Supporting Information

Song et al. 10.1073/pnas.0900527106

Experimental Procedures

Bacterial Invasion Assay. The 5637 human bladder epithelial cells (BECs) were seeded onto 96-well plates at a density of 4×10^4 cells per well and incubated overnight. The cells were infected with 100 MOI bacteria for 1 h. The medium was replaced with fresh culture medium containing 100 μg/mL of the membrane-impermeable antibiotic gentamicin (Invitrogen) to kill extracellular bacteria and incubated at 37 °C for additional 30 min. Each well was washed three times with PBS. To lyse the cells, 100 μL of 0.1% Triton X-100 in PBS was added to each well and incubated for 15 min. Cells were scraped, diluted, and plated onto LB agar plates. Colonies were counted to quantify the number of invading bacteria.

Bacterial Exocytosis Assay. The 5637 human BECs were seeded onto 96-well plates at a density of 4×10^4 cells per well and incubated overnight. Bacteria were diluted in serum free culture media and were added to the monolayer at MOIs of 100:1 or 1,000:1. After 1 h, the cells were washed and treated with 100 μg/mL gentamicin and 100 mM methyl α-D-mannopyranoside (Sigma) for an additional 30 min. Methyl α-D-mannopyranoside was added to media to prevent reattachment and entry of bacteria to BECs. The monolayer was then washed with fresh culture media containing 100 mM methyl α-D-mannopyranoside. Additional culture media (100 μL) plus methyl α-D-mannopyranoside and bacteriostatic agents 25 μg/mL trimethoprim (TMP) and 125 μg/mL sulfamethoxazole (SMZ) (to prevent bacterial growth) were then added to each well and incubated with the monolayer for 4 h. The culture medium was collected, and the cell monolayer was washed with an additional 100 μL of culture media with methyl α-D-mannopyranoside, which was also collected and pooled. Twenty-five microliters of the pooled culture media was plated on LB agar plates. The percentage of extracellular bacteria is determined relative to the intracellular bacterial titer at 0 h. The intracellular bacterial numbers at 0 h are expressed as total numbers of bacteria (100%). When exocytosis is normalized to a control, it is normalized by determining the percentage of extracellular bacteria relative to corresponding intracellular counts at 0 h. Because we noticed that the number of initial intracellular bacteria affects a ratio of bacteria exocytosis, we standardized initial numbers of intracellular bacteria. To make the initial number of intracellular bacteria similar among samples, 200 MOI bacteria for Rab27b siRNA BECs and 400 MOI bacteria for caveolin-1 siRNA BECs were added to the well of BEC culture. For human primary BECs, 1,000 MOI of bacteria were added to the wells. Similar initial intracellular numbers were verified by performing a bacterial invasion assay.

Time Lapse Microscopy. To visualize bacteria, a plasmid expressing GFP (pSMC2) (1) was transformed into *Escherichia coli* CI5. Human primary BECs were cultured on a coverslip in 12-well plate and infected with 500 MOI of *E. coli* CI5 expressing GFP for 1 h. In 1 h, cells were washed with media containing 100 mM methyl a-D-mannopyranoside (Sigma) and treated with 100 μg/mL gentamicin for 30 min to kill extracellular bacteria. Cells were washed and incubated in freshly prepared media containing methyl a-D-mannopyranoside to prevent reentry of bacteria for 3 h. Then, cells were set on a microscope to take a series of images. To distinguish extracellular bacteria from intracellular bacteria, infected cells were immunostained with an Alexa Fluor 546-labeled *E. coli* antibody before time lapse microscopy. An *E. coli* antibody was prelabeled with Alexa Fluor 546 dye using an antibody labeling kit (Molecular Probes).

Kds of Rab27b, Caveolin-1, and MyRIP. Negative control siRNA (siRNA ID no. 120374), Rab27b siRNA (siRNA ID no. 120374), caveolin-1 siRNA (siRNA ID no. 10479), and MyRIP siRNA (siRNA ID no. S24759) were purchased from Ambion and used for knocking down specific genes in BECs according to the vendor's recommendation (Ambion). Briefly BECs were seeded and incubated overnight. On the after day, BECs were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) reagents as recommnended by the vendors (Invitrogen and Ambion). Cells were washed 6 h after transfection and incubated in freshly culture media for additional 48 h. For assays, cells were trypsinized and transferred to appropriate culture dishes 1 day before experiments performed.

