Mechanisms of phagocytosis and host clearance of *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is a ubiquitous gram-negative rod that grows in many environmental sites. Importantly, *P. aeruginosa* has evolved mechanisms to be an opportunistic extracellular pathogen of its human host. This bacterial species is responsible for acute infections commonly associated with burn wounds and invasive instrument procedures and for chronic infections typically in patients with persistent lung disease and compromised immune responses (86, 87). Conditions that permit these long-term infections are exemplified by the diseases chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (86, 87). The high incidences of both acute and chronic respiratory infections, observed in up to 15% of adult COPD cases and >80% of adults with CF (39, 103), have led to the endeavor to understand the underlying conditions that facilitate *P. aeruginosa* infection and colonization, as well as mechanisms of pathogenesis when the bacteria colonize the lungs of mammalian hosts.

Several lines of evidence support the critical importance of innate immunity and, specifically, professional phagocytic cells as a key determinant in the ability of the host to control *P. aeruginosa* infection. Mice, rabbits, and humans that are missing major subsets of phagocytic cells, such as neutrophils or macrophages, or that lack major innate immune response molecules, such as MyD88 (myeloid differentiation primary response gene 88), are highly susceptible to *P. aeruginosa* infection (6, 68, 76, 97). Additionally, patients with CF are well characterized to generate robust antibody responses against *P. aeruginosa*; however, this is insufficient for effective host control of infection and for sterilizing immunity (9, 115, 116). Finally, people with genetically compromised phagocyte responses, such as those with leukocyte adhesion deficiency (LAD), specific granule deficiency, or anhidrotic ectodermal dysplasia with immune deficiency have a marked predisposition to *P. aeruginosa* infection (6). Thus effective control and clearance of *P. aeruginosa* depends on phagocyte recognition, engulfment, and degradation of bacteria in a highly complex and regulated process that works in concert with other innate immune responses, such as inflammatory signals. Although the well-defined opsonin-mediated uptake pathways (5, 40, 147) apply to *P. aeruginosa*, the mechanisms behind *P. aeruginosa* uptake in the absence of effective opsonization, and how this ties into inflammatory cytokine regulation, are only beginning to be elucidated. Nonopsonic phagocytosis is a crucial mechanism for host control during the initial stages of infection, and mouse models indicate that this process may dictate colonization efficiency at the onset of *P. aeruginosa* challenge (4). Whereas recent reports and reviews have detailed various other components of *P. aeruginosa* respiratory disease, such as pulmonary edema, humoral immunity, and therapeutic avenues (10, 16, 20, 31, 32, 46, 51, 61, 73, 88, 96, 102, 110, 122), this review will spotlight the current state of knowledge in nonopsonic phagocytic recognition and response mechanisms to *P. aeruginosa*.
P. aeruginosa. Here, we will focus on mechanisms of cellular internalization and clearance of the bacteria in the context of phagocytic receptors and pathways that modulate uptake. We will highlight bacterial strategies for phagocytic evasion and discuss the known inflammatory mechanisms that alter phagocytic clearance of the bacteria.

**Bacterial Invasion vs. Phagocytic Engulfment**

Cellular invasion by *P. aeruginosa*. A source of controversy and occasional confusion, especially when discussing phagocytic cells, is the concept of bacterial invasion vs. cellular uptake (see Fig. 1). In the first instance, bacteria actively move from an extracellular space into the host cell cytoplasm or cellular compartment, as exhibited by archetypal intracellular pathogens such as *Salmonella enterica* and *Listeria monocytogenes* (37, 119). Consistent with their lifestyle, these microorganisms have evolved mechanisms to facilitate cellular entry and then survive in the intracellular space using machinery and mechanisms specifically designed for that purpose. An example of this is the specialized secretion system (designated Salmonella pathogenicity island-2) that *S. enterica* Typhi utilizes to inject effector proteins across the phagosome membrane and into the host cytosol to modify the phagosome for bacterial replication (37). Another example is the hemolysin listeriolysin O, secreted by *L. monocytogenes*, which allows the bacterium to escape the phagocytic vacuole and thereby enter and replicate in the host cell cytoplasm (119). The demonstration by Fleiszig et al. (41) that *P. aeruginosa* can invade corneal and epithelial cells in vitro led to the proposal that *P. aeruginosa* may analogously invade host cells to facilitate replication (2, 41, 66, 67, 155, 156). Later experiments show *P. aeruginosa* entry into human embryonic kidney and HeLa cells, as well as bacterial traversal across corneal cell barriers in vivo (2, 63, 66, 67). A consistent theme in many of these studies is host cell membrane composition and polarity. Acute lung infection experiments by Zaas et al. (155, 156) demonstrated that lipid raft composition and the caveolin protein Cav-2 were necessary for *P. aeruginosa* invasion into nonphagocytic host cells, but not in alveolar macrophages. These data were later supported by evidence that demonstrated that *P. aeruginosa* binding affects cell polarity and subsequent membrane composition, allowing the bacteria to preferentially invade the basolateral side of Madin-Darby canine kidney cells (67). Bacterial binding may be facilitated through cellular heparan sulfate proteoglycans and N-glycans, though the role of these receptors in subsequent invasion is still fairly preliminary (17, 18, 117). These collective observations are particularly intriguing since *P. aeruginosa* is not known to exhibit specific adaptations for intracellular survival and, as noted below, does employ potent toxins that efficiently kill host cells. When considering bacterial nutrient acquisition and the optimal niches for persistence, a better understanding of how

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**Fig. 1.** Flagellar motility is a key bacterial determinant for phagocytic susceptibility of *Pseudomonas aeruginosa* by both murine and human phagocytes, with motile bacteria phagocytosed ~100-fold more efficiently than nonmotile bacteria (top). Although the cell surface phagocytic receptor(s) for *P. aeruginosa* require further elucidation, independent reports indicate that bacterial engulfment is actin dependent and controlled by the PI3K/Akt signaling pathway. *P. aeruginosa* has also been proposed to invade epithelial cells (bottom), particularly on the basolateral surfaces of these cells, through alterations in cell polarity and in breaches in the monolayer. Proposed mechanisms include invasion via cell surface lipid rafts in conjunction with the caveolin-2 protein, and via heparin sulfate proteoglycans (HSPGs). The HSPG-mediated pathway is subsequently dependent on intracellular PI3K/Akt protein, and RhoA and/or Cdc42 activation. The relationship between the HSPG and lipid raft-mediated pathways is currently unclear.
invasion into host cells is advantageous for the bacteria may elucidate the physiological and clinical relevance of intracellular *P. aeruginosa*.

