Supplemental Experimental Procedures

Mice

TLR4$^{-/-}$ ($Tlr4^{Lps\text{-}del}$) mice originally from the Jackson Laboratory (Bar Harbor, ME) were a kind gift of Kamal Khanna. CD14$^{-/-}$ mice were purchased from the Jackson Laboratory.

Bacterial Strains and Growth Conditions

EHEC (EDL933), ΔLEE and Δ*tir* mutants, *C. rodentium*-expressing shiga toxin (kind gifts of John Leong), *E. coli* BL21, *E. coli* BW25113 and hypovesiculating *E. coli* strains, Δ*pepP*, Δ*ypjA*, Δ*bolA*, and Δ*dsbA,* were grown in LB broth. *E. coli* BW25113 and hypovesiculating *E. coli* strains were obtained from the Keio collection at the Yale Coli Genetic Stock Center. Complements of hypovesiculating mutants were constructed by transforming these mutants with plasmids expressing the corresponding deleted gene as described before (Kailasan Vanaja et al., 2009). MKV15 strain of *E. coli* that produces tetra-acylated lipid A and isogenic WT strain (kind gifts of Egil Lien) were grown in Kozak minimal medium at 30°C as described before (Vorachek-Warren et al., 2002).

Purification and Characterization of Bacterial OMV

OMV were purified from *E. coli* BL21 or *P. aeruginosa* PAK strain as described previously with modifications (Chutkan et al., 2013). Briefly, the bacterial strains were grown in 200 ml of LB till OD_{600} of ~0.5 and the bacteria-free supernatant was collected by centrifugation at 10,000 x g for 10 min at 4°C. This supernatant was further filtered through a 0.45 μm filter and OMV were pelleted by ultracentrifugation at ~400,000 x g for 1.5 h at 4°C in a Beckman NVT™65 rotor. After removing the supernatant OMV were resuspended in 300 μl sterile PBS. The same method was employed for isolating OMV from the peritoneal lavage of mice. Purified OMV were subjected to agar plating to ensure lack of bacterial contamination and Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod) according to the manufacturer's instructions to quantify LPS. The protein content of OMV preparations was assessed by Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. *Neisseria meningitidis* OMV was a kind gift of Dan Granoff (Children's Hospital Oakland Research Institute, CA).

Cell Culture and Stimulations

Bone-marrow derived macrophages (BMDM) and dendritic cells (BMDC) were generated as described previously (Rathinam et al., 2010). THP1 cells were differentiated overnight with 10 ng/ml phorbol 12-myristate 13-acetate (PMA). Peritoneal resident cells were harvested from wild-type mice by lavaging the peritoneal cavity with PBS. Cells used to assess the inflammasome and cell death responses were primed with 400 ng/ml Pam3CSK4 (Invivogen) for 3 h unless otherwise indicated. In experiments with TLR4$^{-/-}$ and CD14$^{-/-}$ cells, priming was performed with poly(I:C) (40 μg/ml; high molecular weight; Invivogen) to bypass the TLR4-TRIF requirement for type I interferon production, which is essential for the noncanonical inflammasome responses to Gram-negative bacteria including EHEC (Rathinam et al., 2012). Cells were infected with overnight grown bacteria at an MOI of 25 unless otherwise indicated for 1 h (30 min for experiments with hypovesiculating mutants and their complements) and then media was replaced with gentamicin (100 μg/ml) containing media or treated with OMV at indicated doses. The supernatants were collected 16 h post-stimulation unless otherwise indicated. For experiments with hypovesiculating mutants and their complements, overnight grown bacterial cultures were re-inoculated in fresh LB and grown until stationary phase (for ~7 h) and then used for infections. In some experiments, OMV were treated with 10 μg/ml polymyxin B for 2 h at 37ºC for neutralizing LPS. For experiments with HeLa cells, cells were infected for 2 h and medium was replaced with gentamicin-containing medium. For control purposes, the cells were transfected with poly(dA:dT) (1 μg/10^6 cells; Sigma-Aldrich) using lipofectamine 2000 or stimulated with ATP (5 mM; Sigma-Aldrich) or nigericin (10 μM; Sigma-Aldrich). In certain experiments, LPS was also transfected using lipofectamine 2000. In certain experiments, BMDM were pre-treated with DMSO, cytochalasin D (2 μM) 45 min prior to infection and the medium containing cytochalasin D was replaced with gentamicin containing medium 1 h after infection. BMDM were also pre-treated with DMSO or Nocodazole (10 μM) in some cases 45 min prior to infection. Heat killed bacteria (Sander et al., 2011) and bacterial lysates (Hagar et al., 2013) were prepared as described previously.

