Supplementary  Materials for

Lipopolysaccharide is Transported to the Cell Surface by a Membrane-to-membrane Protein Bridge

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Materials and Methods

Strains

E. coli was used as a model organism. The *E. coli* NovaBlue strain was used for cloning and plasmid generation. The *E. coli* KRX strain was used for overexpression of His_6-LptBFG, His_6-LptBFGC, and His_6-LptB-E163Q-LptFG. The *E. coli* BL21(λDE3) strain was used for overexpression of LptA-I36*p*BPA-His_6, NusA-His_6-LptA, and LptD-Y112*p*BPA/LptE-His_6. A variant of *E. coli* BL21(λDE3) with the genes *cyoA-E* removed by P1 phage transduction was used for overexpression of C-LptD-His_8/LptE (*33*). The *E. coli* MC4100 strain was used for *in vivo* photocrosslinking of lipopolysaccharide to LptD-Y112*p*BPA. All strains used in this study are listed in Extended Data Table 1. All strains were grown in LB media containing appropriate antibiotics at temperatures ranging from 16-37°C, depending on the experiment.

Plasmid construction

Construction of plasmids used for overexpression of the Lpt inner membrane complex has already been described (*15*). Plasmids and oligonucleotides used in this study are listed in Extended Data Tables 2 and 3. Plasmid pET22/42LptC-T47Am was constructed using site-directed mutagenesis (SDM). Plasmid pET22/42LptC was mutagenized with KOD Hot Start Polymerase (Novagen) using primers T47-amber-f and T47-amber-r. The PCR product of the SDM reaction was digested with DpnI (New England Biolabs) and introduced into NovaBlue competent cells by heat-shock. Transformants were selected in LB media containing carbenicillin (50 μg/mL). After confirming plasmid DNA sequence, the mutagenized plasmid was introduced into NovaBlue and KRX *E. coli* strains.

Construction of plasmids pET23/42LptD-His_8 and pCDFLptE-His_6 has been previously reported (*18, 19*). Plasmid pET23/42LptD-Y112Am-His_8 was made by mutagenizing plasmid pET23/42LptD-His_8 as described above, using primers LptD-Y112Am-f and LptD-Y112Am-r. Likewise, plasmid pET23/42LptD-Y112Am was made by mutagenizing plasmid pET23/42LptD, which has previously been reported (*19*), using primers LptD-Y112Am-f and LptD-Y112Am-r.

Construction of plasmids pET22bLptA-I36Am-His_6 and pET43.1b(+)LptA were previously reported (*22, 24*). Plasmid pET43.1b(+)LptA-I36Am was constructed by mutagenizing plasmid pET43.1b(+)LptA using SDM with primers LptA-I36Am-f and LptA-I36Am-r.

Overexpression and purification of the Lpt inner membrane complex

To overexpress His_6-LptBFG, His_6-LptBFGC, and His_6-LptB-E163Q-LptFGC, KRX cells were transformed with plasmid pCDFDuetHis_6LptBFG or pCDFDuetHis_6LptB-E163Q-LptFG. When LptC was co-overexpressed with LptBFG, this strain was also transformed with pET22/42LptC. To overexpress His_6-LptBFGC-T47*p*BPA, KRX cells were transformed with three plasmids: pCDFDuetHis_6LptBFG, pET22/42LptC-T47*p*BPA, and pSupBpaRS-6TRN *(34)*, which encodes an orthogonal tRNA and aminoacyl-tRNA synthetase to incorporate unnatural amino acid *p*BPA at amber (TAG) stop codons.

Overexpression and purification of each inner membrane complex variant was done as previously reported (*15*). Cultures were grown at 37°C after diluting overnight cultures 1 to 100 into fresh LB broth supplemented with 50 μg/mL spectinomycin (Sigma), 50 μg/mL carbenicillin (Teknova), 30 μg/mL chloramphenicol (Sigma), and 0.5 mM *p*-Benzoyl-L-phenylalanine (*p*BPA, Bachem), as appropriate for each inner membrane complex variant. Expression was induced with 0.02% L-rhamnose at OD_{600} ~ 1, and cultures were grown for 3 h at 37°C. Cells were harvested by centrifugation at 4,200 × g for 20 min and resuspended in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM $MgCl_2$, supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma), 100 μg/mL lysozyme (Sigma), and 100 μg/mL DNase I (Sigma). The resuspended cells were passaged through an EmulsiFlex-C3 high-pressure cell disruptor three times. The cell lysate was centrifuged at 10,000 × g for 10 min to remove unbroken cells. Membranes were isolated by centrifugation at 100,000 × g for 1 h. Membranes were resuspended and solubilized in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM $MgCl_2$, 10% (vol/vol) glycerol, 1% *n*-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 2 mM ATP at 4 °C for 1 h, followed by centrifugation at 100,000 × g for 30 min. The supernatant was applied to TALON metal affinity resin (Clontech), and eluted with 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% (vol/vol) glycerol, 0.05% DDM, 25 mM imidazole. The eluate was concentrated with an Amicon centrifugation filter, 100-kDa molecular weight cutoff (MWCO, Amicon Ultra; Millipore), and then subjected to size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% (vol/vol) glycerol, 0.05% DDM. Fractions were pooled and concentrated to ~ 5 mg/mL. All complexes were analyzed by SDS-PAGE to assess purity. Protein was flash frozen in liquid nitrogen and stored at -80°C until use.

Overexpression and purification of LptA

LptA-I36*p*BPA-His_6 was overexpressed in the periplasm and purified by making spheroplasts, as previously reported (*24*). BL21(λDE3) cells with pSup-BpaRS-6TRN and pET22b-LptA-I36*p*BPA-His_6 were grown to OD_{600} ~ 0.5 in 500 mL LB broth supplemented with 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol, and 0.8 mM *p*BPA. Protein expression was induced with 50 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma) for 2 h at 37°C. The cells were harvested and converted to spheroplasts. The periplasmic fraction was incubated with Ni-NTA Superflow resin (Qiagen), washed with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, and then eluted with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 200 mM imidazole. The purified LptA-I36*p*BPA-His_6 was concentrated with 10 kDa cut-off Amicon centrifugal concentrators (Millipore) to ~2.5 mg/mL, flash frozen with liquid nitrogen and kept at -80°C in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 200 mM imidazole containing 10% glycerol.

To overexpress and purify NusA-His_6-LptA for use in the reconstitution, BL21(λDE3) cells were transformed with pET43.1b(+)LptA. Cultures were grown at 37°C after diluting overnight cultures 1 to 100 into fresh LB broth supplemented with 50 μg/mL carbenicillin. Cells were grown to OD_{600} ~ 0.5, and the temperature was reduced to 16°C. Overexpression was induced by addition of 50 μM IPTG, and the cells were grown for 16 h at 16°C. Cells were harvested by centrifugation at 4,200 × *g* for 20 min.

Cells were resuspended in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol supplemented with 1 mM PMSF, 100 μg/mL lysozyme, and 100 μg/mL DNase I. Cells were lysed by two passages through an EmulsiFlex-C3 high pressure cell disruptor (Avestin Inc.) and centrifuged at 10,000 × *g* for 10 min to remove unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 30 min to remove membranes. Imidazole was then added to the supernatant to a final concentration of 10 mM. Ni-NTA resin was pre-washed with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol in preparation for batch nickel affinity chromatography. The supernatant was then flowed over the pre-washed Ni-NTA resin three times at 4°C. The resin was washed with 20 column volumes of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 20 mM imidazole. The protein was eluted with 4 column volumes of 20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 200 mM imidazole. The eluate was concentrated with an Amicon centrifugation filter, 10 kDa MWCO to ~ 10 mg/mL. Purity was assessed by SDS-PAGE. The concentrated protein was either cleaved immediately with Restriction Grade Thrombin (EMD Millipore) (see below) or flash frozen in liquid nitrogen and stored at -80°C in aliquots until use.

NusA-His_6-LptA-I36*p*BPA was purified as described for the wild-type construct, but the overexpression strain was BL21(λDE3) harboring both pET43.1b(+)LptA-I36Am and pSup-BpaRS-6TRN. Cultures were grown at 37°C after diluting overnight cultures 1 to 100 into fresh LB broth supplemented with 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol, and 0.8 mM *p*BPA. Cells were grown to OD_{600} ~ 1, and the temperature was reduced to 16°C. Overexpression was induced by addition of 10 μM IPTG, and the cells were grown for 16 h at 16°C.

