucoid, alginate-producing form capable of growing with reduced virulence in

Abbreviations: −/−=deficient, asialoGM1=asialoglycam-1, CF=cystic fibrosis, CFTR=cystic fibrosis transmembrane conductance regulator, DC=dendritic cell, ExoS=exoenzyme S, GLP=glycolipoprotein-1, IRAK=IL-1R-associated kinase, IRF=IFN regulatory factor, MD-2=myeloid differentiation protein 2, NOD=nucleotide-binding oligomerization, TAK-1=TGF-β-activated protein kinase 1, TBK=TRAF family member-associated NF-κB activator-binding kinase 1, TIRAP=Toll/IL-1R-containing adaptor protein, TRAM=Toll/IL-1R domain-containing adapter-inducing IFN-β-related adaptor molecule, TRIF=Toll/IL-1R domain-containing adapter-inducing IFN-β, WT=wildtype

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biofilms in the CF host lungs [11]. Mutated bacteria have been isolated from chronically infected CF patient lungs, for example, that lack motility (loss of flagellin and/or pilin) and have altered LPS, loss of type II or type III secretion systems, peptidoglycan modifications, changed pyocyanin production, and antibiotic resistance, among others [11–13]. That there is benefit to the CF patient in eradicating *P. aeruginosa* is borne out in studies showing not only improved lung function but also patient survival [13].

TLRs

The immune system can be divided into two principal arms, the innate and adaptive. The adaptive system includes B and T lymphocytes, both of which possess single antigen-specific receptors that are designed from the recombinations of germline gene segments. These adaptive immune cells possess antigenic memory that results in a more rapid response following re-exposure to the antigen. The innate system includes other leukocyte types and cells at the interface with the environment but also soluble opsonins and antimicrobials and lacks memory. It has long been appreciated that innate cells have receptors for foreign molecules, such as the mannose receptor and formyl-peptide receptors that lead to activation of leukocytes, but a great expansion in the capacity to distinguish microbes has been realized with TLRs. The discovery of TLRs followed the discovery that the IL-1R type I signaling domain was homologous to Drosophila Toll, one role of which is to promote antimicrobial defenses in the fly [14]. The discovery of this homology led to a wider search, and the mammalian TLRs were discovered, also sharing homology with the IL-1R type I. Finding the TLRs solved a long-standing genetic puzzle over the lack of response of the C3H/HeJ strain of mouse to LPS. This strain has a mutation in TLR4, shown to be the main cell receptor for LPS [15]. TLRs are type 1 membrane-spanning proteins and may be found in the cell membrane or intracellular in vesicle membranes. The present number of TLRs has risen to 13. The TLRs are one family of a larger group of recently discovered PRRs that include C-type lectin receptors, NOD1 and -2, NOD-like receptors, and retinoic acid-inducible gene-I-like receptors [3].

The discovery of a broad range of ligands for TLRs has led to the understanding that the receptors are not limited to reacting with microbes but also other host-derived danger signals (reviewed in refs. [3, 16]). The typical outcome of activation through TLRs is the production of inflammatory molecules, and thus, TLRs are appreciated to arm the innate immune cells to react to exposures to foreign microbes. In this regard, a number of *P. aeruginosa* molecules are ligands for various TLRs (**Table 1**), and these have led the investigations into the host response to this bacteria.

CF

CF is a prevalent autosomal-recessive disease, mainly in the Caucasian population. The disease is the manifestation of mutations in a chloride ion channel, the CFTR found on many epithelia. Mutations commonly result in a loss of the protein or deficiency of function [30, 31]. Since its discovery in 1989, over 1000 mutations in the gene have been sequenced; however, a great proportion is ΔF508, a 3-bp deletion, resulting in a protein that fails to mature properly and becomes degraded. Dysfunctional CFTR affects respiratory, pancreatic, hepatic, and gastrointestinal functions. In addition to mutations directly affecting the CFTR, the channel down-regulates a sodium ion channel also present on many mucosal epithelial membranes, responsible for limiting sodium influx into cells. Dysfunction of CFTR consequently results in abnormally high levels of intracellular sodium [32]. The influx of positively charged sodium ions draws negative chloride ions into the cells, while osmotic effects cause dehydration of the mucus layer [33]. In the airways, the consequence is an atypically thick, sticky mucus directly on epithelial cells (the periciliary liquid layer) that flattens cilia on respiratory epithelial cells, leading

