Supporting online material:

Materials and Methods

**Bacterial strains and plasmids.**

*Salmonella typhimurium* wild type SL1344 (*S1*), effectorless SB1304 (which carries loss-of-function mutations in the effectors *sptP, sopE, sopE2, and sopB*, and is unable to induce actin cytoskeleton rearrangements (*S2*)) and *invA* (which is defective in expression of the SPI-1 type III secretion system (*S3*)) were provided by J. Galán, Yale University; GFP-SL1344 was generated by introduction of pAM239 (*S4*) into SL1344. *Yersinia pseudotuberculosis* *yopEH* mutant IP17 (*S5*) and *ysc* mutant IP71 containing p67GFP3.1 (*S6*) were generated in J. Bliska’s laboratory. IP71 contains a Tn5 insertion in the *ysc* operon, which inactivates the type III secretion system. *Listeria monocytogenes* wild type 10403S was provided by D. Portnoy, U.C. Berkeley. *E. coli* K-12 XL1-Blue was obtained from Stratagene. Mammalian transfection plasmids were pEGFP-N2 (Clontech) and Syt VII C₂A cloned into pEGFP-N2 (*S7*).

**Cell culture and *Salmonella/Yersinia* infection.**

Mouse Embryonic Fibroblasts (MEFs) and murine bone marrow-derived macrophages were prepared from wild type and Syt VII-deficient mice as
previously described \((S8)\), and seeded at \(5 \times 10^5\) /well on 35 mm tissue culture dishes 24 h prior to experiments. A 1:30 dilution of an overnight \textit{Salmonella} culture was grown in high salt LB broth for 3 h at 37°C with shaking at 300 rpm (a condition that minimizes induction of cytotoxicity in macrophages \((S9)\), see also (Fig. S2). A 1:40 dilution of an overnight \textit{Yersinia} culture was grown in LB broth for 2 h at 37°C. Bacteria resuspended in HBSS \((\textit{Salmonella})\) or PBS \((\textit{Yersinia})\) were added to cells at a MOI of 10, dishes were centrifuged (50 g 5 min), incubated at 37°C for the desired infection period, washed, and incubated with fresh medium containing gentamicin at 100 µg/ml \((\textit{Salmonella})\) or 8 µg/ml \((\textit{Yersinia})\) for 1 h, followed by 5 µg/ml during the rest of the post-infection period. In some \textit{Salmonella} experiments, 1 mM IPTG was added and maintained during the post-infection period to induce GFP expression. In \textit{Yersinia} experiments, 3 mM IPTG was added 1 h prior to fixation to induce GFP expression only in viable bacteria. For determining colony forming units (CFU), infected monolayers were washed, lysed in PBS 0.1% Triton X-100, and the protein content was determined by BCA (Pierce) to ascertain that no cell loss occurred. The lysates were serially diluted and spread on LB agar plates containing the appropriate antibiotics, and incubated at 37°C \((\textit{Salmonella})\) or 26°C \((\textit{Yersinia})\) for colony development. Release of cytosolic LDH induced by exposure to \textit{Salmonella} was performed as previously described \((S10)\).
Antibodies and fluorescence.

Monoclonal antibodies (mAbs) against the lumenal domain of hamster (UH1) and mouse (1D4B) Lamp 1 (Developmental Studies Hybridoma Bank), anti-Salmonella polyclonal antibodies (Difco Laboratories), goat anti-rabbit, anti-mouse, or anti-rat conjugated to Alexa 488 or 546 (Molecular Probes) and Cy-5 conjugated goat anti rabbit (Zymed) were used. After bacterial infection, cells were washed, fixed with 2% paraformaldehyde in PBS at 4°C overnight, permeabilized in PBS 2% BSA 0.1% saponin (Sigma), and incubated with primary and secondary antibodies. For inside/outside bacterial invasion assays, extracellular bacteria were labeled with primary and Alexa 488 secondary antibodies before permeabilization, and after permeabilization were re-labeled with primary and Alexa 546 secondary antibodies. For co-localization of bacteria with dextran-containing lysosomes, cells were incubated with 0.5 mg/ml lysine-fixable dextran-Texas red (10 kDa, Molecular Probes) for 1h and chased for 3h in fresh media, prior to infection. For live surface staining of Lamp-1 in MEFs, after infection at an MOI of 20 (Salmonella) or 85 (Yersinia), coverslips were incubated with anti-Lamp-1 mAbs at 4°C for 30 min, fixed for 15 min at 4°C, washed and labeled with fluorescent secondary antibody. Images were acquired in a Ziess Axiovert 135 microscope through a 100x objective with a Hamamatsu Orca II cooled CCD camera controlled by Metamorph Software (Universal Imaging), or in a Zeiss LSM 510 laser scanning confocal microscope. The number of intracellular bacteria associated with >200 cells (determined by antibody staining or GFP expression in the presence of extracellular gentamicin)
was determined visually in triplicate. Phagolysosome fusion was quantified by examining all phagosomes in approximately 30 randomly acquired optical Z-stacks, for each experimental condition. GFP-expressing CHO cells were sorted in a Becton-Dickinson Vantage Flow fluorescence activated cell sorter (FACS). TUNEL assays for *in situ* detection of apoptotic macrophages were performed according to the manufacturer’s instructions (Roche). DAPI and TUNEL-positive cells in 10 microscopic fields (>200 macrophages) at 25X magnification were counted, in triplicate.

