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Innate immune responses to *Pseudomonas aeruginosa* infection

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Abstract

Innate immune responses play a critical role in controlling acute infections due to *Pseudomonas aeruginosa* in both mice and in humans. In this review we focus on innate immune recognition and clearance mechanisms that are important for controlling *P. aeruginosa* in the mammalian lung, with particular attention to those that influence the outcome of *in vivo* infection in murine models.

Keywords

Innate immunity; *Pseudomonas aeruginosa*; murine models; pneumonia

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterial pathogen capable of causing a broad range of acute and chronic infections. The organism can be isolated from environmental sources, particularly freshwater and soil, and is a rare skin commensal in some individuals. The intrinsic resistance of *P. aeruginosa* to quaternary amines and other microbicides also makes it difficult to eradicate this organism from equipment and surfaces in healthcare facilities and hospitals.

P. aeruginosa colonization and chronic infection frequently complicate Cystic Fibrosis (CF), a disorder characterized by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. In this population, acquisition of *P. aeruginosa* is associated with declines in pulmonary function, and increased morbidity and mortality [1]. *P. aeruginosa*, however, also causes clinically significant infections in individuals without CF. These include keratitis, skin infections (burn superinfections and folliculitis), urinary tract infections, infections of the upper and lower respiratory tract (tracheobronchitis and pneumonia) and bloodstream infections. Compromise of epithelial barrier function and loss of local immune defenses predisposes individuals to these more acute infections. These important barriers to opportunistic infection are often breached iatrogenically, through urinary tract catheterization and tracheal intubation and mechanical ventilation. Indeed, *P. aeruginosa* is one of the more common causes of intensive care unit infections, and is among the leading causes of ventilator-associated pneumonia [2, 3].

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In this review, we will focus on innate immune responses *to P. aeruginosa* in the setting of acute pulmonary tract infection. In particular, we will examine studies that have exploited murine models of acute pneumonia to identify host immune pathways involved in the recognition and control of *P. aeruginosa* in the lung.

2. *Pseudomonas aeruginosa*: microbial factors relevant for acute infection

P. aeruginosa express several surface structures that are important for motility and adhesion in biotic and abiotic environments. These include a single polar flagellum, polar Type IV pili, and chaperone/usher pili (cup) fimbriae. Although Type IV pili and flagella are required for efficient motility and the initiation and maturation of biofilms, strains isolated from chronically colonized CF patients have often lost expression of these surface structures, through the process of mutation and selection [4]. Even *P. aeruginosa* clinical isolates from individuals without CF show variability in their expression of flagellar and pilus-mediated motility [5]. *P. aeruginosa* can also downregulate synthesis of flagellin and flagella conditionally, upon exposure to purulent sputum [6, 7]. This occurs when neutrophil elastase cleaves the flagellar hook protein, FlgE; in its absence, the anti-sigma factor FlgM accumulates inside the bacterial cell and inhibits transcription of the gene encoding flagellin, *fliC* [8]. Downregulation of flagellin, which is strongly recognized by Toll-like receptor (TLR) 5, might limit the inflammatory response elicited by *P. aeruginosa*.

P. aeruginosa strains also express another TLR agonist, lipopolysaccharide. Environmental strains and those usually isolated from acutely infected individuals express a penta-acylated Lipid A variant which is poorly recognized by human TLR4. In contrast, *P. aeruginosa* isolates from chronically colonized Cystic Fibrosis airways express a greater proportion of more highly acylated Lipid A variants that are more potent human TLR4 ligands. Murine TLR4 discriminates less strongly between variant Lipid A structures in LPS in cell based assays [9].

P. aeruginosa expresses two Type 2 secretion systems (T2SS), Xcp and Hxc, and a Type 3 secretion system (T3SS), which have been reviewed recently [10, 11]. The Xcp T2SS mediates the extracellular secretion of proteases, lipases and toxins that act as virulence factors in various animal models of disease. The contribution of this system to virulence in the acute pneumonia model may be masked to some extent by T3SS effects. Thus, deletion of exotoxin A, a Type 2 secreted AB toxin that ADP-ribosylates elongation factor 2 and inhibits target cell protein synthesis, only modestly attenuated PA103 (T3SS+) clearance from the murine lung and had no significant effect on survival [12]. Recently, Jyot *et al.* systematically compared $T2SS^{-}$, $T3SS^{-}$ and double $T2SS/T3SS^{-}$ mutants in a murine acute pneumonia model [13]. The $T2SS^{-}$ mutant ($T3SS^{+}$) killed all mice rapidly, similar to the wild-type parent, while the $T3SS^{-}$ strain ($T2SS^{+}$) killed ca. 80% with delayed kinetics. Bacterial mutants lacking both secretion systems, however, killed <10% of mice in these experiments, strongly supporting the notion that substrates of the T2SS are responsible for this delayed death. Production of many of the T2SS substrates is regulated by bacterial quorum sensing signals, which have been strongly implicated in *P. aeruginosa* chronic infection and colonization [14].

The T3SS, which translocates a small set of protein substrates across both the bacterial cell envelope and eukaryotic plasma membrane, has been associated with increased *P. aeruginosa* virulence not only in murine acute pneumonia models [15–17], but also in several clinical studies of acute respiratory infection [18–20]. Several of the translocated T3SS effector proteins can modulate innate immune recognition of bacteria or target effector mechanisms of the innate immune system, as has been recently reviewed [11]. These include Exoenzyme U (ExoU), a phospholipase A_2 that can inhibit inflammasome activation by *P.*

aeruginosa [21] and that causes rapid necrotic cell death [22, 23]. Exoenzyme S (ExoS) and ExoT are bifunctional enzymes, exhibiting both GTPase activating protein (GAP) activity toward target Rho-family GTPases, and ADP ribosyl transferase activity toward host proteins involved in endocytic trafficking and cell migration [24]. Both ExoS and ExoT can inhibit migration and phagocytosis of macrophages and neutrophils [25, 26]. These proteins target phagocytes recruited to infected murine airways and can substantially attenuate host responses to infection by inhibiting or killing host immune cells [27, 28].