Lactate Dehydrogenase (LDH) and *E. coli* Survival Assays. LDH release assays were performed to examine bladder cell membrane integrity, according to the manufacturer's instructions (Tox7; Sigma). BECs were uninfected or infected with 100 MOI *E. coli*, and cell viability was measured 4 h after infection. Also, it was examined that the exocytic compartment encasing *E. coli* did not possess bactericidal activity. Inhibitors of lysosome acidification including NH_4Cl (10 mM) and Bafilomycin (1 μM), which neutralize bactericidal activity within lysosomes, caused no improvement in *E. coli* ORN103(pSH2) persistence within 5637 BECs.

Measurement of Intracellular cAMP Levels. The 5637 human BECs were seeded onto 6-well plates and grown overnight. The cells were uninfected or infected with 100 MOI *E. coli* ORN103(pSH2) for the indicated time points. The cells were washed four times with PBS to remove culture media, and lysed in 250 μL of 0.1 M HCl for 10 min. After centrifugation, the supernatant was directly used for the cAMP assay. Intracellular concentrations of cAMP were determined using a cAMP enzyme immunoassay kit (Sigma) according to the manufacturer's instructions.

Creation of Toll-Like Receptor (TLR)4 and Adenylyl Cyclase (AC)3 kds. RNA interference vectors were generated using pQCXIN retroviral vector (BD Biosciences). Briefly, pQCXIN was digested by BamHI and EcoRI and then was religated to generate pQCXIN1. Human U6 small nuclear RNA promoter was PCR-amplified from pTZ U6 + 1 (gift from John Rossi, Beckman Research Institute of the City of Hope, Duarte, CA) with added BglII site (5′ ends), BamHI, and XbaI sites (3′ ends). The PCR product was cloned to the BglII and XbaI sites of pQCXIN1 to generate pQCXIN-U6. The following oligonucleotides were ordered from Integrated DNA Technologies: TLR4a, 5′-GATCCG**TTCCGATTAGCATACTTAG**TTCAAGAGA*CTAAGTATGCTAATCGGAA*CTTTTTTT-3′, and TLR4b, 5′-CTAGAAAAAAAG**TTCCGATTAGCATACTTAG**TCTCTTGAA*CTAAGTATGCTAATCGGAA*CG-3′. The boldface and underlined sequences are forward and reverse sequences, respectively, which correspond to nucleotides 1026–1044 of the human TLR4 gene (GenBank accession no. U88880). The oligos were annealed to form double-stranded DNA and cloned into the BamHI and XbaI sites of pQCXIN-U6 to generate pSi-TLR4. The Amphopack-293 Cell Line (BD Biosciences) was used to produce the viral particles. Production of viral particles, infection of

target cell line (5637), and selection of viral infected cells were performed as recommended by the vendor of the pQCXIN vector (BD Biosciences). The geneticin-resistant stable-transfected cell lines were named TLR4 KD BECs. Knockdowns (kds) were verified by RT-PCR using the specific primers listed below.

RNA Isolation and RT-PCR. Total cellular RNA was isolated using RNeasy purification system (Qiagen); 2 μg of total RNA was reverse transcribed and amplified with gene-specific primers using the RT-PCR System kit (Bio-Rad). The primer sequences for the genes were as follows: 5′-CGATTCCATTGCTTCTTG-3′ (sense) and 5′-GCTCAGGTCCAGGTTCTT-3′ (antisense) for TLR4 and 5′-ATCCCATCACCATCTTCCAG-3′ (sense) and 5′-CCTGCTTCACCACCTTCTTG-3′ (antisense) for GAPDH, 5′- GTGGTTTCTTTGAACCCATA-3′ (sense) and 5′-GAGTTAGGCTTTTGTGATGC-3′ (antisense) for MyRIP, and 5′-TACTCGGTGGAGAAGGAGAAGCAG-3′ (sense) and 5′-CGAAAACGCTTGTGGTCGTATTC-3′ (antisense) for AC-3 (554 bp).

