Supporting Information

Materials & Methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Pseudomonas aeruginosa* were routinely grown in Luria-Bertani (LB) broth at 37°C with shaking at 180 rpm or on LB agar plates. For plasmid selection or maintenance, 50 μg/ml diaminopimelic acid (DAP) or antibiotics were supplemented to the growth media at the following concentration: for *E. coli*, 100 μg/ml ampicillin (Amp), or 25 μg/ml Tetracycline (Tet); and for *P. aeruginosa*, 100 μg/ml Tetracycline or 300 μg/ml carbenicillin (CAR), when required. LPS profiles of *P. aeruginosa* strains were examined by the methods of Hitchcock and Brown (1) using cultures standardized based on the optical density at 600 nm (OD_{600}).

Plasmid construction. The *pcrV* encoding E28-I294 of PcrV was amplified by PCR using the PcrV1/PcrV2 primer set as DNA fragments containing engineered *Eco*RI and *Bam*HI sites at their 5' and 3' ends, and the $hitA_T$ encoding D28-N355 of HitA were amplified from PA103 strain by PCR using HitA1/HitA2 primer sets as fragments containing engineered *Bam*HI and *Hin*dIII sites at their 5' and 3' ends, respectively. Then, PCR fragments encoding truncated PcrV or HitA coding sequences were cloned between *Eco*RI and *Hin*dIII sites of the pYA3494 via three-way ligation, generating the plasmid pSMV81, in which the *pcrV-*$hitA_T$ is fused with *bla ss* (encoding β-lactamase N-terminal signal sequence) driven by strong P_{trc} promoter. To purify PcrV-$HitA_T$ protein and $HitA_T$ protein for immune analysis, the *pcrV-*$hitA_T$ or $hitA_T$ fused to a C-terminal 6×His was amplified from pSMV81 using PcrV3/PcrV4 primers or HitA3/HitA4 primers and cloned into the *Nco*I and *Hin*dIII sites of plasmid pYA3342 to form pSMV82 or pSMV84. To overexpression of the *pcrV-*$hitA_T$ fusion gene in PA103 strain, the P_{trc}-*bla ss-pcrV*-

hitA$_T$ DNA fragment cut from the pSMV81 plasmid was bluntly cloned into an *Escherichia-Pseudomonas* shuttle vector, pUCP20, to construct pSMV83 plasmid (Table 1 and Fig. 2A).

Western blot analysis. Protein samples were boiled for 5 min and then separated by SDS-PAGE. For western blotting, proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 3% skim milk in 10 mM Tris-0.9% NaCl (pH 7.4) and incubated with corresponding primary antibodies: rabbit anti-ExoA (Sigma), rabbit anti-ExoU (from Dr. Alan R. Hauser), mouse anti-PcrV, and mouse anti-HitA$_T$ (lab preparation), and the rabbit polyclonal antibody specific for GroEL (Sigma, St. Louis, MO). Then, the secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit or mouse immunoglobulin G (IgG) (Sigma). Immunoreactive bands were detected by the addition of BCIP/NBT solution (VWR). The reaction was stopped after 2~5 min by washing with large volumes of deionized water.

Bacterial subcellular fractionation analysis. *P. aeruginosa* strains were grown in LB broth at 37°C for 16 h. The bacterial cells were collected by centrifugation (10,000 × g) for 10 minutes. Periplasmic and cytoplasmic fractions were prepared by a lysozyme-osmotic shock method (2, 3). Equal volumes of periplasmic, cytoplasmic, and supernatant fractions and total lysate samples were analyzed using Western blotting.

Protein purification. *E. coli* TOP10 carrying pSMV82 (*pcrV-hitA*$_T$-6xHis) was grown overnight at 37°C in LB broth. The procedures for protein synthesis and purification have been described in our previous study (4). To remove the remaining endotoxin, the purified PcrV-HitA$_T$ (PH) protein was passed through Pierce™ High Capacity Endotoxin Removal Spin Columns (ThermoFisher Scientific).

