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The Effect of Loss of O-antigen Ligase on Phagocytic Susceptibility of Motile and Non-Motile *Pseudomonas aeruginosa*

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Abstract

The bacterial pathogen *Pseudomonas aeruginosa* undergoes adaptation and selection over the course of chronic respiratory tract infections which results in repeatedly-observed phenotypic changes that are proposed to enable its persistence. Two of the clinically significant *P. aeruginosa* phenotypic changes are loss of flagellar motility and modifications to LPS structure, including loss of O-antigen expression. The effect of loss of O-antigen, frequently described as conversion from smooth to rough LPS, and the combined effect of loss of motility and O-antigen on phagocytic susceptibility by immune cells remain unknown. To address this, we generated genetic deletion mutants of *waaL*, which encodes the O-antigen ligase responsible for linking O-antigen to lipid A-core oligosaccharide, in both motile and non-motile *P. aeruginosa* strains. With the use of these bacterial strains we provide the first demonstration that, despite a progressive selection for *P. aeruginosa* with rough LPS during chronic pulmonary infections, loss of the LPS O-antigen does not confer phagocytic resistance *in vitro.* However, use of the *waaLmotABmotCD* mutant revealed that loss of motility confers resistance to phagocytosis regardless of the smooth or rough LPS phenotype. These findings reveal how the O-antigen of *P. aeruginosa* can influence bacterial clearance during infection and expand our current knowledge about the impact of bacterial phenotypic changes during chronic infection.

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Keywords

Pseudomonas aeruginosa; phagocytosis; waaL; O-antigen; O-antigen ligase

1. INTRODUCTION

Pseudomonas aeruginosa is a motile Gram-negative bacterial pathogen that causes opportunistic acute and chronic infections in humans [1, 2]. In particular, this pathogen is responsible for high incidences (>80%) of chronic pulmonary infections and morbidity, in adult patients suffering from cystic fibrosis (CF) [1–3]. Chronic *P. aeruginosa* infections are highly tolerant to standard-of-care clinical antibiotic treatments and are difficult to eradicate [1, 2, 4], highlighting the importance of studying host-pathogen interactions and bacterial adaptations during chronic infections.

Longitudinal studies of *P. aeruginosa* isolates from CF patients with chronic airway infections have revealed characteristic bacterial phenotypic changes that suggest adaptation and selection of the pathogen within the CF lung [5]. Some of these include reduced expression of virulence factors, a switch from non-mucoid to mucoid colony morphology and an increase in expression of efflux pumps [5]. Other important temporal changes of *P. aeruginosa* isolated from the CF lungs include the loss of flagellar motility due to downregulation or absent flagellar gene expression [6, 7], growth of bacteria in microcolonies or biofilms, and modifications of the lipopolysaccharide (LPS) structure [4, 6, 8–10]. *P. aeruginosa* LPS is an integral component of the bacterial outer membrane, has been established as a major virulence factor in *P. aeruginosa*, and provides the bacteria with resistance to host defense mechanisms [9, 11]. LPS modifications have been reported as a conserved theme in infections and are apparently important in the adaptation to *P. aeruginosa* in chronic infections [9, 12, 13]. Importantly, *P. aeruginosa* isolates from chronic infections have rough colony phenotypes with LPS that has short, or no O side chains, rendering the bacteria non-typable and reducing their immunogenicity [14, 15]. Broadly, these changes have been attributed to prolonged inflammation, increased resistance to antibiotic-mediated clearance, and immune system evasion [9, 16]. Innate immunity in the host, specifically through the functions of professional phagocytic cells, is critical for effective control and clearance of *P. aeruginosa*. This is supported by the observations that humans and animal hosts that are lacking phagocytic cells (neutrophils and/or macrophages) are highly susceptible to *P. aeruginosa* infection [17–19].

Although the longitudinal transition to bacteria that lack flagellar motility and/or O-antigen is well established, whether these adaptations confer a selective advantage for phagocytic resistance is incompletely understood. One key insight was the previous demonstration that loss of flagellar motility is a crucial factor that dramatically increases *P. aeruginosa* resistance to phagocytosis (by ~100-fold) by professional phagocytes of the hosts [20, 21]. Another relevant insight is that LPS modifications that reduce its immunostimulatory potential are reported to be prevalent in the adaptation of *P. aeruginosa* to chronic infection [12]. LPS is composed of three distinct regions: lipid A, core oligosaccharide, and O-antigen polysaccharide, where common polysaccharide (CPA, homopolymer) and O-specific antigen

(OSA, heteropolymer) are the two glycoforms of *P. aeruginosa* O antigens [11]. Importantly, during LPS biogenesis, CPA and OSA O antigens are attached onto lipid A-core oligosaccharides in the periplasm by the O-antigen ligase encoded by *waaL* [11, 22]. As a result, *waaL* deletion mutants produce rough LPS lacking O antigen and accumulate polymerized O antigen-linked undecaprenol-phosphate (Und-P) on the periplasmic surface of the cytoplasmic membrane [9, 11, 22]. Genomic analyses of sequential *P. aeruginosa* isolates recovered from CF patients over time reveals modifications in genes involved in biosynthesis of both CPA and OSA O antigens [13, 23, 24]. Interestingly, O-antigen ligase encoded by *waaL* was reported to be one of the few hotspots of gene polymorphisms in an analysis of whole-genome sequence data from *P. aeruginosa* clinical sputum isolates of 32 patients [25]. Many roles have been attributed to O-antigen in the literature; however, the effect of loss of O-antigen with regard to phagocytic susceptibility has never been examined.

In this study, we explored how two of the established temporal trends that occur during chronic infection, loss of motility and O antigen, intersect to alter the host phagocytic response, both individually and in combination. We have previously described the deletion mutant of *motABmotCD*, which encode partially redundant stator complexes required for flagellar rotation and swimming motility, but not flagellar assembly [20, 21, 26]. Of note, all of the aforementioned phagocytic studies using non-motile *P. aeruginosa* were performed using bacteria producing wild-type or smooth LPS with O antigen being expressed. Here, we have generated isogenic Δ*waaL* deletion mutant and a double phenotypic mutant of *waaLmotABmotCD* in two independent strains of *P. aeruginosa*. Quantitative phagocytosis assays were then employed to examine the effect of deletion of *waaL* on susceptibility to phagocytosis in swimming and non-swimming *P. aeruginosa* strains.

