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Pseudomonas aeruginosa Outer Membrane Vesicles Modulate Host Immune Responses by Targeting the Toll-Like Receptor 4 Signaling Pathway

Kelei Zhao,[a,b] Xin Deng,[c] Chuan He,[c] Bisong Yue,[b] Min Wu[a]

Department of Biochemistry and Molecular Biology, School of Medicine and Health Sciences, University of North Dakota, Grand Forks, North Dakota, USA[a]; Key Laboratory of Bio-Resources and Eco-Environment (Ministry of Education), College of Life Science, Sichuan University, Chengdu, Sichuan, People's Republic of China[b]; Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, Chicago, Illinois, USA[c]

Bacteria can naturally secrete outer membrane vesicles (OMVs) as pathogenic factors, while these vesicles may also serve as immunologic regulators if appropriately prepared. However, it is largely unknown whether *Pseudomonas aeruginosa* OMVs can activate inflammatory responses and whether immunization with OMVs can provide immune protection against subsequent infection. We purified and identified OMVs, which were then used to infect lung epithelial cells *in vitro* as well as C57BL/6J mice to investigate the immune response and the underlying signaling pathway. The results showed that OMVs generated from *P. aeruginosa* wild-type strain PAO1 were more cytotoxic to alveolar epithelial cells than those from quorum-sensing (QS)-deficient strain PAO1-Δ*lasR*. The levels of Toll-like receptor 4 (TLR4) and proinflammatory cytokines, including interleukin-1β (IL-1β) and IL-6, increased following OMV infection. Compared with lipopolysaccharide (LPS), lysed OMVs in which the membrane structures were broken induced a weak immune response. Furthermore, expression levels of TLR4-mediated responders (i.e., cytokines) were markedly downregulated by the TLR4 inhibitor E5564. Active immunization with OMVs or passive transfer of sera with a high cytokine quantity acquired from OMV-immunized mice could protect healthy mice against subsequent lethal PAO1 challenges (1.5×10^{11} CFU). Collectively, these findings indicate that naturally secreted *P. aeruginosa* OMVs may trigger significant inflammatory responses via the TLR4 signaling pathway and protect mice against pseudomonal lung infection.

Since 1990, lung infections exceeded HIV/AIDS, cancer, and heart diseases and became the greatest cause of disease worldwide (1). *Pseudomonas aeruginosa* is an important opportunistic pathogen, can cause infections in multiple organ systems, and is extremely invasive in immunocompromised patients with cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis (2–4). Like other Gram-negative bacteria, *P. aeruginosa* can secrete outer membrane vesicles (OMVs) as a potential means to deliver virulence factors to distant locations by fusing with lipid rafts on the host cell membrane (5–7). This has led to growing research interest in the pathogenesis of OMVs in the past few years.

The origination of bacterial OMVs was considered a general envelope stress response (8, 9). Natural OMVs are composed of outer membrane and periplasmic constituents, including phospholipids, lipopolysaccharide (LPS), and proteins as well as several virulence factors (10–13). LPS can be sensed by Toll-like receptor 4 (TLR4) on host cells to mount an innate immune response to Gram-negative bacteria (14, 15). Some LPS can cause TLR2-mediated signaling in human cells, which may be attributed to the structural difference from the typical hexa-acyl bisphosphorylated format of TLR4-activatable LPS (16, 17). Proteins within *P. aeruginosa* secreted vesicles have also been reported to play an important role during infection. Typically, vesicles containing cystic fibrosis transmembrane conductance regulator (CFTR)-inhibitory factor (Cif) can reduce chloride secretion by decreasing the apical expression of CFTR and thereby reducing the mucociliary clearance of pathogens (18, 19).

Previous research reported that OMVs could elicit heightened innate immune responses by the combined sensing of both LPS and protein components (14, 20–22). OMVs were shown to be promising for development of vaccines against infection by the corresponding bacteria (21–27). Remarkably, vaccines developed based on *Neisseria lactamica* OMVs were shown to render effective protection against meningococcal infection and underwent a phase I clinical trial for safety and immunogenicity studies with adult volunteers (27, 28). OMVs from *P. aeruginosa* were also found to trigger the production of cytokines in epithelial cells and macrophages (20, 29). These findings suggest that naturally produced *P. aeruginosa* vesicles have the potential to protect the immunized host against subsequent pseudomonal infection. However, to date, *P. aeruginosa* OMVs have not been tested for immunization as a vaccine candidate. Hence, in this study, we first investigated the possibility that OMVs might activate the intercellular immune response via the TLR4 signaling pathway and then explored the immune protection of OMVs based on a mouse model. Importantly, *P. aeruginosa* OMVs were shown to be capable of protecting immunized mice from lethal challenges with various concentrations of *P. aeruginosa*.

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Address correspondence to Bisong Yue, bsyue@scu.edu.cn, or Min Wu, min.wu@med.und.edu.

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MATERIALS AND METHODS

Bacterial strains and outer membrane vesicle purification. The *Pseudomonas aeruginosa* PAO1 wild-type (WT) strain and quorum-sensing (QS) defective strain PAO1-Δ*lasR* were grown in Luria-Bertani (LB) broth medium. OMVs produced by WT PAO1 and PAO1-Δ*lasR* were purified by using a differential centrifugation and discontinuous Optiprep (Sigma-Aldrich) gradient protocol adapted and modified from a protocol described previously by Bauman and Kuehn (29). Briefly, 500 ml of *P. aeruginosa* cells was grown to the early stationary phase, and bacterial cells were removed by pelleting at 4°C (10,000 × *g* for 10 min). Supernatants were concentrated via a 100-kDa tangential filtration concentration unit (Pall-Gellman), and the retentate was centrifuged and filtered through a 0.45-μm-pore-size Durapore polyvinylidene difluoride filter (Millipore). Vesicles were pelleted (50,000 × *g* for 1 h), resuspended in a discontinuous Optiprep gradient, and centrifuged (100,000 × *g* for 16 h) again. Pure vesicles were recovered from pooled peak fractions by dilution in HEPES and pelleting (150,000 × *g* for 1 h). Pure vesicles were diluted in phosphate-buffered saline (PBS) (pH 7.4) before use. The components of OMVs were visualized by 15% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and Coomassie blue staining.

Cell culture. A549 human lung epithelial cells (ATCC CCL-185) and MLE-12 murine lung epithelial cells (ATCC CRL-2110) were obtained from the American Type Culture Collection (ATCC) and used to examine immune responses induced by the OMVs. A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum plus penicillin-streptomycin-amphotericin B (Fungizone) (Invitrogen, CA). Mouse alveolar macrophage MH-S cells (ATCC CRL-2019) were also obtained from the ATCC and were maintained in RPMI–F-12 medium (50%:50%) and 2 mM HEPES buffer.