OMV isolation and analysis. OMVs were isolated from *P. aeruginosa* strains as previously described with minor modifications (5). Briefly, the strains were grown at 37°C in 1L LB broth (Difco) overnight. The bacterial cultures were supplemented with EDTA (pH 8.0) at 100 mM and kept on ice for 1 h. Then, the bacterial cells were pelleted by centrifugation at 10,000 × g at 4°C for 10 min. The culture supernatant was filtered using a 0.45 μm pore membrane (Millipore) to remove the residual bacterial cells and concentrated with a 100 kDa filter using a Vivaflow 200 system (Sartorius). The OMVs were harvested by ultracentrifugation (120,000 × g) for 2 h at 4°C. The vesicle pellet was washed and resuspended in sterilized 0.1×PBS (pH 7.4), and the ultracentrifugation step was repeated. The final vesicle pellet was resuspended in sterilized 0.1×PBS, filtered with a 0.22 μm pore membrane (Millipore), and stored at -80°C for subsequent studies. The total protein in OMVs was quantified by Pierce™ BCA Protein Assay Kit (Thermo scientific) and the relative lipid contents of the OMVs were determined via an FM4-64 fluorescence dye-binding assay measured by a SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular Devices) as described in the previous report (5).

Transmission electron microscopy (TEM). *P. aeruginosa* cultures were absorbed onto freshly glow-discharged Formvar/carbon-coated copper grids for 10 min. The grids were washed in ddH_2O and stained with 1% aqueous uranyl acetate (Ted Pella, Inc., CA) for 1 min. The excess liquid was gently wicked off, and the grids were allowed to air dry. The samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL Peabody, MA) equipped with an AMT 8-megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA). The OMVs isolated from *P. aeruginosa* and its derived mutant were analyzed by TEM as described in previous reports (5, 6).

Lipid A isolation and analysis by mass spectrometry

Isolation of lipid A species from *P. aeruginosa* OMVs was performed using previously described procedures (7) with minor modifications. Briefly, 40 ml cultures of *P. aeruginosa* were grown at 37°C in LB broth with 180 rpm shaking until an OD_{600} of ~1.5 was reached. Bacteria were harvested by centrifugation, and washed with milliQ H_2O twice. Bacterial pellets were re-suspended in single-phase Bligh-Dyer mixture: 5 ml chloroform, 10 ml methanol and 4 ml H_2O (1 : 2 : 0.8 v/v/v). The resuspended mixture was vortexed vigorously, incubated at room temperature for 30 minutes to ensure cell lysis, and centrifuged at 2,000 × g for 20 minutes. The pellet containing LPS and cell debris was collected and washed with a single-phase Bligh−Dyer mixture one time. Then, the pellet was re-suspended in 2 ml of 25 mM sodium acetate (pH 4.5) and incubated for 30 minutes in boiling water bath to remove the polysaccharide chain from LPS. The hydrolyzed sample was cooled down to room temperature and converted to a two-phase Bligh-Dyer mixture by adding 2 ml chloroform and 2 ml methanol (chloroform: methanol: H_2O, ~2:2:1.8). The sample was mixed by vigorously vortexing and then centrifuged for 10 minutes at 2,000 × g. The lower phase (chloroform portion) containing lipid A was transferred into a clean Teflon centrifuge tube using a glass pipet and dried under a stream of nitrogen using a nitrogen dryer. The dried sample was stored at -20 °C for Mass spectrometry (MS) analysis. For lipid A isolation from OMVs, 2 ml OMVs isolated from 1 L bacteria culture were mixed with 6 ml chloroform: methanol (1:2, v/v) initially. The same procedures were used for lipid A extraction.

Isolated lipid A species were dried under nitrogen and stored at -20°C until MS analysis. For MS analysis, the lipid A extract was re-suspended in 100 µl chloroform-methanol (2:1, v/v), of which 10 µL was injected for each LC/MS analysis. NPLC-ESI/MS was performed as previously

described (8, 9) using an Agilent 1200 Quaternary LC system (Santa Clara, CA) coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). An Ascentis® Si HPLC column (5 μm, 25 cm × 2.1 mm, Sigma-Aldrich) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/ aqueous ammonium hydroxide (600:340:50:5, v/v/v/v.). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v). The elution program was as follows: 100% mobile phase A was held isocratically for 2 min,then linearly increased to 100% mobile phase B for 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C for 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: ion spray voltage (IS) = -4500 V; current gas (CUR) = 20 psi (pressure); gas-1 (GS1) = 20 psi; de-clustering potential (DP) = -55 V; and focusing potential (FP) = -150 V. The MS/MS analysis used nitrogen as the collision gas. Data acquisition and analysis were performed using the Analyst TF1.5 software (Sciex, Framingham, MA).

