Pseudomonas aeruginosa flagellar motility activates the phagocyte PI3K/Akt pathway to induce phagocytic engulfment

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Lovell RR, Hayes SM, O’Toole GA, Berwin B. Pseudomonas aeruginosa flagellar motility activates the phagocyte PI3K/Akt pathway to induce phagocytic engulfment. Am J Physiol Lung Cell Mol Physiol 306: L698–L707, 2014. First published January 31, 2014; doi:10.1152/ajplung.00319.2013.—Phagocytosis of the bacterial pathogen Pseudomonas aeruginosa is the primary means by which the host controls bacterially induced pneumonia during lung infection. Previous studies have identified flagellar swimming motility as a key pathogen-associated molecular pattern (PAMP) recognized by phagocytes to initiate engulfment. Correspondingly, loss of flagellar motility is observed during chronic pulmonary infection with P. aeruginosa, and this likely reflects a selection for bacteria resistant to phagocytic clearance. However, the mechanism underlying the preferential phagocytic response to motile bacteria is unknown. Here we have identified a cellular signaling pathway in alveolar macrophages and other phagocytes that is specifically activated by flagellar motility. Genetic and biochemical methods were employed to identify that phagocyte PI3K/Akt activation is required for bacterial uptake and, importantly, it is specifically activated in response to P. aeruginosa flagellar motility. Based on these observations, the second important finding that emerged from these studies is that titration of the bacterial flagellar motility results in a proportional activation state of Akt. Therefore, the Akt pathway is responsive to, and corresponds with, the degree of bacterial flagellar motility, is independent of the actin polymerization that facilitates phagocytosis, and determines the phagocytic fate of P. aeruginosa. These findings elucidate the mechanism behind motility-dependent phagocytosis of extracellular bacteria and support a model whereby phagocytic clearance exerts a selective pressure on P. aeruginosa populations in vivo, which contributes to changes in pathogenesis during infections.

phagocytosis; Pseudomonas aeruginosa; Akt; flagellar motility

The opportunistic bacterial pathogen Pseudomonas aeruginosa is a highly motile, Gram-negative rod that is ubiquitous within the environment. P. aeruginosa causes acute clinical infections often associated with compromise of lung function, burn wounds, and invasive instrument procedures, and also causes chronic infections in immunocompromised patients (21, 22). The vast majority of patients with the genetic disorder cystic fibrosis (CF) exhibit chronic pulmonary infection with P. aeruginosa, leading to chronic and fatal inflammation and pneumonia (22, 26). For both acute and chronic infections, bacterial flagellar swimming motility is a key determinant of P. aeruginosa colonization of the host (1, 2, 20, 23, 24).

Multiple reports have demonstrated that flagellar function is a critical virulence factor for the establishment, persistence, and inflammatory profile of P. aeruginosa infection (1, 2, 19, 28). P. aeruginosa swims via a single, polar, monotrichous flagellum, which it rotates by proton motive force (33). In the context of disease, this flagellar swimming motility is necessary for the establishment of infection, since P. aeruginosa mutants that lack swimming capability are attenuated in pathogenesis (21). This conforms with the current paradigm that free-swimming bacteria require flagellar motility to initially attach and remain associated, and that loss of motility reflects a strong environmental pressure for the bacteria to convert to a sessile, biofilm lifestyle (2, 7, 21). These nonmotile bacteria are highly resistant to antibiotic treatment and phagocytic host clearance (10, 20, 21).

With regard to the contribution of bacterial motility to the recognition and clearance of P. aeruginosa, we recently identified that bacterial flagellar motility is a pattern-recognition signal for phagocytic engulfment by innate immune cells (1, 19). Bacteria which have lost flagellar motility, either through loss of the flagellar structural protein FliC or the loss of the stator complexes in the flagellar motor, are ~100-fold more resistant to phagocytosis (1, 19) by the cells critical for clearance of P. aeruginosa: neutrophils and macrophages (17). The P. aeruginosa stators are two partially redundant complexes, MotAB and MotCD, which are not necessary for flagellar assembly, but are required for flagellar rotation and swimming motility (5, 33). Genetic deletion of both stator complexes (motABmotCD) leads to a nonswimming mutant, while deletion of single mot complexes leads to intermediate swimming phenotypes, with a motAB mutant displaying greater swimming motility than a motCD mutant (33). Importantly, phagocytosis is proportional to the flagellar swimming capability of the bacteria, and so phagocytic susceptibility is greatest in a highly motile WT strain of P. aeruginosa, and titration of flagellar motility through successive genetic deletion of the stator complexes leads to a progressive increase in phagocytic resistance, independent of the flagellum itself acting as a phagocytic ligand (19). This has prompted the search for specific cellular signaling pathways that are responsive to flagellar motility and that facilitate bacterial engulfment.