**Host phagocytosis of *P. aeruginosa***. In contrast to the above studies that describe a potential intracellular niche, *P. aeruginosa* is generally believed to be an extracellular pathogen, preferring to associate outside of host cell membranes and form extracellular biofilms (153). Entry into neutrophils and macrophages is achieved through active phagocytosis by the host cell. Indeed, this is supported by data linking phagocytosis to bacterial clearance from the host (6, 68, 76). Animal models that lack phagocytic cells quickly succumb to *P. aeruginosa* challenge (68, 76), and people with defects in phagocytic cell function are highly susceptible to *P. aeruginosa* infection (6). Thus internalization is utilized by the host for control of invasive *P. aeruginosa*, and so if the bacterium actively promotes cellular entry, there must be an adaptive benefit that outweighs the cost of potential phagocytic degradation. This leads to a fundamental question in regard to *P. aeruginosa* infections: Are invasion and phagocytosis the same process, and is this merely a semantic, rather than mechanistic, difference? Independent studies have identified that both invasion and phagocytosis rely on phosphoinositide 3-kinase (PI3K) activity in the host cell (62, 66, 67). PI3K converts the host cell membrane lipid phosphatidylinositol (4, 5) bisphosphate (PIP2) into phosphatidylinositol (3, 4, 5) trisphosphate (PIP3) (154), a ligand for Akt recruitment from the cytoplasm. This phosphorylation activity is required for both *P. aeruginosa* invasion into nonphagocytic cells and *P. aeruginosa* engulfment by professional phagocytes (unpublished data). In support of this, the loss of murine PTEN (phosphatase and tensin homolog), the regulatory phosphatase that converts PIP3 back into PIP2, leads to increased phagocytosis of *P. aeruginosa* and in vivo clearance from the lung (60). However, secreted toxins employed by *P. aeruginosa* actively block the activity of certain GTPases, such as Rac1 (Ras-related C3 botulinum toxin substrate 1) and CDC42 (cell division and control protein 42) (64). The functions of these proteins are necessary for phagocytosis by macrophages (discussed below), but the GTPase activities are not necessary for *P. aeruginosa* internalization into epithelial cells (64). Separate reports found that *P. aeruginosa* activates the upstream GTPase RhoA (Ras homolog gene family, member A) in epithelial and endothelial cells, which in turn promotes internalization and vascular permeability (46, 63, 72). Therefore, targeted regulation of host GTPase activity by *P. aeruginosa* can both promote intracellular invasion and inhibit phagocytic ingestion.

**Role of activated protein C pathway**. The RhoA pathway has also garnered recent attention in the context of activated protein C. The ratio of active Rac1 to RhoA in the lung endothelium, which is regulated by the activated protein C pathway, appears to be critical for the regulation of lung edema and protein permeability during *P. aeruginosa* infection (13, 22, 123). Pretreatment of animal models with activated protein C is reported to modulate paracellular permeability and lung injury caused by *P. aeruginosa* through inhibition of Rho [necessary for *P. aeruginosa* internalization into epithelial cells (64)] and activation of Rac1 [necessary for engulfment by phagocytes (80)], but it does not change neutrophil influx or inflammatory cytokine production (13, 22, 123). However, clinical trials using recombinant human protein C for treatment of severe sepsis and septic shock failed to reproducibly decrease patient mortality, and the therapy has subsequently been withdrawn from use (77). Unfortunately, the patient parameters utilized across these trials were inconsistent, making it difficult to reconcile why the treatment failed (77). Recombinant protein C efficacy may be optimized in combination with traditional antibiotic treatment and with pulmonary therapies targeted to patients presenting disorders in pulmonary coagulation. Indeed, there is speculation that patients in the early PROWESS trial, which reported a 6.1% decrease in 28-day mortality, benefited from early antibiotic treatment and volume resuscitation, whereas patients in later trials were selected on the basis of clinical presentation of refractory shock, which may not obligatorily be linked to dysregulated coagulation and is an independent indicator of poor prognosis (77). Therefore, the data to date indicate that, for *P. aeruginosa*-elicited pneumonia, recombinant protein C treatment may be most beneficial if applied during the early stages of infection. Pretreatment has been shown to reduce subsequent pulmonary bleeding and edema and to promote phagocytic clearance through stimulation of Rac1 GTPases in resident alveolar macrophages (13, 123). However, exogenous protein C does not change the host inflammatory response (22), and so efficacy will likely be reduced once *P. aeruginosa* is established and has triggered broad bacterial-derived and immune pathology.