Overexpression and purification of the Lpt outer membrane translocon

To overexpress LptD-Y112*p*BPA/LptE-His_6, BL21(λDE3) harboring pSup-BpaRS-6TRN were transformed with plasmids pET23/42LptD-Y112Am and pCDFLptE-His_6. The reported protocol was followed for overexpressing and purifying this complex (*19*). A 10-mL culture was grown at 26 ºC from a single colony in LB broth supplemented with 50 μg/mL carbenicillin, 50 μg/mL spectinomycin, 30 μg/mL chloramphenicol, 0.2% glucose and 0.5 mM *p*BPA until OD_{600} ~ 0.6. This culture was then used to inoculate a 1.5-L culture that was grown at 26 ºC until OD_{600} ~ 0.6 when expression was induced by addition of 0.1 mM IPTG. After induction of protein expression, cultures were grown an additional 20 h at 26 ºC. Cells were harvested by centrifugation at 4,200 × *g* for 20 min and then resuspended in 60 mL of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl supplemented with 1 mM PMSF, 100 μg/mL lysozyme and 50 μg/mL DNase I. Cells were lysed by two passages through an EmulsiFlex-C3 high pressure cell disruptor (Avestin Inc.) and centrifuged at 10,000 × *g* for 10 min to remove unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 30 min to isolate membranes. The membranes were solubilized in 30 ml of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% N-lauroylsarcosine sodium salt (Sigma) at 4 °C for 1 h and then ultracentrifuged as above. The resulting membrane pellet was resuspended in 30 mL of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl to wash and then recentrifuged as above. The washed pellet was then solubilized with 18 mL of 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1.0% Anzergent 3-14 at 4 °C for 3 h and then ultracentrifuged as above. The supernatant was applied to Ni-NTA metal affinity resin and allowed to incubate at 4 °C

for 1 h. The column was washed with 2 × 10 mL of 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM 1.0% *n*-octylglucoside (OG, Anatrace), and then eluted with 4 mL of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% OG containing 200 mM imidazole. The eluate was concentrated with an Amicon centrifugation filter, 10-kDa molecular weight cutoff (MWCO, Amicon Ultra; Millipore), and then subjected to size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% OG. Fractions were pooled and concentrated to ~ 5 mg/mL. All complexes were analyzed by SDS-PAGE to assess purity. Protein was flash frozen in liquid nitrogen and stored at -80°C until use.

Overexpression and purification of the truncated LptD/E complex (C-LptD-His8/LptE)

To overexpress the outer membrane translocon containing truncated LptD (C-LptD-His_8/LptE), a modified variant of the BL21(λDE3) expression strain, BL21(λDE3) *ΔcyoA-E::Kan* in which the genes, *cyoA-E*, that encode the cytochrome c oxidase proteins have been deleted to improve protein purity, was transformed with pET23/42C-LptD-His_8 and pCDFLptE. C-LptD-His_8/LptE was overexpressed and purified following the wild-type protocol described above, with the inclusion of 30 μg/mL kanamycin (Amresco) in the LB broth in addition to the other reported additives (*19*). Protein was flash frozen in liquid nitrogen and stored at -80°C until use. Purity was assessed by SDS-PAGE.

Preparation of lipid and LPS stock solution

To prepare non-fluorescent proteoliposomes, *E. coli* polar lipid extract (Avanti Polar Lipids, Inc.) was dissolved in water and sonicated for 30 minutes to make a 30 mg/ml aqueous suspension stock. The non-fluorescent lipid stock solution was flash frozen in liquid nitrogen, and stored at -80°C. To prepare fluorescent proteoliposomes, *E. coli* polar lipid extract (Avanti Polar Lipids, Inc.) was dissolved in chloroform (Sigma) with 1% (molar ratio) Atto-488 DPPE (AD 488-15, Atto-tec GmbH, Germany), or 1% (molar ratio) Atto-565 DPPE (AD 565-15, Atto-tec GmbH, Germany), respectively. Lipids were dried into a lipid film, kept *in vacuo* overnight, hydrated with water and lyophilized. The prepared lipid powders were then re-dissolved in water to prepare a 30 mg/ml aqueous suspension containing 1% (molar ratio) Atto-488 DPPE or 1% (molar ratio) Atto-565 DPPE and were then sonicated for 30 minutes. The fluorescent lipid stock solution was flash frozen in liquid nitrogen, and stored at -80°C. To prepare a 2 mg/mL aqueous suspension stock of lipopolysaccharide, LPS from *E. coli* EH100 (Ra mutant, Sigma) was dissolved in water and sonicated for 30 minutes. The lipopolysaccharide stock solution was flash frozen in liquid nitrogen, and stored in aliquots at -80°C.

Inner membrane complex proteoliposome preparation

Aliquots of non-fluorescent or fluorescent lipid stocks and LPS were thawed and sonicated briefly to homogenize. Proteoliposomes containing the Lpt inner membrane complex were prepared by a detergent dilution method (*35, 36*). Prior to dilution, a mixture with the following final concentrations was prepared in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl buffer: 7.5 mg/mL lipid stock, 0.5 mg/mL LPS, 0.25% DDM, and 0.86 μM purified inner membrane complex. While making this mixture, the DDM was first added to the lipid stock solution to make detergent-destabilized liposomes.

Lipopolysaccharide was added to this mixture, which was subsequently kept on ice for 10 min to allow for mixed detergent-phospholipid-LPS micelles to form. The protein complex was added, and the mixture was left on ice for 20 min. The mixture was then transferred to an ultracentrifuge tube and diluted 100× with cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl. After letting the dilute mixture sit on ice for 30 min, the proteoliposomes were pelleted by ultracentrifugation at 300,000 × *g* for 2 h at 4°C. The proteoliposomes were resuspended in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and diluted 100× again, then pelleted by ultracentrifugation at 300,000 × *g* for 2 h at 4°C. Finally, for every 100 µL original mixture (prior to the first dilution step), 250 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol was added. If the resuspended proteoliposomes were not used immediately, they were flash frozen in liquid nitrogen and stored at -80°C. For preparation of empty proteoliposomes, the purified proteins were substituted with equal volume of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol.

ATPase assays

ATPase assays of proteoliposomes were done using a modified molybdate method, as previously reported (*36*). All assays were done in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol (final concentrations). Reactions contained 60% proteoliposomes by volume (prepared as described above, thawed on ice). The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations would be the values listed above. Reactions were initiated at 30°C with the addition of ATP/$MgCl_2$ (final concentrations of ATP varied, 2 mM $MgCl_2$). Aliquots were taken at specified time points and quenched with an equal volume of 12% sodium dodecyl sulfate (SDS, Sigma). Inorganic phosphate was measured using the reported method (*36*). Absorbance values were measured using a Spectramax Plus 384 plate reader (Molecular Devices). For analysis, the mean value for a minus ATP control was subtracted from the mean absorbance value for each replicate. The experiment was repeated three times for each sample. Plotted data represents the mean value and the error bars indicate the standard deviation.

In vivo photo-crosslinking

To photo-crosslink LptD to LPS in *E. coli*, the previously reported method was followed (*24*). To make the strains used for photo-crosslinking, MC4100 harboring pSup-BpaRS-6TRN was transformed with plasmid pET23/42LptD-His_8 or pET23/42LptD-Y112Am-His_8 and grown in LB broth supplemented with carbenicillin (50 µg/mL), chloramphenicol (30 µg/mL), and 0.8 mM *p*BPA. To detect photo-crosslinked adducts, final gel samples were boiled for 10 min and analyzed by SDS-PAGE followed by immunoblotting.

Reconstitution of LPS extraction by LptC

The reconstitution of LPS extraction by LptC in proteoliposomes was performed similarly to the ATPase assay described above. All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Reactions contained 60% LptBFGC-T47*p*BPA proteoliposomes by volume (prepared as described above, thawed on ice). The remaining volume was composed of Tris-HCl, NaCl, and glycerol

such that the final concentrations would be the above values. Reactions were initiated at 30°C with the addition of ATP/$MgCl_2$ or just $MgCl_2$ (final concentrations were 5 mM ATP, 2 mM $MgCl_2$). At specified time points, 30 µL aliquots were removed from the reactions and added to a microtiter plate, which was subsequently irradiated with UV light (365 nm) on ice for 5 min using a B-100AP lamp (UVP). Following UV-irradiation, samples were added to 220 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% DDM. To each sample, 250 µL 20% trichloroacetic acid (TCA, Sigma) was added. The proteins were precipitated on ice, followed by a cold acetone wash. Precipitates were resuspended in 30 µL 2× SDS-PAGE sample buffer supplemented with 5% β-mercaptoethanol (Sigma). Samples were boiled for 10 min and analyzed by SDS-PAGE followed by immunoblotting.