While there is currently no known phagocytic receptor specific for P. aeruginosa, clues to candidate intracellular signaling pathways that may mediate motility-facilitated phagocytosis have emerged. Kierbal et al. (15) observed that blockade of cellular phosphatidylinositol-3-kinase (PI3K) activity prevented P. aeruginosa entry into kidney and HeLa epithelial cells, while Kannan et al. (14) proposed that a PI3K-activator, Lyn kinase, may also regulate the phagocytic fate of P. aeruginosa. Based on these findings, we hypothesized that the PI3K/Akt pathway may be a key link in the
cellular phagocytic response governed by bacterial motility. The PI3K/Akt signaling pathway is commonly initiated by transmembrane receptor signaling, followed by activation of Src kinases such as Lyn, to recruit and activate PI3K (12). This increases cellular plasma membrane concentrations of phosphatidylinositol-(3,4,5)-triphosphate (PIP3), which results in the recruitment, phosphorylation, and activation of the master regulatory kinase Akt (12, 35). Akt controls cellular phagocytic responses through multiple downstream targets that regulate actin polymerization and cytoskeletal arrangements at the target site (31, 35).

Here we have used complementary genetic and biochemical techniques to demonstrate that activation of the cellular PI3K/Akt signaling pathway is a necessary mechanistic component in P. aeruginosa engulfment by alveolar macrophages and other murine and human phagocytes. Importantly, we find that cellular Akt activation, which leads to the phagocytic internalization of the bacteria, is dependent on, and proportional to, the bacterial flagellar motility. These results elucidate a novel regulatory mechanism for phagocytosis of motile pathogens and provide an insight into how P. aeruginosa swimming motility, a necessary virulence factor for the establishment of infection, is recognized by the host immune system and triggers activation of a major innate immune pathway. Correspondingly, this mechanism affords a selective pressure within the host environment for swimming bacteria to downregulate flagellar motility to thereby avoid phagocytic clearance.

MATERIALS AND METHODS

Mice, cells, and bacteria strains. Bone marrow-derived dendritic cells (BMDCs) were cultured from C57BL/6 WT mice obtained from NCI using a modification of Inaba et al. (13) as previously described. Lyn-knockout (Lyn−/−) and TLR5-knockout (TLR5−/−) mice were obtained from Jackson Laboratories. THP-1 cells were provided by Dr. Paul Guyre (Dartmouth) and MH-S cells were provided by Dr. Albert van der Vliet (Univ. of Vermont). All cells were cultured and maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, 5% (vol/vol) Pen/Strep, 5% (vol/vol) L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol until harvest (13). The Pseudomonas aeruginosa strain PA14 is a nonmucoid clinical isolate and is the parental bacterial strain for all of the respective mutants studied. All animal experiments were approved by the Dartmouth Medical School Institutional Animal Care and Use Committee.

Gentamicin protection assays. Phagocytosis of live bacteria was performed as a modified version of published protocols (6) and as previously described (1). Briefly, overnight cultures of P. aeruginosa in LB broth were washed and resuspended in serum-free Hanks’ Balanced Salt Solution (HBSS), and bacteria concentrations were determined by optical density at 600 nm. Where indicated, 2.5 × 10^5 BMDCs of the indicated genotype or THP-1 or MH-S cells were preincubated in 100 μM 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002, Sigma), 5 μM 1,3-dihydro-1-(1-(4-(6-phenyl-1H-imidazol-4-yl)quinolin-7-yl)phenyl)methyl)-4-piperidiny1)-2H-benimidazol-2-one (Akt-Inhibitor VIII, Calbiochem), and Complete protease inhibitor cocktail (Roche) as described by the manufacturers. Cells were analyzed by FACS for acquisition of fluorescence as a function of Akt activation. For forced-contact experiments, an equalized degree of bacteria-host cell contact was achieved by centrifugation of THP-1 cells together with bacteria for 10 min at 715 g as previously described (19), and Akt activation was subsequently measured as described above.