In summary, although invasion and engulfment are distinct cellular processes, there is significant overlap in the machinery that facilitates both activities, which in turn are influenced by both bacterial (e.g., toxins) and host (e.g., protein C) factors. *P. aeruginosa* may coopt cellular uptake mechanisms, evolved to facilitate bacterial clearance by professional phagocytic cells, at certain sites of infection, notably in the eye and potentially in the lungs, to traverse the initial epithelial barriers and promote its own colonization. Since *P. aeruginosa* has adapted to survive in a wide range of environmental niches, specific evolution to invade the human epithelium seems unlikely. Rather, it has likely evolved global mechanisms that can be regulated to promote colonization at sites that provide the most favorable growth conditions. In mammalian hosts, bacterial internalization by host cells at certain anatomical sites may be beneficial and yet must be balanced against phagocytic susceptibility. Indeed, *P. aeruginosa* employs multiple factors, such as exotoxins, alginate secretion, and the regulation of motility (discussed later) to evade phagocytosis during infection and likewise avoid predation by amoeba in the environment. The evolution of these factors suggests that internalization by eukaryotic cells is more often detrimental to *P. aeruginosa* than it is beneficial.

**Receptor-Mediated Internalization of *P. aeruginosa***

The signaling events that govern phagocytic uptake of *P. aeruginosa*, primarily by neutrophils and macrophages, are complex and feature various levels of specificity. Engagement is mediated through bacteria-to-phagocyte contact and binding, in concert with receptor-based recognition of *P. aeruginosa* and downstream signaling to regulate ingestion. As such, engulfment is dictated by numerous factors, including receptor expression on the phagocytes (see Table 1), as well as ligand expression and exposure on the pathogen.
Table 1. Host cell receptors implicated in Pseudomonas aeruginosa invasion or phagocytosis

<table>
<thead>
<tr>
<th>Potential Host Receptors Interacting with P. aeruginosa</th>
<th>Function</th>
<th>Implicated in Invasion or Phagocytosis?</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fcγ-receptors (CD11b/CR3)</td>
<td>Binds Fc portion of host IgG opsonins</td>
<td>phagocytosis</td>
<td>1, 5, 40, 140</td>
</tr>
<tr>
<td>Complement receptors (CD11b/CR3)</td>
<td>Binds host iC3b complement opsonins</td>
<td>phagocytosis</td>
<td>57, 118</td>
</tr>
<tr>
<td>Scavenger receptor A</td>
<td>Binds polyanionic sugars and lipoproteins</td>
<td>neither</td>
<td>3, 30</td>
</tr>
<tr>
<td>MARCO</td>
<td>Binds polyionic ligands</td>
<td>phagocytosis</td>
<td>30</td>
</tr>
<tr>
<td>Toll-like receptors</td>
<td>Binds bacterial extracellular components (e.g., LPS, flagellin, peptidoglycan)</td>
<td>neither</td>
<td>4, 147</td>
</tr>
<tr>
<td>CFTR</td>
<td>N-glycan chloride ion channel</td>
<td>phagocytosis</td>
<td>29, 109, 114, 148</td>
</tr>
<tr>
<td>Caveolin proteins (Cav-1, Cav-2)</td>
<td>Lipid-raft (caveolae) integrated signal transduction</td>
<td>invasion</td>
<td>155, 156</td>
</tr>
<tr>
<td>Heparan sulfate proteoglycans</td>
<td>Binds pilin, low molecular weight OMPs, ionic residues</td>
<td>invasion/adherence</td>
<td>17, 18, 117</td>
</tr>
</tbody>
</table>

MARCO, macrophage receptor with collagenous structure; CFTR, cystic fibrosis transmembrane conductance regulator; OMP, outer membrane protein.

Opsonic phagocytosis. The most elaborated mechanisms of foreign particle engulfment involve phagocytosis driven by antibody-binding of Fc-receptors and complement binding of complement receptors (CRs). Like many gram-negative pathogens, P. aeruginosa is susceptible to opsonic phagocytosis. This process has been extensively covered elsewhere (1, 5, 40, 140) and shares many of the same molecular mechanisms as in nonopsonic uptake. Briefly, receptor engagement leads to recruitment and phosphorylation of kinase signaling molecules, which enable the downstream recruitment and activation of cytoskeletal arrangement proteins such as Rho and Rac GTPases and actin-related proteins; these subsequently coordinate actin polymerization and membrane distortion at the target site (106, 147). Additional host factors, such as surfactant protein A and serum amyloid A, have also been reported to bind P. aeruginosa and facilitate phagocytosis by alveolar macrophages (52, 91). Although opsonic phagocytosis contributes to P. aeruginosa clearance, it is important to note that P. aeruginosa commonly infects sites, such as the lung, where phagocytes express relatively low levels of opsonic receptors (137).