The innate immune responses of A549 and MLE-12 cells were studied in the presence of OMVs (0.25 mg/ml) or LPS (Sigma-Aldrich) from *P. aeruginosa* (100 ng/ml) or lysed OMVs (0.25 mg/ml), followed by incubation until the time of assay. Cells were harvested at designated time points and lysed in chilled radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES [pH 7.5], 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% Na deoxycholate). OMVs were lysed with EDTA (100 mM) at 37°C for 60 min as described previously by Bomberger et al. (6).

Cytotoxicity assay. To determine the strain-specific cytotoxicity of *P. aeruginosa* OMVs on A549 and MLE-12 cells, confluent cells were harvested and seeded into wells of 96-well plates at a concentration of 5×10^4 cells/well. Purified vesicles (0.25 mg/ml) of PAO1 or PAO1-Δ*lasR* or PBS (10 μl each) was added to each well and incubated for the indicated time points. Subsequently, cytotoxicity was determined by measuring the cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Promega) reduction activity according to the method of Abe and Matsuki (30). Briefly, MTT was dissolved in PBS at a concentration of 2.5 mg/ml (6 mM) and added to each well so that the final concentration of MTT in the medium was 0.25 mg/ml (600 μM), and the cells were then incubated for 1 h at 37°C. The reaction was stopped by adding 100 μl of a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (DMF-SDS) (pH 4.7). After overnight incubation at 37°C, the optical density (OD) was read on a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm. The results were calculated as the ratio of the mean OD value of experimental groups to that of the control group treated with PBS.

Proinflammatory responses in mouse models. We studied the immune response against *P. aeruginosa* OMVs in C57BL/6J mice. Mice were obtained from Harlan (Indianapolis, IN) and housed in the animal facility at the University of North Dakota. The animal experiments were approved and performed in accordance with institutional animal care and use committee guidelines (University of North Dakota Institutional Animal Care and Use Committee [IACUC] approval number 1204-4). Mice were lightly anesthetized with an intraperitoneal injection of a mixture of ketamine HCl (30 mg/kg of body weight) and xylazine HCl (15 mg/kg) (Sigma). Mouse lungs were aseptically removed at 12 h and 24 h after intranasal instillation of 0.5 mg purified *P. aeruginosa* OMVs. The lungs were homogenized by the use of a Branson SLPe digital sonifier cell disruptor (Fisher Scientific) in chilled RIPA buffer. The homogenate was centrifuged at 20,000 × *g* for 20 min at 4°C to remove cellular debris.

Inhibition of the TLR4 signaling pathway. To confirm the result that *P. aeruginosa* OMVs could trigger the intracellular inflammatory response via the TLR4 signaling pathway, we used E5564 [α-D-glucopyranose,3-*O*-decyl-2-deoxy-6-*O*-[2-deoxy-3-*O*-[(3R)-3-methoxydecyl]-6-*O*-methyl-2-[[(11Z)-1-oxo-11-octadecenyl]amino]-4-*O*-phosphono-β-D-glucopyranosyl]-2-[(1,3-dioxotetradecyl)amino]-1-(dihydrogen phosphate),tetrasodium salt] (formula weight, 1,401.60), a TLR4-directed endotoxin antagonist (31) synthesized by Eisai Research Institute of Boston (Andover, MA), according to the manufacturer's instructions. MLE-12 cells were seeded in DMEM plus 10% fetal bovine serum and maintained at 37°C. The next day, cells were incubated with the indicated concentrations of E5564 (1 nM and 10 nM) in the presence of *P. aeruginosa* OMVs at a concentration of 0.25 mg/ml for 24 h. Cells treated with same amount of PBS were used as a control group. Cells treated with LPS at a concentrations of 100 ng/ml and 10 nM E5564 were used as a control for E5564. Cells were harvested and lysed in chilled RIPA buffer.

Immunization of mice. To explore the immunoprotective potential of *P. aeruginosa*-derived OMVs, 0.5 mg purified PAO1 OMVs were intranasally instilled into anesthetized C57BL/6J mice on days 1, 3, 7, 14, 21, and 28. At day 29, sera of immunized mice were collected and preserved at −80°C. Other groups of immunized mice (5 mice for each group) were then intranasally challenged with *P. aeruginosa*. To test the protective roles of OMV immunization, two challenge doses were then chosen, a dose (1.5×10^9 CFU) sufficient to induce 100% mortality in the absence of immunization and a dose approximately 100 times (1.5×10^{11} CFU) higher than that. For controls, untreated mice or mice intranasally inoculated with a single dose of OMVs were also challenged with the same amount of *P. aeruginosa*. To test the passive immunity transfer, vesicle-immunized sera with abundant cytokines were diluted into series of concentrations (5-, 10-, 20-, and 50-fold) and were subcutaneously injected into C57BL/6J mice. Each mouse received 0.2 ml diluted serum by subcutaneous injection. The next day, serum-injected mice were challenged by intranasal instillation of the above-mentioned two concentrations of *P. aeruginosa*. Mice subcutaneously injected with either unimmunized sera or the sera immunized with a single dose of OMVs were challenged with the same amount of *P. aeruginosa* as controls. The clinical onset of challenged mice was assessed, from reduced activity, fatigue, lethargy, and messy furs to extremely sick (moribund), which was considered dead on that day, and observation was terminated after 10 days. For each experiment, mice injected with the same amount of PBS were also used as a control.

Western blotting. Rabbit polyclonal antibodies against TLR2, TLR4, and TRIF (TIR domain-containing adaptor protein inducing beta interferon [IFN-β]); mouse monoclonal antibodies against MyD88, Toll/IL-1R domain-containing adapter protein (TIRAP), NF-κB, interleukin-1β (IL-1β), and IL-6; and goat polyclonal IRF-3 (interferon-regulatory factor 3) antibody were purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies were purchased from Rockland Immunochemicals. Western blotting was carried out as described previously (32). Briefly, the samples derived from cells and tissue homogenates were lysed and quantitated, and the lysates were then mixed with a protease inhibitor and boiled for 10 min. Equal amounts of each sample were loaded onto 10% SDS-polyacrylamide minigels and electrophoresed. Subsequently, proteins were transferred onto polyvinylidene difluoride membranes (Pierce) and blocked in 5% nonfat milk blocking buffer for 1 h at room temperature. The membranes were incubated overnight at 4°C with appropriate primary antibodies at the concentrations recommended by the manufacturers and

TABLE 1 Primers for quantitative PCR used in this study[a]

Gene assayed	Primer sequence
IL-1β	5′-TCATTGTGGCTGTGGAGAAG-3′ (forward)
	5′-CATCTCGGAGCCTGTAGTGC-3′ (reverse)
IL-6	5′-CTTGGGACTGATGCTGGTGA-3′ (forward)
	5′-CATTTCCACGATTTCCCAGA-3′ (reverse)
GAPDH	5′-CTGCCCAGAACATCATCCCT-3′ (forward)
	5′-TGAAGTCGCAGGAGACAACC-3′ (reverse)

[a] These primers were designed in this study.

then washed three times with washing solution and incubated for 2 h with the appropriate secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) at the concentrations recommended by the manufacturers. Finally, after washing three times with washing solution, blots were developed with an enhanced chemiluminescence detection kit (SuperSignal West Pico; Pierce) and analyzed by densitometry using ImageJ software.