Bacterial swimming assay. Bacterial swimming assays were performed according to established protocols (3, 33). Briefly, a single bacterial colony was picked and inoculated into the center of an LB plate containing 3.5% agar. Plates were wrapped in parafilm and incubated for 24 h at 37°C and then for 24 h at room temperature. Bacterial swimming capacity is represented by the diameter of colony growth and is visualized by a FluorChem 8900 gel imager using ChemiImager 5000 software.

Statistical analyses. Sample sizes and replicates are indicated in the figure legends. For all graphs, means and SDs are shown. For comparison between two data sets, statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison posttest. Statistical significance is represented by an asterisk and indicates P ≤ 0.05.

RESULTS

Pseudomonas aeruginosa flagellar motility is necessary to activate Akt kinase in phagocytic cells. Since our previous data demonstrated that flagellar motility in P. aeruginosa is necessary for phagocytic uptake of the bacteria both in vitro and in vivo within the lungs (1), we hypothesized that flagellar swimming triggers the activation of intracellular signaling pathways in alveolar phagocytes, and that bacteria that lack flagellar motility do not activate these pathways. The mammalian PI3K/Akt pathway (Fig. 1A) is an important signaling component for phagocytosis or invasion of many bacteria into host cells (15, 35). To test the hypothesis that bacterial swimming motility, specifically, is necessary for cellular Akt activation, primary murine alveolar macrophages or MH-S alveolar macrophages were coincubated with a highly motile wild-type strain of P. aeruginosa (WT PA14) or with a mutant in the same genetic background that lacks the genes necessary for stator function (motABmutCD). The motABmutCD mutant has a fully assembled flagellum, but lacks the ability to produce any torque to

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rotate that flagellum, and thus constitutes a nonswimming phenotype (33). After coincubation of phagocytes with equal numbers of either motile or nonmotile bacteria, we immediately fixed the cells and probed with an antibody specific for the phosphorylated species of Akt and then quantitatively assessed relative Akt activation by FACS analyses. The interaction of phagocytes with WT PA14 elicited robust phosphorylation of Akt; however, exposure to the motABmotCD mutant did not elicit Akt activation, with cellular phospho-Akt levels remaining similar to background (Fig. 1, B and C). To ask if this observation is specific to alveolar macrophages or represents a widespread mechanism in phagocytes, phospho-Akt was assessed in murine BMDCs and human THP-1 monocytic cells (Fig. 2, A and C, respectively). Treatment with phorbol 12-myristate 13-acetate (PMA; a positive control) or WT PA14 elicited robust Akt activation, while coincubation with motABmotCD bacteria revealed that nonmotile bacteria evade phagocyte Akt activation (Fig. 2, A and C). As a control for specificity, cellular PI3K was blocked by LY294002 treatment. Accordingly, this blocked Akt activation even in the presence of motile WT PA14 bacteria, with Akt phosphorylation remaining at background levels in both murine BMDCs (Fig. 2A) and human THP-1 cells (Fig. 2C). The flagellin-recognition receptor TLR5 can potentially modulate signaling activity in the PI3K/Akt pathway (29); therefore we evaluated the contribution of TLR5 to our system by performing parallel Akt activation assays in BMDCs that lack TLR5. We observed that WT PA14, but not motABmotCD, bacteria elicited strong Akt activation in TLR5-deficient cells (Fig. 2B), similar to our findings with WT BMDCs. We therefore conclude that *P. aeruginosa* flagellar motility is necessary to activate mammalian Akt, both in murine pulmonary phagocytes and primary cultured cells, and in human phagocytes, and that this motility-dependent activation is dependent on PI3K but independent of TLR5.

PI3K and Akt activity are required for phagocytosis of *Pseudomonas aeruginosa*. Because the presence or absence of bacterial swimming motility differentially induced the activation of the Akt pathway in both murine and human phagocytes, we next explicitly tested the contribution of the PI3K/Akt pathway to the ingestion of *P. aeruginosa* with the use of a gentamicin protection assay. Equal numbers of WT PA14 or