TABLE 1. Examples of TLR Ligands Produced by *P. aeruginosa*

TLR	*P. aeruginosa* product	Host cell type	Representative references
TLR2/1 or -2/6	Lipoproteins	Airway epithelium Monocytes/macrophages DCs	[17–20]
	LPS	Arterial endothelium Various leukocytes	[21, 22]
	Capsule	Monocytes/macrophages	[23, 24]
	ExoS	Monocytes/macrophages	[25]
	GLP	Monocytes/macrophages	[23, 24]
TLR4	LPS	Arterial endothelium Monocytes/macrophages Various leukocytes	[22, 26, 27]
	Lipoproteins	Airways epithelium DCs	[20]
	Capsule	Monocytes/macrophages	[23, 24]
	ExoS	Monocytes/macrophages	[25]
	GLP	Monocytes/macrophages	[23, 24]
TLR5	Flagellin	Airway epithelium	[28, 29]
TLR9	Unmethylated CpG motif	Airway epithelium	[18]

to poor clearance of mucus and trapped debris, including microorganisms [33]. Examinations of events in the CF patient lung have identified imbalances in pro- and anti-inflammatory cytokines and abnormalities in lipid raft formation, apoptosis, phagosome acidification, and ceramide metabolism in airway cells as possible contributing factors to disease [34]. The static mucosal environment is presumed to render individuals susceptible to opportunistic infections, and CF patients become infected, to some extent in an age-related pattern, by multiple microorganisms but particularly, *Haemophilus influenzae*, *Staphylococcus aureus*, the *Burkholderia cepacia* complex, and ultimately, a high proportion (as many as 80% of adult CF patients) includes *P. aeruginosa* [35]. *P. aeruginosa* becomes challenging to treat, as a result of its physiological properties, pattern of gene expression and antibiotic resistance, and by growing in biofilms, proving significantly different from planktonic cultures [11, 13, 36]. This persistent bacterial infection underlies the chronic lung inflammation that CF patients experience. Understanding the changes in lung innate immune mechanisms as a result of dysfunctional CFTR and the persistent *P. aeruginosa* infection is paramount to changing the natural course of this disease. The TLRs may be the axis point to correct the response, and thus, research has been conducted from a variety of perspectives, including: 1) testing *P. aeruginosa* molecules for potency to activate TLRs in leukocytes or respiratory epithelial cell systems, 2) bacterial challenges in mice deficient in particular TLRs or signaling molecules, and 3) bacterial challenges of cells or mice with deficiencies in CFTR, sometimes combined with deficiencies in TLRs.

SPECIFIC RECOGNITION OF *P. aeruginosa* BY TLRs

TLR2

P. aeruginosa-derived products reported as TLR2 ligands include lipoproteins [17–19], components of the extracellular capsule [23, 24], and the secreted toxin ExoS (Table 1) [25]. LPS, present in the outer membrane of some strains of *P. aeruginosa*, have been reported as a TLR2 ligand in cell lines [21]; however, it is possible that this recognition is a result of contaminating lipoproteins [22]. The extracellular ExoS has been found to ligate multiple TLRs with the C terminus specifically interacting with TLR2 [25]. The intracellular form of ExoS stimulates human monocytes to produce inflammatory cytokines and causes apoptosis in T cells [37]. *P. aeruginosa* lipopeptide I (OprI) binds to TLR2 and -4 on DCs and possibly mucosal epithelia [20]. Mucoid strains of *P. aeruginosa* produce a thick polysaccharide capsule largely comprised of alginate and are associated with poor prognosis during chronic lung infection in CF patients [38]. Mucoid *P. aeruginosa* expresses high levels of lipoproteins [19], which induce TLR2-mediated cytokine responses from airway epithelial cells. TLR2 also contributes to the recognition of mannuronic acid polymers, a major polysaccharide component of the mucoid capsule, as well as slime GLP, which is produced by mucoid and nonmucoid *P. aeruginosa* strains [23, 24].