Generation of BECs Overexpressing GFP-Rab27b. To generate the GFP-Rab27b, the forward primer 5′-GATCTCGAGCTATGACCGATGGAGACTATGAT-3′ and reverse primer 5′-GGTGGATCCCTAGCAGATACA TTTCTTCTCTG-3′ (Integrated DNA Technologies) were used to amplify Rab27b from 5637 BECs by RT-PCR. The RT-PCR products were digested with *Xho*I and BamHI, and then ligated to XhoI/BamHI-digested pLEGFP-C1 (Clontech) to generate pGFP-Rab27b. The Amphopack-293 Cell Line (BD Biosciences) was used to produce the viral particles. Production of viral particles, infection of target cell line (5637), and selection of viral infected cells were performed as recommended by the vendor (BD Biosciences). The geneticin-resistant stable-transfected cell lines were generated and verified under a fluorescence microscope.

Sucrose Density Fractionation of BECs. BECs expressing GFP-fused Rab27b were grown to ≈80% confluence on 15-cm cell culture dishes. Two plates were infected with 100 MOI *E. coli* ORN103(pSH2) in 25 mL of serum-free medium for 2 h. Infected plates, along with uninfected plates, were washed five times with ice-cold PBS and scraped off the plates using a rubber policeman in 2 mL of homogenization buffer [10 mM Tris, pH 7.2/2 mM EDTA/1 mM PMSF, and a 1:100 dilution of mammalian protease inhibitor mixture (Sigma)]. The cell suspension was then passed 20 times through a 21-gauge needle, brought to 45% sucrose by the addition of an equal volume of 90% sucrose, and then overlaid with 4 mL each of 35%, and 5% sucrose. The gradients were centrifuged for 18 h at 39,000 rpm in a SW41Ti rotor (Beckman Instruments), and 12 equal fractions were collected from the top of each gradient and assayed for caveolin-1 and Rab27b by Western blotting using polyclonal anti-caveolin-1 antibody (BD Transduction Laboratories) and monoclonal anti-GFP-antibody (Santa Cruz Biotechnology).

1. Bloemberg G, O'Toole G, Lugtenberg G, Kolter R (1997) Green fluorescent protein as a marker for Pseudomonas spp. *Appl Environ Microbiol* 63:4543–4551.

Immunoprecipitation. GFP or GFP-fused Rab27b were immunoprecipitated from 5637 BECs overexpressing GFP or GFP-Rab27b. Briefly, 5637 BECs were uninfected or infected with 500 MOI *E. coli* ORN103(pSH2). In 2 h, cells were washed and harvested in 0.5 mL of 1× RIPA buffer (Millipore) plus protease inhibitor cocktails (Sigma) and 1 mM PMSF. The harvested cells were then passed 20 times through a 21-gauge needle and microcentrifuged for 10 min at 4 °C. The cell lysates were immediately used for immunoprecipitation assays. For the assay, 200 μL cell lysates were mixed with anti-GFP antibody and incubated with gentle rocking overnight at 4 °C; 50 μL of a 50% suspension of protein A-Sepharose (Upstate) was added, and the sample was further incubated for 2 h at 4 °C. The immunoprecipitates were washed five times with 1 mL of 1× RIPA buffer (Millipore), resuspended in 60 μL of 2× Laemmli sample buffer (Bio-Rad), and boiled for 5 min. Immunoprecipitates were assayed for caveolin-1 using polyclonal anti-caveolin-1 antibody (BD Transduction Laboratories).

To examine that Rab27b, MyRIP, PKA, and caveolin-1 are in the same binding complex in BECs, Rab27b, MyRIP, and PKA were immunoprecipitated from either uninfected or infected 5637 BECs. Bladder cell lysates were prepared as described above and immediately used for an immunoprecipitation assay. Primary antibodies used in this assay were a rabbit anti-Rab27b IgG (IBL), a goat anti-MyRIP IgG (Abcam), and a mouse anti-PKA RIIa IgG (BD Transduction Laboratories). Immunoprecipitates were prepared as described above and assayed for caveolin-1. Each sample was boiled for 5 min and run on a 4–20% Tris-glycine SDS/PAGE gel (Bio-Rad). Interactions among the proteins were revealed with a primary rabbit anti-caveolin-1 antibody (BD Transduction Laboratories) and either a secondary HRP-conjugated Clean-blot IP detection reagent (Thermo Scientific) or a secondary HRP-conjugated mouse IgG (Bio-Rad).