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**Fig. 1.** Akt activation in murine alveolar macrophages by *Pseudomonas aeruginosa* requires functional flagellar motility. A: diagram of mammalian phosphatidylinositol-3-kinase (PI3K)/Akt phosphorylation pathway. B and C: primary murine alveolar macrophages (B) or M-H-S macrophages (C) were coincubated with WT PA14 or motABmotCD bacteria, and then fixed and probed for phospho-Akt. The histograms (left) are representative of Akt activation in primary or M-H-S macrophages as measured by FACS analysis. Quantification of the Akt activation elicited by treatment with WT PA14 or motABmotCD bacteria is shown at right. MFI is normalized to untreated cells (=1 on Y-axis). N = 4 technical replicates of at least 2 biological replicates. *P < 0.05 compared with WT PA14.
motABmotCD bacteria were coincubated with MH-S alveolar macrophages that had been pretreated with the PI3K-inhibitor LY294002, and internalized bacteria were subsequently quantitatively evaluated. From this we found that blockade of PI3K activity for uptake of P. aeruginosa (Fig. 3A). The necessity of PI3K activity for uptake was also confirmed in BMDCs (Fig. 3B). To further evaluate the role of this pathway in P. aeruginosa phagocytosis, we primed BMDCs with the PI3K enzymatic product PIP3 and observed Akt activation drastically reduced phagocytosis of motile WT (Fig. 3B). We have previously shown that uptake of P. aeruginosa, equal numbers of WT PA14 or motABmotCD were coincubated with BMDCs that had been pretreated with the small-molecule Akt-Inhibitor VIII, which selectively blocks the PH domain of Akt and prevents its activity. Inhibition of Akt activity significantly reduced phagocytosis of P. aeruginosa to levels similar to those observed during PI3K inhibition (Fig. 3C). We have previously shown that uptake of P. aeruginosa is not dependent on MyD88 signal transduction (1). To specifically address the contribution of TLR5 to Akt-facilitated phagocytosis of motile bacteria, perhaps through alternative adaptor molecules such as TRIF (4), we compared phagocytosis of P. aeruginosa in WT and TLR5-deficient BMDCs in the presence or absence of Akt inhibition. Loss of TLR5 did not inhibit engulfment of P. aeruginosa (Fig. 3D, inset). Importantly, consistent with experiments that used WT BMDCs, TLR5−/− BMDCs coincubated with motABmotCD displayed a ~100-fold deficit in bacterial phagocytosis compared with those coincubated with WT PA14, and treatment of TLR5−/−

**Fig. 2.** *Pseudomonas aeruginosa* flagellar motility elicits Akt activation in murine and human phagocytes. Murine bone marrow-derived dendritic cells (BMDCs) (A and B) and human THP-1 cells (C) were incubated in the absence or presence of WT PA14 or motABmotCD bacteria and then fixed and probed for phospho-Akt. A: histogram (left) is representative of Akt activation in murine WT BMDCs measured by FACS analysis. Bars (right) exhibit quantification of Akt activation in WT BMDCs elicited by treatment with 40 ng/ml PMA, WT PA14, motABmotCD, or WT PA14 in the presence of 100 μM LY294002. Parallel studies to A were performed with murine TLR5−/− BMDCs (B) and human THP-1 cells (C). MFI is normalized to untreated cells (= 1 on Y-axis). N = 3 replicates of at least 2 biological replicates. *P < 0.05 compared with WT PA14. NS = not significantly different from WT PA14.
BMDCs with Akt-Inhibitor VIII significantly blocked bacterial uptake (Fig. 3D). Therefore, since TLR5-deficient cells are not compromised for phagocytosis of swimming bacteria and are dependent upon motility-based Akt activation, we conclude that TLR5 is not a necessary component in motility-based phagocytic uptake. However, since inhibition of both PI3K and Akt by drug treatment resulted in arrested bacterial uptake, we conclude that this signal transduction pathway, independent of TLR5, is crucial in the phagocytic response to *P. aeruginosa*. Finally, we have previously demonstrated that differences in CFU recovery between bacterial genotypes are not due to any difference in susceptibility to intracellular killing once the bacteria have been internalized (1, 19), and direct treatment of bacteria alone with LY294002, Akt-inhibitor VIII, or PIP3 does not affect bacterial growth dynamics (data not shown). Therefore, these data support a model where a cellular stimulus, initiated by bacterial flagellar motility, initiates a phosphorylation cascade through PI3K that leads to activation of Akt, which is necessary for phagocytosis of the swimming bacteria.

