Naturally Produced Outer Membrane Vesicles from *Pseudomonas aeruginosa* Elicit a Potent Innate Immune Response via Combined Sensing of Both Lipopolysaccharide and Protein Components

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*Pseudomonas aeruginosa* is a prevalent opportunistic human pathogen that, like other Gram-negative pathogens, secretes outer membrane vesicles. Vesicles are complex entities composed of a subset of envelope lipid and protein components that have been observed to interact with and be internalized by host cells. This study characterized the inflammatory responses to naturally produced *P. aeruginosa* vesicles and determined the contribution of vesicle Toll-like receptor (TLR) ligands and vesicle proteins to that response. Analysis of macrophage responses to purified vesicles by real-time PCR and enzyme-linked immunosorbent assay identified proinflammatory cytokines upregulated by vesicles. Intact vesicles were shown to elicit a profoundly greater inflammatory response than the response to purified lipopolysaccharide (LPS). Both TLR ligands LPS and flagellin contributed to specific vesicle cytokine responses, whereas the CpG DNA content of vesicles did not. Neutralization of LPS sensing demonstrated that macrophage responses to the protein composition of vesicles required the adjuvantlike activity of LPS to elicit strain specific responses. Protease treatment to remove proteins from the vesicle surface resulted in decreased interleukin-6 and tumor necrosis factor alpha production, indicating that the production of these specific cytokines may be linked to macrophage recognition of vesicle proteins. Confocal microscopy of vesicle uptake by macrophages revealed that vesicle LPS allows for binding to macrophage surfaces, whereas vesicle protein content is required for internalization. These data demonstrate that macrophage sensing of both LPS and protein components of outer membrane vesicles combine to produce a bacterial strain-specific response that is distinct from those triggered by individual, purified vesicle components.

The innate immune response to Gram-negative bacteria is dominated by recognition of lipopolysaccharide (LPS). LPS is sensed by the Toll-like receptor 4 (TLR4) complex, and numerous studies have focused on both how LPS sensitivity drives effective inflammatory responses, leading to the clearance of infection, as well as how uncontrolled TLR4 signaling can lead to LPS toxicity and play a role in septic shock (20, 39, 48). Much of this research has been performed using purified LPS in the experimental treatments, and yet it is unlikely that pure LPS is shed from bacteria in the context of an infection. Instead, LPS has been found to be shed from the bacteria in the form of outer membrane vesicles.

Outer membrane vesicles are spherical, selective portions of outer membrane and periplasm that are naturally secreted by all Gram-negative bacteria (7, 31, 49). Vesicles are produced at all stages of bacterial growth and have been detected in infected human tissues (7, 23, 28). Vesicle production has been identified as an independent bacterial stress response pathway that is activated when bacteria are exposed to environmental stress, such as might be experienced during colonization of host tissues (35).

Compared to preparations of pure LPS, natural outer membrane vesicles are heterogeneous proteoliposomes of a larger dimension (50 to 250 nm in diameter) than liposomes composed solely of LPS (4, 7). Natural outer membrane vesicles are heterogeneous complexes of pathogen-associated molecular patterns (PAMPs), such as LPS, flagellin, and CpG DNA, as well as other outer membrane proteins, virulence factors, and envelope lipids (7, 31). The molecular composition of vesicles varies with the bacterial strain of origin, while LPS structure remains relatively constant within a bacterial species. The combination of PAMPs, virulence factors, and other outer membrane components results in vesicles that are particularly laden with molecules that can be recognized by the immune system. In the present study we have focused on characterizing macrophage innate immune responses to the combined signals presented by the heterogeneous PAMP ligands presented in the native context of outer membrane vesicles.

Studies of host immune responses to outer membrane vesicles have mainly addressed the generation of antibodies to vesicle components. Notably, a protective antibody response is elicited by outer membrane vesicles generated from *Neisseria meningitidis* and *Vibrio cholerae* (17, 18, 38, 40, 47). In contrast, there are relatively few studies characterizing how vesicles trigger the innate inflammatory response. Outer membrane vesicles from *Helicobacter pylori* and *Pseudomonas aeruginosa* have been shown to elicit interleukin-8 (IL-8) production by epithelial cells (3, 24), and *Salmonella enterica* serovar *Typhimurium* vesicles have been shown to activate dendritic cells to secrete IL-12 and tumor necrosis factor alpha (TNF-α) (1).