2. MATERIALS & METHODS

2.1. Bacteria

P. aeruginosa variants on the PA14 and PAK background (wild-type (wt) and *motABmotCD*) have been previously described and published [20, 21, 26, 27]. Bacteria were cultured overnight at 37°C and subsequently subcultured as indicated for 2 h in lysogeny broth (also commonly called Luria Bertani broth, LB).

2.2. Cells

Bone marrow-derived dendritic cells (BMDCs) were cultured from C57BL/6 WT mice (Charles River Laboratories) as previously described [28]. Six-and seven-day-old BMDCs were used for these studies. Blood was collected from volunteers that include healthy individuals and patients with CF by venipuncture into heparinized tubes. Donor blood samples were procured from three CF patients with the following cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations; R1162X/W1282G (1 patient) and ΔF508/ΔF508 (2 patients). Neutrophils were enriched with a Ficoll-Paque PLUS (GE Healthcare Biosciences) discontinuous gradient followed by dextran sedimentation as previously described, with minor modifications [29]. After Ficoll separation, neutrophils were isolated from the blood pellet using 5% dextran for 75 min and erythrocytes lysed using BD PharmLyse RBC lysing buffer (BD Biosciences).

2.3. Ethics Statement

The Dartmouth Committee for the Protection of Human Subjects (CPHS) approved procurement and use of human cells in this study. All samples were de-identified and obtained with informed consent. This study was conducted in strict compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council and was approved by the Dartmouth Institutional Animal Care and Use Committee (IACUC).

2.4. Mutagenesis of *P. aeruginosa waaL* and PCR diagnosis

The *waaL* gene was deleted from *P. aeruginosa* PA14 and PAK wt and *motABmotCD* strains using homologous recombination with flanking sequences on the non-replicative pMQ30 vector [30]. Briefly, upstream flanking region was amplified using waaL-GOI-F-HindIII (5′-TCACTaagcttATTCCGATGTAGGGCTTCAC-3′) and waaL-SOEGOI-R-EcoRI (5′-TGCttgaattcGCGAACATTCCTTGCTCAAT-3′) and the downstream flanking region was amplified using waaL-SOEGOI-F-EcoRI (5′-GTCCgaattcaaCCAAGGAGCACTGGTTTCTG-3′) and waaL-GOI-R-KpnI (5′-TCACTggtaccCCGTGATCATCGCTTCCTAT-3′), where engineered restriction sites are noted with lowercase bases. Both products were cut with EcoRI, KpnI, and HindIII and ligated into HindIII and KpnI cut pMQ30, which was transformed into DH5α and transformants selected for growth on gentamicin. S17λpir was transformed with the resultant plasmid and transformants selected on gentamicin. The resultant S17λpir strain was mixed with the target *P. aeruginosa* strains for conjugation at 30°C. Single-crossover integrants were selected on PIA (Pseudomonas Isolation Agar) with gentamicin, where the irgosan in PIA selects against the *E. coli* strain. Strains were grown without gentamicin to allow for survival of cells that underwent double-crossover and these were selected for by growth on LB with no NaCl and 5% sucrose at 30°C. Double crossover yielded two distinct colony sizes and both were screened for deletion of *waaL* by two PCR reactions. Amplification with waaL-check-f (5′- AGTGCGGCGATACAGTCC-3′) and waaL-check-R-wt (5′- GGATGTAGAAAAGCCGGTGA-3′) yields a 484-bp fragment in wt and no specific product in the mutant, while amplification with waaL-check-f and waaL-check-R-delta (5′- TGGCTACGTCATCGACATTC-3′) yields a 760-bp fragment in the deletion mutant and an 1846 bp fragment in wt. Amplification with the waaL-check-R-wt primer is inefficient in PAK strains due to 3 SNPs within the chosen primer sequence, which was designed with PA14 and PAO1 genomes. Using this PCR diagnostic check, the small colonies from the double crossover step were determined to be *waaL* deletion mutants (Fig 1a). These strains grow well on LB and appear to only have the small-colony phenotype on the LB with no NaCl and sucrose.

2.5. Lipopolysaccharide isolation and immunoblot analysis

Before LPS isolation, 10^9 bacteria were pelleted from *P. aeruginosa* wt PA14 and PA14 *waaL* overnight cultures as measured by the optical density (OD 600nm). The LPS from these strains was prepared by proteinase-K digestion [31]. The bacterial LPS preparations were subjected to SDS-PAGE on 10% polyacrylamide gels. Western immunoblotting of the isolated LPS was performed using rabbit polyclonal antisera raised against the serotype O-19 strain (1:100) (generated by the Lam lab), followed by peroxidase affinipure goat anti-

rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch) diluted to 1:5000 (incubated for 1h). The blot was developed using Pierce ECL substrate (Thermo Scientific) and visualized using autoradiography film (Genesee Scientific).

2.6. Bacterial swimming motility and biofilm formation assays

The phenotypic assay for swimming motility was performed as previously described in 0.3% LB agar plates [21, 32]. Images were collected at 48-h post inoculation to monitor the formation of bacterial halos to assess the relative swimming motilities of the bacterial strains. Biofilm formation assays were performed using 96-well round-bottom microtiter plates (CoStar 2797) as previously described utilizing M63 medium supplemented with 1 mM $MgSO_4$ and arginine [33, 34]. Following incubation inside a humidified chamber at 37°C for 18 h, the supernatants were removed and the wells were stained with 0.1% (w/v) crystal violet solution and rinsed with distilled water [33]. This assay was conducted three times for each strain and the representative images are shown.

2.7. Gentamicin protection assay

Phagocytosis of live bacteria was performed as previously described [21, 28]. Briefly, subcultured bacteria of the indicated genotypes were washed, resuspended in serum-free Hanks balanced salt solution (HBSS) (Corning Cellgro, Manassas, VA), and their concentrations were determined at OD600nm. Where indicated, 10% (vol/vol) of complement-inactivated human serum from a healthy donor was included throughout the assay. A total of 2.5×10^5 cells (BMDCs or neutrophils) were incubated with the indicated bacterial genotype at an MOI of 10 (for BMDCs) or 25 (for human neutrophils) for 45 min at 37°C, followed by incubation with 100 μg/ml gentamicin for 20 min at 37°C. The cells were washed twice in HBSS and lysed with 500 μl 0.1% Triton X-100 solution in 1X Phosphate Buffered Saline (PBS) (HyClone Laboratories, Logan, UT). Lysates were plated on LB plates and incubated overnight at 37°C. CFUs were subsequently enumerated and are represented as percentage of mean wt or *waaL* bacteria phagocytosis as denoted.