Lastly, *P. aeruginosa* produces a number of small molecules that are directly inhibitory or toxic to immune cells. Pyocyanin, a redox-active phenazine, can trigger neutrophil apoptosis *in vitro* [29] and *in vivo* [30]; bacteria that cannot produce pyocyanin are cleared more efficiently from the acutely infected mouse lung than isogenic wild-type organisms [30, 31]. Another secreted small molecule, rhamnolipid, causes neutrophil necrosis; bacterial mutants deficient in rhamnolipid synthesis are more rapidly cleared from mouse lungs in a model of persistent colonization in which animals are infected with bacteria encapsulated in agar beads and evaluated 3 days post infection [32]. Pyocyanin-deficient bacterial strains are also cleared more efficiently than isogenic wild-type strains in such a persistent colonization model at 7 days post infection [33]. Pyocyanin and rhamnolipid production are positively regulated by quorum sensing signals, and their expression is greater when bacteria are in stationary phase or high-density growth conditions, such as biofilms [34]. Their relative importance in acute *P. aeruginosa* infections appears to be less than that of the T3SS or T2SS substrates discussed above [35].

Finally, it is important to point out that *P. aeruginosa* clinical and laboratory strains show significant variability in the complement of surface structures, exoproteins and exotoxins that they express. It is well-appreciated that strains associated from chronically colonized CF patients acquire mutations that downregulate not only expression of flagella and type IV pili, but also expression of pyocyanin, pyoverdin, quorum sensing signals and T2SS and T3SS substrates, and upregulate the expression of exopolysaccharides (recently reviewed in [1]). However, clinical strains isolated from acutely infected patients without CF also show significant variation in their expression of these virulence factors [5]: for example, T3SS-positive strains express different complements of substrates, and some 30–50% of isolates are phenotypically T3SS-negative [5, 35]. Variation in virulence factor expression is also exhibited by the clinically derived laboratory strains used in many of the animal studies reviewed below (PAO1, PAK, PA14, PA103). Often, effects of such bacterial variation are not explicitly considered in interpreting experimental outcomes, and increase the challenge of generalizing these findings.

3. Recognition of *P. aeruginosa* in the respiratory tract: the role of pattern recognition receptors

Several conserved microbial structures, collectively known as microbe-associated molecular patterns (MAMPs), have been implicated in activating the host innate immune response to *P. aeruginosa*. MAMPs are sensed by a set of germ-line encoded receptors called pattern recognition receptors (PRRs) that include cell surface and endosomal Toll-like receptors (TLRs), and cytosolic Nod-like receptors (NLRs). MyD88, an adaptor molecule for almost all Toll-like receptors (TLRs), is required for the control of *P. aeruginosa* in the lung [36, 37]. Both TLR4 and TLR5, which recognize LPS and flagellin respectively, can initiate protective responses to *P. aeruginosa* infection. This is illustrated by similar survival of singly deficient TLR4 or TLR5 mice as compared to wild-type animals following infection with a flagellated (Fla^+) *P. aeruginosa* strain PAK, versus decreased survival of TLR4/5 double knockout mice [38–40]. However, when animals are infected by a Fla^- strain, such as PA103, TLR4 signaling becomes essential [41]. Other TLRs that are expressed by lung

epithelium and alveolar macrophages, such as TLR2 and TLR9, do not appear to mediate *P. aeruginosa* recognition in the lung.

4. *P. aeruginosa* recognition by the inflammasome

A prominent early host response to *P. aeruginosa* pulmonary infection is secretion of the pro-inflammatory cytokine interleukin-1β (IL-1β) [37, 40, 42]. Pro-IL-1β is processed into its active, secreted form by the cysteine protease caspase-1 [43]. This requires the assembly of a macromolecular inflammasome complex to which procaspase 1 is recruited and activated in presence of a protein from the NLR family [44]. Although many NLRs can activate caspase-1, *P. aeruginosa* induced caspase-1 activation requires NLRC4 (also known as IPAF) in alveolar [45], bone marrow-derived [46] and thioglycollate-elicited peritoneal macrophages [21]. An intact *P. aeruginosa* T3SS is necessary for NLRC4 dependent caspase-1 activation. Of note, the T3SS effectors ExoU [21] and ExoS [47] can inhibit caspase-1 activation, by mechanisms that require their respective phospholipase A_2 and ADP-ribosyltransferase activities.

Several mechanisms for inflammasome activation by T3SS positive pathogens have been proposed based on *in vitro* work. Initial papers proposed TLR5-independent recognition of flagellin by the NLRC4 inflammasome [48]. Subsequent studies, however, demonstrated NLRC4 inflammasome activation by nonflagellated *P. aeruginosa* strains in a T3SS-dependent manner [21]. As transfection of the T3SS rod protein from *P. aeruginosa* (PscI) or from other Gram-negative bacteria into macrophages is sufficient to activate the NLRC4 inflammasome, one model proposes that the NLRC4 inflammasome recognizes structural motifs common to these T3SS inner rod proteins and flagellin, if they gain access to the macrophage cytosol [49]. The observation, by other investigators, that very high extracellular $[K^+]$ can attenuate flagellin-independent activation of NLRC4 and caspase-1 is the basis for another model in which T3SS intoxication of host cells leads to a selective potassium efflux that activates the inflammasome [50]. Whether efflux is directly mediated by the T3SS apparatus or a molecule it transports is not addressed by this study.

In vivo, caspase-1 activation and subsequent IL-1R dependent signaling are required for rapid neutrophil recruitment to the site of infection [36, 42]. Of note, IL-1R signaling in non-bone marrow derived cells, such as airway epithelial cells, is necessary and sufficient for these early host responses [36, 51]. The absence of IL-1R signaling measurably diminishes early *P. aeruginosa* clearance in the lung [42, 52], and may be most important for rapid recognition of relatively small inocula of bacteria in the airways. Caspase-1 knockout mice, IL-1R knockout mice, and multiple TLR knockout mice are all able to control *P. aeruginosa* replication in the lung, however, unlike their MyD88 knockout counterparts. This forcefully illustrates the fact that multiple MyD88-dependent pathways are capable of responding to *P. aeruginosa* during infection, and that each appears capable of eliciting responses that lead to pathogen clearance.