This research has focused on outer membrane vesicles from the opportunistic pathogen *P. aeruginosa*. *P. aeruginosa* infects...
the respiratory tract of patients with nosocomial pneumonia, cystic fibrosis, or acute respiratory distress syndrome (14). Host responses to acute pulmonary infections with P. aeruginosa are characterized by an intense inflammatory response. Macrophage recognition of bacterial components initiates a cascade of proinflammatory cytokine secretion, resulting in large numbers of neutrophils infiltrating the lung to clear the bacteria (44).

We hypothesize that naturally produced vesicles may play an important role in activating innate immune responses. Given their small size and high proportion of PAMPs, it is reasonable to expect that vesicles could easily infiltrate into infected tissues and stimulate widespread inflammation. In addition to TLR ligands, vesicles also contain a variety of protein virulence factors that may specialize vesicles for specific functions or types of host cell damage. P. aeruginosa vesicles have been shown to contain the adhesins OprF, OprG, and OprH, as well as virulence factors that include β-lactamase, hemolysin, phospholipase C, antimicrobial polynucleotides, and quorum-sensing molecules (3, 12, 25, 31, 34). P. aeruginosa vesicle-associated proteins have also been shown to directly affect host cells. Vesicle-packaged CIF protein downregulates lung epithelial expression of the cysitic fibrosis transmembrane conductance regulator (CFTR) protein (10, 33), while aminopeptidase enriched in vesicles increases association of vesicles with cultured epithelial cells (2).

The present study describes how vesicles, with their heterogeneous mixture of PAMPs, contribute to the innate immune response during P. aeruginosa infection. We have focused on vesicle-macrophage interactions, since macrophages are sentinel cells that initiate inflammatory defenses against colonizing bacteria. Our data demonstrate that macrophages are more sensitive to the potent stimulus of outer membrane vesicles from P. aeruginosa compared to equivalent levels of pure LPS. Neutralizing the LPS reactivity of the vesicles or altering the protein composition of the vesicles significantly impacted both the macrophage-vesicle interactions and the cytokine responses to the vesicles. Based on our findings, we conclude that a potent and distinct inflammatory response to vesicles is the cumulative effect of sensing vesicle-associated heterogeneous protein and LPS ligands by macrophages.

MATERIALS AND METHODS

Bacterial strains. The P. aeruginosa strains used were the laboratory strains PAO1 and PAOLΔ/hc and the clinical isolate S470. PAO1Δ/hc was provided by Dan Wozniak (Ohio State University). Strain S470 was provided by J. R. Wright (Duke University) and is a minimally passaged, nonmucoid cystic fibrosis clinical isolate.

Vesicle preparation. Vesicles were purified from cell free supernatants by the method described by Bauman and Kuehn (3). Briefly, P. aeruginosa was grown in LB broth to early stationary phase. Cells were pelleted and supernatants were concentrated via a 100-kDa tangential filtration concentration unit (Pall-Gelli-man) to ~500 ml. The retentate was centrifuged and filtered through a 0.45-μm-pore-size Durapore polypyrrolidene dihydrox filter (Millipore) to remove the remaining bacteria. Vesicles were precipitated from the cell-free supernatant with ammonium sulfate, pelleted, dialyzed overnight with HEPES (50 mM, pH 7), concentrated (50-kDa MWCO Centriplus; Millipore), and adjusted to 45% Opti-Prep/HEPES-NaCl. Opti-Prep gradients were layered over the 2-ml crude vesicle samples at concentrations of 40, 35, 30, 25, and 20%. Gradients were ultracentrifuged (100,000 × g, 16 h), and 1-ml fractions were removed from the top. Fractions were analyzed for vesicle protein content by SDS–15% PAGE and Ruby Red staining. Pure vesicles were recovered from pooled peak fractions of the Opti-Prep gradients, reconstituted into phosphate-buffered saline (PBS; 130 mM NaCl, 8 mM NaHPO4, 2 mM KCl, 1 mM KH2PO4 [pH 7.4]). The protein concentration of the vesicle preparations was determined by Bradford assay.