The efficient recognition of certain *P. aeruginosa* ligands by TLR2 may require the presence of coreceptors or heterodimers with TLR1 or -6 [39]. TLR2 reportedly collaborates with asialoGM1 signaling on host cells in response to *P. aeruginosa* flagella in vitro [28] and following phagocytosis of *P. aeruginosa* ligand binding to mannose receptors, and TLR2, in monocyte endosomes, leads to synergistic activation of downstream signaling cascades [40]. Whereas TLR2 contributes to the inflammation as a result of *P. aeruginosa* pulmonary infection in murine models [41, 42], including knockout mice exhibiting excessive inflammation to some strains [42, 43], it appears not to be critical to ultimately clearing the infection.

TLR4

TLR4 appears to share several *P. aeruginosa* ligands with TLR2, including possibly LPS [22, 26, 44], certain outer-membrane proteins [38], ExoS [25], alginate capsule [23], and to a lesser extent, slime GLP (Table 1) [24]. TLR4 binds to the lipid A component of LPS on the outer membrane of gram-negative bacteria [3]. This event induces a potent immune response, potentially underlying severe inflammation and sepsis in the host [45].

P. aeruginosa LPS ligation of TLR4 depends on the structure of lipid A produced by a particular strain and species-specific variation in the receptor-binding domain. Over the course of an infection in CF patients or depending on the growth condition of the bacteria, *P. aeruginosa* may produce several different varieties of lipid A [46] and also as it chronically infects the host. It appears that certain forms of lipid A confer a selective advantage to bacteria colonizing the CF lung by allowing for increased resistance to cationic antimicrobial peptides [47]. Many laboratory and environmental strains synthesize a pentacylated lipid A, which does not activate human TLR4 [44]. However, strains adapted to chronic infection of CF patients often produce hexacylated lipid A, which is a more potent agonist of TLR4 [26, 27, 47]. The observation that hexacylated lipid A is a strong TLR4 agonist has been confirmed for a variety of bacterial species [48]. However, this relationship does not appear to be true of the murine TLR4 signaling complex. TLR4, on murine macrophages, appears to react to pentacylated and hexacylated forms [27], whereas human TLR4 signaling complexes react strongly to the hexacylated form [26]. This difference in specificity is a result of sequence variations in a hypervariable region of the TLR4-binding site. Overall, the patient lung becomes colonized with *P. aeruginosa* strains expressing a variety of lipid A types [49].

TLR4 requires the presence of several coreceptors, CD14 and MD-2, for efficient interaction with LPS [50]. Bacterial LPS, complexed with soluble LPS-binding protein is recognized by CD14 on cell surfaces [46]. The LPS is then transferred to TLR4 in the presence of MD-2, finally leading to intracellular signaling. It is important that all factors of this signaling complex be present for efficient TLR4-mediated responses to LPS, including that of *P. aeruginosa*. Another possible coreceptor for TLR4 in the context of *P. aeruginosa* recognition may be the CFTR. In this case, the authors suggested that CFTR and TLR4 may contribute to the phagocytosis of *P. aeruginosa* [51].

TLR4 localization appears to depend on the cell type in question as well as the environment of those cells. TLR4 is readily accessible to *P. aeruginosa* ligands that come into contact with the surface of airway epithelial cells as well as cells of the myeloid lineage, such as macrophages and neutrophils [52–55].

TLR4, together with TLR5, is critical to resistance to *P. aeruginosa* pulmonary infection in healthy mice. Mice deficient in TLR4 exhibit poor neutrophil recruitment and higher bacterial loads following infection [41, 42]. Additionally, the lack of TLR4/LPS or TLR5/flagellin interactions leads to impaired neutrophil chemokine, TNF-α, and IL-6 responses from murine airway epithelial cells and alveolar macrophages [56]. Consistent findings have been observed in vivo through the combination of various TLR knockout animals and bacterial strains [42, 57, 58]. This redundancy between TLR4 and -5 is important to consider in experimental design. At least one study has underestimated the importance of TLR2/4-dependent recognition of *P. aeruginosa* LPS by inoculating mice lacking these receptors with a strain of bacteria that expressed normal flagellin [59]. Given that TLR5 was still functional in these animals, this remaining receptor/ligand interaction was sufficient for a protective effect.

TLR5

Many strains of *P. aeruginosa* possess a single flagellum for swimming motility, and it is an important virulence factor [60]. TLR5 specifically binds to flagellin, the primary component of bacterial flagella [61]. The TLR5-binding site of *P. aeruginosa* flagellin is located within aa residues 88–97, and mutations within this region may drastically reduce the interaction of the molecule with TLR5 without impacting bacterial motility [29]. A loss of glycosylation of *P. aeruginosa* flagellin is also sufficient to decrease TLR5-mediated responses, although glycosylation does not appear to affect TLR5 recognition of flagellin from other species.