Cell estimation in bronchoalveolar lavage fluid and histological analysis. Lungs of WT and immunized mouse after a 12-h *P. aeruginosa* challenge were lavaged 5 times by instilling and withdrawing 1 ml of PBS with an aseptic syringe. Bronchoalveolar lavage fluid (BALF) samples were collected, mixed to ensure an even suspension, and then stained with HEMA-3 (Fisher, Rockford, IL) according to the manufacturer's instructions. Subsequently, 10 μl of the mixture was pipetted into the wells of a hemocytometer for cell differential counting using a light microscope. After bronchoalveolar lavage procedures, the lungs of WT and OMV-immunized mice were aseptically harvested and fixed in 10% formalin. The paraffin-embedded tissue sections were prepared on a rotary microtome and stained with hematoxylin-eosin by using standard techniques (33). All sections were examined by light microscopy. To assess bacterial deposition, 1 g lungs or spleens was homogenized in liquid nitrogen, followed by a brief sonication. Homogenized tissues were fixed in PBS and spread onto LB plates for quantitative bacterial culture. Triplicates were done for each sample and control.

Quantitative PCR. Specific primers (Table 1) for IL-1β, IL-6, and GAPDH were designed by using Primer 3.0 software (http://frodo.wi.mit.edu/primer3/), based on the consensus of sequences that are deposited in GenBank. Mouse lungs were collected after challenge with 0.5 mg PAO1 OMVs for 12 and 24 h. Lungs were lysed and quantitated, and the lysates were ultrasonically disrupted. Total RNA was extracted by using TRIzol reagent, followed by reverse transcription and quantitative PCR using a Qiagen OneStep RT-PCR kit in accordance with the manufacturer's instructions. All experiments were performed in triplicate. Gene expression was calculated by using the $2^{-\Delta CT}$ method (34) and normalized to GAPDH levels in each sample.

Enzyme-linked immunosorbent assay. Cytokine protein production (tumor necrosis factor alpha [TNF-α], IL-1β, and IL-6) in the serum of OMV-challenged mice was quantified by an enzyme-linked immunosorbent assay (ELISA), using ELISA kits (eBioscience Co., San Diego, CA) according to the manufacturer's instructions. All the experiments were performed in triplicate.

Confocal fluorescence microscopy. Purified OMVs were resuspended in labeling buffer (50 mM Na_2CO_3, 100 mM NaCl [pH 9.2]), and X-rhodamine-5-(and -6)-isothiocyanate [5(6)-XRITC] (Molecular Probes) was added at a concentration of 1 mg/ml for 1 h at 25°C, followed by ultracentrifugation at 52,000 × *g* for 30 min at 4°C. To visualize the phagocytosis of murine alveolar macrophages toward *P. aeruginosa* OMVs, 0.1×10^6 MH-S alveolar macrophages were seeded overnight on collagen-coated, glass-bottom MatTek dishes (MatTek, Ashland, MA) and then exposed to labeled vesicles for 5 min and 15 min. The unbound vesicles were washed off with PBS, and the cells were surface stained with the lipid raft marker Alexa Fluor 488 (green; Molecular Probes) and visualized by confocal microscopy.

Data analysis and statistics. The density of the Western blot bands was determined by using ImageJ software. Data and statistical tests were computed by using Graphpad Prism version 5.0 (Graphpad, San Diego, CA). Means were compared by using Student's *t* test or one-way analysis of variance (ANOVA), followed by a Tukey-Kramer *post hoc* test using a 95% confidence interval. Data are presented as means ± standard errors of the means (SEM). A chi-square test with Yates' correction was used to compare the survival rates between immunized mice and the unimmunized group.

RESULTS

OMVs generated from *P. aeruginosa* PAO1 or PAO1-Δ*lasR* were cytotoxic to epithelial cells. In order to study the pathogenic role of OMVs, we used a protocol established previously by Bauman and Kuehn (29) to purify and characterize OMVs. To account for the impact of pathogenic factors on immunogenicity, we isolated OMVs from *P. aeruginosa* WT strain PAO1 and a quorum-sensing-deficient strain, PAO1-Δ*lasR*. SDS-PAGE results showed that the components of the two purified OMVs were almost identical, except for a band corresponding to the known molecular mass of OprD (48 kDa), as also previously reported by Bauman and Kuehn (29), in the low-density fractions of PAO1 OMVs (Fig. 1A and B). To test the cytotoxic effect of the naturally secreted OMVs, we employed human alveolar epithelial A549 cells and murine alveolar epithelial MLE-12 cells, as both cell types are models commonly used in culture to test host-pathogen interactions (35, 36). OMVs derived from either PAO1 or PAO1-Δ*lasR* were cytotoxic to both A549 and MLE-12 cells after a delay of 8 h of incubation (Fig. 1C and D). These results were consistent with data reported previously by Bomberger et al. (6), showing that the *P. aeruginosa*-produced OMVs were capable of causing cytotoxicity on epithelial cells. Moreover, it seemed that the OMVs from PAO1 were more cytotoxic to A549 cells than those from PAO1-Δ*lasR* after 10 h of treatment (Fig. 1C). Therefore, we chose WT *P. aeruginosa* for the following experiments, which might elicit vigorous cellular proinflammatory responses.