To calculate the quantity of LPS in each sample, serial dilutions of LPS standards and purified vesicles were separated by SDS–15% PAGE and stained for LPS with a PRO-Q Emerald LPS staining kit (Molecular Probes). The density of the LPS B bands was determined by using ImageJ software, and the LPS content in the vesicles was calculated by using the standard curve generated from the pure LPS samples.

LPS purification. LPS from P. aeruginosa was purified by a modified version of the method of Zaidi et al. (55). Briefly, cells were lysed in 10% sarcosine, debris was pelleted, and LPS was precipitated overnight with 4 volumes of 95% ethanol. LPS was pelleted by ultracentrifugation, resuspended in PBS, and digested with DNase, RNase, and promace. The digest was ultracentrifuged to pellet the LPS, and the LPS was hyphylated.

Fluorescent labeling. Purified vesicles were fluorescently labeled by incubation with Alexa Fluor 555-succinimidyl ester (AF555 [Invitrogen], in 0.1 M Na2CO3 [pH 9]) according to the manufacturer’s instructions for 1 h at 25°C with mixing. Free AF555 was removed from labeled vesicles by three washes in PBS (150,000 × g, 30 min).

Cell culture. RAW264.7 or MH-S mouse macrophage cells were grown in Kaighn’s F-12K media with 10% fetal bovine sera and penicillin-streptomycin-ampicillin (Fungizone). Confluent cells were seeded into wells of a 96-well plates at a concentration of 5 × 104 cells/well. Vesicles, purified LPS, or sham PBS treatment (10-μl doses of each) were added to each well, followed by incubation until time of assay.

Polymyxin B treatment. Purified vesicles or LPS were preincubated with 0.1 mg/ml polymyxin B for 30 min at room temperature prior to addition to macrophages. Polymyxin B (50 μg/ml) was also added to the macrophage cell culture media for experiments using the polymyxin B-pretreated vesicles or LPS.

Protease K treatment. Purified vesicles were treated with 0.1 mg of proteinase K/ml overnight at 37°C with agitation. Postincubation, soluble proteins and excess proteinase K were removed from vesicles by three washes in PBS (150,000 × g, 30 min).

Inhibition of TLR9. RAW264.7 cells were pretreated for 1 h with 3 μg of nuclease-resistant phosphorothioate oligodeoxycytidylate (iCpG DNA; ODN 2088 5′-TCCTGCGGCGGAATG-3′) to inhibit TLR9 signaling (50).

Real-time PCR. Total RNA was harvested from cells by using a Qiagen RNeasy kit and DNase treated to remove contaminating DNA. Harvested RNA was quantitated, and 1 μg of RNA was reverse transcribed to cDNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). This cDNA was purified from reaction components by using a Qiagen DNA clean-up kit and reconstituted in a final volume of 50 μl. Then, 1 μl of purified cDNA was used as the template for each real-time PCR. Real-time PCR was performed on a Bio-Rad iCycler using the iQ SYBR green Supermix. Primers for target genes are presented in Table 1. Gene expression was normalized to actin production in each sample, and the fold induction was determined by using the ΔΔCT method (23).

ELISA. Cytokine protein production (macrophage inflammatory protein 2 [MIP-2], IL-1β, IL-6, and TNF-α) was quantified by enzyme-linked immunosorbent assay (ELISA; R&D Systems/BD) according to the manufacturer’s instructions.