Although flagellin is a well-established ligand for TLR5, there is debate over how the characteristic of motility shapes host responses. One study compared the efficiency with which human monocytes and macrophages phagocytosed *P. aeruginosa* mutants and found that motile bacteria were more readily engulfed than those that expressed flagellin but were nonmotile (as a result of mutations of another flagellum component) [62]. In view of this observation, loss of motility, even without a complete loss of the flagellum, may be a mechanism of immune evasion for *P. aeruginosa*, given that many clinical isolates are nonmotile [63, 64]. However, the biological significance of this finding is drawn into question by the results of an in vivo study. In a murine *Pseudomonas* lung infection, nonmotile bacteria, which expressed flagellin, were cleared as effectively as WT bacteria, and the presence or absence of flagellin was the more important factor in determining host responses [65]. Bacterial mutants, which overproduced flagellin, caused severe inflammation, whereas those that lacked flagellin appeared to evade immune control and were cleared from the lungs relatively slowly. This outcome suggests that the motility function of flagellin is relatively insignificant in the course of infection compared with its proinflammatory effect through TLR5.

That the host response depends on the presence of flagellin versus motility is supported further by observations that TLR5 is critical to macrophage phagocytic destruction of *P. aeruginosa*. Unprimed mouse alveolar macrophages challenged with WT strain *P. aeruginosa*-secreted IL-1β, which in turn, proved essential for acidification of phagolysomes and intracellular bacterial killing [66]. This mechanism was compromised by bacteria mutated in the TLR5 recognition site of flagella, by bacteria lacking flagella, or during WT bacterial infection of $TLR5^{-/-}$ but not $TLR4^{-/-}$ cells. Thus, the loss of the TLR5 response would have direct and indirect consequences on the host response, which results in an impairment of antimicrobial effectors.

A further confounder in hematopoietic cell TLR5 use comes from model systems that have shown that TLR5 may not be immediately accessible to its ligand. For example, in resting human neutrophils, TLR5 localization appears to be mainly intracellular but may be recruited to the plasma membrane following TLR1/2 heterodimer activation [67].

The expression of TLR5 on nonhematopoietic cells is also an important component of innate immunity to *P. aeruginosa* [68]. However, there is conflicting evidence regarding the localization of TLR5 in airway epithelial cells. TLR5 has been detected on the apical surface of human tracheal epithelial cells in situ [69], on these cells in primary cultures [70], and on mouse airway epithelium [71]. Contrasting reports of apical expression, polarized cultures of human nasal and bronchial epithelium were found predominantly expressing TLR5 basolaterally but recruited TLR5 to the apical surface following the colocalization of flagellin with asialoGM1 [28, 72]. A CF nasal epithelial line growing polarized on Transwell filters responded with higher cytokine levels when exposed to *P. aeruginosa* from the basal versus the apical side, and flagellin-deficient bacteria failed to stimulate cells, measured as nuclear-translocated NF-κB [73]. These discrepancies in the pattern of TLR5 detection may be a result of the specific cell source, or TLR5 possibly becomes basolateral with cultivation of cells but remains capable of translocating apically with various stimuli and/or particular manipulations in culture. Nevertheless, epithelial cells are responsive to *P. aeruginosa* flagellin, including through TLR5, which remains an important receptor for the protective response to *P. aeruginosa*.

TLR9

Unlike the other TLRs discussed to this point, TLR9 functions intracellularly, where it detects unmethylated CpG motifs abundant in bacterial DNA [74, 75]. The role of TLR9 in recognition of and response to *P. aeruginosa* in pulmonary infection is not yet characterized thoroughly, although it reportedly contributes to the inflammatory response, up-regulation of other TLRs, and bacterial clearance [18]. However, neutrophils appear to be capable of responding to *P. aeruginosa* DNA in a TLR9-independent manner [76]. Thus, the importance of this receptor may vary based on cell type.