***P. aeruginosa* OMVs triggered proinflammatory responses *in vitro* and *in vivo* via the TLR4 signaling pathway.** To determine the innate immune response induced by *P. aeruginosa* OMVs, we first detected the expression of TLR2 and TLR4 after challenging respiratory epithelial cells with LPS and OMVs, respectively. We noted that the expression of TLR4 (but not TLR2) increased significantly in A549 cells with challenge of OMVs or LPS from *P. aeruginosa* (see Fig. S1 in the supplemental material). These findings suggested that the purified OMVs could induce innate immune responses via the TLR4 signaling pathway, which might be attributed to the properties of LPS. To further test this hypothesis, we evaluated several signaling proteins associated with the TLR4 pathway using Western blotting. As expected, we found that the expression levels of TLR4, MyD88, and NF-κB increased in both A549 and MLE-12 cells after incubation with *P. aeruginosa* OMVs for 24 h, and the level of IL-1β was increased in MLE-12 cells (Fig. 2A to D). However, no significant difference in the expression levels of TRIF and IRF-3 was observed (see Fig. S2 in the supplemental material). These results indicated that an OMV complex of *P. aeruginosa* might be required to elicit a vigorous immune response in epithelial cells *in vitro* via a MyD88-dependent TLR4 signaling pathway. Since E5564 is a widely used TLR4-directed endotoxin antagonist (31), we set up to confirm the *P. aeruginosa* OMV-induced TLR4 signaling pathway with E5564 to examine the impact when blocking TLR4. The expression of TLR4-related signaling proteins, such as MyD88, NF-κB, and IL-

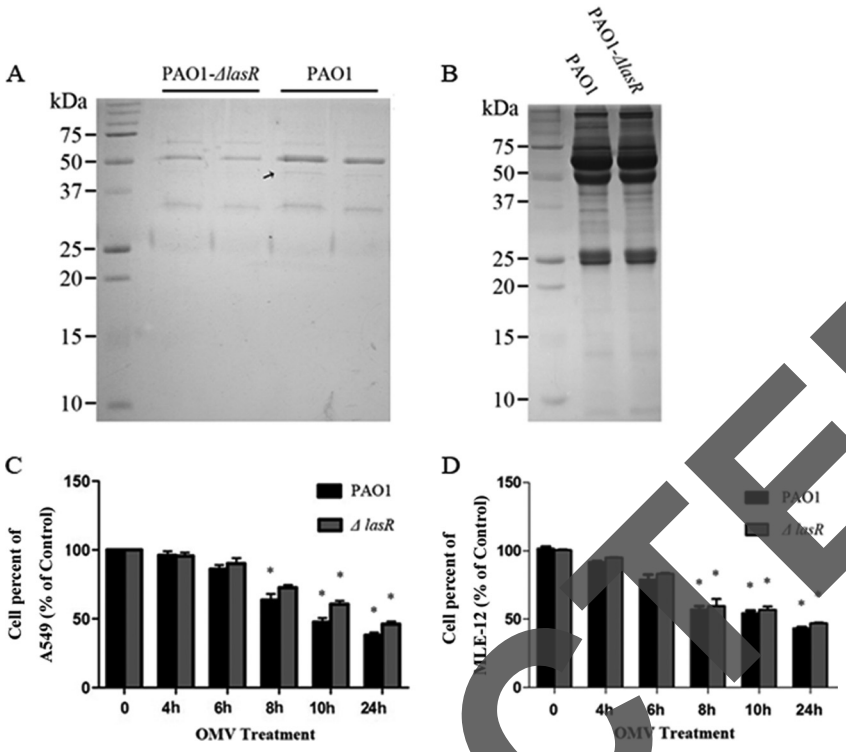

FIG 1 *P. aeruginosa* PAO1- and PAO1-Δ*lasR*-generated OMVs elicited time-dependent cytotoxicity in lung epithelial cells. (A and B) Fractions sequentially removed from the top of each gradient were analyzed with Coomassie-stained SDS-PAGE gels. (A) Low-density fractions; (B) high-density fractions. The black arrow indicates the different band between the components of PAO1- and PAO-Δ*lasR*-generated OMVs. (C and D) Cytotoxicity was determined in A549 cells (C) and MLE-12 cells (D) by an MTT assay. Ten microliters of purified vesicles (0.25 mg/ml) from PAO1 or PAO1-Δ*lasR* was added to each well and incubated for the indicated time points. Cells treated with an equal amount of PBS were used as a control. Data are presented as means ± SEM, and each column is compared with the 0-h time point (*, $P < 0.05$, determined by one-way ANOVA followed by a Tukey-Kramer *post hoc* test).

1β, was abolished by incubation with 10 nM E5564 for 24 h compared with sham controls. Indeed, blocking of TLR4 led MLE-12 cells to fail to respond against the challenge of *P. aeruginosa* OMVs (Fig. 2E and F). To further determine the downstream factors of the OMV-induced immune response, we explored the OMV-elicited signaling pathway using mouse models. After intranasally challenging C57BL/6J mice with OMVs 6 times, as described in Materials and Methods, we determined the levels of production of TLR4, TIRAP, and NF-κB, which increased 1.5- to 4-fold at 12 and 24 h in comparison with the sham-treated mice (Fig. 3). These data were consistent with data reported previously by Lee et al. concerning the OMVs of *Escherichia coli* (21). Due to the consistent observations from both *in vitro* and *in vivo* studies, we conclude that *P. aeruginosa* OMVs could efficiently stimulate the release of inflammatory mediators in the mouse model and that the immune response might be dependent on the TLR4 pathway.

These studies prompted us to surmise that the activation of immune responses was attributed primarily to the existence of LPS rather than intracellular virulence factors within OMVs. To test our hypothesis, OMVs were lysed with 100 mM EDTA and then used to stimulate A549 and MLE-12 cells in parallel with LPS. Interestingly, in contrast to LPS, the production of cytokines such as IL-1β and IL-6 was impaired by lysed OMVs (Fig. 4), suggesting that the development of cellular proinflammatory responses requires intact OMVs. Furthermore, we also found that there were DNA species by agarose electrophoresis (data not shown), using the boiled vesicles generated from both *P. aeruginosa* strains, which is consistent with studies reported previously by Renelli et al. (37). A large body of literature reported that the presence of the CpG motif in bacterial DNA could be recognized by TLR9 to trigger inflammatory responses (37–40). Thus, we evaluated the induction of TLR9 using OMV-derived DNA and OMV lysates. However, no significant changes in TLR9 expression and cytokine production were observed by using various concentrations of DNA and OMV lysates (data not shown), and the function of OMV-associated DNA remains unknown (53).