Confocal microscopy. All fluorescence microscopy reagents were purchased from Molecular Probes/Invitrogen unless otherwise stated. MH-S mouse alveolar macrophages were seeded overnight onto coverslips in 24-well tissue culture plates (106 cells per well), followed by incubation with fluorescently labeled vesicles for 15 min at 37°C. All subsequent steps were carried out on ice using ice-cold Dulbecco phosphate-buffered saline (PBS) for washes. After incubation with vesicles, cells were washed twice to remove unbound vesicles or LPS. Cell exteriors were labeled with the FluoReporter cell surface biotinylation kit, washed twice, and incubated with Alexa633-conjugated streptavidin. Cells were then washed, fixed in neutral buffered formalin, mounted with ProLong AntiFade reagent, and visualized on a Leica SPS confocal microscope.

Statistics. All experiments were performed with n ≥ 4. Statistical comparisons between groups were performed using single-factor analysis of variance, followed by Tukey’s post hoc test. The statistical significance was set at P ≤ 0.05.

RESULTS

Macrophages are more sensitive to outer membrane vesicles than to pure LPS. Within the lung, macrophages are critical to sensing and initiation of the inflammatory host response to P. aeruginosa (22). In order to analyze how sentinel macrophages...
trigger inflammation in response to outer membrane vesicles secreted by *P. aeruginosa*, we developed an in vitro assay to measure proinflammatory cytokine gene expression by macrophages. Outer membrane vesicles were collected from the overnight culture supernatant of clinical *P. aeruginosa* strain S470 and purified by density gradient. Proinflammatory genes (MIP-2, KC, TNF-α, IL-1β, IL-6, and iNOS) were selected to be assayed based on their previously described upregulation during active *P. aeruginosa* infection (5, 11, 21, 22, 41, 42, 45, 51, 52). RAW 264.7 macrophages were coincubated with vesicles for 3 h, and gene expression was assayed by real-time PCR and normalized to actin production in each sample. The fold induction was determined by using the ΔΔCt method (32). To determine the minimal dose of vesicles required to trigger a response, we used a 10-fold dilution series of vesicle doses which were standardized to LPS content. A 3-h exposure elicited optimal upregulation of gene expression (data not shown), and therefore this condition was used in subsequent experiments.

Outer membrane vesicles contain significant quantities of LPS; however, the membrane context of vesicle LPS might activate a different cytokine response than extracted, pure LPS. To compare both the amount and the type of response, cytokine levels were measured for macrophages exposed to equivalent concentrations of LPS either purified or as part of a pure vesicle preparation. As seen in Fig. 1A, both vesicles and pure LPS stimulated significant transcription of MIP-2, TNF-α, IL-1β, and IL-6. Other genes assayed, such as KC and iNOS, did not exhibit significant upregulation in response to either vesicles or LPS (data not shown). Proinflammatory gene expression was clearly dose dependent. At the highest dose (1 μg of LPS content), both treatments triggered similar expression levels by all cytokines assayed, indicating that this may be a saturation level response (data not shown). However, sequential dilution of doses demonstrated an increasing divergence in the response with vesicles triggering a more profound cytokine response than pure LPS as the dose decreased. Since secreted vesicles comprise a small fraction (ca. 1 to 2%) of the total outer membrane protein of a growing culture (3), these lower doses represent a physiologically relevant point of comparison.

The four cytokines that demonstrated significant transcriptional upregulation were further analyzed by ELISA for protein secretion into culture supernatant over the course of a 3-h exposure to either vesicles or purified LPS. Figure 1B shows that similar levels of MIP-2 were secreted over this time period in response to both LPS and vesicles, indicating that recognition of LPS within vesicles may be the dominant stimulus needed to generate this cytokine. Neither treatment stimulated significant production of IL-1β, even through significant amounts of IL-1β RNA transcripts were present, as assayed by real-time PCR. In contrast, both TNF-α and IL-6 were secreted at significantly higher levels in response to outer membrane vesicles than LPS. Thus, *P. aeruginosa* vesicles are potent stimulators of macrophages that lead to the upregulated secretion of MIP-2, TNF-α, and IL-6.