Nonopsonic phagocytosis. Importantly, phagocytosis of P. aeruginosa and some other bacteria also occurs when opsonization is absent or ineffective, and this is independent of Fc-receptor or CR activation (89, 106, 125, 133, 134). Nonopsonic phagocytosis likely evolved as a frontline mechanism for the immune system to recognize and respond to pathogens at the very onset of infection. Likewise, this mechanism is critical for bacterial clearance at anatomical sites of endogenously low levels of antibody and complement, such as the bronchopulmonary tree and the urinary tract (134). Although this ingestion process uses much of the same intracellular machinery as opsonization-based phagocytosis, the mechanism is not well understood. The process is further complicated by redundant receptor activity and differential expression, a dependence on the type of particle being engulfed, and promiscuous receptor binding to bacterial ligands (3, 40, 106). Early studies by Heale et al. (57) and Pollard et al. (118) suggested that the integrin CD11b (CR3) as well as CD14 mediate phagocytosis of P. aeruginosa, but the evidence indicates that this is strain dependent and seems to be highly variable on the basis of the ligand moieties and polysaccharide architecture presented by the bacteria. For example, Pollard et al. observed that a P. aeruginosa strain isolated from a CF patient was not efficiently engulfed by neutrophils isolated from a patient with LAD (deficient in CR3), yet a strain isolated from the LAD patient was efficiently engulfed by LAD phagocytes, thus pointing to the variability and specificity in phagocyte-strain interactions (118). This variability in receptor-ligand interaction for effective phagocytosis extends to other bacterial species as well. Some outer membrane proteins, such as OmpA, in virulent strains of Escherichia coli and Klebsiella pneumoniae dictate recognition and internalization by macrophages (59, 141), but not necessarily neutrophils (45), and loss of the OmpA ortholog, OprF, in P. aeruginosa may affect both association with host cells and the ability of the bacteria to ultimately form a biofilm (38, 75). Likewise, we recently showed that scavenger receptor A (SR-A) on mouse phagocytes is the major phagocytic receptor engaged by specific strains of γ-proteobacteria (e.g., DH5α E. coli), but it is not singularly necessary for uptake of the parental strain K12 E. coli, nor is it needed for uptake of PA14 P. aeruginosa (3). However, a recent report by Domingo-Gonzalez et al. (30) demonstrated that treatment of alveolar macrophages with soluble MARCO (macrophage receptor with collagenous structure), which is also a class-A scavenger receptor, inhibited phagocytosis of P. aeruginosa by ~50%. Observations such as these highlight the complexity and heterogeneity of receptor signaling and ligand specificity during nonopsonic engulfment processes. Lastly, it is important to distinguish phagocytic uptake from bactericidal killing. For example, the loss or blockade of active CFTR (cystic fibrosis transmembrane conductance regulator) protein has been interpreted as being directly responsible for the defective clearance of P. aeruginosa by phagocytes (148) and also for epithelial cells (114), although the contribution of epithelial cell phagocytic activity to clearance of P. aeruginosa is likely minimal. Subsequent experimentation has called into question whether CFTR specifically facilitates engulfment of P. aeruginosa, since the data support an alternative explanation that CFTR activity may instead facilitate phagolysosomal degradation of the bacteria (26, 29, 109).

An additional complication in the search for a specific phagocytic receptor for P. aeruginosa is that many of the pathogen-associated molecular patterns (PAMPs) that might be expected to facilitate phagocytosis, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin, also stimulate host immune signaling receptors, particularly the Toll-like receptors (TLRs) (128). The TLRs responsible for recognition of these components, particularly TLR2, TLR4, and TLR5, play a critical role in leukocyte stimulation and activation, yet these do not appear to directly facilitate ingestion (147). Additionally, the loss of MyD88, a signaling adaptor for many TLRs, does not alter the phagocytic capacity of mouse macrophages for P. aeruginosa (4). Taken together, the accumulated data indicate that the stimulus for phagocytosis during P. aerugi-
nosa infection involves multiple mechanisms of uptake that can work independent of, or synergistically with, each other and can potentially involve the stimulation of multiple families of receptors. Thus a current and challenging avenue of investigation is to understand the underlying mechanisms of phagocytosis during host clearance of P. aeruginosa and to clarify how differential ligand expression patterns and receptor-ligand interactions between phagocytes and bacterial strains change the phagocytic dynamic, in terms of both recognition and killing.

Bacterial Strategies for Phagocytic Evasion

Although P. aeruginosa is susceptible to phagocytic engulfment and degradation, multiple tactics have been identified by which the pathogen attenuates vulnerability to phagocytosis. One such phenotypic trait is the conversion from a free-swimming, planktonic lifestyle to a biofilm-associated, sessile lifestyle (85). This transition correlates with long-standing clinical observations that P. aeruginosa downregulates flagellar motility and converts to a strong mucoid phenotype. Additionally, most P. aeruginosa strains express potent toxin secretion systems which, along with nonmotility and exopolysaccharide secretion, are believed to cumulatively contribute to phagocytic resistance and subsequent bacterial persistence.

Loss of flagellar motility by P. aeruginosa. The majority of laboratory and environmental P. aeruginosa strains are highly motile, utilizing a single, polar, montrichous flagellum for swimming. Infectivity studies have found that flagellar functionality is necessary for P. aeruginosa to initially colonize the host and establish infection (8, 115). Lillehoj et al. (82, 83) demonstrated that the P. aeruginosa flagellum binds to host cell Muc1 mucin, which is produced in the upper airway endothelium, whereas later studies confirmed that flagellar motility is required for P. aeruginosa to establish stable colonization within the host (86, 105, 115). This colonization corresponds to a conversion to a biofilm phenotype in which the bacteria are typically nonmotile, since reports that examine bacterial isolates recovered from long-term, chronic respiratory disease have shown that P. aeruginosa populations in these infections have absent or downregulated flagellar gene expression (42, 85, 104). The basis for this loss of flagellar motility is multifaceted. For the pathogen, conversion to a sessile lifestyle can be metabolically beneficial if the bacteria are in a nutrient-rich site, and the advantages to bacterial populations living in close-knit aggregates (e.g., quorum sensing) have been well documented (126). Additionally, host immune factors may also contribute to this conversion. Neutrophil elastase, a serine proteinase secreted during inflammation, has been shown to repress expression of P. aeruginosa flagellin (132). The observed conversion to a nonmotile biofilm during chronic infection may also reflect selection for this state. P. aeruginosa that form biofilms are much more resistant to standard-of-care antibiotics used in the clinic (100, 101, 151). Additionally, the progressive recovery of nonmotile bacterial isolates may reflect selection of bacteria resistant to phagocytosis. We recently identified that flagellar motility in P. aeruginosa is a critical phagocytic activation pattern both in vitro and in vivo (4). Loss of flagellar motility, independent of the flagellum itself, provides the bacteria with a ~100-fold increase in resistance to phagocytic uptake by macrophages, neutrophils, and dendritic cells (see Fig. 1) (4). This is independent of other bacterial virulence factors or host opsonization and is due to bacterial activation of the host cell PI3K/Akt signaling pathway specifically by swimming motility (Ref. 84 and Lovewell et al., unpublished data). Flagellar swimming motility results in cellular Akt activation, which in turn regulates phagocytic recognition in the host cell and subsequent phagocytosis of the bacteria (unpublished data). This is supported by separate studies illustrating that the PI3K/Akt pathway is necessary for both TLR-based activation and bacterial internalization into mammalian cells in various models of P. aeruginosa infection (49, 60, 66, 67). Furthermore, deletion of PTEN phosphatase, an enzyme which has the opposite activity of PI3K and dephosphorylates PIP3, leads to increased phagocytic activity of P. aeruginosa in alveolar macrophages (60). How the host response to bacterial motility is transduced through the plasma membrane is unknown, but we speculate that it may reflect the ability of swimming bacteria to induce receptor clustering or a membrane perturbation. Regardless, it is clear that the down-regulation of flagellar motility, which is observed in chronic lung infections, directly contributes to P. aeruginosa persistence by providing the pathogen a potent means of phagocytic evasion.