Reconstitution of LPS release to LptA

The reconstitution of LPS release from proteoliposomes to LptA was conducted similarly to the LPS extraction reconstitution described above. All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Reactions contained 60% IM proteoliposomes by volume (prepared as described above, thawed on ice). The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations were the above values. LptA-I36*p*BPA-His_6 was added to a final concentration of 2 µM prior to starting the assay. Assays were initiated at 30°C with the addition of ATP/$MgCl_2$ (final concentrations were 5 mM ATP, 2 mM $MgCl_2$). At specified time points, 25 µL aliquots were removed from the reactions and added to a microtiter plate, which was subsequently irradiated with UV light (365 nm) on ice for 3 min using a B-100AP lamp. Following UV-irradiation, samples were added to 225 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl supplemented with 0.2% DDM. To each sample, 250 µL 20% TCA was added. The proteins were precipitated and gel samples prepared as described above. Samples were boiled and subject to SDS-PAGE followed by immunoblotting.

Outer membrane translocon proteoliposome preparation

Aliquots of either non-fluorescent or fluorescent liposomes and LPS were thawed and sonicated briefly to homogenize before use. Proteoliposomes containing the Lpt inner membrane complex were prepared by a detergent dilution method (*35, 36*). A mixture with the following final concentrations was prepared in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl buffer: 9 mg/mL liposomes, 1.25% OG, and 1.5 µM purified LptD-Y112*p*BPA/LptE. While making this mixture, first the OG was added to the liposomes to make detergent-destabilized liposomes. The mixture was kept on ice for 10 min. The protein complex was added, and the mixture was left on ice for 20 min. The mixture was then added to an ultracentrifuge tube and diluted 100× with cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl. After letting the dilute mixture sit on ice for 30 min, the proteoliposomes were pelleted by ultracentrifugation at 300,000 × *g* for 2 h at 4°C. For every 100 µL original mixture (prior to the dilution step), 200 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol was added. If the resuspended proteoliposomes were not used immediately, they were flash frozen in liquid nitrogen and stored at -80°C. For preparation of empty proteoliposomes, the purified proteins were substituted with equal volume of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl,10% glycerol. For preparation

of outer membrane proteoliposomes containing truncated LptD/E complex, the LptD/E complex used in the preparation steps were substituted with equimolar of C-LptD/E-His_8.

Thrombin cleavage of NusA-His6-LptA

NusA-His_6-LptA was cleaved with Restriction Grade Thrombin (EMD Millipore) for 16 hour at room temperature (~ 22°C). NusA-His_6-LptA was either cleaved immediately after purification, or an aliquot was thawed on ice and cleaved. For the cleavage reaction, 0.00396 U enzyme was used per µg NusA-His_6-LptA. The cleavage mixture was used immediately.

Reconstitution of LPS transport to LptD

Prior to reconstitution experiments, 100 µL proteoliposomes containing LptD-Y112*p*BPA/LptE were incubated with 113 µg protein from the LptA thrombin cleavage reaction for 30 min at 4°C or uncleaved NusA-His_6-LptA as a control. We decided to incubate OM proteoliposomes rather than IM proteoliposomes with LptA because wild-type LptA has been found to preferentially associate with OM fractions when whole cell lysates are fractionated (*22*). The proteoliposomes and any associated LptA were pelleted by ultracentrifugation at ~ 300,000 × *g* for 3 h at 4°C in an Optima™ MAX-E (Beckman Coulter Inc.). After removing the supernatant, the pellet was resuspended in 36.5 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol (for a 1:1 ratio of OM:IM proteins in the assay) and either used immediately or flash frozen in liquid nitrogen and stored at -80°C until use.

All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Assay mixtures contained 60% LptBFGC proteoliposomes by volume (prepared as described above, thawed on ice) and 10% resuspended outer membrane proteoliposomes (with any associated LptA) by volume. This allowed for approximately equimolar amounts of inner membrane and outer membrane complexes in the final assay mixture. The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations would be the values mentioned above. The mixture was kept at 4°C for 15 min prior to initiating the assay. Reactions were initiated at 30°C with the addition of ATP/$MgCl_2$ or just $MgCl_2$ (final concentrations were either 0.5 mM or 5 mM ATP, as specified, and 2 mM $MgCl_2$). At specific time points, 25 µL aliquots were removed from the reactions and added to a microtiter plate, which was subsequently irradiated with UV light (365 nm) on ice for 5 min using a B-100AP lamp. Following UV-irradiation, aliquots were added to 225 µL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, supplemented with 0.1% Anzergent 3-14. To each sample, 250 µL 20% TCA was added. The proteins were precipitated on ice, followed by a cold acetone wash. Precipitates were resuspended in 30 µL 2× SDS-PAGE sample buffer supplemented with 5% β-mercaptoethanol. Samples were boiled for 10 min and analyzed by SDS-PAGE followed by immunoblotting.

SDS-PAGE and immunoblotting

Home-made Tris-HCl 4-20% polyacrylamide gradient gels and Tris-glycine running buffer were used for SDS-PAGE/immunoblotting experiments. To analyze purified protein complexes or contents of proteoliposomes, home-made Tris-HCl 14% polyacrylamide gels and Tris-glycine running buffer were used followed by staining with

Coomassie Brilliant Blue (Alfa Aesar). For immunoblotting, proteins were transferred onto Immun-Blot® PVDF membranes (Bio-Rad). Mouse monoclonal antiserum against the LPS core was purchased from Hycult Biotechnology. Rabbit anti-LptD antiserum was provided by Shin-ichi Matsuyama (Rikkyo University, Tokyo, Japan). The sources of rabbit anti-LptC, LptA, and LptB antisera have been previously reported (*15, 22, 23*). LPS crosslinking was detected using a sheep anti-mouse horseradish peroxidase (HRP) conjugate secondary antibody (GE Amersham). Lpt proteins were detected by a donkey anti-rabbit HRP conjugate secondary antibody (GE Amersham). Bands were visualized using ECL™ Prime Western Blotting Detection Reagent (GE Amersham) and Biomax Light Film (Kodak).

Reconstitution of LPS transport for flow cytometric analysis

Reconstitution of LPS transport to LptD was done as described above, with minor modifications to optimize for FACS analysis. Pre-incubation of LptD-Y112pBPA/LptE (or controls) with LptA or NusA-His_6-LptA was done as described above. For NusA-His_6-LptA samples, uncleaved LptA was directly used in an equimolar ratio. All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Assay mixtures contained 30% Atto-565-PE labeled LptBFGC proteoliposomes by volume (prepared as described above, thawed on ice) and 10% resuspended Atto-488-PE labeled outer membrane proteoliposomes (with any associated LptA) by volume. This change in reconstitution reaction composition allowed for approximately equimolar concentrations of fluorescent Atto-488 DPPE and Atto-565 DPPE proteoliposomes in the final mixture shown in the flow cytometry. The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations would be the values mentioned above. The mixture was kept at 4°C for 15 min prior to initiating the assay. Reactions were initiated at 30°C with the addition of $MgCl_2$ (to a final concentrations of 2 mM). Samples were incubated at 30°C for 15 min and then kept at room temperature until FACS analysis. Samples were then diluted 1 to 10 with FACS buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol), and analyzed by flow cytometry.

Flow cytometry

Flow cytometric studies were performed on a BD FACSAria flow cytometer. Samples were excited on a 488-nm excitation laser source, band-pass filter 515-545 nm (green fluorescence) for Atto-488 detection, and excited on a 561-nm excitation laser source, band-pass filter 564-606 nm (red fluorescence) for Atto-565 detection. Auto-compensation was conducted in FACSDiva™ software using Atto-488 outer membrane proteoliposomes and Atto-565 inner membrane proteoliposomes as single stain controls. The forward scatter (FSC) and side scatter (SSC) thresholds were both set as 200 to include all proteoliposomes in the dot plot. For cytometric sorting, the populations of interest shown in the dot plot were selected, and a 4-way sorting module with 70 µm nozzle at 60 psi was used to collect the corresponding gated populations. For each condition, reaction mixtures consisting of three biological replicates of proteoliposomes were independently sorted. The resulting dot plots were analyzed using an identical gating strategy mentioned above. The number of particles sorting into each population for

each condition is the mean of three biological replicates and standard deviation. The flow cytometric data was analyzed on FlowJo X 10.0.7r2.