2.8. Akt activation assay

Akt activation assays were performed as previously described [32]. Briefly, a total of 3×10^5 BMDCs were incubated in serum-free HBSS for 90 min at 37°C. The cells were then incubated with the indicated subcultured bacterial strains at MOI = 10 for 45 min at 37°C, followed by blocking for Fc receptor with monoclonal antibody 2.4G2. BMDCs were subsequently fixed in 300 μl of 4% paraformaldehyde in PBS for 10 min at 37°C, washed in cold PBS, and permeabilized in 500 μl of 100% cold methanol for 10 min. After washing with cold PBS to remove the methanol, the cells were probed with phospho-Akt antibody in cold PBS containing 3% BSA, 1mM PMSF (Sigma), and 1X MS-SAFE protease and phosphatase inhibitor (Sigma) as described by manufacturers. The antibody used was mouse anti-phospho-Akt (Ser473), clone 6F5 IgGK (Millipore), which was labeled with Zenon One Alexfluor-647 mouse IgG (Molecular Probes) as described in the manufacturer's instructions. Akt activation was quantified using FACS to acquire fluorescence.

2.9. *In vitro* inflammation assay

A total of 2.5×10^5 BMDCs per well in a 24-well plate were infected with subcultured bacteria (MOI = 1) to a final volume of 400 μl Roswell Park Memorial Institute 1640 (RPMI-1640) containing 10% Fetal Bovine Serum (FBS) (HyClone Laboratories, Logan, UT). Bacteria were incubated with the cells for 3 h at 37 ℃ and 5%CO2. Cell-free supernatants were collected 3-h post infection (pi) and production of Interleukin-1β (IL-1β) and IL-6 cytokines was analyzed by ELISA. The DuoSet enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-1β and IL-6 were acquired from R&D Systems (Minneapolis, MN).

2.10. LDH assay

Human neutrophils (healthy and from CF patients) were infected with subcultured bacteria at the indicated MOI in a total volume of 300 μl HBSS with 2% FBS for 2 h at 37°C and $5\%CO_2$. The infections were carried out in 24-well plates using 2.5×10^5 neutrophils per well. The CytoTox kit (Promega) was used according to the manufacturer's protocol to measure lactate dehydrogenase (LDH) release from cell-free supernatants, representing cytotoxicity.

2.11. Statistical analyses

Means ± standard deviations (SD) derived from multiple independent experiments with technical replicates are shown for each graph. Sample sizes for each experiment are noted in the figure legends. As indicated, unpaired Student's *t* test with Welch's correction or one-way ANOVA with Tukey's post hoc analyses were performed using Prism 5.0a to determine statistical significance of the data. Statistical significance is represented in figures by asterisks.

3. RESULTS

3.1. Creation and validation of *P. aeruginosa waaL* mutants

To assess the functional roles of the *waaL* gene in *P. aeruginosa*, deletion mutants in the swimming-competent PA14 and PAK wildtype (wt) strains were generated using homologous recombination as described in the Methods section. Isogenic *ΔwaaL* (deletion mutants) were also generated in the PA14 *motABmotCD* and PAK *motABmotCD* backgrounds to assess the role of *waaL* in non-motile strains. The presence of the mutant gene in PA14 and PAK was verified by PCR screening of the double crossover products (Fig. 1A).

Validation of the Δ*waaL* phenotype was confirmed by loss of O-antigen on the LPS of the mutant strains. LPS from wt PA14 and PA14 *waaL* was analyzed by Western blotting using polyclonal antisera raised against the serotype O-19 strain. As expected, we observed O antigen-positive LPS bands in wt PA14, while PA14 *waaL* was defective in the production of CPA and OSA LPS (Fig. 1B) [22].

3.2. Δ*waaL* mutation in *P. aeruginosa* did not abrogate swimming motility nor ability of the bacteria to form biofilms

To determine the relationship between motility and O-antigen expression with regard to phagocytic susceptibility, we first sought to determine the effect of the *ΔwaaL* mutation on bacterial swimming motility. To test this, the strains were inoculated by stabbing puncture into 0.3% LB agar plates to monitor the zones surrounding the point of inoculation as an indication of swimming motility. In accordance with previous results, there was a complete lack of swimming motility in the PA14 and PAK *motABmotCD* stator-deficient mutants, compared to the swimming-competent wt strains (Fig. 2A). Importantly, PA14 *waaL* and PAK *waaL* exhibited swimming motility, while PA14 *waaLmotABmotCD* and PAK *waaLmotABmotCD* were completely non-motile. To functionally confirm the motility assay, the strains were evaluated for the capability to form biofilms: previous research has demonstrated that flagella-mediated motility in *P. aeruginosa* is required to initiate biofilm formation [35, 36]. The biofilm phenotypes corroborate the motility phenotypes observed with the mutants. PA14Δ*waaL* and PAKΔ*waaL* were able to form biofilms, while PA14 *waaLmotABmotCD* and PAK *waaLmotABmotCD* were not (Fig. 2B). Notably, these results were reiterated in multiple strains (PA14 and PAK) suggesting that they may be broadly applicable in *P. aeruginosa*. Overall, these data indicate that mutants deficient for O-antigen expression are capable of swimming motility and biofilm formation.

3.3. Phagocytosis of *P. aeruginosa* by BMDCs is independent of O-antigen ligase expression in the bacteria

To evaluate the effect of loss of O-antigen ligase expression on phagocytic uptake of *P. aeruginosa*, we employed a quantitative gentamicin protection phagocytic assay with murine BMDCs. Interestingly, PA14 *waaL* and PAK *waaL* were phagocytosed by BMDCs at levels comparable or slightly higher than those for the wt strains. In comparison, and as a control, both *motABmotCD* strains exhibited a dramatic reduction in their phagocytic uptake compared to the respective parental strain (Fig. 3A and B) [20, 21, 27]. In order to account for the possibility that differences in intracellular killing of bacteria could be contributing to our phagocytosis results, we incubated the indicated bacteria with murine BMDCs for a shorter amount of time (20 minutes instead of 45 minutes) before addition of gentamicin. Indeed, similar to the previous results (Figs. 3A and B), the shorter incubation led to equivalent or greater phagocytosis of PA14 *waaL* and PAK *waaL* by BMDCs, compared to their wt strains (Fig. 3C and D). These data support that differential intracellular killing of bacteria is not responsible for the observed phagocytic phenotypes.