P. aeruginosa exopolysaccharide secretion. A second phenotype commonly observed in chronic P. aeruginosa infections is exopolysaccharide secretion (EPS). P. aeruginosa contains a number of genetic loci that can generate or modify exopolysaccharides (psl locus), alginate production (alg and muc loci), and a mucoidy phenotype (98, 107, 127). These gene products elicit the EPS matrix and canonical biofilms commonly associated with P. aeruginosa establishment in the lungs and elsewhere. Importantly, genetic analyses have also identified that expression of some of these gene products provides for phagocytic resistance (19, 74, 108). During initial host colonization, nonmucoid strains expressing the polysaccharide Psl effectively limit deposition of complement molecules C3, C5, and C7, presumably by hindering access of the complement to the bacterial outer membrane (98). This provides the bacteria with measurable resistance to CR-mediated phagocytosis and an early advantage against the innate immune system (98). Moreover, once P. aeruginosa has established persistent infection in the host, it converts to an increasingly mucoid phenotype, characterized by the upregulation of alginate production (87, 92, 93). Leid et al. (81) and others have illustrated that mucoid strains and biofilm-associated P. aeruginosa resist phagocyte activation and uptake in both opsonized and nonopsonized conditions, supporting an initial finding by Eftekhar and Speert (35) that the phagocytic defect in alginate-positive strains can be rescued by treatment with alginate to degrade the mucoid EPS. Unfortunately, the direct mechanism of alginate-based phagocytic inhibition remains unclear, since conflicting interpretations suggest that alginate may sterically inhibit bacteria-to-phagocyte binding and influence engulfment processes (108), or alternatively it may inhibit GTPase activation of signal transduction pathways (69), which are believed to contribute to P. aeruginosa engulfment. Again, although many of the underlying mechanisms remain to be elucidated, it is clear that P. aeruginosa has evolved strategies to limit the host phagocytic response at the onset of infection (Ps1 production), during establishment within the host (pro-
P. aeruginosa type-III secretion system. Although EPS production by bacterial pathogens has been well documented in many systems, secretion of anti-phagocytic molecules by P. aeruginosa is not limited to sugars. P. aeruginosa has a fully functional type-III secretion system (T3SS) by which it can inject effector proteins ExoT, ExoS, ExoY, and ExoU directly into host cells (12, 36, 79, 120). Relative expression of these effectors is strain dependent, with different P. aeruginosa strains exhibiting different combinations of effectors (55, 129). ExoY is a nonspecific cyclase that increases vascular permeability but is not directly cytotoxic, whereas ExoU is a potent phospholipase that compromises mammalian cell membranes (36, 54). Although neither of these bacterial proteins has directly been shown to affect phagocytic functionality, the toxins ExoT and ExoS have been shown to limit uptake of P. aeruginosa by macrophages (54). The inhibitory mechanism of these two effectors is based on targeting host Rho, Rac, and CDC42 GTPases. Mechanistically, these host G proteins regulate various cellular functions including cytoskeletal organization and their activity is necessary for P. aeruginosa internalization. When injected into host cells, ExoT and ExoS act as GTPase-activating proteins that provide for the loss of GTP-bound Rac1 and CDC42 in macrophages (47, 63, 143). Thus these toxins shift the equilibrium of host GTPases into the inactive, GDP-bound form, functionally locking out the major regulators of cytoskeleton rearrangement and hindering phagocytic capability (27, 47). Additionally, these exotoxins can induce host cell death, particularly in epithelial cells, which promotes tissue damage and limits cellular immune responses (36). During P. aeruginosa-induced respiratory disease and infection, these effectors contribute to persistence by attenuation of ingestion and clearance by phagocytes and also facilitate cell death, thereby exacerbating infection severity.

P. aeruginosa inhibition of PAR2. Another virulence factor of note that has recently been identified to functionally inhibit host phagocytosis is the elastolytic metalloproteinase LasB (bacterial elastase, not to be confused with neutrophil elastase), which cleaves host protease-activated receptors such as PAR2 (34). PAR2 signaling is described to regulate host inflammatory responses, particularly in the lung, and a recent paper by Moraes et al. (99) demonstrated that loss of PAR2 on neutrophils significantly decreased phagocytic uptake of P. aeruginosa through an undefined mechanism. Activation of PAR2 has been reported to recruit phagocytic machinery, such as MAP kinases, Rho-Rac GTPases, and actin (136), and the protein could therefore be acting as a direct phagocytic receptor. Conversely, stimulation could simply lead to indirect immunomodulatory effects that activate phagocytes for enhanced phagocytic capability. Regardless, the capacity for PAR2 as a regulator of phagocytic uptake for P. aeruginosa remains intriguing, especially when considering that expression of elastase by virulent strains of P. aeruginosa could potentially modulate both inflammatory responses and phagocytosis through PAR2 inhibition.

Taken together, it is apparent that P. aeruginosa utilizes toxin secretion in concert with alginate production and alterations in motility to limit effective host responses. These observations highlight the adaptations P. aeruginosa has developed to enhance colonization and persistence in human hosts, and consequently the recent mechanistic findings are now clarifying the bases of the bacterial phenotypes documented in chronic pulmonary infections.