Immunofluorescence Microscopy. The 5637 BECs overexpressing GFP-Rab27b were seeded onto 12-mm diameter glass coverslips and grown overnight. The cells were fixed overnight in 1% paraformaldehyde in PBS. After removing the fixative, the cells were permeabilized and blocked with saponin buffer (0.05% saponin/10 mM Hepes/10 mM glycine/10% goat serum). The cells incubated with primary antibodies diluted in saponin buffer for 30 min at room temperature (RT), washed three times with saponin buffer, and incubated with secondary antibodies in saponin buffer for 30 min at RT. A primary polyclonal antibody against caveolin-1 (BD Transduction Laboratories) was revealed with donkey anti-rabbit IgG Cy5 (Molecular Probes). Coverslips were mounted with Prolong Gold antifade reagent (Molecular Probes) and examined using a Nikon confocal laser scanning instrument with appropriate filter sets.

Statistical Analysis. Unpaired *t* tests were preformed on all datasets to determine the statistical significance of experimental changes from control values.

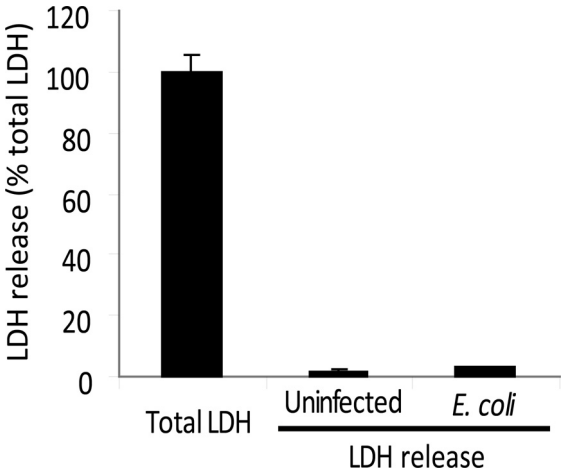


Fig. S1. The 5637 BEC membrane integrity was assessed by a LDH release assay. *E. coli* infection to BECs for 4 h caused no difference in LDH releases from unfected BECs.