Protective roles of *P. aeruginosa* OMVs against experimental infection with *P. aeruginosa*. To further characterize the immune properties of the isolated OMVs, we next tested the hypothesis that *P. aeruginosa* OMV-induced serum could protect rodents from subsequent infection by *P. aeruginosa* strains. First, sera of *P. aeruginosa* OMV-challenged mice were collected to measure the inflammatory cytokine levels. We found that the concentrations of TNF-α, IL-1β, and IL-6 increased significantly at 24 h after the last OMV challenge (Fig. 5). As shown in Table 2, *P. aeruginosa* OMV-immunized C57BL/6J mice (5 per group) survived until the end of the experiments after challenge with *P. aeruginosa* at a lethal concentration (1.5×10^9 CFU) and even at concentrations 100-fold higher than this dose. The progression of infection in unimmunized mice or mice immunized with a single dose of OMVs was rapid, with no survivors at 24 h postchallenge, whereas mice immunized with OMVs 6 times survived the bacterial challenges. These results indicate that *P. aeruginosa* OMVs could efficiently stimulate the release of inflammatory factors in mouse models to

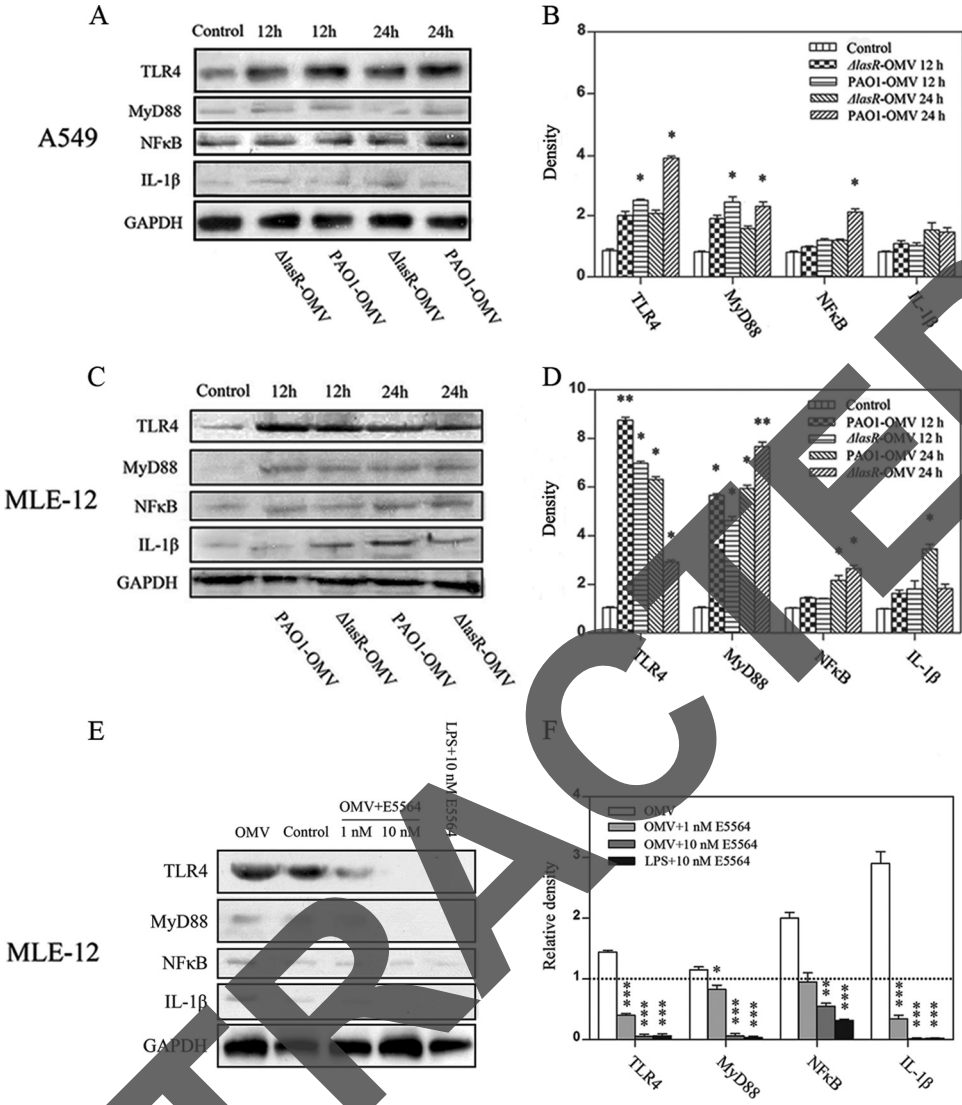

FIG 2 *P. aeruginosa* OMVs trigger intracellular inflammatory responses via the TLR4 signaling pathway. (A to D) Released *P. aeruginosa* OMVs at a concentration of 0.25 mg/ml increased the expression levels of TLR4 and immunogenic proteins MyD88 and NF-κB, finally causing the release of proinflammatory factors in A549 cells (A and B) and MLE-12 cells (C and D), as determined by Western blotting. Cells treated with the same amount of PBS were used as a control. (E and F) Production of TLR4 immunogenically related proteins was significantly impaired when TLR4 was inhibited by E5564 at a concentration of 10 nM. Cells treated with the same amount of PBS were used as a control group. Cells treated with LPS at a concentration of 100 ng/ml and 10 nM E5564 were used as a control for E5564. Representative gels are depicted with a graph that shows the means ± SEM from 3 experiments in which protein levels were normalized to GAPDH levels (B and D) and then to levels of a PBS-treated control (F). Levels shown in each column were significantly decreased compared with the levels of the control (B and D) or natural OMVs (F) (*, $P < 0.05$; **, $P < 0.001$ [determined by one-way ANOVA followed by a Tukey-Kramer *post hoc* test]).

protect the mice from the high-dose pathogen challenge. Moreover, the immunized mice significantly eliminated invading bacteria and recruited neutrophils and macrophages to the lungs against *P. aeruginosa* challenge compared to unimmunized control mice (Fig. 6). To evaluate the mechanism of immune protection, we attempted to determine the protective potential of OMVs in naive mice by a passive immunity transfer of the sera of OMV-immunized mice. The serum of an individual mouse with high-level cytokine production was then serially diluted and injected into C57BL/6J mice subcutaneously. We found that serum-vaccinated mice were protected from *P. aeruginosa* challenges with various CFU. After the serum vaccination, mice were again challenged with a lethal dose (1.5×10^9 CFU) of *P. aeruginosa* and were completely protected from death versus the control mice that received sham treatments (PBS, unimmunized serum, or serum from mice immunized with a single dose of OMVs). We then increased the amount of *P. aeruginosa* to 1.5×10^{11} CFU to further assess the protection efficacy. Mice were also completely protected by the serum, which was diluted 5- and 10-fold. However, for the mice that received serum that was diluted 20-fold, 1 mouse out of 5 mice died. In contrast, control mice that received unimmunized serum and serum from mice immunized with only one dose of OMVs were all dead after challenge with either concentration of *P. aeruginosa* (Table 3). Therefore, *P. aeruginosa*-generated OMVs may be capable of protecting mice from *P. aeruginosa* lung infection.