Models of Phagocytosis of P. aeruginosa

P. aeruginosa can infect human hosts through multiple routes. Acute infections are commonly found in COPD-based pulmonary disease, burn wounds, and keratitis of the eye, and with invasive instrument procedures such as bronchial tubes, catheters, and intravenous needles (86). Chronic P. aeruginosa infection, particularly in the lungs, has been extensively studied and is usually limited to immune-compromised patients, and the associated bacterial pneumonia in patients with CF is often the ultimate cause of mortality (48, 87, 104). Since the host phagocytic response is believed to be a key determinant in the subsequent pathology, multiple in vitro and animal models of phagocytic cell responses to P. aeruginosa, with various strengths and weaknesses, have been developed to help elucidate how this pathogen interacts with this critical arm of the innate immune system.

In vitro models of phagocytosis. Labeling of P. aeruginosa with fluorescence and antibiotic-resistance genes is the basis for many of the classic phagocytic assays. These assays are typically assessed by either microscopy or antibiotic protection following coinubcation with phagocytic cells derived from the lung or elsewhere. These in vitro assays are well suited to provide information on P. aeruginosa-host-phagocyte interactions and have been extensively used to contribute to our understanding of the mechanisms of binding and the downstream effects leading to ingestion, both through bacterial adhesins such as pili and flagellin and through cellular integrins, scavenger receptors, and opsonic receptors. Much of our understanding of phagocytic components and regulation involved in P. aeruginosa infection, as well as the aforementioned bacterial mechanisms of phagocyte evasion, also come from standard coinubcation experiments. These continue to be the easiest means to tease apart the basic biology of P. aeruginosa-phagocyte interactions, but unfortunately these experiments are also limited. During P. aeruginosa infection of the lungs or at other sites, bacteria interact with highly heterogeneous cell populations that cannot be completely reproduced in vitro. Similarly, the dynamic interactions between humoral components, such as opsonins, proteases, and extracellular fibers (e.g., neutrophil NETS) and pathogens make comprehensive in vitro modeling very challenging. Concomitantly, extracellular factors expressed by P. aeruginosa, such as the previously mentioned mucoid phenotype, are not static and can change on the basis of environmental factors. Therefore, modeling phagocytosis in vitro is useful in elucidating basic mechanisms but is limited, as all in vitro experiments are, in terms of scope and translation to natural disease.

Murine models of phagocytosis. The more clinically relevant in vivo models of infection have also proven useful in understanding many various P. aeruginosa interactions, although there continue to be many challenges with execution and interpretation of the phagocytosis and bacterial clearance experiments. Mice, and to a lesser extent rats, infected with P. aeruginosa via the lung (inhalation/aspiration), eye (corneal scratch), and peritoneum (direct injection) are the most widely used models. A major strength of these murine models is that
mice display a robust acute response as measured by neutrophil and macrophage cellularity and proinflammatory cytokine production in a matter of hours postinfection (111, 149, 150). When mice are challenged with a sublethal dose, phagocytes will rapidly clear the commonly used laboratory strains of *P. aeruginosa*, whereas increasing dosages of bacteria lead to exacerbation of bacterial sepsis, cytokine storm, and increased host lethality, but not necessarily chronic infection (68, 111, 139, 149). As such, these murine infections are well suited models for acute pneumonia and instrument-based and wound-associated infections. A recent advance in assessment of in vivo phagocytosis, which was subsequently adapted for use with *Borrelia burgdorferi* (56), was the use of a modified gentamicin-protection assay employed to quantify phagocytosis of *P. aeruginosa* in both the murine lung and peritoneal cavity over a short, single-dose infection period (4). This assay is useful in modeling initial phagocytic and innate immune responses but is a poor representation of long-term exposure to *P. aeruginosa*, since the infection is typically resolved by mice within 48 h.

**Zebrafish models of phagocytosis.** Like the murine model, the newly emerging zebrafish model of *P. aeruginosa* infection is an exciting tool for studying host-pathogen interactions in acute infections. Zebrafish can be genetically manipulated, are susceptible to *P. aeruginosa* infection, and rely on innate immune mechanisms for control of bacterial infections. Thus, with the advent of transparent fish lines that express fluorescent neutrophils and macrophages, investigators have recently been able to perform intravital imaging of *P. aeruginosa* interactions with phagocytes in real time (15). Moreover, the immune cell profile in zebrafish embryos can be manipulated through morpholino-based gene regulation tools, allowing investigators to tease apart the relative spatiotemporal contribution of various effector cells during *P. aeruginosa* infection (23). These advances provide a powerful tool for probing the dynamics of *P. aeruginosa*-host cell interactions, trafficking, and phagocytosis in vivo. Two major limitations of this model are the disparities in anatomy (e.g., lungs) and that, as in murine infections, *P. aeruginosa* does not chronically infect zebrafish and the model does not effectively replicate persistent human disease.

**Models of phagocytosis in chronic infections.** Studies of chronic *P. aeruginosa* infection, especially in the context of CF, are some of the most pursued avenues in *P. aeruginosa* research. CF is a genetic disorder caused by a mutation in the CFTR gene, which codes for an ion transport protein. In humans, loss of CFTR function leads to numerous physiological defects, most notably defective mucociliary clearance in the lungs and the buildup of mucus and alveolar fluid (121). This allows for eventual chronic colonization by *P. aeruginosa*, leading to inflammation and fatal pneumonia (121). Modeling the disease in murine hosts is complicated by the well-documented observation that mice with a deletion in the CFTR gene do not recapitulate human CF disease, which includes susceptibility to chronic *P. aeruginosa* colonization (50, 65). Therefore, although acute infections in mice retain a high degree of relevance for *P. aeruginosa* in humans, including in vivo experiments of phagocytic clearance, a major risk factor in chronic *P. aeruginosa* infection is not well represented in classic murine models.