Particle counts of 100,000 were recorded for cytometric analysis. Proteoliposome mixtures were first selected based on forward and side scatter (FSC-A/SSC-A), then excluded over-sized aggregates using forward and side scatter width/height plot (FSC-W/FSC-H and SSC-W/SSC-H). The remaining particle population was then used for further analysis with specific fluorophores. All flow cytometry data in the manuscript were presented as percent in FlowJo data analysis with averages and standard deviation of at least three experiments.

Liposome imaging by confocal microscopy

Samples were prepared for imaging by squashing 3 μL of FACS sorted proteoliposomes between a slide and an 18 ×18 mm coverslip (VWR). Liposomes were imaged using spinning disk confocal microscopy (Nikon TE2000-E microscope, Yokogawa CSU-X1 spinning disk, Hamamatsu ImagEM EMCCD, μManager acquisition software, and either a 100× 1.40 NA objective for representative images shown in Fig 4 or a 60× 1.20 NA objective for colocalization quantification shown in Fig S10). Fluorescently labeled lipids incorporated into the liposomes were excited at 488 nm or 565 nm for inner-membrane and outer-membrane proteoliposomes respectively. Acquired images were cropped and the contrast was adjusted using ImageJ.

For each image and channel, particle centroids were located using a MATLAB implementation of the Crocker and Grier particle tracking algorithm (*38*). For each identified centroid, the distance from all centroids in the opposite channel was calculated. Pairs of particles between the channels were considered to be colocalized if the distance between their centroids was less than 2 pixels (~0.5 μm). Thus, for each pair of images, the number of particles in the inner-membrane channel, the number of particles in the outer-membrane channel, and the number of colocalized particles could be measured. The code used is freely available at https://github.com/peterjfoster/Proteoliposome_colocalization. Plotted data represents the mean value and the error bars indicate the standard deviation.

Liposome fusion assay

The extent of liposome fusion occurring during the reconstitution and FACS experiments was assessed using a previous reported method (*31*). Briefly, *E.coli* polar lipid extract containing 1.5% (molar ratio) of 16:0 NBD-PE (Avanti Polar Lipids) and 1.5% (molar ratio) 16:0 Liss Rhod-PE (Avanti Polar Lipids) were dissolved in chloroform, dried into a lipid film, rehydrated and lyophilized to get a fluorescent lipid stock. The lipid powder was then used to prepare a 30 mg/ml sonicated aqueous suspension of fluorescent *E.coli* polar lipids. The inner membrane proteoliposome and outer membrane proteoliposomes were next prepared as previously described. NBD-PE and Liss Rhod-PE form a FRET pair. Liposome fusion was assessed by monitoring an increase in NBD fluorescence that occurs as the fluorescent lipid concentration is diluted and de-quenching occurs.

All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations) as previously described. Assay mixtures contained 30% NBD/Liss Rhod-labeled LptBFGC proteoliposomes by volume (prepared as described above,

thawed on ice) and 10% resuspended outer membrane proteoliposomes (with any associated LptA) by volume, or contained 30% LptBFGC proteoliposomes by volume (prepared as described above, thawed on ice) and 10% resuspended NBD/Liss-Rhod-labeled outer membrane proteoliposomes (with any associated LptA) by volume. The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations would be the values mentioned above. The mixture was kept at 4°C for 15 min prior to initiating the assay. Reactions were initiated at 30°C with the addition of $MgCl_2$ (final concentration of 2 mM).

The fluorescence of NBD-PE was monitored on a Spectramax i3 - UV/VIS multimode absorbance/fluorescence 96-well plate reader. The NBD was excited at 460 nm and the emission was read at 538 nm. The 96-well plate (Costar 3603, Corning) was read at 5 min interval for 1h. Fluorescence after complete de-quenching of the fluorescent lipids was estimated by adding an equal volume of 2.5% Triton-X 100 (Sigma). The experiment was repeated three times for each sample. Plotted data represents the mean value and the error bars indicate the standard deviation.

Reconstitution of LPS transport after FACS

Atto-488-labeled OM proteoliposomes with LptA were incubated with Atto-565-labeled IM proteoliposomes and sorted by gating based on fluorescence thresholds using a BD FACSAria flow cytometer. Populations A and B were isolated and LPS transport was initiated by addition of ATP (1 mM) and incubation at 30°C. An aliquot of the reaction mixture prior to sorting (either undiluted or diluted ten-fold according to our FACS protocol) were included as controls. UV crosslinking, TCA precipitation, sample preparation, and immunoblotting were done as described above.

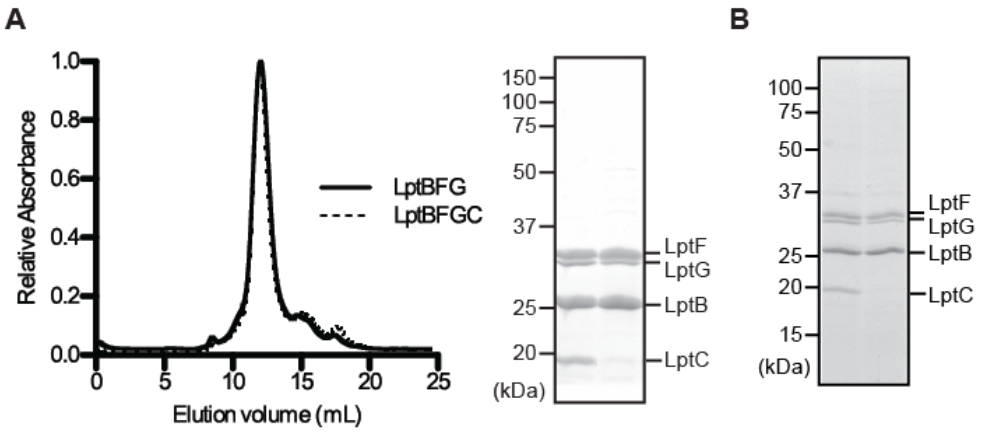

Fig S1. Lpt inner membrane (IM) protein complexes can be overexpressed, purified, and incorporated into liposomes.

(A) Gel filtration chromatograms and SDS-PAGE analysis of inner membrane LptBFG or LptBFGC complexes.
(B) SDS-PAGE analysis of TCA-precipitated proteoliposomes containing phospholipids, LPS, and purified inner membrane LptBFG or LptBFGC complexes.

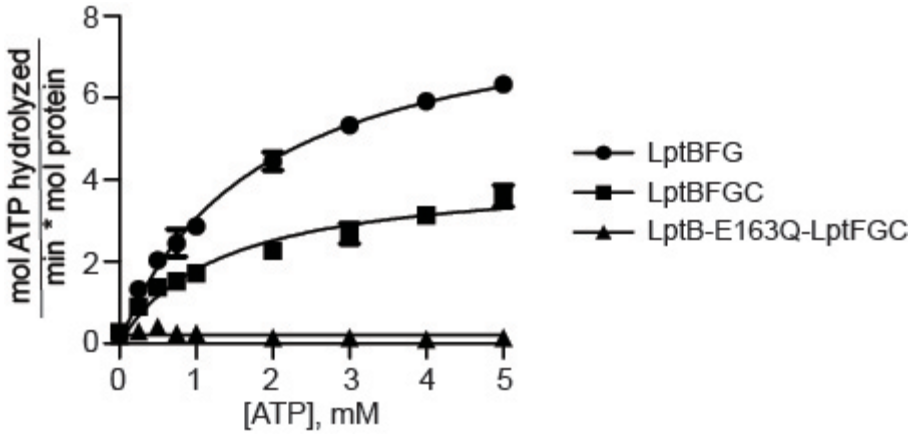

Fig S2. ATPase activity of inner membrane proteoliposomes containing LPS.

The ATPase activity of proteoliposomes containing LPS and either LptBFG or LptBFGC was measured using an inorganic phosphate release assay (*37*). Data represent the averages and standard deviations of three independent experiments.