**Forced contact between phagocytes and nonswimming motABmotCD bacteria is not sufficient to elicit Akt activation.** We have previously shown that nonswimming *P. aeruginosa* do not initially bind to the cell surfaces of phagocytes as well as motile bacteria, and while forced contact between bacteria and phagocytes will overcome the binding deficit, it will not overcome the phagocytic deficit of the nonswimming mutant (19). Because artificially equalized contact between BMDCs and swimming or nonswimming bacteria does not elicit equal degrees of phagocytosis (19), we asked whether the preferential cellular Akt response to swimming *P. aeruginosa* was in response to phagocyte interactions with the bacteria or was directly reflective of bacterial flagellar motility. Therefore, we tested the hypothesis that forced contact between BMDCs and motABmotCD would be insufficient to activate Akt to the levels observed in equalized contact with WT PA14. To examine this, WT PA14 or motABmotCD were centrifuged on top of layered murine BMDCs to force equal cell-to-bacteria contact (18) between both bacterial genotypes and the BMDCs. Following coincubation of the BMDCs and bacteria, Akt activation, assessed by induction of phospho-Akt, was determined by FACS analysis. Forced contact of motABmotCD provided for a small increase in Akt phosphorylation levels above background; however, it did not elicit the same levels as WT PA14 (Fig. 4). These results support the hypothesis that cellular Akt activation is in response to bacterial flagellar motility and that cellular contact is not solely sufficient to induce Akt activation by nonswimming *P. aeruginosa* mutants.

**Cellular Lyn kinase is dispensable for phagocytic engulfment of Pseudomonas aeruginosa.** Lyn kinase is a member of the Src-kinase family of protein kinases that can contribute to
the upstream recruitment and activation of PI3K and has been implicated as an important signaling molecule in the cellular internalization of *P. aeruginosa* (14). To specifically test the contribution of Lyn kinase to motility-dependent phagocytosis, we coincubated WT BMDCs or Lyn$^{-/-}$ BMDCs with WT PA14 or motABmotCD bacteria and then assayed for Akt activation and phagocytic engulfment. We observed a modest decrease in Akt phosphorylation in BMDCs lacking Lyn compared with WT BMDCs both at steady state and when the cells were incubated with WT PA14, and the total increase in phospho-Akt upon incubation with WT PA14 was similar between WT and Lyn$^{-/-}$ BMDCs (Fig. 5A). The small difference in Akt activation in Lyn$^{-/-}$ cells did not translate into a phagocytic defect, as bacterial uptake by WT cells was not significantly different from that of Lyn$^{-/-}$ BMDCs for either the WT PA14 or the motABmotCD bacteria (Fig. 5B). These data demonstrate that the activity of Lyn kinase is not critical to the phagocytic response to *P. aeruginosa*. A likely possibility is that there is functional redundancy for Lyn kinase in the phagocytic pathway, with Lyn contributing to Akt activation in the presence of bacterial stimuli, but not acting as the unique transducer for initiation of phagocytic ingestion.

Akt activation by swimming *Pseudomonas aeruginosa* is not dependent on actin polymerization. Since phagocytosis of motile *P. aeruginosa* was found to specifically trigger, and be dependent on, activation of cellular Akt, we sought to determine if Akt phosphorylation is directly coupled to actin polymerization during phagocytic uptake. We hypothesized that Akt activation occurs prior to actin-based closure of the phagocytic cup, and that phosphorylation is a downstream consequence of phagocytosis-Akt activity. To test this, we treated human THP-1 phagocytes with the mycotoxin cytochalasin B, which inhibits actin polymerization, and assayed for phosphorylation and phospho-Akt elicited by swimming *P. aeruginosa*. Consistent with previous results (19), pretreatment of cells with cytochalasin D significantly blocked phagocytic uptake of WT PA14 (Fig. 6A), but did not change the growth dynamics of *P. aeruginosa* on LB media (data not shown), confirming that engulfment of *P. aeruginosa* is an actin-dependent process. Importantly, even in the presence of cytochalasin D we observed that coincubation with WT PA14 elicited a significant increase in phospho-Akt (Fig. 6B) comparable to that observed with no drug treatment (Fig. 6B, inset). This indicates that Akt is activated by swimming *P. aeruginosa* independent of actin polymerization, even though both cellular processes are necessary for phagocytosis. These data are consistent with other models of bacterial internalization, which support that cytochalasin D treatment marginally attenuates total phospho-Akt levels, but that bacterial entry is not necessary to activate mammalian Akt (32). Thus these results clarify the order of events during phagocytosis, showing that bacterial swimming motility triggers host Akt phosphorylation, which then coordinates downstream actin-based phagocytic uptake.