**CpG DNA does not significantly contribute to the inflammatory response to vesicles.** Outer membrane vesicles from *Pseudomonas* have been observed to contain bacterial DNA, which is a ligand for endosomal TLR9 (43). To investigate the contribution of CpG DNA in the response to vesicles, TLR9 signaling was inhibited by pretreatment of RAW 264.7 cells with an inhibitory oligonucleotide (iCpG), which is a competitive inhibitor of CpG binding to TLR9. As seen in Fig. 2, inhibition of TLR9 signaling with iCpG had no effect on cytokine responses to S470 vesicles. No significant change in the cytokine transcriptional profiles was seen at any of the vesicle doses tested, indicating that CpG DNA is not a significant trigger of inflammatory responses to vesicles.

**Flagellin stimulates MIP-2 and IL-6 secretion in response to outer membrane vesicles.** Since flagellin is present in the protein profiles of *P. aeruginosa* outer membrane vesicles and is a potent innate immune stimulus via both TLR5 and the NOD-like receptor IPAF (3), we investigated the contribution of flagellin to the cytokine response to vesicles using vesicles from strain PAO1 and its isogenic flagellin mutant (ΔfliC). We quantified the flagellin bands from SDS-PAGE profiles of both PAO1 and S470 derived vesicles using densitometric analysis and demonstrated that these preparations contained equivalent levels of flagellin (data not shown). We could not use RAW 264.7 cells for these experiments because these cells do not express TLR5 (53). Therefore, we used the MH-S alveolar macrophage cell line, which is known to express TLR5. We noted first that the MH-S cell line exhibited much lower sensitivity to vesicles than did RAW 264.7 cells: the lowest vesicle dose (0.001 μg of LPS content) elicited only modest transcriptional increases for any of the genes assayed, these increases did not result in measurable cytokine levels by ELISA and, unlike RAW 264.7 cells, the MH-S cells did not secrete TNF-α in response to any of the vesicle doses (Fig. 3 and data not shown). However, ELISA analysis indicated that vesicles did induce both MIP-2 and IL-6 secretion and that these were significantly lower in response to ΔfliC vesicles across multiple doses. Thus, the flagellin component of vesicles contributes

### Table 1. RT-PCR primers used in these studies

<table>
<thead>
<tr>
<th>Gene assayed</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>MIP-2</td>
<td>TCCATGAAAGCCATCCGACTGAT</td>
<td>ACATCCCACCCACACAGTGAAGA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ACCACCTCTCCCTTGCAACTCA</td>
<td>TCTCATGCACACCATCAAGGACT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGTGTGAGGGTGCTGATGACCA</td>
<td>TGGAGATTGTGGATGTCAGCAACAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>AAGCCGACTAGTTGCCCAGTAGA</td>
<td>TGGCTAAAGGCAAGACCATCCTCA</td>
</tr>
<tr>
<td>KC</td>
<td>AAGTTGTCAGAGAACGAGGTTTCC</td>
<td>ACCACAAAAGGTGATCATAGCCCA</td>
</tr>
<tr>
<td>iNOS</td>
<td>ATGTCATGAGCAAAAGGGCCAGAAC</td>
<td>CTGCTGTGTTGGTGACAAAGACCATTT</td>
</tr>
<tr>
<td>Actin</td>
<td>AGAGGGAAATCGTGGTGAC</td>
<td>CAATAGTGATGACCTGGCCTT</td>
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significantly to the stimulation of MIP-2 and IL-6 secretion by macrophages.

Strain-specific proteins impact the inflammatory responses to vesicles. We previously demonstrated that the protein composition of vesicles varied with bacterial strain and can affect their association with host cells (2, 3). Therefore, we investigated whether the macrophage responses would also differ for vesicles from different strains of *P. aeruginosa*. A proteomic comparison of vesicles from the clinical isolate S470 and lab strain PAO1 demonstrated some significant differences in their protein composition (3). The ratios of LPS to protein content for these two vesicle preparations were highly similar (data not shown), so any observed differences in the responses would likely be the result of protein composition rather than LPS concentration.

Cytokine upregulation and secretion were assayed in response to vesicles from S470 and PAO1. As shown in Fig. 4A significant differences in gene expression by real-time PCR analysis were detected for the lowest dose (0.001 μg of LPS content). At this dose, vesicles from strain S470 upregulated significantly higher levels of all three cytokines assayed.