5. Innate immune cells: roles in *P. aeruginosa* recognition and clearance

Polymorphonuclear Neutrophils

Lung infection by *Pseudomonas aeruginosa* leads to the massive recruitment of neutrophils into infected airways. Neutrophils play a primary and unambiguous role in *P. aeruginosa* clearance during acute pulmonary infection, as clearly demonstrated by the extreme susceptibility of neutropenic mice to this pathogen. For example, neutrophil depletion effected via either cyclophosphamide treatment or administration of anti-Ly6 (Gr1) monoclonal antibodies rendered C57Bl/6 mice susceptible to very low inocula (10–100 CFU/mouse) of several different *P. aeruginosa* strains, which corresponded to a decrease in

the LD_{50} for strain PAO1 of ca. 10^5 [53]. Moreover, granulocyte killing of *P. aeruginosa* in immunocompetent mouse models of pneumonia appears to be "saturable": low bacterial inocula are cleared more effectively than intermediate inocula, while infection with higher bacterial numbers results in net increases in bacterial lung burden [54]. Thus important factors that determine the outcome of *P. aeruginosa* pneumonia are first, the ability to rapidly recruit sufficient numbers of neutrophils to the airways and second, the ability of the recruited neutrophils to kill *P. aeruginosa*. The latter can be altered by both host and bacterial factors that directly impact neutrophil phagocytosis and survival [28, 55].

Neutrophil recruitment to the lungs following *P. aeruginosa* infection can be significantly inhibited by prior intratracheal administration of an anti-CXCR2 monoclonal antibody that inhibits binding of ELR+ CXC chemokine ligands such as MIP-2 and KC. Mice treated with an anti-CXCR2 mAb showed diminished survival and increased bacterial burden in a *P. aeruginosa* acute pneumonia model [56]. Neutrophil recruitment to airways following *P. aeruginosa* infection is also dependent on β_2 integrins (e.g. CD11b/CD18, CD3). Blockade of CD18 or CD11b by systemic administration of blocking antibodies reduced neutrophil recruitment in a *P. aeruginosa* pulmonary infection model [57]. A complementary experimental system in which both wild-type and CD18 deficient hematopoietic cells were used to reconstitute lethally irradiated mice demonstrated preferential recruitment of wild-type neutrophils to *P. aeruginosa* infected airways, consistent with the notion that CD18 is required for this response [58]. CD11b/CD18 dependent neutrophil recruitment is also impaired in mice lacking the urokinase receptor (uPAR, CD87), which is a modulator of β_2 integrin function and CD11b/CD18 in particular. In one study, $uPAR^{-/-}$ mice showed a ca. 10-fold defect in neutrophil recruitment to the airways and ca. 2-fold greater bacterial numbers at 4h post infection (hpi) [59].

A primary function of recruited neutrophils is pathogen elimination. Neutrophil serine proteases play important roles in *P. aeruginosa* clearance, as demonstrated by the studies of Hirche *et al.* with mice lacking neutrophil elastase ($NE^{-/-}$). $NE^{-/-}$ mice showed increased susceptibility to *P. aeruginosa* in an acute pneumonia model, and bacterial clearance from lungs was impaired in knockout animals as compared to wild-type controls [60]. These authors found that the bacterial outer membrane porin, OprF, was a primary target of neutrophil elastase activity. *P. aeruginosa* incubated with purified NE showed diminished growth and viability and increased outer membrane permeability; no such effects were observed on bacteria lacking OprF. $NE^{-/-}$ and wild-type mice were equally susceptible to infection by OprF-mutant *P. aeruginosa*, supporting the authors' hypothesis that this is a primary target of NE-mediated clearance. Interestingly, the OprF mutant bacteria were less virulent in the acute pneumonia model, suggesting that the altered outer membrane of these mutant bacteria may make them increasingly susceptible to NE-independent host clearance mechanisms.

Neutrophil serine proteases (NE, cathepsin G, proteinase 3) target host cell, as well as bacterial proteins, and can themselves contribute to tissue inflammation and damage during bacterial infection. Serine protease inhibitors modulate the activity of these enzymes *in vivo*. Spi6, the murine homolog of the human ova-serpin proteinase inhibitor PI9, appears to be a weak inhibitor of NE [61]. Deletion of Spi6 rendered neutrophils activated by *P. aeruginosa* infection more susceptible to cell necrosis; this was not observed in doubly deficient $NE^{-/-}$ $Spi6^{-/-}$ neutrophils [62]. Thus Spi6 appears to protect neutrophils from NE-mediated lysis. $Spi6^{-/-}$ animals showed improved survival and diminished bacterial burden in the lung in an acute pneumonia model, associated with a modest (2.6-fold) increase in extracellular NE activity in the lung. In this model, therefore, increased neutrophil lysis appeared to be beneficial for pathogen clearance, possibly through increased release of extracellularly active enzymes (serine proteases, lysozyme) and factors associated with neutrophil

extracellular traps, but did not carry an obvious cost for the host vis a vis increased tissue damage.

A different outcome, however, was observed in mice deficient for *serpinb1*, a potent inhibitor of multiple neutrophil serine proteases. *Serpinb1*$^{-/-}$ mice were comparable to wild-type controls in their ability to recruit neutrophils to the airways and control bacterial replication at an early timepoint (6 hpi) after intranasal infection with PAO1, but had ca. 5-log greater numbers of lung bacteria by 24 hpi and significantly increased overall mortality [63]. The absence of *serpinb1* increased neutrophil serine protease activity toward host molecules implicated in *P. aeruginosa* defense, such as surfactant protein-D (SP-D), and also appeared to enhance neutrophil necrosis and apoptosis, even in the absence of active bacterial infection. As a consequence, the number of viable neutrophils in infected lung and airways was markedly diminished in knockout animals by 24 hpi and bacterial clearance was impaired.