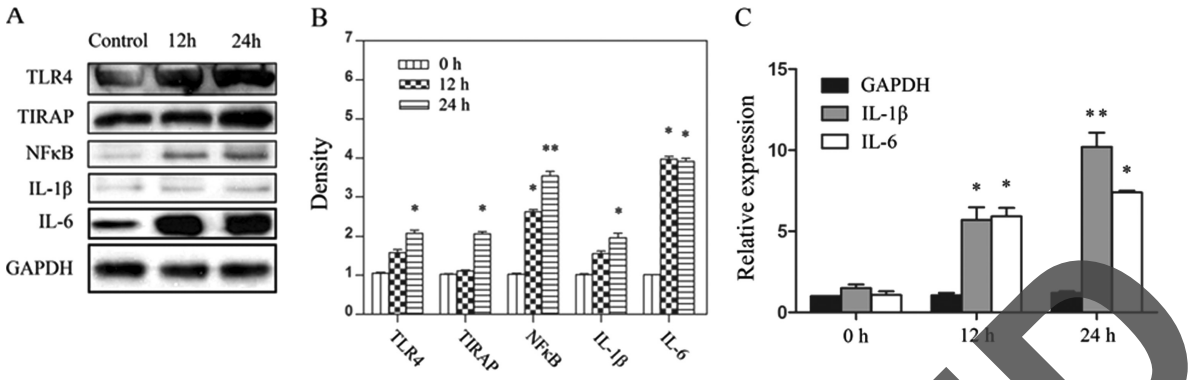

FIG 3 *P. aeruginosa* PAO1-generated OMVs increase the expression levels of TLR4 pathway immunogenically related proteins, including TLR4, TIRAP, NF-κB, IL-1β, and IL-6, in C57BL/6J mouse lungs, as determined by Western blotting (A and B) and quantitative PCR (C). WT mouse models were intranasally instilled with 0.5 mg purified OMVs and the same amount of PBS as a control. Lungs were harvested at the designated time points. Data are presented as means ± SEM from 3 experiments in which protein levels were normalized to GAPDH levels. Data from quantitative PCR were calculated by using the $2^{-\Delta\Delta C_T}$ method (34) and normalized to GAPDH levels. Values in each column were compared with the control values (*, $P < 0.05$; **, $P < 0.001$ [determined by one-way ANOVA followed by a Tukey-Kramer *post hoc* test]).

DISCUSSION

Outer membrane vesicles, which act as vanguard units in the battle between Gram-negative bacteria and hosts, provide a great convenience for the development of bacterial infection. Despite intense interest, the precise role of OMVs in inducing host pathophysiology remains to be fully determined, and *P. aeruginosa* OMVs have not been tested for immunizing potency and vaccine purposes. The current study demonstrated for the first time that *P.*

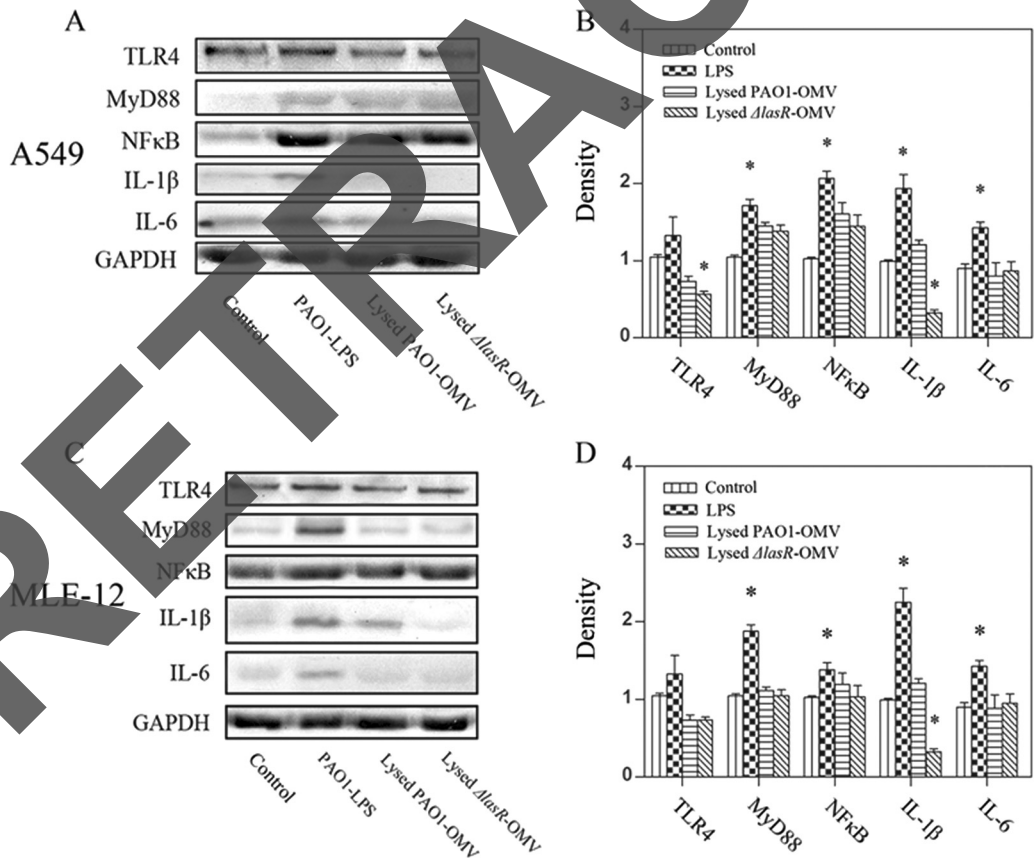

FIG 4 Lysed *P. aeruginosa* OMVs inefficiently activate the expression of TLR4 pathway immunogenically related proteins, including TLR4, MyD88, NF-κB, IL-1β, and IL-6, in A549 (A and B) and MLE-12 (C and D) cells, as determined by Western blotting. Cells were treated with lysed OMVs at a concentration of 0.25 mg/ml and with LPS at a concentration of 100 ng/ml for 24 h. Cells treated with 100 nM EDTA were used as a control group. A representative gel is depicted with a graph that shows the means ± SEM from 3 experiments in which protein levels were normalized to GAPDH levels. Data for each column were compared with data for the control (*, $P < 0.05$ [determined by one-way ANOVA followed by a Tukey-Kramer *post hoc* test]).