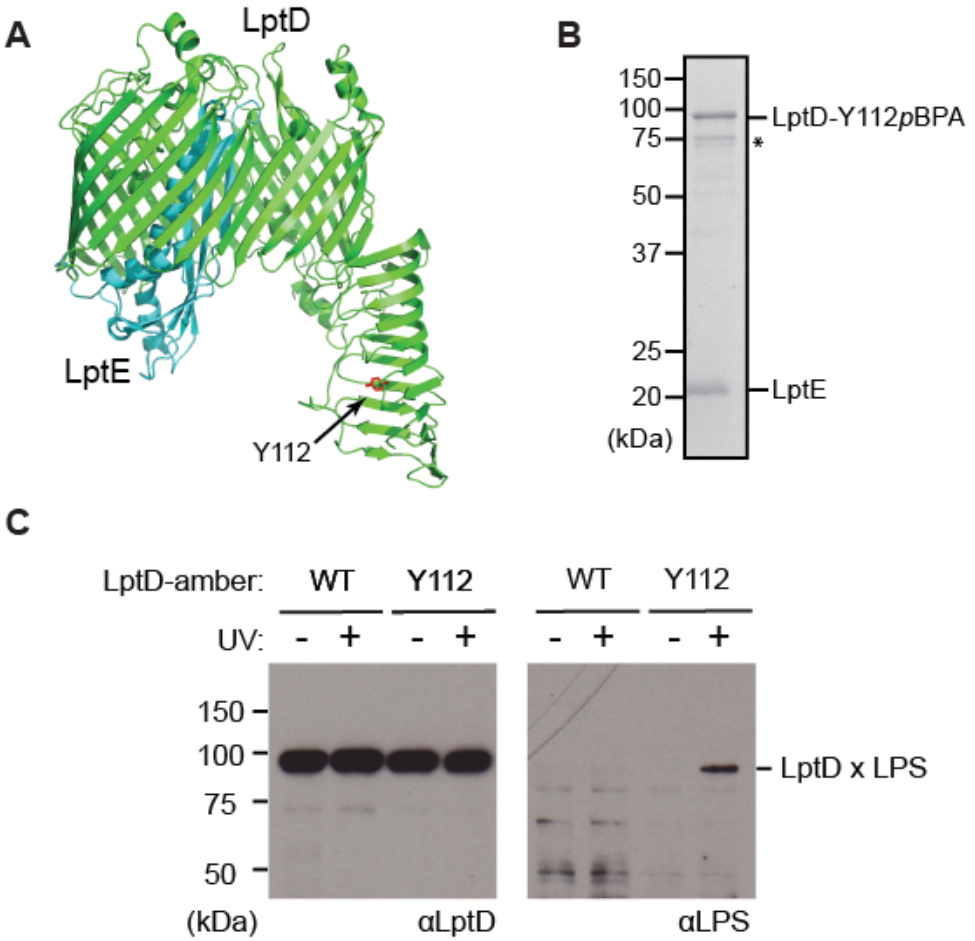

Fig S3. LptD-Y112pBPA/LptE, which crosslinks to LPS *in vivo*, can be overexpressed, purified, and incorporated into liposomes.

(A) The crystal structure of *S. flexneri* LptDE (PDB ID: 4q35) shows amino acid Y112 in LptD facing the interior of the hydrophobic β-jellyroll of LptD (left). This position crosslinks to LPS after UV-irradiation of the cells.

(B) SDS-PAGE analysis of TCA- precipitated proteoliposomes containing phospholipids and the purified outer membrane translocon with a photo-crosslinkable amino acid (*p*BPA) in LptD. The asterisk (*) indicates a 75-kDa band that corresponds to a truncation of LptD that is missing the N-terminal domain. This species is present at low concentrations in all protein preps of LptD.

(C) The wild-type His- tagged protein ("WT") does not contain *p*BPA. The crosslinked adduct (LptD × LPS) was detected by nickel-affinity chromatography followed by immunoblotting with anti-LPS antiserum.

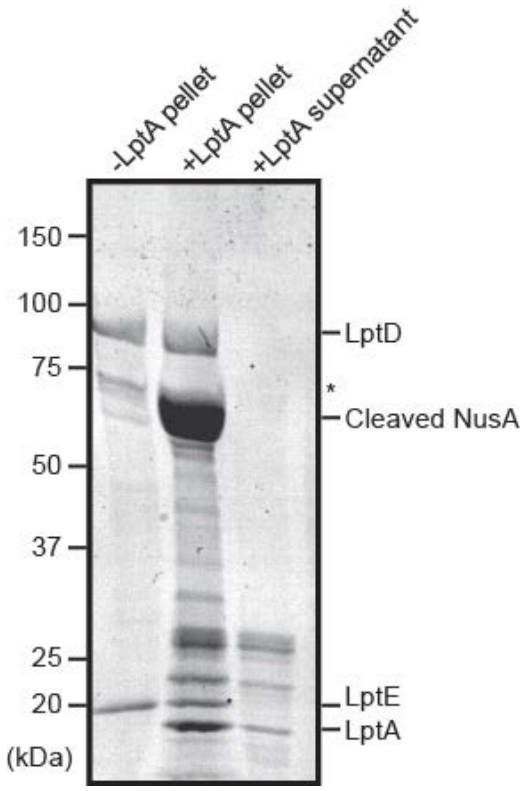

Fig. S4. Coomassie of LptA associated with LptDE

Proteoliposomes containing LptD-Y112*p*BPA/LptE were incubated with buffer or excess LptA to allow association. Samples were ultracentrifuged isolate the proteoliposomes and any associated LptA. Pellet and supernatant fractions were separated, TCA-precipitated, and visualized by Coomassie gel stain. The asterisk (*) indicates an N-terminal truncation of LptD that is present in most protein preps of LptDE at low abundance.

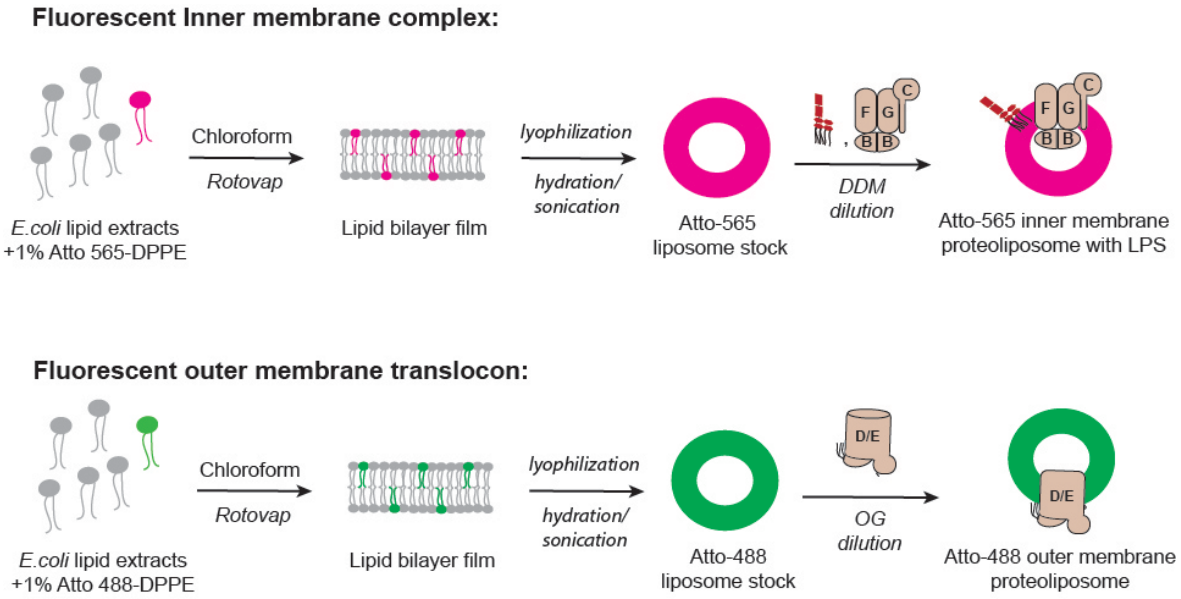

Fig S5. Schematic of fluorescent proteoliposome preparation.

Proteins and LPS can be inserted into liposomes in either orientation, but only the productive orientation is shown for simplicity.