*Pseudomonas aeruginosa* swimming capability determines the degree of Akt activation and phagocytic susceptibility. We previously demonstrated a direct correlation between flagellar
swimming motility and phagocytic susceptibility using a series of mutations in *P. aeruginosa* that progressively attenuate flagellar torque generation, and by a biochemical titration of *V. cholerae* flagellar motility (19). We therefore hypothesized that if the cellular Akt pathway is responsive to bacterial motility, then the stepwise loss of swimming motility by the bacteria will cause a commensurate decrease in cellular Akt activation. To test this, we used mutations in the stator genes of *P. aeruginosa* to control the degree of flagellar motility. These mutations lead to decreasing bacterial motility in the order of WT PA14 > motAB > motCD > motABmotCD (Fig. 7A), and we have previously found that each of these successive mutations leads to a significant decrease in phagocytic susceptibility (19). Murine BMDCs were coincubated with highly motile WT PA14, partially motile motAB, severely repressed motCD, or nonmotile motABmotCD, and subsequently fixed and probed for phospho-Akt. We observed that WT PA14 elicited the highest level of Akt activation, while the mot series of mutants induced progressively less Akt activation in BMDCs (Fig. 7B), corresponding to the phenotypic attenuation of motility. Plotting the average phospho-Akt induction for each respective genotype against the corresponding values for phagocytic susceptibility (19) indicates a remarkably high correlation ($R^2 = 0.9645$) between activation of Akt and subsequent phagocytosis as a function of flagellar motility (Fig. 7C). These results extend and support our previous studies connecting flagellar swimming capacity with phagocytic susceptibility (1, 19), and identify the Akt pathway as the mechanism by which phagocytic cells respond to swimming bacterial pathogens.

**DISCUSSION**

In this work we have discovered a new role for a major signal transduction pathway as a regulatory response mechanism in the phagocytosis of the bacterial pathogen *P. aeruginosa*. While previous studies have identified that the flagellar motility of *P. aeruginosa* is a potent activator of phagocytic engulfment, the major signaling pathway(s) responsible for motility-driven bacterial ingestion remained unclear. Here we have utilized genetic and biochemical methods to delineate that bacterial swimming motility activates the host PI3K/Akt phosphorylation pathway in phagocytic cells. Activation of the master regulatory kinase Akt is necessary for engulfment of the extracellular pathogen *P. aeruginosa*, and loss of bacterial motility leads to a corresponding loss of Akt stimulation and phagocytosis. This is independent of the flagellum itself acting as a phagocytic ligand, independent of TLR5 signaling, and cell-bacteria contact alone is not sufficient to induce Akt phosphorylation and bacterial ingestion. Our data indicate that the major stimulus for phagocytic uptake of *P. aeruginosa* is flagellar motility, and we show for the first time that flagellar torsion acts as a biological rheostat for induction of Akt activation and subsequent actin coordination for phagocytic ingestion.