Neutrophil serine proteases may also target the family of protease-activated receptors (PAR) expressed by lung epithelium, neutrophils and macrophages. Deletion of one of the 4 known PARs, PAR2, resulted in a slight increase in the number of bacteria recovered from the airways of PAR2$^{-/-}$ mice at 24h post *P. aeruginosa* infection, despite the apparent recruitment of greater numbers of neutrophils to this site in the knockout mice. PAR2$^{-/-}$ neutrophils showed reduced phagocytosis of both opsonized latex beads and *P. aeruginosa*, but no difference in neutrophil oxidative burst in response to phorbol myristate acetate (PMA) stimulation. A similar defect in PAR2$^{-/-}$ macrophage phagocytosis was also observed [64].

Nitric oxide and derived reactive nitrogen species are important immune mediators [65]. Nitric oxide (NO) appears to be a significant potential bactericide for *P. aeruginosa*: growth of bacteria lacking nitric oxide reductase is strongly inhibited by exogenous NO and these mutant organisms are killed more efficiently than an isogenic wild-type control by LPS activated RAW 264.7 macrophages [66]. Inhalation of exogenous NO was shown to reduce bacterial load at 24 hpi in a rat model of *P. aeruginosa* pneumonia [67], while the inhibition of nitric oxide synthase (NOS) with S-methyl-isothiourea decreased mouse survival and increased lung bacterial burden at 24 hpi in *P. aeruginosa* intranasally infected mice [68]. A recent study demonstrated the protective role of inducible nitric oxide synthase (iNOS) in acute *P. aeruginosa* murine pneumonia by examining the phenotype of Ksr1 (kinase suppressor of Ras-1) deficient mice [69]. Ksr1$^{-/-}$ mice showed significantly increased mortality after pulmonary infection with *P. aeruginosa*. Infected mice appeared to recruit adequate numbers of neutrophils to the lung, but failed to control bacterial replication. Isolated Ksr1-deficient macrophages and neutrophils could phagocytose bacteria, but failed to kill internalized organisms. The authors demonstrated impaired activation of iNOS by *P. aeruginosa* infection in the absence of Ksr1, and suggested that Ksr1 functions as a scaffold that promotes iNOS interaction with and activation by Hsp90. Importantly, exogenous supplementation of NO by NO donors (such as DETA-NONOate) increased survival of Ksr1$^{-/-}$ mice after *P. aeruginosa* infection.

Alveolar macrophages

The first immune cells likely to encounter *P. aeruginosa* in the lung are resident alveolar macrophages. Macrophages can internalize and kill bacterial pathogens; however, their role in pathogen sensing is also of primary importance during *P. aeruginosa* infections. *In vitro*, murine alveolar macrophages secrete chemokines (KC) and cytokines (TNF-α and IL-6) following activation of TLR-4 and TLR-5 by *P. aeruginosa* LPS and flagellin, respectively [70]; thus macrophages are capable of producing neutrophil recruiting chemokines. As discussed above, alveolar macrophages also respond to a T3SS associated *P. aeruginosa*

signal by activating caspase-1. IL-1β produced by macrophages can be sensed by airway epithelial cells, which in turn secrete neutrophil chemokines [36].

Depletion of alveolar macrophages via aerosol administration of clodronate (Cl_2MDP)-liposomes in rat and mouse models of *P. aeruginosa* acute lung infection significantly attenuated inflammatory responses such as secretion of the chemokines MIP-2 and KC. Both neutrophil recruitment and bacterial clearance were impaired in these studies [71, 72]. Other investigators failed to observe significant effects on bacterial clearance [73] or animal survival [53] when intranasal, rather than aerosol, instillation of Cl_2MDP-liposomes was employed to deplete alveolar macrophages. In these latter studies, residual alveolar macrophage counts were greater, which may account for the authors' failure to observe effects associated with depletion.

Another important role of alveolar macrophages during pulmonary infection is phagocytosis of dying neutrophils and initiation of resolution and repair. The cytokine MCP-1, which is secreted by type II alveolar epithelial cells in response to *P. aeruginosa* infection, stimulates alveolar macrophage migration and phagocytosis *in vitro* and *in vivo* [74]. Infection of MCP-1 deficient mice with *P. aeruginosa* results in small decreases in bacterial clearance at ca. 18 hpi, but also appears to lead to increased lung cell death and lung injury [75]. The authors also suggest that macrophages may play a direct role in bacterial phagocytosis and clearance, as MCP-1 stimulated macrophages demonstrate increased phagocytosis of opsonized *Escherichia coli* particles and increased superoxide production [75]. A potential role for macrophages in the clearance of *P. aeruginosa* is also supported by studies with mice deficient in epilysin ($Mmp28^{-/-}$). This matrix metalloproteinase is expressed by Clara cells in lung airways and is downregulated by *P. aeruginosa* infection. Mmp28-deficient mice show a more rapid recruitment of macrophages to the lung than wild-type controls; this is accompanied by a modest decrease in bacterial burden at this timepoint (4 hpi) in mutant animals [76]. Depletion of macrophages with systemic and intranasal administration of clodronate-liposomes resulted in increased lung bacterial burden in both wild-type and mutant animals, supporting the notion that macrophages can make a small but measurable contribution to early bacterial clearance.

Dendritic cells

Dendritic cells are professional antigen presenting cells that link innate and adaptive immunity. The role of DCs in the response to *P. aeruginosa* pulmonary infection has only been examined in the context of a post-sepsis model. In this study, animals that underwent cecal ligation and puncture (CLP) eight days prior to intratracheal inoculation with *P. aeruginosa* showed high (80%) mortality, while sham-operated animals uniformly survived subsequent infection [77]. Infected post-CLP mice had lower IL-12(p70) and higher IL-10 levels in BAL than sham-operated controls, suggesting an ineffective Th1 response. The authors had observed that BM derived DCs (BMDCs) from post-CLP mice showed *ex vivo* defects in maturation and T cell priming. Therefore, they administered BMDC from control mice intratracheally along with the *P. aeruginosa* inoculum, and found that this improved murine survival. Intratracheal BMDCs did not alter bacterial clearance, but did modulate the balance between IL-12 and IL-10, suggesting that they play a role in inflammatory responses to pulmonary *P. aeruginosa* in this post-sepsis model [77].