Measurement of antibody responses. An enzyme-linked immunosorbent assay (ELISA) was used to assay antibody titers against PcrV-HitA$_T$ or PA whole cell lysates (PCL) in serum. Polystyrene 96-well flat-bottom microtiter plates were coated with 100 ng/well of purified PcrV-HitA$_T$ or PCL. Antigens suspended in sodium carbonate bicarbonate coating buffer (pH 9.6) were applied in a 100 μl volume in each well. The coated plates were incubated overnight at 4°C. The procedures for measuring the antibody titers were described in our previous report. The highest dilution of sera resulting in an OD_{405} value at least 2-fold higher than that obtained from the sham mouse serum was considered the antibody titer (10).

Inhibition of PA cytotoxic assay. Sera from immunized mice for inhibiting cytotoxicity of *P. aeruginosa* were assayed as described previously (11) with minor modifications. HeLa cells were seeded in 48 well plates ($2.5*10^4$ cells/well). *P. aeruginosa* PA103 were grown to log phase and diluted to obtain the desired MOI (1:10). The serum was heat inactivated at 56°C for 30 min and incubated with the PA103 for 15 min at room temperature at a dilution of 1:500. HeLa cells were infected with the bacteria incubated with different sera or PBS as a control. The plates were incubated at 37°C, 5% CO_2 for 4 h, and the release of lactate dehydrogenase (LDH) in the supernatant of infected cells was determined using a CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega). The percent cytotoxicity was calculated using the formula: (Experimental-ES-TS+M)/(T_{max}-TS); where Experimental is OD_{490} from the well being tested, ES is OD_{490} from bacteria only, TS is OD_{490} from untreated cells, M is OD_{490} from media and T_{max} is OD_{490} from lysed cells (obtained by adding Lysis solution 45 min prior to end of incubation).

Reference

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Table S1. Primers used in the present study.

Primer name	Sequence [a] (5' to 3')
exoT-UF	cgggagctctatccatcgggttctccgccccgg
exoT-UR	tggcaacgccggggtcccgggaggggcaggcggcgcgtcctgacggga
exoT-DF	tcccgtcaggacgcgccgcctgcccctcccgggaccccggcgttgcca
exoT-DR	cggtctagatgactgcgtctcgttcg
exoA-UF	cgggagctcgacagctcggcgtagaccagc
exoA-UR	acccatcacaggagccatcgcggtggtgattccctcggcgatc
exoA-DF	gatcgccgagggaatcaccaccgcgatggctcctgtgatgggt
exoA-DR	cggtctagagcgacgctcgacaatgctct
lasA-UF	cgggagctcgtcggcggcttcttcgggccgc
lasA-UR	ttcgatgaccaggagctacccgtcggcgcggggcccggctcca
lasA-DF	tggagccgggccccgcgccgacgggtagctcctggtcatcgaa
lasA-DR	cggtctagaagccggacgaggacgacggtta
lasB-UF	cgggagctcgatgttccacggggtgttcca
lasB-UR	tgctggccgggggccaccgagcttacttgttcagttctcctggtttttttc
lasB-DF	gaaaaaaccaggagaactgaacaagtaagctcggtggccccggccagca
lasB-DR	cggtctagaggtcgtgtgctggggatcgaa
wbjA-UF	cgggagctcgctgctacttcacccatagctagcg
wbjA-UR	ctttctatcgagaacccccttccagactgcgctacaaggccggccagga
wbjA-DF	tcctggccggccttgtagcgcagtctggaaggggggttctcgatagaaag
wbjA-DR	cggtctagacccaccataacaccatatgcggtca