Greater differences were detected when cytokine levels were measured by ELISA (Fig. 4B). MIP-2 secretion was significantly higher in response to PAO1 vesicles, whereas the secretion of both TNF-α and IL-6 was greater in response to S470 vesicles. TNF-α secretion above the baseline of untreated cells was not detected at the lowest dose for either vesicle treatment, nor were significant IL-6 levels detected in response to the lowest dose of PAO1 vesicles. These data indicate that strain-specific vesicle components affect both the nature and the sensitivity of the macrophage proinflammatory cytokine response.

LPS and vesicle proteins act synergistically to generate a distinct inflammatory response. Two different biochemical treatments were used to investigate the contribution of particular vesicle components in more detail. First, vesicles were pretreated with polymyxin B to neutralize the contribution of LPS. Polymyxin B binds to the lipid A portion of LPS, inhibiting sensing by the TLR4 complex (27). Second, vesicles were incubated overnight with proteinase K to degrade vesicle proteins. Figure 5A shows the effect of proteinase K treatment on the vesicle proteins. The protein profile of the vesicles was...
significantly altered, with many high-molecular-weight bands disappearing after protease treatment. All proteins were not fully degraded, as they were likely to be protected from protease treatment by the vesicular membrane.

Macrophages were incubated with either of these treated vesicle preparations, and cytokine expression was assayed by real-time PCR and ELISA. As seen in Fig. 5B and C, polymyxin B treatment of vesicles dramatically depressed both cytokine transcriptional and protein responses of all genes assayed. The significant inhibition of cytokine transcription by polymyxin B treatment was surprising given the higher potency of whole vesicles compared to LPS (Fig. 1B). Polymyxin B-treated LPS, used as a control for these experiments, generated minimal transcriptional responses by the macrophages (data not shown). These results indicate that LPS acts as an adjuvant in the reaction of macrophages toward vesicles, greatly enhancing the response to the non-LPS components of vesicles.

In comparison to polymyxin B, proteinase K treatment of vesicles resulted in a more selective dampening of cytokine responses. Proteinase K treatment depressed IL-6 production most significantly for all tested doses of vesicles, as measured by both reverse transcription-PCR and ELISA (Fig. 5B and C), indicating the likelihood that the production of this cytokine is triggered in response to outer membrane protein recognition. However, for several cytokines, induction levels increased with protease treatment of the vesicles. Secreted MIP-2 levels were higher than that seen in response to untreated vesicles (Fig. 5C). TNF-α secretion was most dramatically impacted by proteinase K treatment at the lowest vesicle dose (0.001 μg of LPS content), whereas significantly higher TNF-α secretion was seen compared to the response to either intact vesicles or pure LPS. Together, these data suggest that vesicles interact with macrophages in a manner that triggers a distinct, strain-specific response that reflects recognition of a combination of vesicle constituents.

Sensing of both LPS and vesicle proteins is required for internalization by macrophages. Based on their activation of distinct cytokine responses, we predicted that LPS, vesicles, and treated vesicles might interact differently with macrophages. We developed a confocal microscopy assay to compare how our preparations of vesicles associated with macrophages. MH-S alveolar macrophages were treated with untreated, proteinase K-treated, or polymyxin B-treated, Alexa Fluor 555-labeled S470 vesicles for 15 min. Vesicles were fluorescently labeled after proteinase K treatment, whereas polymyxin B treatment was performed on fluorescently labeled vesicles. After vesicle coincubation, the cells were washed to remove unbound material, surface biotinylated, and incubated with Alexa Fluor 633-streptavidin.

The different LPS-containing reagents yielded substantially distinct localization profiles (Fig. 6), giving insight into the molecular basis for the distinct pattern of activation of the macrophages. Untreated S470 vesicles appeared as punctate particles that were not labeled by streptavidin and thus were likely to have been internalized by the macrophages. Polymyxin B treatment resulted in few vesicles interacting with macrophages. The few vesicles that were observed were not labeled by streptavidin and thus were internalized by the macrophages. Proteinase K-treated vesicles adhered to the surfaces of macrophages and were labeled by streptavidin and thus not internalized. These data indicate that vesicle LPS is required for initial surface binding of vesicles to the macrophage surface, whereas recognition of vesicle protein triggers internalization.