Based on the observed phagocytosis phenotype, we assessed the cellular Akt activation upon incubation of BMDCs with PA14Δ*waaL* compared to wt PA14. Previous data demonstrated that the PI3K/Akt pathway, which is an important mammalian intracellular signaling pathway for phagocytosis of many bacteria by host cells, is activated in response to motile bacteria but not by non-motile bacteria [32, 37]. Relative Akt activation was quantitatively measured by FACS analysis following intracellular staining for phosphorylated Akt (phospho-Akt). The exposure of BMDCs with PA14 *waaL* elicited phosphorylation of Akt that was comparable to that of wt PA14. In comparison, and as a negative control, bacteria that lack flagellar motility (*motABmotCD*) did not elicit Akt activation, such that cellular

phospho-Akt levels remained similar to untreated cells (Fig. 3E). Together, these data provided the evidence that leads to two initial insights. First, that phagocytosis of *P. aeruginosa* and the associated signaling are relatively unaffected by loss of O-antigen ligase expression and that loss of O-antigen is unlikely to correspond to selection for phagocytic resistance. Second, these data support that the *P. aeruginosa* O-antigen is not the predominant bacterial ligand recognized by the host cell phagocytic receptors.

3.4. *P. aeruginosa* defective in swimming motility are resistant to phagocytosis by BMDCs independent of rough LPS phenotype

Previous experiments have demonstrated that loss of bacterial motility enables phagocytic evasion by *P. aeruginosa* with the smooth LPS phenotype, however it is unknown whether O-antigen of LPS affects the phagocytosis of non-motile *P. aeruginosa.* We hypothesized that loss of motility would enable phagocytic evasion regardless of change in the LPS structure from smooth to rough. To address this, we tested the *waaLmotABmotCD* double phenotypic mutants in our BMDC phagocytic assay and compared the uptake to Δ*waaL* single mutants in the PA14 and PAK backgrounds. When compared to the Δ*waaL* mutant in wt PA14 and PAK, Δ*waaL* in non-motile mutant backgrounds exhibited phagocytic resistance (Fig. 4A and B), similar to smooth non-motile bacteria as demonstrated in Fig 3. Moreover, analogous to results from Fig. 3, uptake of PA14 and PAK *waaLmotABmotCD* compared to the *waaL* mutants after 20 minutes incubation mirrored the data obtained after a a 45 minute co-incubation (Fig. 4C and D). This suggests that the phagocytic resistance exhibited by the non-motile bacteria with rough LPS is also independent of differential intracellular bacterial killing kinetics. To validate and extend these results, we assessed cellular Akt activation following infection of BMDCs with PA14 *waaLmotABmotCD*. The *waaLmotABmotCD* did not stimulate phosphorylation of Akt above the levels of untreated cells (Fig. 4E). These results suggest that loss of flagellar motility enables resistance of bacteria to uptake by phagocytic cells, independent of the presence or absence of O-antigen.

Although the precise contribution of opsonins during *P. aeruginosa* chronic lung infections is unclear, we wanted to examine whether the presence of opsonin-containing human serum in the assay media would impact relative phagocytosis of the non-motile and O-antigen deficient mutants compared to wt strains. Therefore, we performed phagocytosis assays in media containing 10% human serum and compared them to media without serum. Results using serum-containing assay media recapitulated those obtained in serum-free conditions, such that *waaL* phagocytosis is comparable to wt, while phagocytosis of *waaLmotABmotCD* resembles *motABmotCD* (Fig. 5). This suggests that serum does not affect our relative phagocytic phenotypes.

3.5. *P. aeruginosa* that lack O-antigen ligase elicit similar levels of pro-inflammatory cytokine production by BMDCs

We previously reported that loss of flagellar motility results in decreased cell-surface interactions with macrophages and thereby reduces bacterial type 3 secretion system (T3SS)-dependent inflammasome activation and IL-1β production in response to *P. aeruginosa* infection [27]. However, while TLR4 and TLR5 are integral to inflammatory cytokine responses to *P. aeruginosa* infection, neither is necessary for direct phagocytic

recognition and uptake of *P. aeruginosa* in *in vitro* assays [21]. Therefore, we sought to evaluate whether waaL was required for IL-1β and IL-6 production in response to *P. aeruginosa* infection, the former dependent upon bacterial contact with the host cell and the latter dependent upon TLR signaling. BMDCs exhibited comparable elicited IL-1β responses following infection with wt PA14 and PA14 *waaL*. As a control, BMDCs infected with the *motABmotCD* mutant produced lower quantities of IL-1β, consistent with previous observations (Fig. 6A) [27]. Similar to the results observed for IL-1β, equivalent levels of IL-6 were produced by BMDCs infected with wt PA14 and Δ*waaL*. Interestingly, infection with PA14 *motABmotCD* also elicited similar IL-6 production, consistent with the previous observation that induction of pro-inflammatory TLR signaling is independent of bacterial binding and phagocytosis (Fig. 6B) [21]. These findings demonstrate that cellular inflammatory cytokine responses to *P. aeruginosa* infection do not require bacterial O-antigen ligase expression nor does loss of O-antigen result in evasion of an inflammatory response.

3.6. O-antigen-deficient but motile *P. aeruginosa* exhibit phagocytic susceptibility to human blood-derived neutrophils

We extended our studies to human primary neutrophils in order to examine the phagocytic susceptibilities of the aforementioned bacterial strains and to validate the phagocytic phenotypes observed with murine BMDCs. Neutrophils play an essential role in clearing *P. aeruginosa* infections and neutropenic patients and mice are highly susceptible to those infections [17, 19]. Indeed, gentamicin protection assays employing purified human healthy neutrophils validated the mouse studies, such that motile rough LPS mutants (PA14Δ*waaL*) were phagocytosed to a greater extent compared to wt PA14 in both serum-free and 10%human serum-containing assay media (Fig. 7A and B). On the other hand, non-motile rough LPS mutants (PA14 *waaLmotABmotCD*) were as resistant to phagocytosis by neutrophils as PA14 *motABmotCD* in serum-free as well as serum-containing media (Fig. 7A and B). Consistent with Fig. 5, addition of serum to the gentamicin assay media did not seem to alter the observed phagocytic phenotypes. Of specific interest, similar results were obtained with blood-derived neutrophils obtained from CF patients, although phagocytosis was not significantly higher for PA14 *waaL* compared to wt PA14 (Fig. 7C). Thus, the similar phagocytic outcomes comparing those observed with murine BMDC to those with human neutrophils indicate that this phenotype is consistent across multiple cell types and species and that it is likely to be relevant to interactions with human phagocytic cells.