TLR9 has been implicated further in resistance to respiratory *P. aeruginosa* in an interesting approach to assessing the contribution of TLRs. Following up earlier data showing that inhaled bacterial lysates provided protection, Duggan and colleagues [77] systematically applied aerosolized TLR agonists to mice that were challenged subsequently with lethal bacterial infections. They found that a combination of palmitoyl-2-cysteine-serine-lysine-4 (TLR2/6 agonist) and oligodeoxynucleotide ODN2395 (TLR9 agonist) synergized to protect the mice from an otherwise lethal *P. aeruginosa* challenge, as well as from other bacteria. Despite this combination of TLR agonists stimulating neutrophil infiltration into the lungs, protection was patent in mice depleted of neutrophils. The authors subsequently determined that ex vivo mouse tracheal epithelial cells stimulated through TLRs2/6 and -9 could resist infection by *Streptococcus pneumoniae* [77]. One limi-

tation in the application of these findings was the need for specific TLR9 ligands, as some failed to synergize with TLR2/6. This finding is particularly interesting in view of the apparent requirement for neutrophils for protection against *P. aeruginosa* pneumonia in mice [78]. It remains to be determined if the synergy among these TLRs similarly protects CF mice.

Together, these studies indicate that TLR2, -4, -5, and -9 contribute to the recognition of *P. aeruginosa* by a variety of cell types and presumably the induction of a protective innate immune response. That TLRs alone can provide protection has been demonstrated by the synergistic benefit by combining TLR2/6 and -9 stimulation and by showing increased susceptibility in mice lacking signaling molecules common to multiple TLRs, such as MyD88.

TLR SIGNALING IN RESPONSE TO *P. aeruginosa*

The binding of a TLR to its ligand triggers a complex series of signal transduction events aimed at providing an appropriate immune response to the stimulus [79, 80] (**Fig. 1**). The initial binding event is followed by the recruitment of an adaptor protein, which determines the downstream signaling pathway.

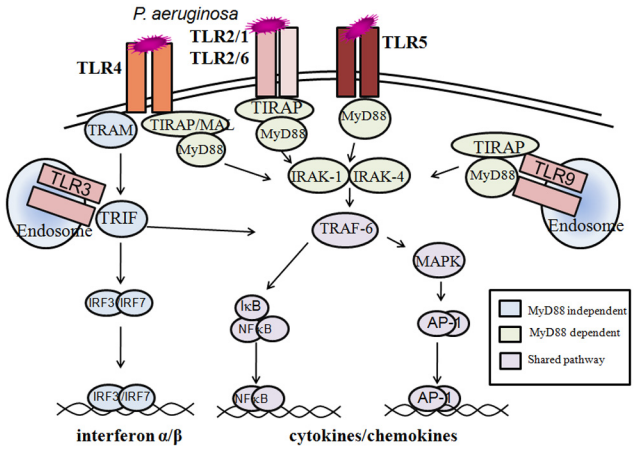

Figure 1. Binding of ligand to the TLRs triggers a series of molecular signaling events, the principal outcome of which are soluble mediators of the inflammatory/immune response. TLR2, -4, -5, and -9 have been shown to serve as receptors for *P. aeruginosa*. All TLRs, with the exception of TLR3, use MyD88 as an adaptor protein. TLR3 is included to show the related signaling events. Although many TLRs recruit MyD88 directly upon ligand binding, TLR1, -2, -4, and -6 require TIRAP for MyD88 recruitment. The initial steps of the MyD88-dependent signaling cascade lead to the sequential phosphorylation of IRAK-4, IRAK-1, TRAF-6, and TAK-1 and subsequent NF-κB activation. TRAF-6 may activate p38, JNK, and/or ERK1/2. The effects of MyD88-dependent TLR signaling are diverse, encompassing the downstream signaling pathways of several MAPKs. TLR3 and -4 also activate a distinct pathway; TLR3 recruits TRIF directly, whereas TLR4 requires the prior binding of TRAM. Either complex activates TBK1 kinase, which phosphorylates IRF3 and -7. These transcription factors mediate the expression of a variety of genes, including IFN-α and -β. MyD88- and TRIF-dependent pathways are required for host defense against *P. aeruginosa* infection. MAL = MyD88 adapter-like.