pchA-UF	cgggagctccacctgttcgtctccgcccatc
pchA-UR	ggccgcaggggtcttcgtttgcggcaccccgtgtctggcgc
pchA-DF	gcgccagacacggggtgccgcaaacgaagacccccctgcggcc
pchA-DR	cggtctagaaactaatcgccatgaatgaaaa
phzM-UF	cgggagctcgctgccggaggacgtggagaac
phzM-UR	tggccttcgagatctttcagggatcggaactctcaacggttggc
phzM-DF	gccaaccgttgagagttccgatccctgaaagatctcgaaggcca
phzM-DR	cggtctagaaaggcaataggagtttcatccag
alg-UF	cgggagctcgacgtgctgctcaacctggcttcc
alg-UR	catcttcatggtcgggtaccggtaggatgttttctctgcgaggg
alg-DF	ccctcgcagagaaaacatcctaccggtacccgaccatgaagatg
alg-DR	cggtctagacgccctggtcgggatagtcgta
rhlAB-UF	cgggagctcctgcctgggcaagagcacctac
rhlAB-UR	tatctgttatgccagcaccgtttcacacctcccaaaaatttt
rhlAB-DF	aaaatttttgggaggtgtgaaacggtgctggcataacagata
rhlAB-DR	cggtctagaggcgatttccccggaactcttg
pvdA-UF	cgggagctctggaacgcctgctcgccgctca
pvdA-UR	gccaatccagaggaactggaatcggcgccacgccgccacgc
pvdA-DF	gcgtggcggcgtggcgccgattccagttcctctggattggc
pvdA-DR	cggtctagatgtcttcatcgagggttccagtta
plcH-UF	cgggagctcttgacttccggtgggtaggtttcg
plcH-UR	accacccgggaaataaaacgagcgaggagtccatcgcatga
plcH-DF	tcatgcgatggactcctcgctcgttttatttcccgggtggt

plcH-DR	cggtctagaggagtagtggccgatgatccct
htrB2-UF	cgggagctcgcgcaccggagtcttcaccacctt
htrB2-UR	cgcgtccggaatgcccgtccggacggttccgacgacgatca
htrB2-DF	tgatcgtcgtcggaaccgtccggacgggcattccggacgcg
htrB2-DR	cggtctagatcgccgaagtactcgcggttga
phoA-UF	cgggagctcctgtgcaaattgttgcgcacat
phoA-UR	ccttttcgttctggtccgagacgcatttccctatgttgag
phoA-DF	ctcaacatagggaaatgcgtctcggaccagaacgaaaaagg
phoA-DR	cggtctagagcgccctgcaacgactgctgtt
PcrV1	cgggaattcgaacaggaagaactgctg
PcrV2	cggggatccggatccaatggcactcagaatatca
HitA1	cggggatccggtggcggcggtagcg
HitA2	cggaagcttttaatggtgatgatgatg
PcrV3	cggccatggttgaacaggaagaactgctg
HitA3	cggaagcttttaagtgatgatgatgatgatgtggtgatgatgatg

a, Underlining indicates restriction endonuclease recognition sequences.

Supplementary Figure Legends

Fig. S1. Analysis of LPS in *P. aeruginosa* and its OMVs. (A) LPS profile of wild-type (WT) *P. aeruginosa* PA103 and its derived mutants (PA-m6, PA-m13 and PA-m14, see Table 1) with the *wbjA* mutation. Samples were separated on 12% SDS-PAGE gel and stained by silver nitrate (1). Loading of the samples was adjusted to give approximately equal staining of the lipid A-core bands at the bottom of the gel. (B) Lipid A profiles of OMVs isolated from WT PA103 and PA-m14 mutant strains by MALDI-TOF analysis. Strains were grown in LB broth at 37°C overnight, and OMVs were isolated as described in the SI Materials and Methods. (C) Structures of hepta- and hexa-acylated lipid A species in OMVs from WT *P. aeruginosa* PA103 and PA-m14 mutant with the *lpxL1*(*htrB1*) deletion. LpxL1 (Htrb1) catalyzes the addition of a secondary laurate acyl chain to lipid A. Various modifications shift the peak by the indicated m/z values. The hydroxyl (+ OH) was added to the 2 position R-3-hydroxylaurate chain or the 3 position laurate chain.

Fig. S2. Transmission electron microscopy (TEM)**.** (B) Comparison of morphological alterations between wild-type (WT) *P. aeruginosa* PA103 and PA-m14 mutant by TEM. Bars, 1 μm. (C) TEM image of OMVs purified from WT PA103 and PA-m14 strains. The samples were prepared by conventional staining with 1% aqueous uranyl acetate as described in the SI Materials and Methods. Bars, 500 nm. The results are representative of three repeated experiments.