A potential workaround in mice is the agarose-bead model of *P. aeruginosa* infection. Developed by Cash et al. (21) and modified for mice by Starke et al. (135), the protocol utilizes bacteria embedded in small agarose polymer plugs. The polymer physically retards bacterial clearance in vivo and allows for a longer, more chroniclike infection. This model has been used to examine various immune and drug responses to persistent *P. aeruginosa* in vivo, such as mortality and cytokine induction between CF-related mouse genotypes (58, 145). However, the use of the beads inhibits phagocyte association with bacteria and does not enable bacterial properties, such as motility, binding, and adhesion, that influence interactions with host cells and therefore initiation and persistence of infection (58, 135). Also, the scope and duration of infection is on the order of days and weeks, and longer infection studies require reinfection of the mice (58, 135). Thus the model is still limited in clinical relevance to natural chronic *P. aeruginosa* infection in humans, especially in terms of phagocytic clearance.

The recently developed porcine model of CF and, to a degree, the recently reported ferret model have many advantages in studying lung disease in vivo, including pneumonia associated with *P. aeruginosa* infection (65, 124, 142). Mutation of the CFTR gene in pigs and ferrets leads to pathology more closely resembling that observed in humans (65). Specific to *P. aeruginosa* engulfment, several reports have identified impaired bacterial clearance from CFTR-deficient pigs (113, 124, 138). So far the evidence indicates that this is not due to inherent phagocytic deficits conferred by loss of CFTR, but rather due to impaired bacterial killing elicited from misregulated pH of the alveolar fluid and insufficient oxidation of the lysosomal compartment in the alveolar phagocytes (113, 124). This is supported by corresponding in vitro work using murine and human *cfr−/−* phagocytes that demonstrate that *CFTR* does not play a role in the actual engulfment process but instead contributes to phagolysosomal degradation by modulation of compartmental ion gradients (26, 29, 109). Thus it is likely that the chronic *P. aeruginosa* colonization in *cfr−/−* pigs, as well as human patients, is due to multiple contributing factors such as impaired mucociliary clearance, phagocytic evasion mechanisms of the bacteria, and inhibition of bactericidal activity in alveolar phagocytes. In both the pig and ferret models, the eventual development and availability of genetic and immunological tools for these animals will hopefully better delineate the in vivo contribution of bacterial motility, alginate production, and toxin secretion to phagocytosis and pathogen clearance in the context of CF.

**Ties between phagocytosis and inflammation.** The host inflammatory response to *P. aeruginosa* is intimately connected to the phagocytic clearance of the bacteria. *P. aeruginosa* expresses a variety of PAMPs (e.g., LPS, flagellin) that lead to a robust inflammatory response. This response is critical for control of the bacterial infection through recruitment and activation of phagocytic cells. The inflammatory response has to be tightly regulated, however, since lack of an efficient inflammatory response can lead to reduced infiltration of phagocytes, resulting in reduced bacterial clearance via phagocytosis and other bactericidal mechanisms (71). Conversely, disproportionate inflammation can damage the host tissue due to excessive infiltration and activation of phagocytes and in some cases may even be coupled to reduced bacterial clearance (24, 130). Although there is certainly evidence that alveolar macrophages are direct contributors to bacterial clearance
through phagocytosis (4, 60), they are also essential for mounting early inflammatory responses and recruitment of neutrophils for subsequent bacterial uptake and clearance. In support of this, depletion of alveolar macrophages prior to intratracheal instillation of bacteria in an acute model of infection led to delayed bacterial clearance kinetics, likely as a consequence of impaired neutrophil influx (71).

**PRRs and PAMPs in *P. aeruginosa*-induced inflammation.**

*P. aeruginosa* expresses potent TLR2, TLR4, TLR5, and TLR9 agonists, and the TLR4-dependent inflammatory response to LPS, in particular, is critical to clear infection (78, 131). Since the loss of MyD88-dependent responses leads to lower inflammatory cytokine production and reduced bacterial clearance in an acute infection model (131), many of the early studies focused on the roles of TNF-α and (to a lesser degree) IL-6. Interestingly, the magnitude of the TNF-α response to *P. aeruginosa* is modulated by the phagocytic class-A scavenger receptor (SR-A or MSR1), with loss of SR-A expression associated with delayed bacterial clearance kinetics, likely as a consequence of impaired neutrophil influx (71).

**Inflammasome activation during *P. aeruginosa* infection.**

An emergent mechanism for the relationship between inflammation and phagocytic response to *P. aeruginosa* focuses on the role of the cytokine IL-1β. IL-1β is increased in the sputum and bronchoalveolar lavage fluid of CF patients with *P. aeruginosa* infections (14, 53). Likewise, clearance of *P. aeruginosa* with antibiotics in children is correlated with reduced IL-1β production (33). As discussed previously, *P. aeruginosa* uses its T3SS to inject effector proteins into host cells (54). Yet along with effector proteins, potent *P. aeruginosa* PAMPs such as flagellin, the T3SS rod-protein PscI, and pilin can also be translocated through the T3SS, which leads to cellular activation of the NLRC4 (NLR family, CARD domain 4) inflammasome and the release of IL-1β (and IL-18) (7, 44, 94, 95, 144). The fundamental mechanisms underlying NLRC4-dependent release of IL-1β in response to *P. aeruginosa* has recently been the subject of several excellent reviews (25, 43, 112); here we will focus on the role of the elicited IL-1β in relation to subsequent bacterial phagocytosis, killing, and clearance.