**DISCUSSION**

The goal of the present study was to characterize both the inflammatory response to *P. aeruginosa* outer membrane vesicles and the molecular components key to this response. Outer membrane vesicles triggered a distinct, strain-specific pattern of cytokine production in macrophages compared to...
that of pure LPS. LPS and flagellin both played roles in triggering cytokine production, whereas CpG DNA did not. Proteinase K digested vesicles bound to the surface of macrophages without internalization and exhibited reduced levels of IL-6. Further, the response to surface proteins depended on the presence of LPS, since a lack of LPS sensing abrogated the majority of the response.

It was anticipated that the macrophage response to vesicles would closely resemble that to pure LPS; however, this was clearly not the case. The higher potency of vesicles to trigger cytokine production was particularly evident at low vesicle doses. A titration of vesicle dose was similarly used by Alaniz et al. (1) to study the ability of vesicles from Salmonella to activate dendritic cells. Our study supports their conclusion that vesicles are potent stimulators of proinflammatory cytokines. However, since Alaniz et al. standardized their vesicle and LPS doses to total dry weight rather than LPS content, these responses cannot be compared directly in terms of the relative contribution of LPS to this response. Therefore, while both studies clearly demonstrate the potency of vesicles to trigger inflammatory responses, our study defines the contribution of LPS to the macrophage response and demonstrates that the context of heterogeneous components present in the outer membrane is critical to the generation of a robust response.

This study supports the concept that the three-dimensional configuration in which vesicle ligands (including LPS) are presented to macrophages impacts both the type and sensitivity of their response. Beyond acting as stimuli, vesicle proteins exhibit the ability to block or hinder vesicle interactions with surface receptors such as TLR4 or MD2. The pattern of TNF-α secretion observed in our studies illustrate this ability, since TNF-α secretion was enhanced by protease treatment (Fig. 5B and C), whereas protein-intact vesicles required higher doses to trigger similar levels of TNF-α production (Fig. 1B).

Several other studies have further suggested that the spatial orientation of LPS impacts its interactions with host cells. Analysis of CD14-dependent internalization of LPS has shown that aggregate size of LPS complexes may directly affect the

![FIG. 3. Flagellin contributes to the MIP-2 and IL-6 responses to outer membrane vesicles. The contribution of flagellin was determined using MH-S mouse macrophage cells incubated with vesicles from the PAO1 or PAO1ΔfliC strains. (A) Fold changes in gene expression were assessed by real-time PCR normalized to actin production. (B) Protein secretion was assayed from culture supernatants by ELISA. The bars represent the SEM (n = 4). *, P < 0.05; **, P < 0.01.](http://iai.asm.org/.../Downloaded from)
rate of recognition and uptake (30). Further, Elsbach concluded that the binding of and responses to LPS by inflammatory cells critically depend on the context and presence of other bacterial compounds (such as proteins) in which the LPS was presented (16).

The dramatic effect proteinase K treatment had on the response to vesicles demonstrates that the protein content of vesicles include key ligands of significant cytokine responses. Flagellin monomers, ligands for both TLR5 and NOD-like receptors, have been detected in purified *P. aeruginosa* vesicles (3, 36). Transcriptional upregulation of IL-1β was detected in response to intact vesicles; however, no mature protein was secreted, indicating that modification by caspase-1 did not occur. Since previous studies demonstrated that LPS increases transcription of the IL-1β gene without active secretion of the protein (54), we propose a similar effect occurs for vesicles.