All TLRs except TLR3 use MyD88 as the adaptor protein. Additionally, TLR1, -2, -4, and -6 require a second factor, TIRAP, for MyD88 recruitment. MyD88-dependent signaling leads to the sequential phosphorylation of IRAK-4, IRAK-1, TRAF-6, and TAK-1. At this point, TAK-1 activates a cascade of IKKs, leading to the eventual ubiquitination and degradation of IκB, which then liberates NF-κB to move into the nucleus, where it drives transcription. NF-κB is the "master" regulator of host immunity; targeting genes include cytokines, chemotactic factors, and adhesion molecules important in launching inflammation. The MyD88-dependent TLR signaling pathway may also activate the MAPK family, p38, ERK, and JNK. Like NF-κB, these kinases mediate a constellation of cellular processes and gene expression through AP-1 transcription factors [81]. Following TLR activation, TRAF-6 may activate any and all of p38, JNK, or ERK1/2. TLR3 and -4 have been reported to activate a second pathway of TLR signaling, which depends on TRIF (Fig. 1) [80]. TLR4 requires the previous binding of TRAM [82]. Either complex activates the TBK1 kinase, which in turn, phosphorylates IRF3 and -7, which mediate the expression of a variety of genes, including IFN-α and -β.

There is another layer of complexity involved in signaling through TLR4 as well, given that it may activate NF-κB in a MyD88-independent manner [80, 82]. Specifically, the complex of TLR4, TRAM, and TRIF is capable of activating TRAF6 downstream of the MyD88-dependent signaling cascade [80]. Through this unique combination of adaptor proteins, TLR4 is able to mediate delayed NF-κB activation, even in the absence of MyD88 (Fig. 1).

Predicted from the intracellular signaling pathways, MyD88 is critical for TLR-driven NF-κB activation early during infection, and consequently, studies have shown that mice lacking MyD88 are more susceptible to pulmonary infection with *P. aeruginosa* than are WT mice [41, 42, 83–85]. The presence of MyD88 in nonhematopoietic cells is crucial for the recruitment of neutrophils [86]. Accordingly, whole *P. aeruginosa* and products have been shown to induce MyD88-dependent signaling in specific cell types; for example, ExoS triggers cytokine production from human monocytes [25]. Following exposure to whole bacteria, the signaling pathway from MyD88 to TRAF6 has been shown to be essential for cytokine production from murine airway epithelial cells and alveolar macrophages [69]. Thus, TLR signaling through MyD88 appears to be essential in mediating host innate immune responses to *P. aeruginosa* in vitro and in vivo.

Noteworthy is that in contrast to the presumed activation of TLRs, certain components of MyD88-dependent TLR signaling pathways have been reported to be down-regulated following exposure to whole or components of *P. aeruginosa*. This state of tolerance may contribute to a reduced inflammatory state in the host during infection and thus, less tissue damage. Tolerance to whole *P. aeruginosa* in human airway epithelium is mediated by inhibition of IRAK-1, resulting in less NF-κB transcriptional activity [87].

The importance of TIRAP (Fig. 1) in the context of cell signaling in response to *P. aeruginosa* infection depends on the strain of bacteria involved and thus, by extension, the TLR ligands present. TIRAP is not crucial for cytokine production

in response to WT *P. aeruginosa* in cultured human airway epithelium or for controlling the course of pulmonary infection in mice [69, 84]. However, TIRAP$^{-/-}$ mice display impaired neutrophil accumulation and increased bacterial load and bacterial dissemination when infected with a mutant strain of *P. aeruginosa* lacking flagella. These results again underscore that TLR5, which binds flagellin and does not require TIRAP for MyD88 recruitment, may be sufficient to trigger the host immune responses to flagellated *P. aeruginosa*. Thus, it appears that TIRAP-dependent pathways, possibly involving TLR2 and -4, are only critical for an effective host response to nonflagellated *P. aeruginosa*, as TIRAP-independent pathways can serve as a substitute when flagellin is present.

ACTIVATION OF MAPK CASCADES IN *P. aeruginosa* INFECTION

The activation of MAPKs in a TLR-dependent manner appears to contribute to the expression of certain innate immune factors, such as human β-defensin 2, TNF-α, and CXCL8. *P. aeruginosa* slime-GLP, ExoS, and flagellin lead to the activation of MAPK; for example, slime-GLP stimulates TNF-α through TLR2 and -4 and is ERK1/2- and p38-dependent [24, 88–90]. ExoS, also a ligand for TLR2 and -4, appears to contribute to ERK1/2 phosphorylation in airway epithelial cells [88]. Maximal MAPK activation in response to *P. aeruginosa* may depend on a synergistic relationship between TLR2 and the mannose receptor, which binds to bacterial molecules within the phagosome [25]. Flagellin activates p38 through its interaction with TLR5, and in one report, activated p38 was shown to enhance NF-κB-mediated expression of CXCL8 and COX-2 [89].