Lymphocytes

Several papers have suggested that lymphocytes might play a role in *P. aeruginosa* infection. Koh *et al.* infected $Rag2^{-/-}$ animals and demonstrated increased mortality in these mice as compared to wild-type controls following intranasal infection with PAO1 [53]. This survival difference was only observed at one bacterial dose, and disappeared with inocula

ca. 2-fold lower or higher. Nieuwenhuis *et al.* made a similar observation in $Rag2^{-/-}$ mice, reporting impaired bacterial clearance in knockout animals after infection with *P. aeruginosa* strain D4 [78]. These authors went on to ask whether natural killer T (NKT) cells, a subgroup of CD1d-restricted T cells that produce large amounts of cytokines upon activation, participated in immune responses to *P. aeruginosa*. They observed that CD1d deficient mice had reduced bacterial clearance, attenuated early neutrophil recruitment and increased mortality as compared to wild-type controls [78]. However, another study using $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice (lacking the major NKT subset of $V\alpha14^{+}$ cells) found no differences in bacterial clearance or neutrophil recruitment after PAO1 infection between these knockout strains and wild-type controls [79]. The different outcomes observed by these two groups may be the result of differences in the bacterial strains used for infection, or may be due to use of $CD1d^{-/-}$ mice backcrossed onto different (Balb/c vs. C57Bl/6) backgrounds.

Natural killer (NK) cells, NKT cells and $CD8^{+}$ T cells all express the NKG2D receptor, which can directly recognize transformed or infected cells that express structurally related surface ligands in the context of cell stress. Borchers and colleagues demonstrated that *P. aeruginosa* pulmonary infection induced expression of the RAE-1 family of NKG2D ligands by airway and alveolar epithelium and alveolar macrophages [80]. Antibody blockade of the NKG2D receptor, which inhibits NK cell-mediated cytotoxicity against NKG2D ligand-bearing cells *in vitro*, decreased pro-inflammatory cytokine and nitric oxide levels in BAL and increased lung bacterial burden some 20-fold at 24 hpi [80]. A follow-up study by these authors employed a mouse model in which the RAE-1 ligand (*Raet1a*) was conditionally expressed from a doxycycline-inducible promoter in airway epithelial cells [81]. Induction of the transgene prior to PAO1 infection improved bacterial clearance and murine survival. NK cells harvested from doxycycline treated mice were more sensitive to *in vitro* stimulation with LPS, as measured by IFN-γ production, than cells harvested from control mice. One interesting aspect of these studies is their suggestion that cross-talk between epithelial cells and immune cells may lower the threshold at which the NKG2D-expressing immune cell respond to TLR ligation, and thus prime the immune system's responses to pathogen.

Many types of T cells (e.g. $CD4^{+}$ Th17 cells, γδ T cells, $CD8^{+}$ T cells and NKT cells) produce the proinflammatory cytokine IL-17. A recent paper examined whether IL-17 influenced murine pulmonary responses to acute PAO1 infection in C57Bl/6 mice [82]. The authors demonstrated that IL-17 mRNA and protein levels increased significantly within 4–8 hpi in lung and BAL, respectively. Intraperitoneal administration of a monoclonal anti-IL-17 antibody prior to infection was associated with diminished G-CSF and KC levels and attenuated neutrophil recruitment to airways at 8 hpi; treated animals showed impaired bacterial clearance at 8 and 16 hpi. $CD4^{+}$ Th17 cells were shown to be one source of IL-17 in the *P. aeruginosa* infected mouse lung.

6. Epithelial cells: sensing and signaling

Isolated airway epithelial cells respond to *P. aeruginosa* in a MyD88- dependent manner, producing chemokines and cytokines such as KC and IL-6. Either TLR4 recognition of LPS or TLR5 recognition of flagellin are sufficient to elicit this response [70]. Epithelial cells are also implicated in *in vivo* innate immune responses to *P. aeruginosa* pulmonary infection. Sadikot and colleagues demonstrated this by targeted modulation of the NF-κB pathway in respiratory epithelium, using adenoviral constructs that expressed either RelA (AdRelA) or a dominant negative allele of IκB (AdIκBdn) to activate or inhibit NF-κB activity, respectively [83]. Mice pre-treated with AdRelA and subsequently infected with *P. aeruginosa* PA103 showed increased bacterial clearance at 24 and 48 hpi, as compared to

mice pre-treated with a control adenovirus. Mice pretreated with the inhibitory AdIκBdn showed impaired bacterial clearance at 48 hpi, and a ca. 30% decrease in recruited airway neutrophils at 24 hpi. Increased TNF-α levels were observed in the infected AdRelA-treated mice, while decreased MIP-2 levels were observed in AdIκBdn-treated mice. Thus, manipulating activity of the NF-κB pathway in epithelial cells was sufficient to alter the innate immune response to *P. aeruginosa*.