Previous data have demonstrated that motility-deficient *P. aeruginosa* is less capable of inducing cell death of BMDCs in a process that is dependent upon bacterial interactions with the host cell and expression of the bacterial T3SS [27]. It is important to note that *motABmotCD* and wt bacteria have similar expression of T3SS-associated genes [20, 27]. However, the effect of O-antigen expression on bacterial-induced cytotoxicity is unknown. Therefore, we tested the effect of the *ΔwaaL* mutation, and therefore the loss of O-antigen, on human neutrophil viability following infection with *P. aeruginosa*. Human neutrophils from healthy (non-CF) individuals and CF patients were infected with PA14 and its mutants *motABmotCD, waaL* and *waaLmotABmotCD* at MOI = 15 and cytotoxicity was assessed by LDH release after 2 h. Robust, but comparable, cytotoxicity was observed in neutrophils

infected with wt and *waaL* bacteria at the indicated MOIs, which contrasted with the lower cytotoxicity elicited by the non-motile mutants. The non-motile *motABmotCD* mutant triggered very low levels of cytotoxicity while cytotoxicity with *waaLmotABmotCD* was slightly higher (Fig. 7D and E). To confirm that cytotoxicity of neutrophils was dependent on bacterial T3SS activity, we also infected neutrophils with PA14 *popB*, which lacks a functional T3SS translocon. As expected, there was almost no cytotoxicity observed with the *popB* mutant (Fig. 7D and E). Overall, these results support that the interactions of O-antigen - deficient *P. aeruginosa* with host cells confers higher or similar phagocytic susceptibility and comparable induced cytotoxicity to that observed with O-antigen-expressing bacteria. However, loss of flagellar motility enables the bacteria, regardless of O-antigen expression, to evade phagocytosis.

4. DISCUSSION

Two of the established temporal trends observed from longitudinal samples of bacteria isolated from chronic pulmonary *P. aeruginosa* infection of patients with CF or COPD include loss of flagellar motility and alterations to the LPS structure [4, 6–8, 10]. We have previously determined that the loss of flagellar swimming motility, regardless of flagellar expression, is a critical factor that contributes to bacterial resistance to clearance through phagocytosis [20, 21, 27]. However, the contributions of other identified bacterial adaptations, with loss of LPS O-antigen being pertinent to these studies, to evasion of phagocytic recognition and clearance remain enigmatic. Therefore, based on the clinical observations that *P. aeruginosa* isolates acquire the rough LPS phenotype over the course of chronic infection [14], we have investigated whether LPS O-antigen loss confers an advantage to the bacteria in the context of phagocytic evasion of innate immune cells. Additionally, we have addressed independent and combined effects of losing flagellar swimming motility and O-antigen expression with regard to phagocytic resistance.

We employed a bacterial genetic approach to generate Δ*waaL* constructs in both wild-type and non-swimming mutant backgrounds. Our results showed that the *ΔwaaL* mutants produce rough LPS lacking O-antigen, which is consistent with published literature [22]. Interestingly, deletion of the *waaL* gene in different bacterial species has been reported to have disparate effects on swimming motility [22, 38, 39]. For instance, a *P. aeruginosa* PAO1 *waaL* transposon-insertion mutant and a Δ*waaL* mutant in an avian pathogenic *Escherichia coli* (APEC) were shown to exhibit partially-diminished swimming motility, while the Δ*waaL* mutant in *Proteus mirabilis* had no impact on swimming motility in that species [22, 38, 39]. By generating independent *ΔwaaL* mutants in the PAK and PA14 strains of *P. aeruginosa*, we were able to show that these mutants only exhibited relatively minor decreases in flagellar motility as assessed by using swimming assays; however, we note that the assay could not discern whether this is due to alterations in flagellar rotation or, potentially, alterations to the exterior of the bacteria that diminish the ability of the bacteria to spread through the agar. However, consistent with the retention of flagellar motility, we showed that bacteria of *ΔwaaL* form robust biofilms. These data are supported by previous studies that showed that the *P. aeruginosa* PAO1 *waaL* strain forms thick biofilms [40] and *waaL* deletion in APEC actually increased the ability to form biofilms [38]. However, *waaLmotABmotCD* mutants are non-motile and are unable to form biofilms, which provides

both a control for motility and an opportunity to assess whether there is an association between swimming motility and O-antigen phenotypes together. Our results indicate that flagella-mediated swimming motility and biofilm formation are largely independent of the presence or absence of O antigen on *P. aeruginosa.*

Importantly, our findings demonstrate that the level of phagocytosis of Δ*waaL* mutant *P. aeruginosa* by dendritic cells and neutrophils is similar or slightly higher compared to phagocytosis of wt bacteria. Deletion of O-antigen ligase (and thus the presence of O-antigen, indirectly) in motile strains is dispensable for phagocytosis by neutrophils and dendritic cells, and may even provide a modest additional susceptibility. Thus, despite the clinical observation of selection for O antigen-deficiency in *P. aeruginosa* during chronic infection, the phenotype change to producing rough LPS did not confer evasion of phagocytosis by innate immune cells. Our results contrast with studies done using O antigen-deficient *Burkholderia cenocepacia*, which are more susceptible to phagocytic internalization [41]. However, it is noteworthy that *Burkholderia* has an intracellular lifecycle and is a non-obligate intracellular pathogen, while *P. aeruginosa* is largely regarded as an extracellular pathogen. Therefore, with regard to phagocytosis, loss of O-antigen may benefit the ability of *Burkholderia* to access an intracellular environment, while *P. aeruginosa* would not share similar benefits [41].