TRIF-DEPENDENT PATHWAYS IN *P. aeruginosa* INFECTION

TRIF-dependent signaling pathways also play a role in the response to *P. aeruginosa* infection, as TLR4 may recruit the TRAM/TRIF complex that facilitates delayed NF-κB activation [24]. This pathway may allow for efficient, albeit delayed clearance of pulmonary *P. aeruginosa* infection, even in MyD88 knockout mice [91]. Secondly, TRIF and the downstream IRF transcription factors mediate the production of certain cytokines important in bacterial clearance, such as CCL5, in response to *P. aeruginosa*. TRIF and IRF3 contribute to CCL5 expression in murine macrophages, whereas IRF1 is required for the expression of other cytokines in response to *P. aeruginosa* LPS [92–94]. Additionally, IRF3 appears to be important for macrophage and neutrophil recruitment to the lungs of infected mice, whereas IRF1 mediates CD8$^+$ T cell and NK recruitment [93, 94]. Thus, TRIF activation of IRF transcription factors is important in controlling antimicrobial responses to *P. aeruginosa*.

MODIFICATION OF THE TLR RESPONSE TO *P. aeruginosa* IN CF

The emerging understanding is that in healthy individuals, respiratory infections with *P. aeruginosa* are essentially prevented by innate mechanisms stimulated by the bacteria attaching to epithelial cells, including possibly via asialoGM1 and the CFTR, and provoking an acute inflammatory response, which includes neutrophils capable of clearing the infection. Infected epithelial cells slough as a final defense. In the CF patients who lack surface CFTR, concentrated mucus results in declining lung function, reduced mucociliary clearance, and ineffective antimicrobials, all of which contribute to early *P. aeruginosa* persistence. Then, with reduced adhesion to host cells and adaptations, such as the loss of virulence factors, the bacteria become established and resistant to inflammatory cells, including neutrophils, which subsequently damage the lungs, for example, as a result of neutrophil elastase activating TLR4 (**Fig. 2**) [10, 95, 96]. This speaks to the fact that there appears not to be a lack of a response but that the response is ineffective against the highly adapted bacteria. A comprehensive discussion of whether the CF patient experiences an exaggerated inflammatory response in the respiratory tract can be found elsewhere [97]. CFTR mutations may impact cells directly by heightening constitutive and induced cytokines and interfering with microbicidal processes in leukocytes, possibly despite TLR activation [98]. One example of a change in constitutive mediator production was a report showing that primary bronchial epithelial cells from ΔF508 homozygous CF patients showed heightened, spontaneous CXCL8 secretion associated with reduced levels of IKK, which is responsible for

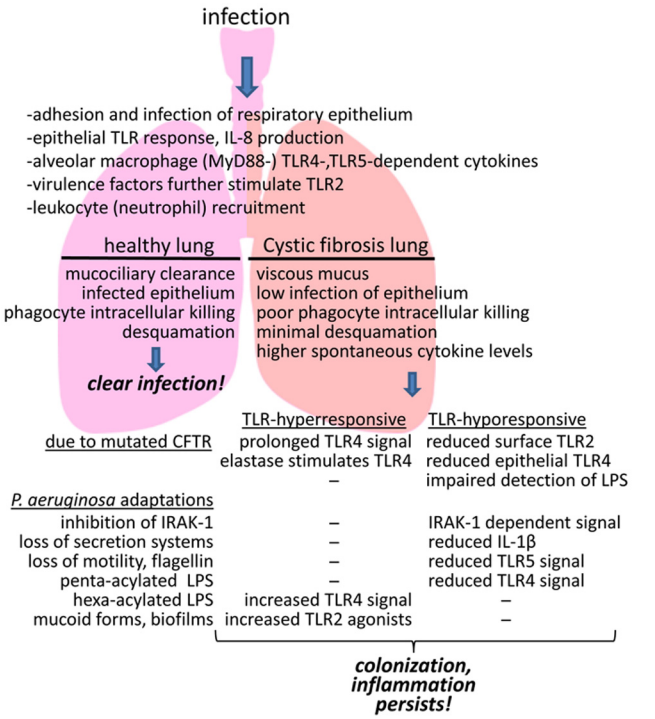

Figure 2. The healthy and CF lung response to *P. aeruginosa* infection shares some common elements but also has considerable divergences, including in magnitude and specifically involving various TLRs. From the available literature, mutations in CFTR impact TLR responses directly and indirectly in negative and positive mechanisms, and the net outcome is chronic infection and inflammation.