Fig. S3. Protective efficacy against *P. aeruginosa* by subcutaneous infection and antibody opsonophagocytic killing capacity. BALB/c mice (n=10) were immunized with

PBS/Alhydrogel, 10μg of PH/Alhydrogel, 10μg of PH/dmLT, 50μg of OMV-NA or 50μg of OMV-PH by i.m. injection and boosted on 21 days after the initial immunization. Mouse weight was monitored and recorded for 6 weeks; (A) Anti-PH total IgG titers at days 14, 28, and 42 in 10μg of PH/Alhydrogel- or 10μg of PH/dmLT- immunized mice. On 42 days after initial immunization, mice were challenged with 6.5×10^6 CFUs of wild-type PA103 (~30 LD_{50}) by i.n. administration, and animal survival was recorded for 15 days. (B) On 42 days after initial immunization, mice were challenged with 7.4×10^7 CFUs of wild-type PA103 (10 LD_{50}) by subcutaneous administration, and animal survival was recorded for 15 days. Statistical significance was analyzed by Log-rank (Mantel-Cox) test; (C) Comparative analysis of opsonophagocytic killing activity against *P. aeruginosa* PAO1 using anti-sera from different immunized mice. Data were shown as the mean ± SD; (D) Comparative analysis of opsonophagocytic killing activity against a clinic *P. aeruginosa* isolate, AMC-PA10 using anti-sera from different immunized mice. Data were shown as the mean ± SD. The statistical significance among groups were analyzed by two-way multivariant ANOVA with a Tukey post hoc test.

Fig. S4. Analysis of antigen-specific lung $CD8^+$ T-cell responses in immunized mice. BALB/c mice (n = 4) were immunized with PBS, 10μg of PH plus adjuvant, 50μg of OMV-NA or 50μg of OMV-PH by i.m. administration. On day 42 after the initial immunization, lymphocytes from the lungs were aseptically isolated from mice and stimulated in vitro with 20 μg/ml purified recombinant PcrV-$HitA_T$ fusion protein for 48 h to detect specific $CD8^+$ T cells producing IFN-γ, TNF-α, and IL-17A. PBS-immunized mice lung cells were considered as controls. (A) Representative flow cytometry profiles of $CD8^+$ T cells producing IFN-γ, TNF-α,

and IL-17A in the lungs of different immunized mice. (B) Quantification of $CD8^+$ IFN-γ^+-, $CD8^+$ TNF-α^+-, and $CD8^+$ IL-$17A^+$-positive cell numbers in the lungs of mice. Each symbol represents a data point obtained from an individual mouse, with a mean ± SD. The experiments were performed twice, and data were combined for analysis. The statistical significance among the groups were analyzed by two-way multivariant ANOVA with a Tukey post hoc test: ns, no significance; *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$; ****, $p< 0.0001$. Abbreviations: interferon (INF)-γ; tumor necrosis factor (TNF)-α.

Fig. S5. Analysis of antigen-specific spleen $CD8^+$ T-cell responses in immunized mice. BALB/c mice (n = 5) were immunized with PBS, 10μg of PH plus adjuvant, 50μg of OMV-NA or 50μg of OMV-PH by i.m. administration. On day 42 after the initial immunization, lymphocytes from the spleen were aseptically isolated from mice and stimulated in vitro with 20 μg/ml purified recombinant PcrV-$HitA_T$ fusion protein for 48 h to detect specific $CD8^+$ T cells producing IFN-γ, TNF-α, and IL-17A. PBS-immunized mice spleen cells were considered as controls. (C) Representative flow cytometry profiles of $CD8^+$ T cells producing IFN-γ, TNF-α, and IL-17A in the spleens of different immunized mice. (D) Quantification of $CD8^+$ IFN-γ^+-, $CD8^+$ TNF-α^+-, and $CD8^+$ IL-$17A^+$-positive cell numbers in the spleens of mice. Each symbol represents a data point obtained from an individual mouse, with a mean ± SD. The experiments were performed twice, and data were combined for analysis. The statistical significance among the groups were analyzed by two-way multivariant ANOVA with a Tukey post hoc test: ns, no significance; *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$; ****, $p< 0.0001$.