IL-1β is predominantly produced by macrophages and, in response to *P. aeruginosa*, is dependent on the NLRC4 inflammasome. In the early phases of infection, IL-1β is sensed by resident epithelial cells through the IL-1 receptor (IL-1R) leading to the upregulation of other neutrophil chemottractants such as macrophage inflammatory protein 2 (MIP-2) and KC/IL-8 (70, 146). An effective immune response depends on the early recruitment of neutrophils through these chemokines, since blockade of CXCR (chemokine C-X-C motif receptor)-dependent signaling leads to reduced neutrophil cellularity and bacterial clearance (146). Although cleavage of monomeric flagellin in the environment by the bacterial protease AprA is thought to limit TLR5-dependent responses (11), recent evidence suggests that the TLR5-MyD88 axis also plays an important role in bacterial phagocytosis and killing in alveolar macrophages through an IL-1R-dependent pathway in response to swimming-competent *P. aeruginosa* (28).

The use of genetic knockout mice and Anakirna (IL-1R antagonist effective on both humans and mice) to elucidate the bactericidal mechanisms involved has indicated that IL-1R signaling is crucial to control bacterial growth in an acute lung infection model (97, 152) but could lead to deleterious effects on the host during the later phases of infection (24, 130). Independent studies by Cohen and Prince (24) and Schultz et al. (130) demonstrated the role of IL-1-driven pathology during the early course of infection, leading to delayed clearance of bacteria from the murine lungs. It should be noted that these results are in the context of an acute *P. aeruginosa* infection in IL-1R⁻/⁻ mice, which may have compensatory or interrelated mechanisms (such as reduced autophagic degradation of inflammatory cytokines) leading to excessive inflammation and delayed bacterial clearance. Although Schultz et al. pharmacologically blocked IL-1R signaling in wild-type (WT) mice and obtained similar data compared with the IL-1R⁻/⁻ experiments, the differences in bacterial survival in the lungs of WT mice and IL-1R⁻/⁻ mice were dependent on the dose of infection as well as the time at which bacterial survival was measured (130). Another explanation for the reduced bacterial burden in the lungs of IL-1R⁻/⁻ mice compared with the WT controls may be that the excessive inflammation elicited by *P. aeruginosa* could have deleterious effects on the WT host, thereby providing the bacteria with a niche to survive and multiply. This could account for the differences seen in bacterial burden in WT and IL-1R⁻/⁻ mice at later time points [18 or 24 h postinfection (hpi)] (24, 130). In contrast, IL-1R⁻/⁻ mice displayed reduced neutrophil influx and bacterial clearance compared with WT mice at 4 hpi (152). Similarly, pharmacological blockade of IL-1R signaling in a mouse model where MyD88 expression was restricted to airway epithelial cells led to reduced neutrophil recruitment and reduced bacterial clearance from the lung up to 24 hpi (97). In this model, only airway epithelial cells could signal through IL-1R owing to the presence of MyD88, whereas IL-1R signaling was deficient in the hematopoietic compartment. Cumulatively, these data indicate that IL-1β is required for the early clearance of *P. aeruginosa* and can lead to the secretion of IL-1R-dependent chemokines such as KC/IL-8 and MIP-2 via epithelial cells to control infection through recruitment of neutrophils (97, 152). Thus potent IL-1β response to *P. aeruginosa* is pivotal since these models demonstrate the protective as well as the deleterious effects downstream of IL-1R signaling.

An additional layer of complexity was recently revealed that ties in to the ability of *P. aeruginosa* to evade the host innate immune response. IL-1β production in response to *P. aeruginosa* is independent of phagocytosis but requires both bacterial contact with host phagocytes and a functional T3SS (111). However, phagocytic evasion by *P. aeruginosa* through loss of flagellar motility is conferred, in part, by reduced bacterial...
interaction with the phagocyte cell surface (84). This results in reduced in vitro and in vivo IL-1β responses to nonmotile P. aeruginosa and is correlated with neutrophil recruitment and subsequent bacterial clearance (111). Thus loss of bacterial flagellar expression or motility likely confers an advantage to the bacteria and enables chronic infection through evasion of phagocytosis by resident cells and, through minimization of the IL-1β response, a synergistic amelioration of subsequent IL-1β-dependent bacterialicidal responses including recruitment and activation of additional phagocytes.

The threshold of IL-1β required for clearance of bacteria through recruitment of phagocytes without causation of excessive damage to host tissue is currently not well understood. In the case of chronic infection, understanding the kinetics of IL-1R signaling and bacterial clearance will be critical since excessive or reduced IL-1β production could both lead to increased bacterial survival, albeit through different signaling mechanisms. Finally, understanding the pivotal effects of the magnitude of IL-1β in response to bacterial load on epithelial and hematopoietic cells, as well as subsequent chemokine release and the downstream effector responses, will be essential toward gaining a more comprehensive understanding of the extent to which different cell types dictate bacterial clearance vs. pathology.

Summary

The interactions between host and pathogen are often complex and dynamic, even when simplified to a particular immunological response to a specific bacteria. Although phagocytosis is a critical process within the innate repertoire of defenses against P. aeruginosa in the lung and elsewhere, understanding how it is regulated has not been simple. Care must be taken to differentiate between bacterial invasion and host internalization, because the two processes can share similar components and outcomes but are operationally different, especially in terms of pathogenesis. Likewise, the cellular structures and receptors that govern phagocytosis of P. aeruginosa, and how these interact with pathogen effectors and virulence factors, will need to be further elucidated before we can fully understand why phagocytosis is such an important factor in P. aeruginosa infection and, mechanistically, how it is initiated and regulated both by the host and the pathogen. Lastly, effective clearance of P. aeruginosa must be considered in the context of inflammatory responses. Although uptake and inflammation are not necessarily dependent on each other, they can influence the efficacy of one another and are indirectly linked as the major mechanisms of control, and successful pathogen clearance requires both processes to work synergistically. Current and emerging models of P. aeruginosa disease, especially in the context of chronic pulmonary infection, will provide further insights into how effective phagocytic clearance during infection is achieved.

REFERENCES


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