phosphorylating IκBα [99]. With regard to TLRs, an example is prolonged TLR4 signaling as a result of the failure of early endosomes to fuse with lysosomes [100, 101]. Pseudomonas conversion to the mucoid form is associated with increased lipoprotein production and a coincidental increase in TLR2 responsiveness by human respiratory epithelial cells ex vivo [19]. TLR5 possibly contributes to an exaggerated response in CF, with a report that the expression of this receptor is elevated on neutrophils in the airways of patients [67], although the finding of increased TLR5 has been disputed [18]. Regardless, TLR5 presumably becomes less important when the *P. aeruginosa* lose expression of flagellin. Such mechanisms would presumably contribute to the hyper-responsiveness and chronic inflammation seen in patients with long-standing *P. aeruginosa* infections (Fig. 2).

Contrasting findings of high constitutive mediator expression and enhanced TLR responses, which may explain a hyperinflammatory state, are polarized cultures of CF bronchial epithelium in which TLR4 expression and the capacity to induce cytokine production appear compromised [54]. Transfecting these cells with functional CFTR restored TLR4 expression and functionality. Furthermore, the expression of TLR2 and -4 on the surface of bronchial biopsies obtained from CF patients was lower than in healthy controls, although a relatively high number of TLR4-positive neutrophils were detected in the submucosa [52]. It appears that CFTR directly may contribute to the detection of bacterial LPS and phagocytosis by epithelial cells, possibly in a synergistic manner with TLR4 [51, 102, 103], and thus, these interactions would be lost with mutant CFTR. These negative effects of CFTR mutations on TLR expression and function could contribute to the susceptibility to bacterial infection experienced by CF patients by dampening inflammatory signals. The balance of the seemingly paradoxical mechanisms is ongoing inflammation that becomes harmful to the lungs and failure to clear the infection (Fig. 2).

Finally, much of the research using cell cultures has intentionally focused on the TLR response alone, yet it is understood that other humoral mediators interact and impact TLR signaling. This added layer of complexity may begin to close the gap in our understanding of the paradoxical hypoinflammatory responses (mostly observed in vitro) versus hyperinflammatory responses. One published example from the CF literature is mucin 1 interfering with TLR5 signaling by binding MyD88 in cells [71]. Still another example of mediator interactions that has not been investigated thoroughly in CF is the effect of the complement anaphylatoxins on TLR activity. *P. aeruginosa* (and other organisms) activate complement [104], and split complement components modify TLR signaling [105]. It has been reported that $C5aR^{-/-}$ mice are highly susceptible to respiratory *P. aeruginosa* infection [106], so these mediators most certainly interact but have yet to be examined in the context of the CF lung.

CONCLUDING REMARKS

P. aeruginosa possesses suitable TLR ligands, and these ligands are used during active (model) infections to elicit TLR-mediated responses in the lung. Through model studies, TLRs can be suitably stimulated to provide adequate innate immunity to resist respiratory infection, confirming that TLRs can be an axis to achieve immunity. These findings should encourage more research into similarly using TLRs to bolster innate mechanisms in the susceptible human host. Yet, in the healthy human respiratory tract, innate mechanisms are sufficient to manage infection by *P. aeruginosa*. These mechanisms become disabled in the CF patient, permitting chronic infection, which is exacerbated further by dysfunctional cellular killing mechanisms and direct effects of the mutated CFTR on TLR function. As the bacteria mutate and adapt, TLR stimulation may be intact and even exaggerated, which further promotes inflammation, manifesting as the chronic sequelae and particularly, failing lung function seen in these patients. This paradigm should spawn research into the relationship between the evolving bacterial adaptations and the CF host response, where manipulating, including down-regulating, the TLR response may, in fact, prove to be beneficial [107].

AUTHORSHIP

S.M.M. wrote the first submission, prepared Table 1 and Fig. 1, and contributed to editing the second submission of the review. A.W.S. prepared Fig. 2, and A.W.S. and T-J.L. edited the manuscript drafts.

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