We found that vesicle proteins other than flagellin contribute to the IL-6 response via recognition within the macrophage. The expression profiles of IL-6 secretion to protease-treated vesicles and flagellin-deficient vesicles differed significantly and cannot only be explained by protease degradation of surface-associated flagellin. In addition, the responses to S470 and PAO1 vesicles differed, whereas the responses to purified LPS from both strains did not (data not shown). Macrophage-bound vesicles, inhibited from entering by cytochalasin D, trigger MIP-2 and TNF-α production but not IL-6, confirming the role of vesicle proteins and internalization in triggering an IL-6 response (data not shown). MIP-2 and TNF-α can be stimulated by binding of outer surface receptors such as TLR4 and TLR5.

Each cytokine analyzed exhibited a distinct pattern of responses to vesicles. MIP-2, a neutrophil chemoattractant, was secreted in a pattern that most closely matched TLR triggered responses to purified LPS. Diminished production of MIP-2 by deletion of flagellin further indicates that surface TLR receptors are engaged to stimulate production (6). Protease treatment actually enhanced MIP-2 secretion, indicating that vesicle proteins may physically block TLR receptor engagement of LPS. In contrast, IL-6 production appears to be predominantly a response to vesicle proteins, since it dramatically decreased following either the deletion of flagellin or general protease treatment. The observed TNF-α response to vesicles was intermediate to those of MIP-2 and IL-6.

Analysis of the cytokine promoter regions indicates that the

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**FIG. 4.** The cytokine responses to outer membrane vesicles are bacterial strain dependent. RAW 264.7 mouse macrophage cells were incubated with vesicles from clinical isolate S470 or lab strain PAO1 for 3 h. Gene expression was assayed by real-time PCR (A), and protein secretion was assayed from culture supernatants by ELISA (B). The bars represent the SEM (n = 4). *, P < 0.05; **, P < 0.01.
MIP-2 response to vesicles may be triggered primarily by NF-κB-dependent pathways, since the MIP-2 promoter only contains binding sites for AP-1 and NF-κB (29). In contrast, the TNF-α and IL-6 promoters contain a larger variety of regulatory binding sites. The presence of sites responsive to prostaglandins or cyclic AMP, in addition to AP-1 and NF-κB sites (13, 46), results in sensitivity to other ligands such as vesicle proteins.

The data presented here indicate that vesicle LPS acts as an adjuvant for other components. This effect was most dramatically demonstrated by the lack of response to vesicle proteins in polymyxin B treatment experiments. Vesicle localization further support the key contributions of vesicle proteins to internalization and the generation of a robust cytokine response. Thus, vesicle proteins are particularly effective stimuli of cytokine production when recognized in the context of other PAMPs such as LPS.

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Recent mechanistic studies have provided support for the adjuvanticity of TLR ligands. Sensing of TLR ligands directly impacts phagosomal maturation, selection of antigens for MHC presentation, and maturation of dendritic cells (8, 9). Further, the presence of LPS during an initial antigen exposure changed the mechanism and magnitude of T-cell trafficking upon subsequent LPS-free antigen challenge, as well as amplified the phagocytic inflammatory response (37). These studies suggest that the response to the LPS-protein mixture in vesicles may translate to protective downstream T-cell and memory responses. Further, LPS or other TLR ligands greatly enhanced protective responses to N. meningitis Vesicle-based vaccines (15, 19). Our demonstration that low doses of LPS within a vesicle context augment protein specific responses could be used to refine efforts to engineer outer membrane vesicles into vaccines.

In summary, the data presented here demonstrate that naturally secreted outer membrane vesicles of P. aeruginosa are potent stimulators of strain-specific proinflammatory responses. Macrophage responses to a complex of heterogeneous PAMPs are distinct from the responses to the individual components, require the presence of LPS for maximal potency, and trigger intense responses via combined sensing of multiple pathogen-specific stimuli. Future studies will focus on determining which outer membrane proteins are critical to the observed responses.
FIG. 6. Macrophage recognition of outer membrane vesicle proteins is required for internalization by macrophages. MH-S alveolar macrophages were exposed to native, proteinase K-treated, or polymyxin B-treated Alexa Fluor 555-labeled vesicles for 15 min. At the indicated time points, the unbound label was washed off, and the cells were surface labeled with biotin and Alexa Fluor 633-streptavidin (blue) and visualized by confocal microscopy.

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