While the studies presented in this manuscript test one aspect of the host innate immune response to *P. aeruginosa* lacking O-antigen, it is reasonable to suggest that WaaL (O-antigen ligase) likely plays an important role in the regulation of other factors that contribute to *P. aeruginosa* virulence. For instance, it has been previously shown that *P. aeruginosa* O-antigen-deficient isolates were more sensitive to *in vitro* killing by serum complement and oxidative stress (H2O2) [14, 42]. Moreover, there are data to support that some adaptations associated with modifications of LPS of *P. aeruginosa* that favor persistence in the CF airways confers reduced virulence and pathogenicity, and these studies are corroborated in experiments that tested *waaL* mutants of several bacterial species within a variety of *in vivo* infection models [38, 42–45]. However, it is noteworthy that many of the reports that describe adaptations of *P. aeruginosa* resulting in modification in the LPS were focused on changes to the lipid-A portion rather than the O-antigen, and observed changes in lipid A likely reflect an adaptation or selection to evade inflammatory responses [9]. Importantly, we also demonstrate that *P. aeruginosa* strains that are defective in swimming motility are resistant to phagocytosis regardless of whether smooth LPS or rough LPS is produced on the cell surface of the bacteria. These data are the first test of the contribution of O-antigen to susceptibility to phagocytic activities in swimming competent and swimming-defective *P. aeruginosa* backgrounds. We conclude that loss of swimming motility confers phagocytic evasion regardless of the presence or absence of O-antigen on the bacteria, and in some cases, may even overcome additional phagocytic susceptibility conferred by loss of O-antigen.

In summary, there is a need to understand how these adaptive or selective changes to the bacteria affect immune evasion in the hopes of developing strategies to effectively eliminate the notoriously difficult to eradicate chronic infections. Using *P. aeruginosa* bacteria with genetic deletions of the *waaL* gene, we demonstrate that the loss of O-antigen (ligase) does

not confer phagocytic evasion of the motile bacteria by murine and human phagocytic cells. Importantly, we also identify that the resistance to phagocytosis exhibited by non-swimming mutants is independent of LPS O-antigen composition. Overall, these findings provide key insights into how *P. aeruginosa* adaptations observed during chronic infections of the CF lung environment contribute to host phagocytic evasion.

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Highlights

- New bacterial mutants enable combinatorial test of motility and O-antigen loss on phagocytosis.
- Phagocytosis of *P. aeruginosa* is independent of presence of O-antigen.
- Non-motile *P. aeruginosa*, regardless of LPS architecture, are resistant to phagocytosis.
- Outcomes validated with primary human neutrophils.

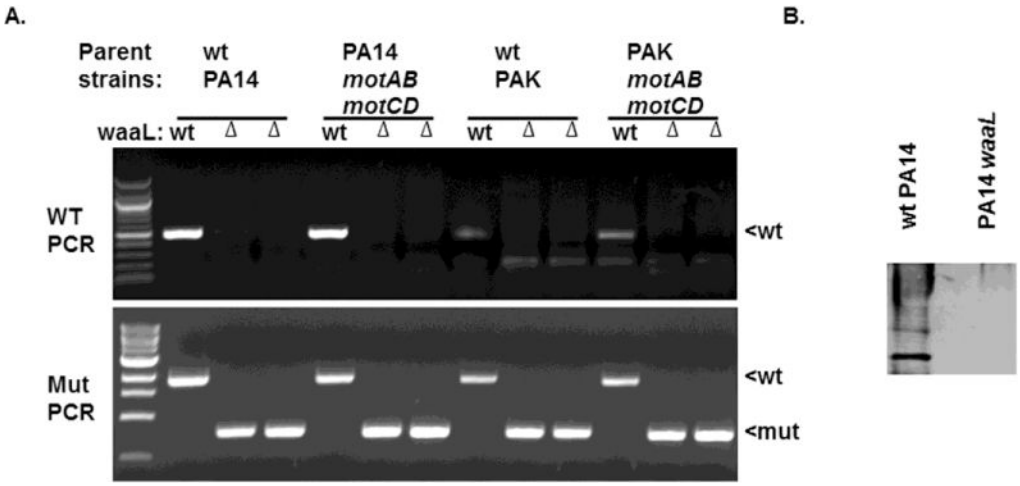

Figure 1. PCR and western immunoblotting analyses of *P. aeruginosa* strains

(A) Diagnostic PCR amplification of waaL deletion (*waaL*) in the wt and *motABmotCD* backgrounds of the PA14 and PAK strains of *P. aeruginosa*. Using the primers described in the materials and methods, deletion of the native locus was tested with a PCR reaction containing a reverse primer within the waaL coding sequence (top panel) and one using flanking sequences that shows the decrease in size from the deletion of the waaL coding sequence. (B) LPS from wt PA14 and PA14 *waaL* was prepared by proteinase-K digestion and probed with rabbit polyclonal antisera raised against the serotype O-19 strain.

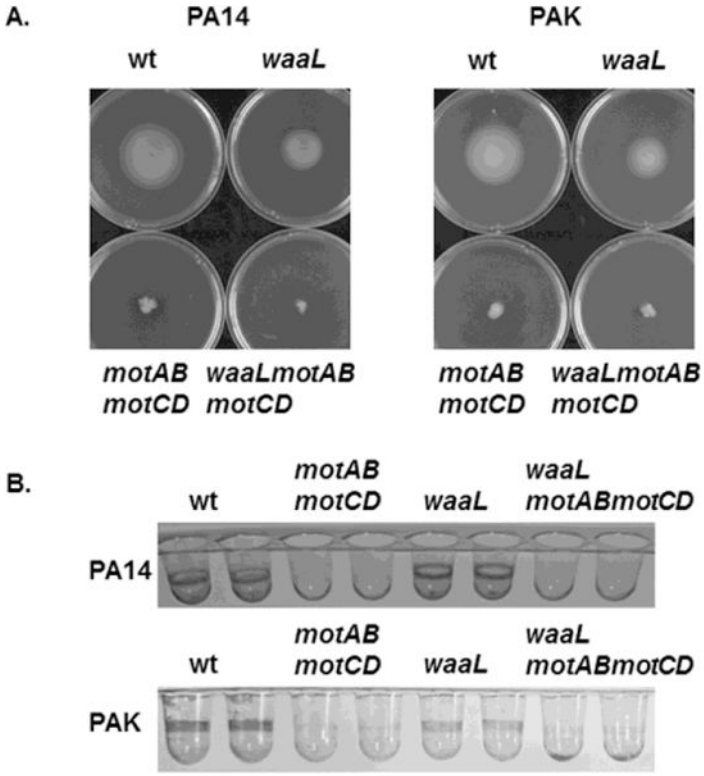

Figure 2. *P. aeruginosa* deficient in O-antigen ligase expression exhibit swimming motility and biofilm formation

(A) wt and mutant *P. aeruginosa* strains were assayed for swimming motility in LB media containing 0.3% agar. wt PA14, wt PAK and single mutant waaL-deficient strains are fully competent at swimming motility, while the mutants deficient in the flagellar stator proteins (*motABmotCD*) and the double phenotypic mutants *waaLmotABmotCD* are completely impaired at swimming motility. The images shown are representative swimming plates of the indicated strains. (B) The *P. aeruginosa* strains described in (A) were assessed for biofilm formation following overnight culture in supplemented M63 minimal medium. Staining with 0.1% crystal violet was used to view the biofilms. Images of representative wells of a 96-well biofilm assay for each strain are shown.