Flagellar motility has long been recognized as a major virulence factor in *P. aeruginosa* and other pathogens (2, 7, 20, 23, 27). Numerous studies have reported that bacterial pathogens that are typically motile but have genetically or physiologically lost flagellar function subsequently fail to establish an infection during host challenge (11, 18, 21). The precise underlying mechanisms for the motility dependence are complex and remain to be clarified, but infection is likely promoted by motility-based signaling, tissue tropism, coregulation with alternative virulence factors, and flagellar surface adhesion. Importantly, once bacterial colonization is established, there is a strong phenotypic prejudice toward the loss of motility (2, 7, 21). This likely represents a global trend in host-bacteria interactions, as this loss of flagellar motility has been documented in many different pathogenic and symbiotic systems; for example, *V. fischeri* colonization of squid ducts requires flagellar motility, but once established in the host, the bacteria then lose flagellar function and must convert to a sessile lifestyle to exhibit their canonical bioluminescence (30). For the pathogen *P. aeruginosa*, flagellar motility is required to initially establish the microcolonies and biofilms observed in cystic fibrosis patients, yet clinical isolates recovered from patients suffering from chronic, long-term infections are largely nonswimming, having downregulated flagellar gene expression or phenotypically lost the flagellum entirely (20, 23). This coincides with bacterial resistance to host immune strategies, particularly phagocytosis.
The necessity of phagocytic cells for host defense against *P. aeruginosa* has been well documented, as mice lacking phagocytes and mediators of innate immunity quickly succumb to *P. aeruginosa* challenge (17, 25). Phagocytic engulfment and degradation of bacteria by neutrophils and macrophages appears to be the major mechanism of host control of *P. aeruginosa* (17). Correspondingly, phagocytic evasion by *P. aeruginosa* and its persistence in chronic infections coincides with the loss of swimming motility (23, 24). The physiological process of phagocytosis itself is a highly coordinated series of events that can result from a multitude of different stimuli (9). Ingestion of nonopsonized bacteria is commonly mediated by a variety of receptors, such as scavenger receptors and integrins, which bind to foreign sugars and protein structures, yet due to a high degree of redundancy in downstream effects, identification of specific receptors for individual pathogens has proven difficult (9). As such, there is currently no known phagocytic receptor specific for *P. aeruginosa*, yet phagocytosis readily occurs both in vitro and in vivo with a preferential response to bacteria that retain active flagellar motility (1, 19, 23, 24). This motility-dependent phagocytic recognition is not due to a simple deficiency in bacteria-cell association, since nonswimming mutants maintain phagocytic resistance even after cellular contact and binding, nor is it due to changes in expression of any recognized pathogen-associated molecular pattern (PAMP), secreted molecules, susceptibility to bactericidal degradation, or growth defect (19). Furthermore, flagellar motility also dictates the phagocytic susceptibility of *V. cholerae* and *E. coli* to varying degrees (19). Since the phenotype of flagellar swimming is prominent among bacterial species, and the host response to it is conserved between mice and humans (1, 19), we believe that it is probable that flagellar motility activates specific fundamental cellular processes that lead to bacterial engulfment and clearance. This likely reflects a widespread mechanism of pattern recognition utilized by the innate immune system for defense against motile bacterial pathogens in the lungs and elsewhere.

Since swimming competency is required for *P. aeruginosa* to induce Akt phosphorylation, which is necessary for engulfment of the bacteria, this supports the hypothesis that the PI3K/Akt pathway is the major regulatory process for motility-driven engulfment. This is also in agreement with separate models showing that motile *P. aeruginosa* will invade epithelial cells at preferentially localized PI(3)P-rich membrane sites and in an Akt-

![Image](https://example.com/image.png)

**Fig. 7.** Phagocyte Akt activation correlates with *Pseudomonas aeruginosa* swimming motility. **A:** WT PA14, motAB, motCD, or motABmotCD bacteria were assayed for swimming motility in LB media containing 3.5% agar. **B:** murine BMDCs were coincubated with WT PA14, motAB, motCD, or motABmotCD bacteria and then fixed and probed for phospho-Akt. Akt activation was quantified by FACS analysis and MFI was normalized to untreated cells (= 1 on Y-axis). **C:** relative phospho-Akt elicitation quantified by coincubation of murine BMDCs with the indicated genotype of *P. aeruginosa* is plotted against relative phagocytic uptake for each respective genotype. Average phospho-Akt is quantified by FACS analysis and MFI is normalized to untreated cells. Phagocytosis is represented as a percent of WT PA14 susceptibility, assayed by gentamicin protection assay, and normalized for input bacteria. *N* = 4 replicates from at least 2 independent experiments. *P* < 0.05 compared with WT PA14.
dependent manner (16). Likewise, we were able to stimulate uptake of *P. aeruginosa* by exogenous treatment of cells with PIPs, which likely increased the recruitment of total Akt from the cell cytoplasm and thus primed those cells for phagocytosis. Consequently, we observed increased phagocytosis of both WT PA14 and motABmotCD, but the observation that phagocytosis of motABmotCD did not reach the same level as WT (even at the level of lipid saturation) suggests that the phagocytic signal in motility-dependent uptake is at least partially localized to the site of bacteria-cell contact, yet is still dependent upon PI3K activity. A potential explanation is that receptor activation at the site of bacteria-cell contact, elicited through flagellar swimming, induces localized activation of PI3K and subsequently Akt, which in turn directs ingestion at that target site.