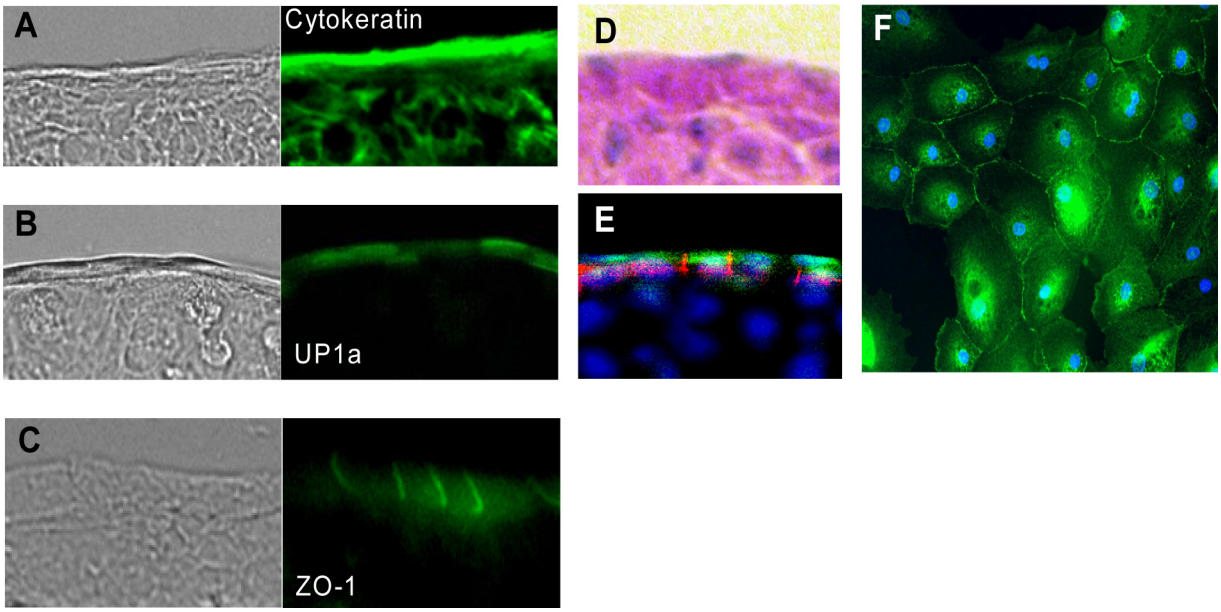


Fig. S2. Cultured human primary bladder cells appear fully differentiated. (*A*–*E*) Human primary bladder cells were grown on transwells for 7 days in keratinocyte-SFM medium supplemented with 2.0 mM Ca^{2+} and 5% FBS. Cryostat sections were stained for pan-cytokeratin, uroplakin 1a, and ZO-1 (*Right* panel of *A*, *B*, and *C*, respectively). The *Left* panels show the corresponding DIC images. Notice that the cells are several layers thick with the upper layer staining positive for markers of terminal differentiation. Notice the ZO-1 staining of tight junctions between superficial epithelial cells in *C*. (*D* and *E*) Images of the same section. (*D*) histologically stained, (*E*) confocal image stained for nucleus (blue), Uroplakin 1a (green) and ZO-1 (red). (*F*) Same cells grown in monolayer were stained for ZO-1. Notice the ZO-1 staining between cells.