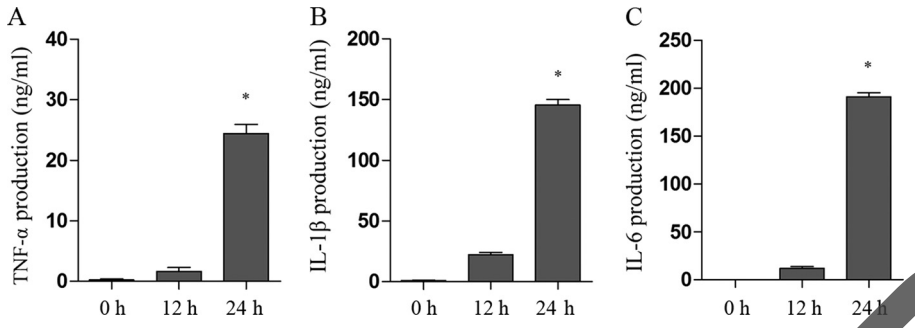

FIG 5 Production of proinflammatory cytokines in sera of OMV-treated mice, as determined by ELISA. WT mouse models were intranasally instilled with 0.5 mg purified OMVs and the same amount of PBS as a control. Sera were harvested at the designated time points. Data are presented as means ± SEM, and data from each column are compared with data from the 0-h time point (*, $P < 0.001$ [determined by one-way ANOVA followed by a Tukey-Kramer *post hoc* test]).

aeruginosa OMVs can activate the immune response via the TLR4 signaling pathway and provide protection against pseudomonal lung disease in a murine infection model.

The components of *P. aeruginosa* OMVs are yet to be fully defined and may vary among different isolation or culture conditions due to the envelope stress-facilitated OMV production (8, 9). Bauman and Kuehn found previously that the aminopeptidase PaAP (PA2939) or PepB was highly enriched in vesicles generated from a cystic fibrosis (CF) isolate (29). *P. aeruginosa* possesses two hierarchically superimposed and well-characterized QS systems, *las* and *rhl*, controlling the synthesis and secretion of a number of virulence factors during infection. Especially, the existence of the QS molecule 2-heptyl-3-hydroxy-4-quinolone in OMVs suggested that the QS system might be implicated in the packaging of OMVs (13, 41). In this study, the difference between the OMVs generated by PAO1 and those generated by PAO1-Δ*lasR* was analyzed by SDS-PAGE, and the components were almost identical except for a band of approximately 48 kDa, which might be OprD in PAO1 OMVs (Fig. 1A). Previously, porin was shown to be related to imipenem and carbapenem resistance (42, 43). It is currently unclear why the vesicles from WT PAO1 caused more cytotoxicity to alveolar epithelial cells than did those from PAO1-Δ*lasR* (Fig. 1C). It is likely that additional virulence factors in OMVs have yet to be identified. In addition, complicated bacterial virulence factors, including unannotated proteins, may be responsible for environmental stresses and can also influence the outcome of infection (44). Nevertheless, our results suggested that the QS system might be involved in the process of OMV packaging.

Whole bacterial organisms can activate TLR4 signaling and initiate vigorous immune responses, including phagocytosis and cytokine production, following *P. aeruginosa* infection, while TLR2 may have different immune functions (45–47). The literature also showed that OMVs generated from a wide range of bacteria can elicit the production of various cytokines by epithelial cells (48, 49). In *Neisseria meningitides*, LPS acts as an adjuvant to the components of vesicles and can profoundly modulate inflammatory responses of macrophages in a TLR4-dependent manner (21, 22, 25). The current study provided a detailed *in vitro* and *in vivo* mechanism for the stimulation of proinflammatory mediators by *P. aeruginosa* OMVs that was TLR4 dependent (Fig. 2 and 3). The OMV-triggered signaling pathway observed was essentially the same as purified *P. aeruginosa* LPS (Fig. 2 and 4). Interestingly, no significant difference was observed for the production of immune mediators triggered by PAO1 OMVs and PAO1-Δ*lasR* (Fig. 2), indicating that although QS might be associated with the packaging and cytotoxicity of OMVs, this master QS regulator may not be a key factor for activating proinflammatory responses. Our previous study indicated that *P. aeruginosa* infection was dependent on lipid raft-mediated membrane signals (50). Bomberger et al. also found that OMV virulence factors were delivered into the host cell cytoplasm by fusing with lipid rafts (6). As shown by our confocal microcopy results, vesicles were initially colocalized with lipid raft microdomains on the cell surface and then rapidly internalized into the cytoplasm (see Fig. S3 in the supplemental material). When the lysed OMVs of *P. aeruginosa* were incubated with A549 and MLE-12 cells, the expression levels of cytokines, particularly IL-1β, decreased significantly (Fig. 4). However, the changes in expression levels of MyD88, NF-κB, and IL-6 were less prominent. Although there was also some residual LPS, the OMVs with lysed membranes could not efficiently activate immune responses. In addition to the significantly increased level of production of TLR4-mediated responders elicited by intact OMVs (Fig. 2), we reasoned that the OMVs lacking an intact membrane may have lost the ability to target lipid rafts, which in turn prevents the vesicle components from being delivered into the cytoplasm to induce the production of proinflammatory factors. Therefore, the activation of the host immune response elicited by *P. aeruginosa* OMVs is dependent primarily on LPS via the TLR4 signaling pathway but requires an intact membrane struc-

TABLE 2 Immunization of mice with *P. aeruginosa* OMVs protects against intranasal challenge with PAO1[b]

Challenge dose (CFU) of PAO1 or PBS	Mouse	No. of survivors ($n = 5$)			
		24 h	48 h	72 h	7 days later
1.5×10^9	OMV immunized	5	5	5	5
1.5×10^9	Control[a]	1	0	0	0
1.5×10^{11}	OMV immunized	5	5	5	5
1.5×10^{11}	Control[a]	0	0	0	0
1.5×10^9	Wild type	0	0	0	0
PBS	Wild type	5	5	5	5

[a] Mice inoculated intranasally with a single dose of OMVs.
[b] Protection was significant for immunized mice compared with the unimmunized group ($P < 0.001$, determined by the chi-square test with Yates' correction).