MyD88, the adaptor for most TLRs and IL-1R/IL-18R, lies upstream of NF-κB. Mice lacking MyD88 are highly susceptible to *P. aeruginosa* infection, showing delayed and ultimately inadequate recruitment of innate defenses to the lung. Skerrett and colleagues asked whether MyD88 expression in bone-marrow derived cells was sufficient to generate a wild-type response to *P. aeruginosa* pulmonary infection by constructing radiation chimeras between MyD88$^{-/-}$ and wild-type mice [51]. They observed that mice that had received a wild-type bone marrow, but lacked MyD88 expression in stromal cells, had delayed recruitment of neutrophils to the airways following *P. aeruginosa* infection. Conversely, wild-type recipients of a MyD88$^{-/-}$ bone marrow demonstrated early (4 hpi) pulmonary production of KC and MIP-2 and robust neutrophil recruitment similar to that observed in wild-type controls. These findings suggested that non-bone marrow cells play a non-redundant role in initiating innate immune responses to *P. aeruginosa*. More recently, experiments carried out with MyD88$^{-/-}$ mice that express a *CC10-MyD88* transgene demonstrated that expression of MyD88 only in airway epithelial cells was sufficient to control *P. aeruginosa* pulmonary infection [36]. The authors used the IL-1R antagonist, IL-1Ra (anakinra), to demonstrate that IL-1R signaling was required for early neutrophil recruitment and bacterial control in transgenic animals. Anakinra-treated transgenic mice showed an intermediate defect in bacterial clearance at 24 hpi as compared to transgene-plus or transgene-negative animals, suggesting that epithelial cells may also respond to *P. aeruginosa* via other MyD88-dependent pathways *in vivo*, such as those downstream of TLRs. Collectively, these studies suggest that the airway epithelium is a key source of neutrophil chemokines, produced in response to IL-1R ligation. This likely allows the epithelium to “amplify” signals produced by macrophages exposed to T3SS-positive *P. aeruginosa*, such as IL-1β, leading to more rapid and robust neutrophil recruitment [42].

Epithelial cells participate in innate immune responses not only by producing signals that recruit and modulate the activity of leukocytes, but also by secreting products with anti-microbial activities. Airway and alveolar epithelial cells produce surfactant proteins, collectin family members that play roles in innate host defense against bacterial and viral pathogens. Intratracheal infection experiments carried out with surfactant protein (SP)-A and SP-D singly and doubly deficient mice demonstrated modest, but statistically significant decreases in clearance of *P. aeruginosa* PAK at 6 hpi by all mutant mice [84]. SP-A and SP-D can opsonize *P. aeruginosa*, and Giannoni *et al.* went on to demonstrate reduced *in vivo* phagocytosis of infecting bacteria by alveolar macrophages lavaged from mice lacking either or both surfactant proteins. SP-C deficient mice also show subtle defects in *P. aeruginosa* clearance; this protein, however, does not appear to opsonize or kill bacteria directly. Nonetheless, SP-C deficiency was associated with altered macrophage activation and impaired phagocytosis [85]. Many *P. aeruginosa* strains, however, secrete proteases that degrade surfactant proteins; these are produced in response to the quorum-sensing (QS) molecules N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL). These include the zinc metalloprotease, elastase, and protease IV, a serine protease [86, 87]. A recent paper shows that secretion of these exoproteases by wild-type PAO1 during *in vivo* pulmonary infection significantly reduces the content of SP-A and lysozyme in bronchoalveolar lavage fluid, and that a non-flagellated (Δ*fliC*) mutant is defective in exoprotease secretion—and thus, attenuated for virulence in wild-type, but not SP-A$^{-/-}$, mice. This defect in exoprotease secretion was correlated with

defective QS molecule production by the Δ*fliC* mutant, and could be complemented by the addition of synthetic 3-oxo-C12-HSL or C4-HSL [88].

7. Complement System

The complement system is an important effector mechanism of the innate immune system, capable of direct bacterial killing through assembly of the membrane attack complex (MAC; C5b-C9) on the bacterial cell envelope. Murine serum was unable to kill *P. aeruginosa* strain UI-18, despite assembly and activation of the MAC on the bacterial surface; in contrast, human serum was able to kill UI-18, suggesting a species-specific difference in the function of the MAC, or differences in bactericidal enzymes that take advantage of MAC induced cell wall defects [89]. Nonetheless, C5 deficient mice showed increased susceptibility to pulmonary challenge with *P. aeruginosa*, as did mice depleted of complement by the administration of cobra venom factor [89]. Neutrophil recruitment to the lung was not affected by complement depletion, but lung bacterial clearance was markedly impaired, suggesting a defect in bacterial phagocytosis or killing. Activation of complement leads not only to formation of the C5b-containing MAC, but also to production of two potent activation fragments, C3a and C5a, whose inflammatory effects are mediated by binding to their respective receptors, C3aR and C5aR. A prominent role for the C5a anaphylotoxin had already been suggested by Höpken et al, who observed that $C5aR^{-/-}$ mice were profoundly susceptible to intratracheal infection with *P. aeruginosa* PAO1. These mice exhibited massive neutrophil recruitment to the airways, but were unable to clear bacteria from the lungs [90]. This phenotype appeared to be relatively lung specific, as no difference in bacterial clearance or neutrophil recruitment was observed in $C5aR^{-/-}$ versus wild-type mice following peritoneal infection.

C5aR modulates the relative cell surface expression levels of FcγRII and FcγRIII, which have immunosuppressive and immunostimulatory functions, respectively. Rhein *et al.* demonstrated that *P. aeruginosa* infection normally leads to an increase in the FcγRIII/FcγRII ratio by generating C5a; this change in expression ratio is not observed in infected $C5aR^{-/-}$ mice [91]. PAO1 infected FcγRIII-deficient mice phenocopied infected $C5aR^{-/-}$ mice, suggesting that defective modulation of FcγR may underlie the increased susceptibility to *P. aeruginosa*. However, the mechanistic basis for susceptibility was not defined by these studies, as peritoneal macrophages harvested from wild-type and FcγRIII-deficient mice showed no differences in phagocytic activity or production of reactive oxygen species following PMA stimulation [91].

Complement-depleted mice are less susceptible to *P. aeruginosa* infection than C5-deficient mice [89]. Complement-depleted mice cannot produce C3a after *P. aeruginosa* infection, and this may well account for the milder phenotype observed in these animals. $C3aR^{-/-}$ mice intranasally infected with PA103 show the opposite phenotype of $C5aR^{-/-}$ animals: they have increased bacterial clearance, diminished neutrophil recruitment and lower levels of chemokines and pro-inflammatory cytokines in bronchoalveolar lavage fluid 24 hpi [92]. These studies therefore reveal important roles for the complement activation factors C3a and C5a in control of *P. aeruginosa* in the murine pneumonia model, and also illustrate the engagement of both pro- and anti-inflammatory innate responses following pulmonary infection.