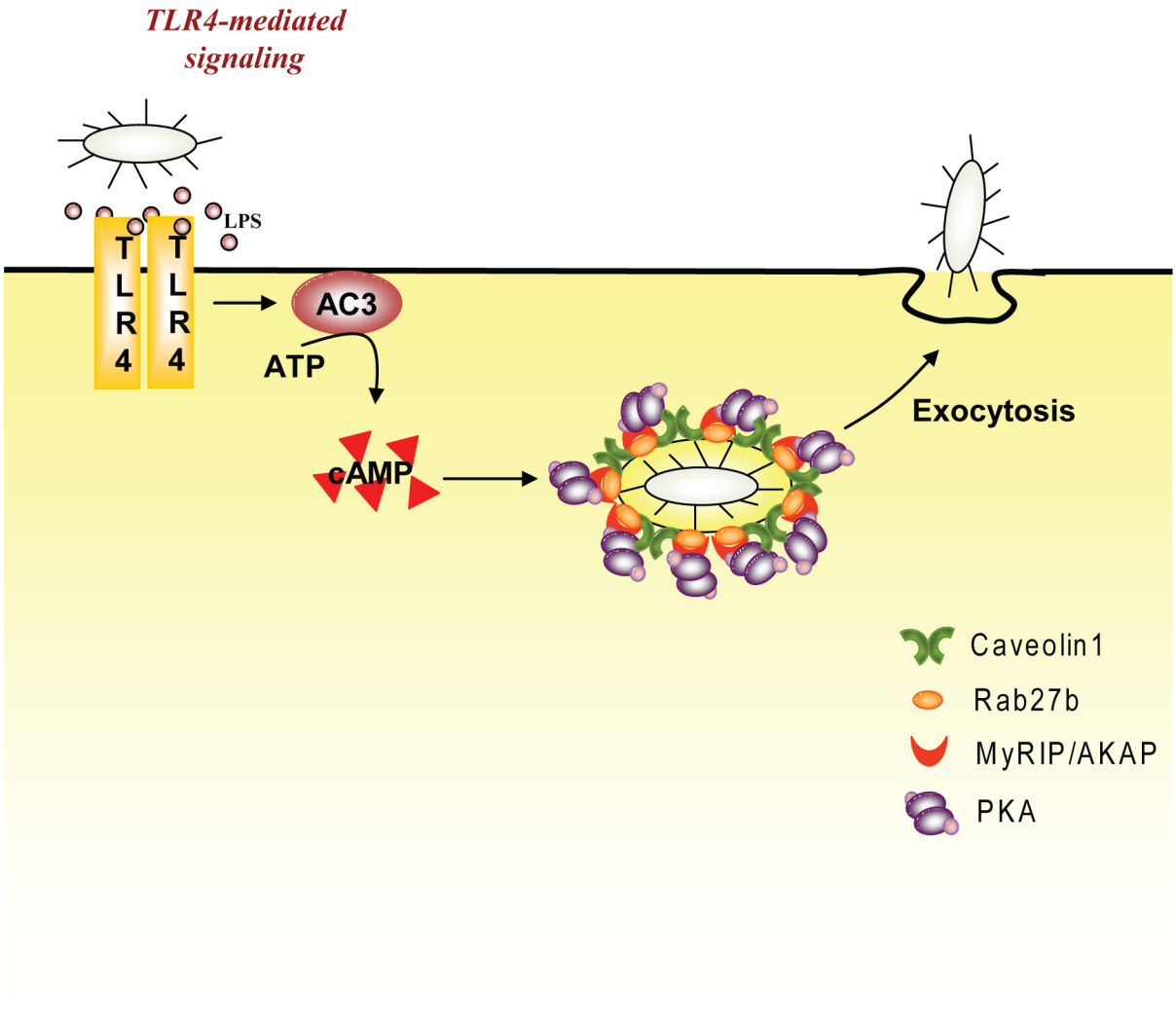
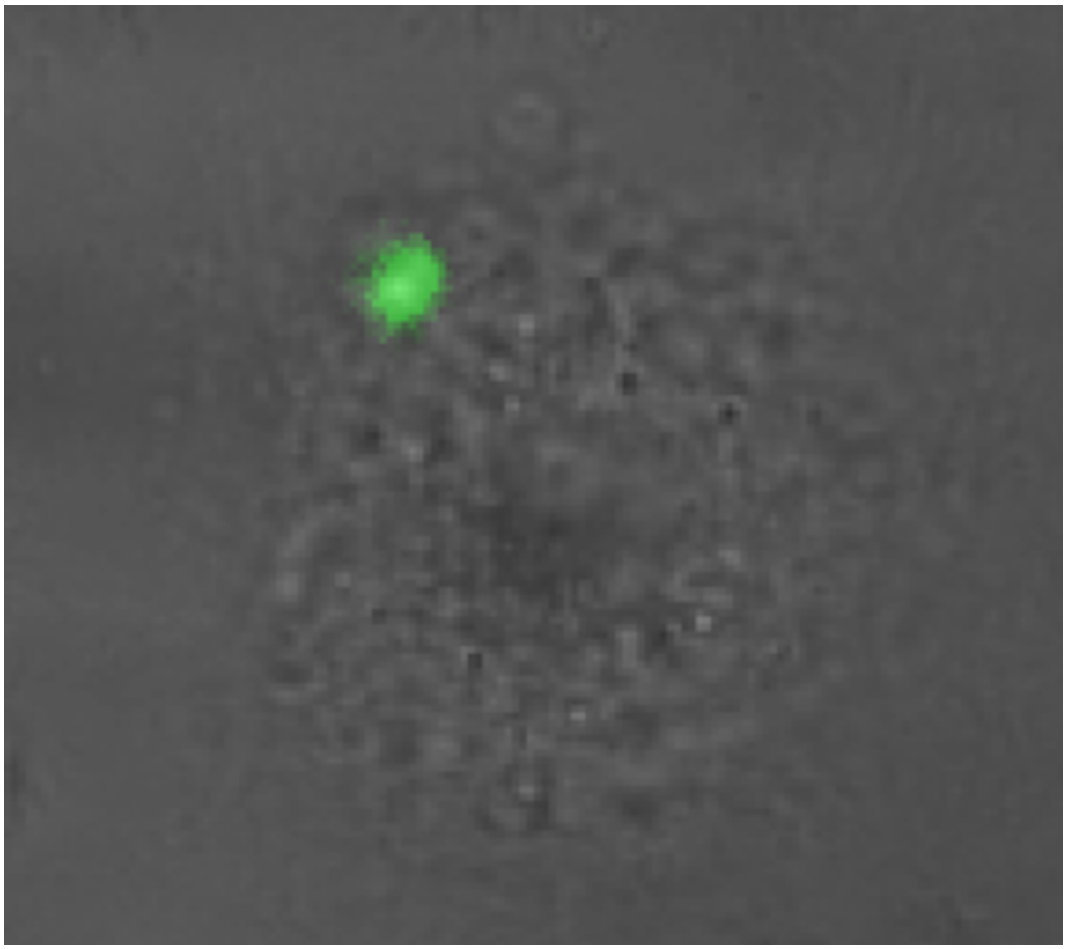


Fig. S3. Schematic representation of the TLR4-mediated mechanism for expulsion of bacteria from infected BECs. The TLR4 initiated and AC-3, cAMP, and PKA-dependent signaling pathway regulates bacteria exocytosis from infected BECs. Caveolin-1, Rab27b, MyRIP, and PKA are found in the same binding complexes.



Movie S1. A series of time lapse images showing expulsion of *E. coli* from an infected BEC. Video microscopy was used to visualize exocytosis of bacteria from infected human primary BECs. Primary BECs were infected with *E. coli* CI5 expressing GFP for 1 h, after which gentamicin was added to the culture media to kill extracellular baceria. Infected BECs were then incubated in a fresh medium and used for video microscopy. Images shown here are representative images from three independent experiments.

Movie S1 (GIF)