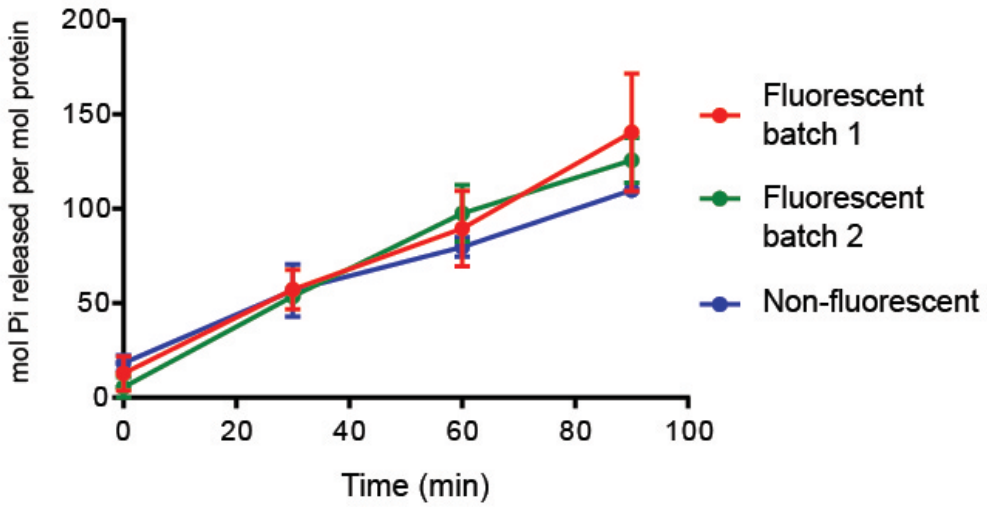

Fig S6. Comparison of ATPase activity of fluorescent and non-fluorescent proteoliposomes containing LptBFGC and LPS.

ATPase activity was measured using an inorganic phosphate release assay with an ATP concentration of 5 mM (*37*). Data represent the averages and standard deviations of three independent experiments.

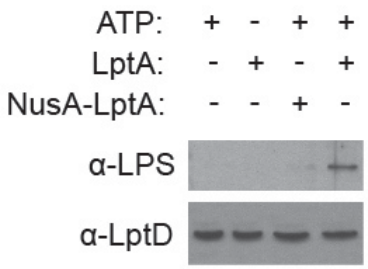

Fig S7. Fluorescent proteoliposomes can transport LPS to LptD in the presence of LptA and ATP.

Proteoliposomes containing purified LptD-Y112*p*BPA/LptE were pre-incubated with buffer, LptA, or NusA-LptA and then were incubated with proteoliposomes containing LPS and LptBFGC. The assay was initiated with 1 mM ATP and aliquots were taken after 60 min and UV-irradiated. Crosslinking was detected by immunoblotting.

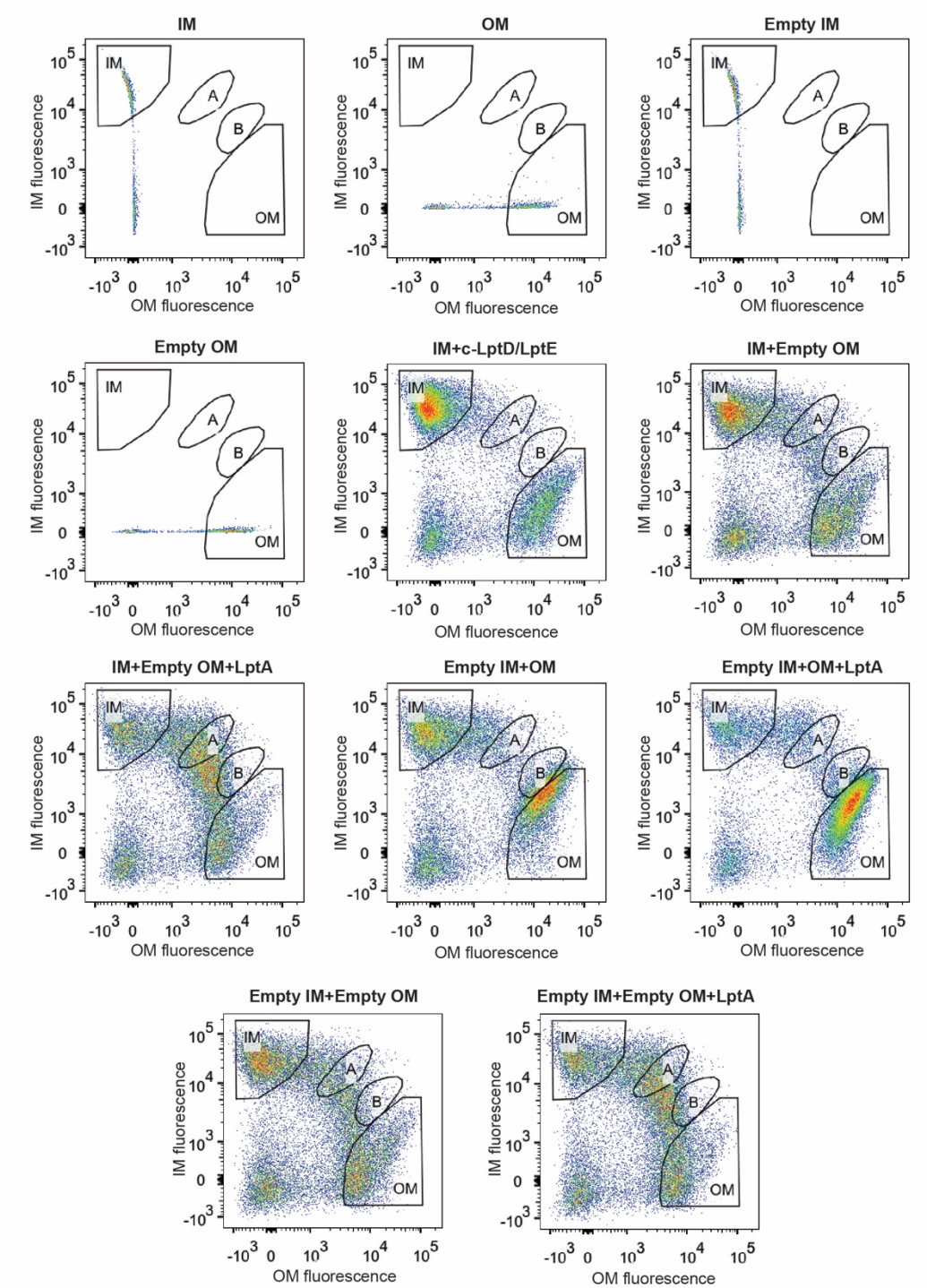

Fig S8. Representative flow cytometric analysis of controls.

Flow cytometric analysis of reaction mixtures containing fluorescent proteoliposomes. Proteoliposome incubation was done as described for crosslinking experiment, but initiating with buffer instead of ATP (equivalent particle distributions were observed in the presence of ATP). All reconstituted samples were analyzed using the same cytometric gating strategies in Figure S10.