8. Signals that modulate the innate immune response to *P. aeruginosa*

We have already discussed many of the chemokines and cytokines produced by lung resident cells upon encountering *P. aeruginosa*, and have described their roles in recruiting and activating immune cells to the site of infection. In the following sections we will focus

on two signaling pathways that have been considered as targets for manipulating the innate response to *P. aeruginosa*.

TNF-α

TNF-α is primarily produced by bone-marrow derived cells in a MyD88-dependent fashion following *P. aeruginosa* acute pulmonary infection [36, 51]. Data regarding the role of TNF-α in *P. aeruginosa* pulmonary infection are derived from rodent models in which either TNF-α or its receptors (TNFR1 and TNFR2) are genetically deficient, or TNF-α interactions with its receptor are acutely inhibited. The findings from these studies are not consistent. Skerrett *et al.* reported that TNFR1/TNFR2 deficient mice (on C57Bl/6) clear the flagellated T3SS-positive strain PAK from their lungs slightly faster than wild-type controls, and neutrophil numbers are 2-fold greater at 4 hpi in receptor-deficient mice [93]. This contrasts markedly with the findings of Lee *et al.*, who reported that TNF-α knockout mice fail to recruit neutrophils to the airways after *P. aeruginosa* infection, and showed a 5-log increase in bacterial burden at 24 hpi (as well as decreased survival) when compared to TNF-$\alpha^{+/+}$ littermates [94]. This second study uses TNF-α mice on a different genetic background (B612SF2/J) and an incompletely characterized *P. aeruginosa* strain (ATCC 33358); these are variables that might influence the relative importance of TNF-α during infection. For example, anti-TNF-α impaired clearance of *P. aeruginosa* in BALB/c mice, in which TNF-α secretion is strongly and rapidly induced after infection, but was without effect in DBA/2 animals, which—like C57Bl/6 mice—showed modest upregulation of TNF-α post infection [95]. Variable expression of bacterial virulence factors can also influence TNF-α induction, e.g. wild-type PA103 induced >20-fold more TNF-α than a T3SS-negative mutant when inoculated into C57Bl/6 airways [42]. It is also possible that lifelong absence of TNF-α promotes compensatory mechanisms in these knockout mice that are absent in TNFR deficient animals or anti-TNF-α treated mice, and that these compensatory mechanisms influence the response to pulmonary *P. aeruginosa*.

In aggregate, TNF-α signals appear to favor bacterial clearance from the lung [83, 95–98], though the magnitude of this effect is relatively modest in all studies except for that of Lee *et al.* In part, this may be due to the fact that TNF-α not only has pro-inflammatory effects, but also induces the expression of anti-inflammatory molecules, such as Muc1 and IL-10, through TNFR1 [99, 100]. Muc1 suppresses TLR signaling [101] and promotes neutrophil apoptosis [99], promoting the resolution of airway inflammation. Like TNFR1$^{-/-}$ mice, Muc1$^{-/-}$ animals show a modest increase in bacterial clearance and increased numbers of neutrophils in the airways [99].

IL-10

IL-10 is an anti-inflammatory cytokine whose production peaks relatively late (24 hpi) after acute *P. aeruginosa* pulmonary infection, at a time when pro-inflammatory cytokine and chemokine levels are diminishing [102]. The role of IL-10 in pulmonary infection is complex: an excess of the molecule, as seen in post-sepsis immunosuppression models [103, 104] or transgenic mice overexpressing IL-10 in the lungs [105] attenuates protective pro-inflammatory responses to *P. aeruginosa* pulmonary infection and diminishes murine survival. Deficiency of IL-10, on the other hand, would be expected to increase pro-inflammatory responses to endotoxin [106] and to infecting pathogens. Indeed, infection of IL-10$^{-/-}$ mice with a mucoid isolate, M5715 (which would likely not express T3SS exotoxins, or pro-inflammatory surface structures such as flagellin), resulted in a prolonged and slightly more exuberant pro-inflammatory response in mutant mice as compared to controls [107]. However, both wild-type and IL-10$^{-/-}$ mice cleared *P. aeruginosa* by day 6, with identical kinetics, in this model. These results contrast with an earlier study by Sawa *et al.* [108], in which administration of recombinant IL-10 one hour prior to and 8 hpi with *P.*

aeruginosa showed a partial improvement in survival and a delay in murine death. In this model, the T3SS-positive, ExoU producing strain PA103 was used, at a dose that killed 80% of mice by 24 and all animals by 48 hpi; mice infected with this high inoculum of PA103 rapidly recruit large numbers of neutrophils to the lung and show substantial pulmonary inflammation ([52]; Lavoie and Kazmierczak, unpublished data). Although the authors did not report the effects of IL-10 administration on the inflammatory response to PA103 infection, the improvement in survival may be due to amelioration of the tissue damage that accompanies this neutrophilic response. In aggregate, these studies are consistent with the notion that rapid recruitment of neutrophils is necessary for the clearance of *P. aeruginosa* from the lung, and that an excess of IL-10 versus pro-inflammatory cytokines is detrimental to control and clearance of this pathogen. However, they also illustrate the negative consequences of an overly exuberant and dysregulated innate immune response on pulmonary function and bacterial clearance—a scenario that appears particularly relevant to the pathogenesis of chronic *P. aeruginosa* infection in CF patients.