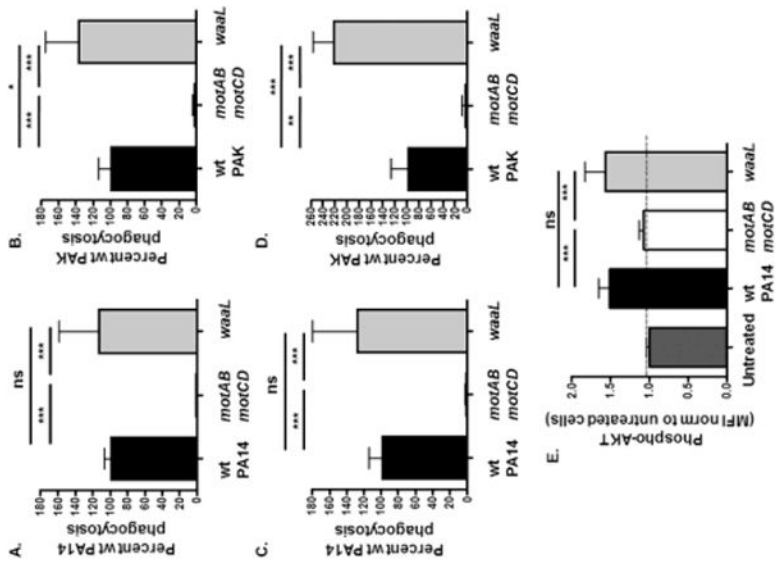

Figure 3. Phagocytic susceptibility of O-antigen -deficient *P. aeruginosa* is similar to that of bacteria with O-antigen

Murine bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice were assayed for relative *in vitro* phagocytosis of (A and C) wt PA14, *motABmotCD*, or *waaL* and (B and D) wt PAK, *motABmotCD*, or *waaL* bacteria by gentamicin protection assay incubated for 45 minutes (A and B) or 20 minutes (C and D) at MOI =10. Phagocytic uptake levels were normalized as percentages of respective mean wt phagocytosis. (E) Murine BMDCs were coincubated with wt PA14, *motABmotCD, or waaL* bacteria, and then fixed and probed for phospho-Akt. Akt activation (phospho-Akt) was quantified by FACS analysis and mean fluorescence intensity (MFI) was normalized to untreated cells (= 1 on *Y*-axis). All data are analyzed using one-way ANOVA with Tukey's post hoc analyses and are representative of at least two independent biological experiments ($n \geq 4$). ***, $p \leq 0.0005$; *, $p \leq 0.05$; ns, not significant.

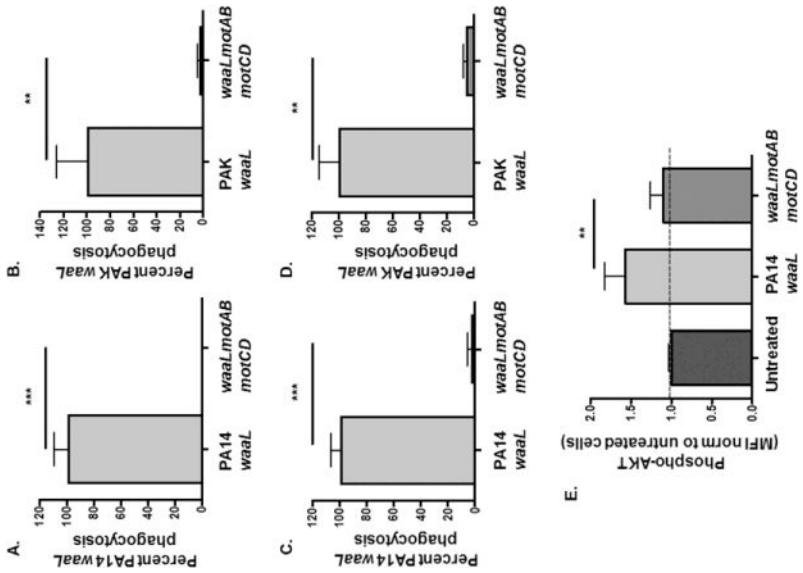

Figure 4. Non-motile *P. aeruginosa* with rough LPS architecture is resistant to phagocytosis by murine phagocytes

Murine BMDCs were assayed for relative *in vitro* phagocytosis of (A and C) PA14 *waaL or waaLmotABmotCD* and (B and D) PAK *waaL or waaLmotABmotCD* bacteria by gentamicin protection assay incubated for 45 minutes (A and B) or 20 minutes (C and D) at MOI=10. Phagocytic uptake levels were normalized as percentages of respective mean *waaL* phagocytosis. (E) Murine BMDCs were coincubated with PA14 *waaL or waaLmotABmotCD* bacteria, and then fixed and probed for phospho-Akt. Akt activation (p-Akt) was quantified by FACS analysis and MFI was normalized to untreated cells (= 1 on *Y*-axis). Data for panels A-D are analyzed using the unpaired t-test with Welch's correction, while data for panel E are analyzed using one-way ANOVA with Tukey's post hoc analyses. All data are representative of at least two independent biological experiments ($n \geq 4$). ***, $p \leq 0.0005$; **, $p \leq 0.005$.