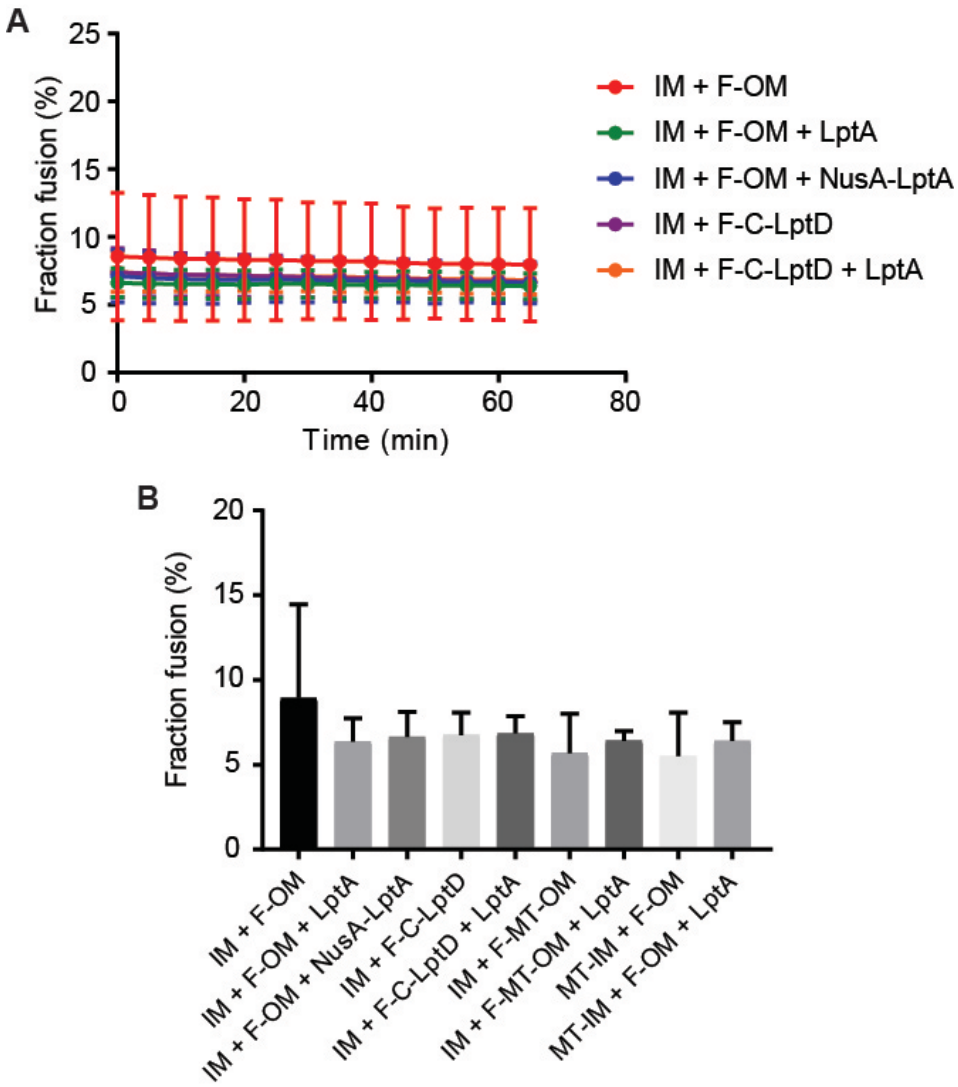

Fig S9. Liposome fusion assay shows limited fusion during lipopolysaccharide transport.

Fusion was measured by a fluorescence de-quenching assay (*31*). Data represent the averages and standard deviations of three independent experiments.

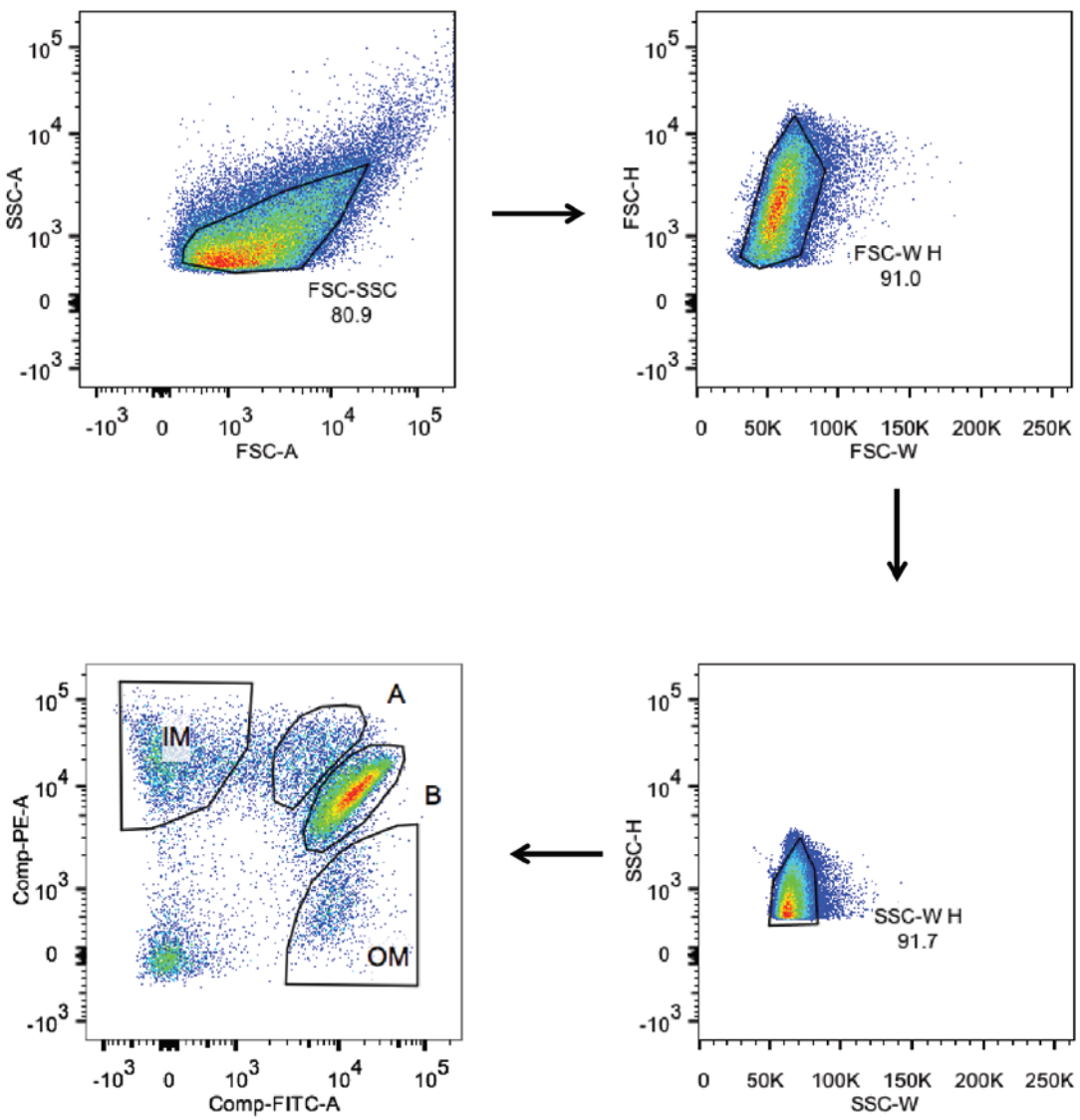

Fig S10. Gating strategy and analysis for FACS sorting.

Proteoliposome mixtures were initially selected based on forward and side scatter area (FSC-A/SSC-A) to gate the majority of the detected particles. Then over-sized aggregates and debris were excluded by gating first on a forward scatter width/height (FSC-W/FSC-H) plot and then on a side scatter width/height (SSC-W/SSC-H) plot. The selected particle population was displayed on the FITC/PE dot plot for further analysis of the fluorescent proteoliposome populations. Flow cytometry data were presented as percentages using FlowJo data analysis.