Isolation of Cytosol Fraction from BMDM and HeLa cells

Subcellular fractionation of BMDM was conducted by a digitonin-based fractionation method as described previously with modifications (Ramsby and Makowski, 2005). Briefly, 4×10^6 cells were infected with EHEC or treated with 50 μg of OMV (200 μg for HeLa cells) or left untreated in a 6-well plate. For EHEC-infected cells medium was replaced with gentamicin containing medium at 1 h of infection for BMDMs and 2 h of infection for HeLa cells. After 4 h of treatment, the cells were washed with sterile cold PBS 4 times on a platform shaker on ice to remove any attached bacteria or OMV. Cells were subsequently treated with 300 μl of 0.005% digitonin extraction buffer for 8 min and the supernatant containing cytosol was collected. The residual cell fraction containing cell membrane, organelles and nucleus was collected in 300 μl of 0.1% CHAPS buffer. Cytosol and residual fractions were subjected to agar plating to detect bacteria, BCA assay for protein quantification and LAL assay for LPS quantification. Additionally the fractions were immunoblotted for Na^+/K^+ ATPase, EEA1, Rab7, LAMP1, and GAPDH to confirm the purity of cytosol fraction. Non-detergent based fractionation was conducted as described before (Sun et al., 2013).

Confocal Microscopy

BMDM were stimulated with EHEC or OMV or left untreated for various durations as indicated. The cells were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X and blocked with 5% goat serum prior to staining overnight at 4°C with antibodies against LPS (clone 2D7/1; Abcam), Lipid A (clone 26-5; Hycult Biotech), clathrin (clone D3C6; Cell Signaling), EEA1 (clone C45B10; Cell Signaling), Rab7 (clone D95F2; Cell Signaling), LAMP1 (clone 1D4B; eBioscience), LAMP2 (clone ABL-93; eBioscience) or caspase-11 (17D9; Sigma-Aldrich) followed by staining with fluorescently labeled secondary antibodies for 1 h. Plasma membrane was stained with cholera toxin B Alexa fluor 647 conjugate (CTB) (Life Technologies). The cells were visualized using a Zeiss LSM 780 microscope.

Transmission Electron Microscopy (TEM)

For negative staining of OMV, 5 μl of OMV sample was added to carbon coated copper mesh grid, stained with 1% uranyl acetate for 1 minute and imaged with a Hitachi H-7650 Transmission Electron Microscope. For immunogold staining of OMV, 5 μl of OMV was placed on a Ni coated glow discharged grid for 1 min, blocked with 5% goat serum and stained with mouse anti-LPS antibody (Abcam) or rat anti-OmpF antibody (kind gift of Justin Radolf). Samples were then stained with goat anti-mouse or goat anti-rat antibodies conjugated with 10 nm gold particles (Electron Microscopy Sciences) and counter stained with 1% uranyl acetate. To ensure the specificity of immunogold labeling, staining was performed with secondary antibodies alone without primary antibodies.

For TEM of intestinal sections, C57BL/6 mice were orally gavaged with 1×10^9 CFU of *C. rodentium*-Stx. On day 7 cecal samples were collected, rinsed thoroughly, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS, dehydrated in methanol and embedded in LR Gold resin. Ultra thin sections (70 nm) were cut and immunogold stained using a rabbit anti-*E. coli* LPS antibody (Bioss Antibodies) and goat anti-rabbit antibody conjugated with 10 nm gold particles (Electron Microscopy Sciences). To ensure the specificity of immunogold labeling, staining was performed with secondary antibodies alone without primary antibodies.

For immunogold staining of macrophages to detect cytosolic LPS, BMDMs infected with EHEC for 4 h or left uninfected were collected by centrifugation after washing thoroughly to remove extracellular bacteria. The cell pellet was then fixed and processed similar to the intestinal sections as described above.

Antibodies for Immunoblotting

The following antibodies were used for immunoblotting: caspase-1 p20 (clone casper1; Adipogen), IL-1β (AF-401-NA; R&D Systems), Na^+/K^+ ATPase (clone 464.6; Novus Biologicals), EEA1 (clone C45B10; Cell Signaling Technologies), Rab7 (clone D95F2; Cell Signaling Technologies), LAMP1 (clone 1D4B; eBioscience), AP2 (clone 31/AP50; BD Biosciences), GAPDH (clone 71.1; Sigma-Aldrich), β-actin (Sigma-Aldrich), and OmpF (kind gift of Justin Radolf).

Quantitative RT-PCR

RNA was extracted using RNeasy kit (QIAGEN). cDNA was synthesized from total RNA using the iScript Select cDNA synthesis kit (Bio-Rad). Real time quantitative PCR for indicated genes was performed using

iQ SYBR green supermix (Bio-Rad) and primers designed from PrimerBank (https://pga.mgh.harvard.edu/primerbank/index.html; Spandidos et al., 2010). β-actin expression was used for normalizing the target gene expression and the fold difference in transcript levels was analyzed by Livak's method (Livak and Schmittgen, 2001).

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