9. Relevance of murine studies to understanding human susceptibility to acute *P. aeruginosa* infection

The mammalian lung possesses many different innate mechanisms for recognizing and responding to *P. aeruginosa*. These responses are, in large part, overlapping and redundant, with the result that *P. aeruginosa* infections are predominantly restricted to individuals with some sort of systemic or local immunocompromise. In the murine studies discussed above, TLR and IL-1R signaling pathways play key roles in initial recognition of *P. aeruginosa*, and mice lacking the MyD88 adaptor common to these pathways are profoundly susceptible to infection with this organism. Mutations in the adaptor MyD88 and the downstream kinase IRAK-4 have also been described in humans [109, 110]. Of note, these individuals had an increased susceptibility to a narrow range of bacterial pathogens, specifically *Streptococcus pneumoniae*, *Staphylococcus aureus* and *P. aeruginosa*. In the study of Picard *et al.*, *P. aeruginosa* was responsible for 17% of all invasive and 21% of all non-invasive bacterial infections in MyD88 and IRAK-4 deficient individuals. The consanguineous kindred described by Conway *et al.*, characterized by MyD88 deficiency, contained 7 children, 4 of whom had multiple invasive *P. aeruginosa* infections. This association of susceptibility to *S. pneumoniae*, *S. aureus* and *P. aeruginosa* was also seen in deficiencies of two other innate immune signaling proteins, NEMO and IκBα: although their spectrum of infectious disease is somewhat broader, affected individuals rarely suffer from mycobacterial, fungal or viral disease (reviewed in [111]).

In the series of both Conway *et al.* and Picard *et al.*, severe life-threatening infections were seen in infancy and childhood, but were rare after the teenage years. This improvement in prognosis with age—an unusual feature for a primary immunodeficiency—might suggest that innate immune responses are particularly important before the development of adaptive B- and T-cell responses. Other innate pathways, independent of MyD88/IRAK, might also mature with age, eventually compensating for the lack of TLR and IL-1/IL-18 signaling.

Some acquired and primary immunodeficiencies that affect phagocyte function and/or number are also associated with increased incidence of severe infections due to *P. aeruginosa*. These include congenital neutropenia [112], as well as acquired neutropenias resulting from hematologic malignancies or treatment with bone-marrow suppressing agents [113]. Indeed, empiric antibiotic regimens for neutropenic patients with suspected infections now routinely include anti-Pseudomonal agents, with the result that mortality due to *P. aeruginosa* has significantly declined in this patient population [113].

Leukocyte adhesion deficiencies (LAD), which result in defective neutrophil chemotaxis to sites of infection, are also associated with increased susceptibility to *P. aeruginosa* [114]. LAD type I is characterized by an absence of CD18, which is also a major receptor (CD18/CD11b, CR3) for complement-opsonized particles. Thus increased susceptibility might not only result from impaired neutrophil recruitment, but also from defective non-opsonic phagocytosis of *P. aeruginosa*, as shown by Pollard *et al.* [114]. Whether this defect is of particular significance for alveolar macrophages, functioning in the absence of serum opsonins, is a matter of speculation—but it is interesting to note that the murine studies described earlier revealed a significant role for complement-opsonization in a pulmonary infection model, but not after intraperitoneal infection [90].

10. Conclusions and future directions

Innate immune responses are a primary means of controlling *P. aeruginosa*. Their engagement, particularly in the respiratory tract, requires careful control to achieve rapid recognition and clearance of pathogen while minimizing inflammation-mediated damage to airways and impairment of alveolar gas-exchange. Work with murine models of acute pulmonary infection has revealed a large number of innate immune pathways that can participate in the response to this pathogen. Understanding the relative importance of these pathways and how they are integrated *in vivo*, however, remains a challenge.

One particularly exciting aspect of these studies is our increasing appreciation of the variability of bacterial factors that alter and modulate the host immune response following infection. The expression and even structure of supposedly immutable ligands for pattern recognition receptors, such as LPS and flagellin, can be modulated by *P. aeruginosa* in the environment of the mammalian respiratory tract, varying the capacity of the innate immune system to respond to different strains and isolates of the “same” organism. Genotypic variation in the toxins, exoenzymes, secretion systems and surface structures produced by clinical and environmental isolates affects not only the virulence of these organisms, but also profoundly alters the ways in which they interact with the innate immune system. Quorum-sensing signals and responses to host tissue metabolites lead to further phenotypic variation in bacterial expression of such “virulence factors” and influence the ultimate outcome of these host-pathogen interactions. Many studies, however, have been carried out with relatively little consideration of how these pathogen associated variables influence observed immune responses. This may limit the generalizability of conclusions drawn from such studies.

Transgenic and knockout murine models have served as incomparable resources for studying the *in vivo* contribution of defined innate immune pathways to control of pulmonary infection. Nonetheless, many differences between the murine and human innate immune systems are already appreciated, and others will undoubtedly be described—these will clearly pose challenges as we attempt to apply what we have learned from murine models to improving our ability to manage and cure human *P. aeruginosa* infections. There are other, perhaps less obvious, challenges to interpreting findings from these murine studies. Our review of the literature has clearly shown that still unappreciated differences between inbred mouse lines can profoundly influence the relative importance of a particular host immune pathway, which should lead to caution in interpreting the results of any particular study. *In vivo* outcomes are also sensitive to how infection is initiated (i.e. inoculum size) and when its progression is assayed. If carefully modulated, these variables can provide tremendous insight into likely mechanisms for the complex *in vivo* progression from pathogen recognition, response initiation and amplification, phagocyte recruitment and activation, pathogen clearance, and resolution of inflammation. If incompletely considered, however, they limit our ability to judge cause and effect in a complex *in vivo* model.

Lastly, mice fail to recapitulate many of the more chronic manifestations of human *P. aeruginosa* respiratory tract infections. While this may be relatively unimportant for understanding responses to *P. aeruginosa* acquisition, mechanisms that might lead to persistent colonization in the context of chronic airway disease or CF are not easily studied in this model. In this regard, the recent development and characterization of porcine models of CF that spontaneously develop classic features of human CF disease and have apparent deficits in lung bacterial clearance soon after birth is quite exciting [115]. Though more expensive and technically challenging than rodent models, studies with CF pigs may finally allow investigators to understand the causal roles of inflammation and infection in the development of the profoundly debilitating respiratory tract pathology that characterizes this disease in humans.

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