While we identified PI3K and Akt as two vital components in the signaling cascade initiated by bacterial flagellar motility, there are likely multiple adapter proteins that contribute in various degrees to the overall phagocytic stimulus. Previous work using broad-spectrum Src kinase inhibitors and RNAi has suggested that Lyn kinase may be an important signaling molecule, upstream of PI3K, in the phagocytic response to *P. aeruginosa* (14). To rigorously test this we used murine genetic knockouts for Lyn but did not identify a significant defect in phagocytic engulfment of the bacteria. Thus, while it is possible that Lyn kinase makes a contribution to the signaling cascade that leads to phagocytosis, it appears that functional redundancy may exclude an identifiable phenotype in regard to phagocytosis of motile or nonmotile *P. aeruginosa*. Focusing downstream of Akt, we observed that phagocytosis did not occur when actin polymerization or Akt kinase activity was inhibited, but Akt was still activated by swimming *P. aeruginosa* in the presence of cytochalasin D, indicating that the order of events is that actin-based engulfment was facilitated by Akt activity. This is supported by independent studies of the intracellular bacterium *Salmonella typhimurium*, which show that treatment with cytochalasin D diminishes total phospho-Akt in epithelial cells, but Akt is still activated, even in the presence of cytochalasin D, by *Salmonella* coinfection (32). We therefore propose a model where flagellar motility facilitates mammalian Akt activation, which in turn coordinates actin-regulated engulfment of the swimming bacterium.

As previously noted, the identification of a cell-surface phagocytic receptor specific for *P. aeruginosa* has yet to be elucidated. We have previously observed that deletion of the adaptor protein MyD88 did not affect phagocytic uptake of *P. aeruginosa*, and interpreted this result as indicative of TLR5 being dispensable for motility-driven phagocytosis (1). However, work in other systems using epithelial cells has suggested that TLR5 can potentially signal through an alternative adaptor protein TRIF, and that PI3K activation can be regulated through TLR5 (4, 29). Thus, we specifically tested the role of this receptor using a TLR5 genetic knockout, and found that loss of TLR5 did not inhibit phagocytosis of WT PA14; it slightly (∼2×) increased uptake. Moreover, TLR5−/− BMDCs responded to treatment with Akt-Inhibitor VIII which blocked phagocytosis of *P. aeruginosa*, which indicates that even in the absence of TLR5 Akt functions to promote bacterial phagocytosis. Therefore, TLR5 does not appear to be the receptor responsible for sensing bacterial flagellar motility. It remains to be determined if the stimulus for motility-driven phagocytosis occurs though a novel, motility/tension-sensing receptor, or is due to multiple receptors being activated in a relatively short time period through repeated association and contact derived from the action of bacterial swimming. While there are examples of tension-sensing receptors in other systems, such as catch-bond receptors in rolling leukocytes (8), to date there is no recognized pattern recognition receptor that responds to bacterial motion. However, there are examples of immune receptor clustering, and receptor signaling in trans, leading to signal amplification and subsequent downstream effects (34). A potential scenario is that flagellar motility allows multiple receptors to cluster and quickly activate in cis and in trans to overcome a hypothetical signaling threshold, which in turn allows for the recruitment and activation of PI3K, initiation of a kinase cascade, activation of regulatory Akt, and facilitation of actin polymerization for phagocytic uptake.

In summary, this work has identified for the first time that the PI3K/Akt pathway mediates the motility-driven phagocytosis of *P. aeruginosa*. Uptake of swimming bacteria is not a simple function of host-bacteria association, as normalized bacteria-cell contact does not elicit similar levels of Akt activation or phagocytosis. Moreover, the stepwise loss of bacterial flagellar motility is proportional to the degree of Akt activation in host cells, in parallel to the phagocytic susceptibility. These data clarify the mechanism behind bacterial flagellar motility eliciting host phagocytosis, and support a model whereby the innate immune system contributes to a selective pressure for the loss of bacterial motility within the host environment. This is consistent with clinical observations of nonswimming phenotypes persisting in long-term, chronic infections.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


Olsen JE, Hoegh-Andersen KH, Casadesus J, Rosenkranz J, Chadfield MS, Thomsen LE. The role of flagella and chemotaxis genes in host-pathogen interaction of the host adapted Salmonella enterica serovar Dublin compared with the broad host range serovar S. typhimurium. BMC Microbiol 2013; 13: 67.


Shiratsuchi H, Basson MD. Akt2, but not Akt1 or Akt3 mediates pressure-stimulated serum-opsonized latex bead phagocytosis through activating mTOR and p70 S6 kinase. J Cell Biol 2006; 174: 533–537.