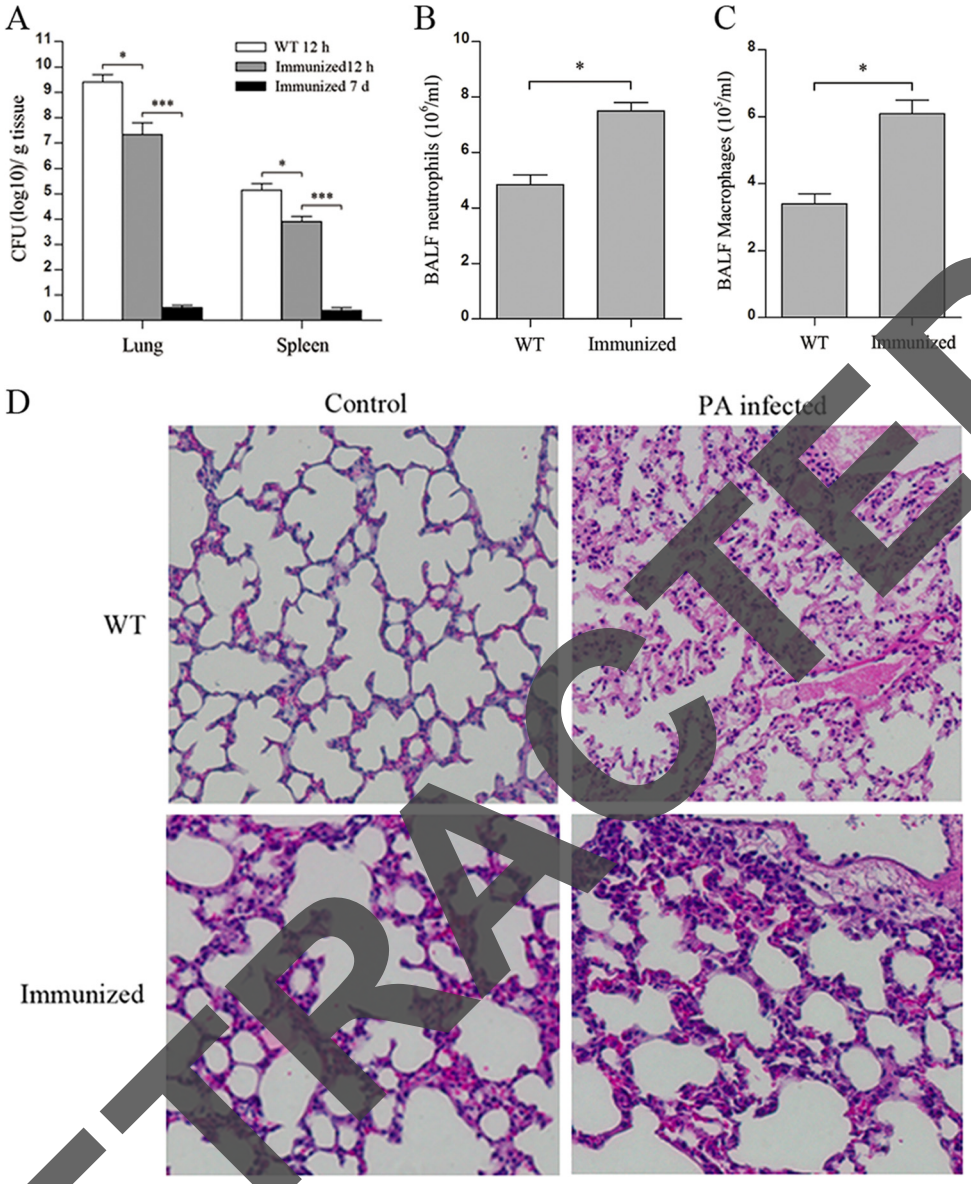

FIG 6 Bacterial burden and BALF cell counts following *P. aeruginosa* challenge. WT and OMV-immunized mice were exposed to *P. aeruginosa* (1.5×10^9 CFU). (A) Lungs and spleens were harvested at 12 h and 7 days to enumerate invading bacteria, while all WT mice were dead at 24 h. (B to D) BALF was harvested at 12 h for counting neutrophils (B) and macrophages (C), while lungs were collected for evaluation of histology alteration (D). Data are representative of two experiments and are presented as means ± SEM (*, $P < 0.05$; ***, $P < 0.001$ [determined by Student's *t* test]).

ture to fuse to the host cell membrane (see Fig. S3 in the supplemental material).

Our study provides further evidence that development of a pseudomonal vaccine based on OMVs of *P. aeruginosa* is a promising approach. Vaccines that effectively prevent *P. aeruginosa* pulmonary infections could be very useful, and previous studies suggested that LPS is an influential effector in the development of vaccines in mice (20–22, 51). Ramphal et al. also showed that *Pseudomonas* LPS is not a key virulence factor in acute pneumonia and thus can be used as an adjuvant to trigger a prominent proinflammatory response and effectively defend the lung from *P. aeruginosa* infection (52). Thus, in this study, we immunized mice with a series of intranasal challenges to evaluate the vaccine potential of OMVs against pseudomonal lung infection. We found that *P. aeruginosa* OMVs almost fully protected mice against *P. aeruginosa* challenge at 1.5×10^9 CFU and even at a 100-fold-higher concentration of this inoculum. All the OMV-immunized mice survived after challenge with a lethal concentration of *P. aeruginosa* (Table 2). Additionally, the sera of OMV-immunized mice contained increased levels of proinflammatory cytokines (Fig. 5) and could also be passively transferred to protect other mice from death against subsequent *P. aeruginosa* challenges (Table 3). Currently, the detailed mechanism for OMVs in inducing enhanced immunity remains to be fully determined. As our study is the first to clearly reveal the immune protective effect of *P. aeruginosa* OMVs, further mechanistic studies of their immunization potential might facilitate the development of more efficient and broadly protective vaccines for pseudomonal diseases.

TABLE 3 Immunized serum protects mice against intranasal challenge with PAO1[c]

Challenge dose (CFU) of PAO1 or PBS	Serum dose (fold dilution)	No. of survivors ($n = 5$)			
		24 h	48 h	72 h	7 days later
1.5×10^9	5	5	5	5	5
	10	5	5	5	5
	20	5	5	5	5
	50	4	4	4	4
	Control[a]	0	0	0	0
	Control[b]	0	0	0	0
1.5×10^{11}	5	5	5	5	5
	10	5	5	5	5
	20	5	4	4	4
	50	1	1	0	0
	Control[a]	0	0	0	0
	Control[b]	0	0	0	0
PBS	Not added	5	5	5	5

[a] Unimmunized serum.
[b] Serum from mice inoculated intranasally with a single dose of OMVs.
[c] Protection was significant for serum-injected mice compared with the uninjected group ($P < 0.001$, determined by the chi-square test with Yates' correction).

In summary, the data presented here demonstrated that naturally secreted OMVs of *P. aeruginosa* were cytotoxic to alveolar epithelial cells. *P. aeruginosa*-generated OMVs also efficiently stimulated the release of murine inflammatory factors via the TLR4 signaling pathway requiring LPS. Importantly, *P. aeruginosa* OMVs were confirmed to be capable of protecting immunized mice from a subsequent lethal *P. aeruginosa* challenge. Unlike *P. aeruginosa*, OMVs had a significantly reduced virulence repertoire, thus drastically reducing safety concerns if applied in clinics. Therefore, a prospective vaccine based on OMVs may also offer enhanced safety, improved immunogenicity, and elevated efficacy for prevention of pseudomonal diseases.

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K.Z. performed all experiments, K.Z., B.Y., and M.W. designed studies and wrote the paper. C.H. provided critical reagents. M.W. conceived of the studies.

All authors have submitted the ICMJE form for disclosure of potential conflicts of interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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