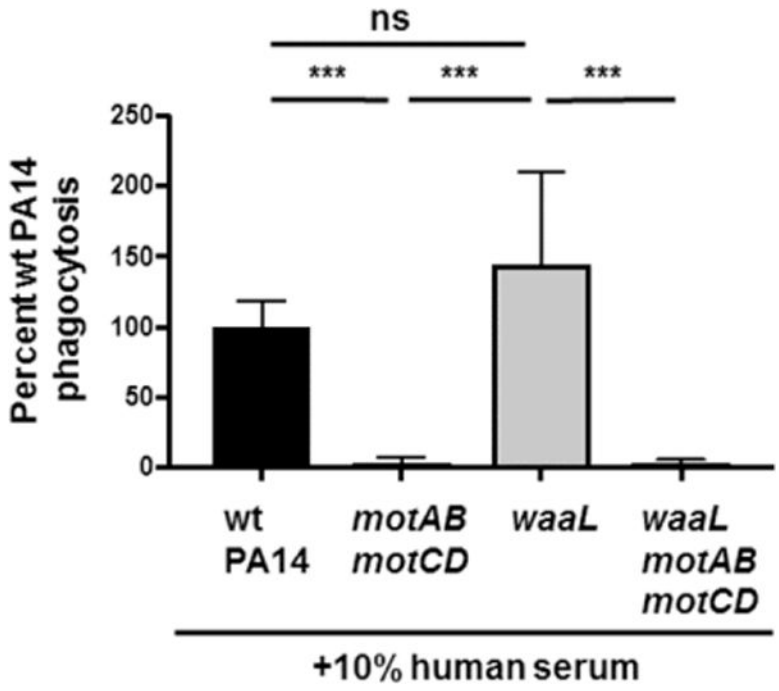

Figure 5. Phagocytosis phenotypes of *waaL* mutants are recapitulated in the presence of serum

Murine BMDCs were assayed for relative *in vitro* phagocytosis of wt PA14, *motABmotCD, waaL, or waaLmotABmotCD* in the absence or presence of 10% human serum by gentamicin protection assay at MOI=10. Phagocytic uptake levels were normalized as percentages of mean wt phagocytosis. Data are accumulated from three independent biological experiments and analyzed using one-way ANOVA with Tukey's post hoc analyses ($n = 6$). ***, $p \leq 0.0005$; ns, not significant.

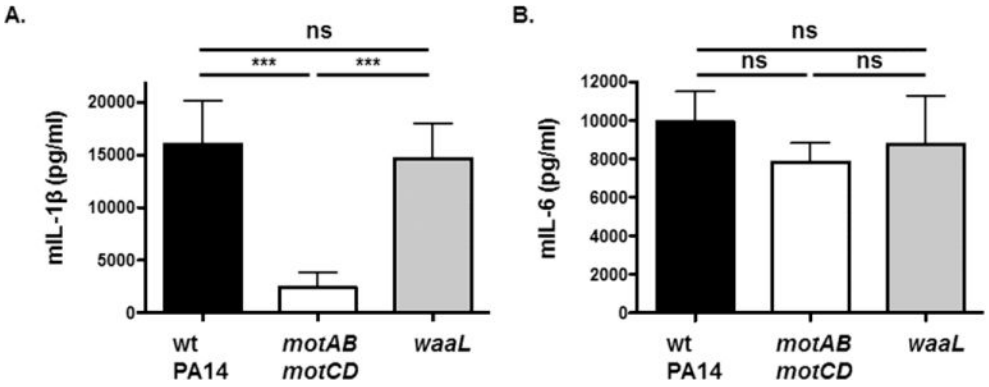

Figure 6. Infection of BMDCs with waaL-deficient bacteria elicit comparable levels of inflammatory cytokines to wt bacteria

Murine BMDCs were infected with wt PA14, *motABmotCD, waaL*, or *waaLmotABmotCD* at a MOI of 1. Culture supernatants were collected 3h postinfection and analyzed by ELISA for (A) IL-1β and (B) IL-6 production. Data are accumulated from at least two independent biological experiments and analyzed using one-way ANOVA with Tukey's post hoc analyses ($n \geq 4$). ***, $p \leq 0.0005$; ns, not significant.

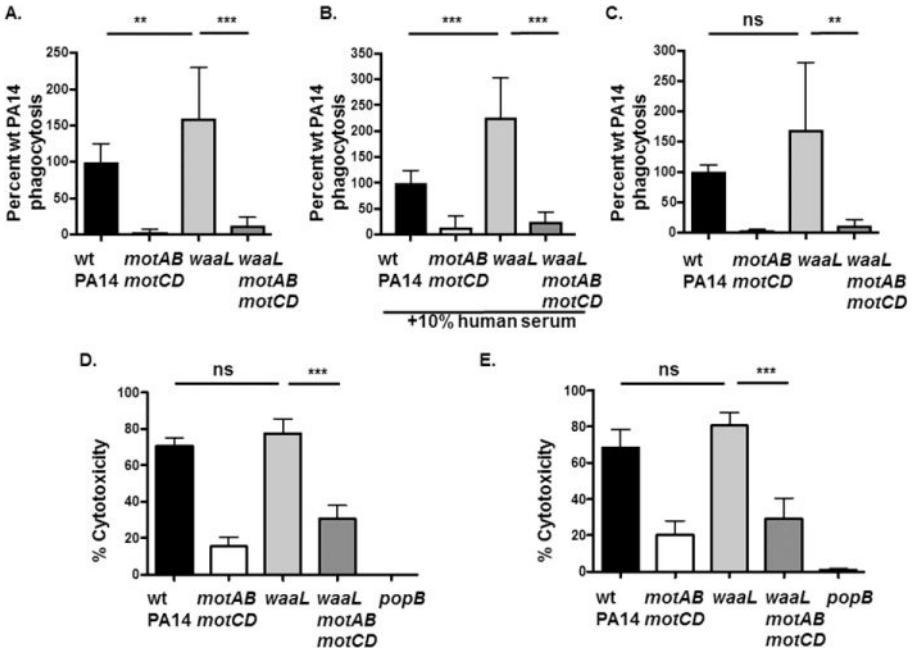

Figure 7. Phagocytosis and cytotoxicity elicited by O-antigen ligase deficient *P. aeruginosa* in human neutrophils

(A and B) Primary human blood neutrophils from healthy individuals or (C) from CF patients were assayed for relative *in vitro* phagocytosis of wt PA14, *motABmotCD, waaL*, or *waaLmotABmotCD* by gentamicin protection assay at MOI=25 in the (A) absence or (B) presence of 10% human serum. Phagocytic uptake levels were normalized as percentages of mean wt phagocytosis. (D) Cytotoxicity of neutrophils from healthy patients or (E) from CF patients following infection with wt PA14, *motABmotCD, waaL*, or *waaLmotABmotCD* (MOI=15) was assayed using the LDH assay. Release of LDH into the culture supernatants was measured at 2h post infection. All data are analyzed using one-way ANOVA with Tukey's post hoc analyses and are representative of at least three independent biological experiments ($n \geq 5$). ***, $p \leq 0.0005$; **, $p \leq 0.005$; ns, not significant.