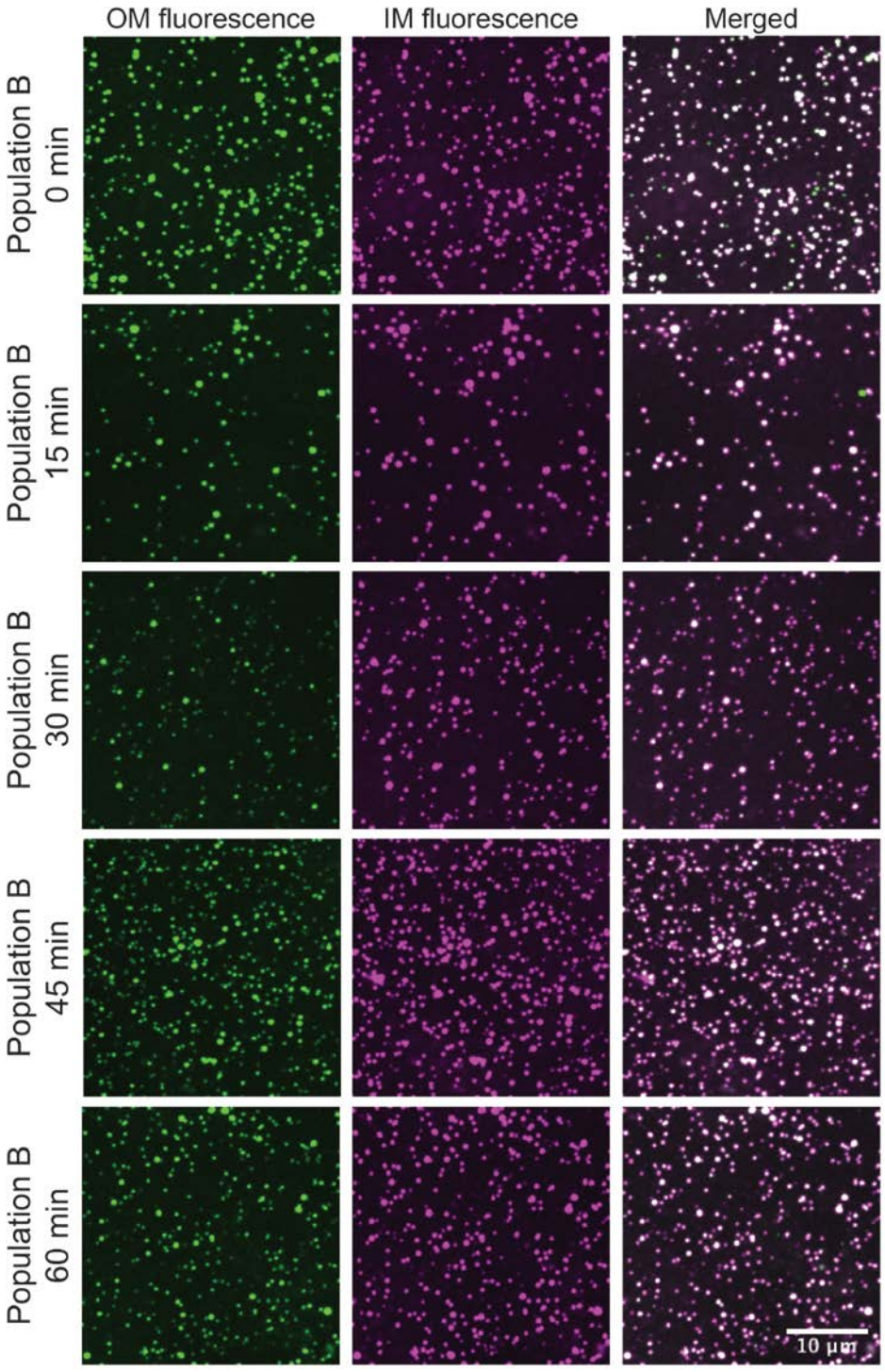

Fig. S11 Time course of imaging of the Population B

Representative confocal microscope images of Population B. Aliquots of a sorted sample were imaged over the course of 60 minutes as indicated above. Sorting parameters were the same as described in Figure 4. Scale bar: 10 μm.

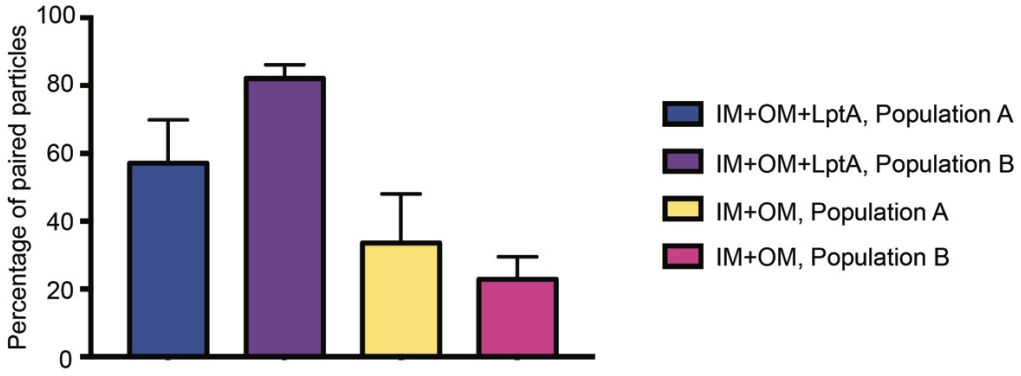

Fig S12. Quantification of paired particles based on a particle tracking algorithm.

For each image and channel, particle centroids were located using a MATLAB implementation of the Crocker and Grier particle tracking algorithm (*38*). Pairs of particles between the channels were considered to be colocalized if the distance between their centroids was less than 2 pixels (~0.5 μm). Plotted data represents the mean value and the error bars indicate the standard deviation.

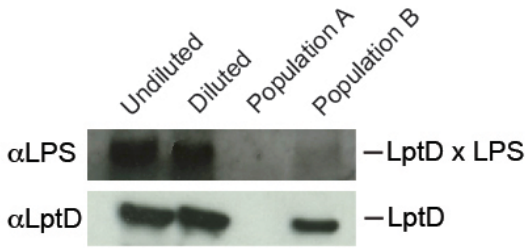

Fig S13. Addition of ATP to Population B stimulates LPS crosslinking to LptD.

Atto-488-labeled OM proteoliposomes with LptA were incubated with Atto-565-labeled IM proteoliposomes and sorted by gating based on fluorescence thresholds using a BD FACSAria flow cytometer. Populations A and B were isolated and LPS transport was initiated by addition of ATP (1 mM) and incubation at 30°C. An aliquot of the reaction mixture prior to sorting (either undiluted or diluted ten-fold according to our FACS protocol) were included as controls.

Table S1. Particle counts distribution in gated populations shown in Figure S7.

Data were normalized such that percentages of counts represent the portion of the total counts in all gated sub-populations.

Particle counts distribution in gated populations

Percentage (%)	Population IM	Population A	Population B	Population OM
IM	71.1	0	0	0
OM	0	0	0	72.4
Empty IM	70.8	0	0	0
Empty OM	0	0	0	70.9
IM+cDE	50.7	1.69	0.82	24.4
IM+Empty OM	30.7	4.10	2.39	26.2
IM+Empty OM+LptA	16.6	11.8	4.85	18.0
Empty IM+OM	26.8	3.33	8.24	28.0
Empty IM+OM+LptA	12.1	3.52	5.1	60.7
Empty IM+Empty OM	23.8	5.84	2.78	23.2
Empty IM+Empty OM+LptA	17.4	10.7	4.87	20.2

Extended Data Table 1. Strains used in this study.

Strains	Source	Genotype
E. coli NovaBlue	Novagen	*endA1 hsd*R17 (r_K^-, m_K^+) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* F′[*pro*A^+B^+ *lac*I^qZΔM15::Tn*10*]
E. coli KRX	Promega	[F', *tra*D36, Δ*omp*P, *pro*A^+B^+, *lac*I^q, Δ(*lac*Z)M15] Δ*omp*T, *end*A1, *rec*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), e14– (McrA–), *rel*A1, *sup*E44, Δ(*lac-pro*AB), Δ(*rha*BAD)::T7 gene 1
E. coli BL21(λDE3)	Novagen	F^- *omp*T *gal dcm lon hsd*S_B(r_B^-, m_B^-) λ(DE3)
E. coli BL21(λDE3) Δ*cyo*	Dr. Aaron Garner at Harvard University	F^- *omp*T *gal dcm lon hsd*S_B(r_B^-, m_B^-) λ(DE3) Δ*cyoA-E::Kan*
E. coli MC4100	Novagen	F^- *araD139 Δ(argF-lac) U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 ptsF25 thi*

Extended Data Table 2. Plasmids used in this study.

Name	Reference
pCDFDuetHis$_6$LptBFG	(*15*)
pCDFDuetHis$_6$LptB-E163Q-LptFG	(*15*)
pET22/42LptC	(*15*)
pET22/42LptC-T47Am	This study
pET22bLptA-I36Am-His$_6$	(*24*)
pET43.1b(+)LptA	(*22*)
pET43.1b(+)LptA-I36Am	This study
pET23/42LptD-His$_8$	(*18*)
pET23/42LptD-Y112Am-His$_8$	This study
pET23/42LptD	(*19*)
pET23/42LptD-Y112Am	This study
pET23/42CLptD-His$_8$	(*19*)
pCDFLptE	(*19*)
pCDFLptE-His$_6$	(*19*)
pSupBpaRS-6TRN	(*34*)

Extended Data Table 3. Oligonucleotides used in this study.

Name	**Sequence**(5'-to-3')
T47-amber-f	TATAAAAGCGAGCATTAGGACACGCTCGTCTAT
T47-amber-r	ATAGACGAGCGTGTCCTAATGCTCGCTTTTATA
LptD-Y112Am-f	CTCGGTAATGTCCATTAGGACGATAACCAGGTG
LptD-Y112Am-r	GGACATTACCGAGCGCATCAACGGTAC
LptA-I36Am-f	CACTGATCAGCCGTAGCACATTGAATCG
LptA-I36Am-r	CGATTCAATGTGCTACGGCTGATCAGTG