**Listeria monocytogenes intracellular growth assay**

Bone marrow macrophages from wild-type and Syt VII -/- mice were plated 48 h before infection. One ml of a $1.4 \times 10^9$/ml *Listeria monocytogenes* culture in BHI broth was centrifuged, washed, and used to infect macrophages at a MOI = 0.1. After 30 min the dishes were washed, and after an additional 30 min in fresh media 25 µg/ml gentamicin was added. At different times post-infection, infected macrophages were washed and lysed in 1 ml sterile water for 10 min at room temperature. Serial dilutions of the lysates were plated to determine CFUs. To visualize polymerized actin, macrophages were infected at a MOI = 1 for 30 min, incubated for 2 h with 25 µg/ml gentamicin, washed, fixed for 20 min in 4 % paraformaldehyde, and stained with phalloidin-Alexa Fluor 546 (Molecular Probes; 1:40 dilution). At least 825 bacteria from 150 infected macrophages were analyzed for each experimental group.
**EGF Degradation Assay**

Wild type and Syt VII -/- MEFs were plated on 35 mm dishes, washed with binding medium (DMEM, 20mM HEPES pH 7.4, 1 mg/ml BSA) and incubated with 4 ng/ml $^{125}$I-EGF (ICN, 100 µCi/µg) for 1 h at 37°C. Labeled cells were placed on ice and washed four times with ice-cold HBSS containing 1 mg/ml BSA. Pre-warmed complete medium (DMEM, 10% FCS, 0.1 µM β-mercaptoethanol, 2 mM L-glutamine) was added and the cells were incubated at 37°C for 30-180 min. At each time point, the chase medium was removed and the cells solubilized in 1 ml 1N NaOH/1% Triton X100. The intact $^{125}$I-EGF in the chase medium was separated from low molecular weight degradation products by precipitation with 15% trichloroacetic acid. The precipitation mixture was incubated for 30 min on ice followed by centrifugation at 14,000g for 30 min at 4°C. The supernatant was removed and the TCA precipitate was resuspended in 1N NaOH. Radioactivity in the cell lysate, TCA supernatant, and TCA precipitate was determined by scintillation counting. The amounts of degraded $^{125}$I-EGF were calculated as the counts in the TCA soluble fraction as a percentage of the total radioactivity in all three fractions (TCA soluble, TCA precipitate, cell lysate).

**E. coli Killing Assay**

Wild type and Syt VII -/- bone marrow macrophages were plated at 2.5 x $10^5$ per 35 mm dish. One ml of an overnight *E. coli* culture was washed, resuspended in 1 ml PBS, added to macrophages at a MOI = 5 followed by centrifugation (130 g, 5 min) and incubation at 37°C for 5 min. After four washes
fresh media was added and the cells were further incubated at 37°C for 0, 15, 30, or 60 min. At each time point, cells were lysed in 1 ml sterile water for 10 min at room temperature, and serial dilutions were plated to determine CFUs.

**NO and reactive oxygen species detection assays**

The concentration of nitrite (NO$_2^-$), the stable oxidized derivative of NO, was determined in 100 µl aliquots of cell culture supernatants transferred to 96-well plates. 100 µl of Griess reagent solution (1% Sulfanilamide, 0.1% napthylene diamine dihydrochloride, 2% H$_3$PO$_4$) was added per well, and the absorbance was measured at 540 nm in a microplate ELISA reader. Sodium nitrite (Sigma) diluted in culture medium was used as standard (S11).

Bone marrow macrophages obtained from wild type and Syt VII -/- mice were loaded with 2 mM DHE (dihydroethidium, Molecular Probes) or 25 mM H$_2$DCFDA (2',7'- dichlorodihydrofluorescein diacetate, Molecular Probes) for 20 min at room temperature, prior to exposure to either opsonized zymosan (Molecular Probes) or GFP-Salmonella at 37°C. The superoxide-specific oxidation product of intracellular DHE (S12) can be visualized at 567 nm (593 nm when bound to DNA). Following exposure to reactive oxygen species, de-esterified H$_2$DCFDA (2',7'-dichlorofluorescein, DCF) fluoresces at 530 nm (S13). Data were collected from macrophages 30 min after particle uptake using a Leica SP2-AOBS laser-scanning confocal microscope (Exton, PA, USA) equipped with 9 laser lines (488 nm for H$_2$DCFDA and 543 nm for DHE), a 63X planApo (NA 1.4) objective and differential interference optics. During confocal observation, cells were kept at 37
°C using a temperature-controlled heating stage (Brook Industries). Images were analyzed using Leica Confocal Software, and Image J (Wayne Rasband, Research Services Branch, National Institute of Mental Health).

**Statistical analysis**

Data sets in triplicate or quadruplicate were compared using Student’s unpaired t test for independent samples. P values <0.05 were considered significant.
Legends for Supporting Figures

**Figure S1. *Salmonella* resides intracellularly in vacuoles containing Lamp-1, in cells with or without normal Syt VII function.** (A) CHO cells expressing GFP (left) or Syt VII C2A-GFP (right) were infected with *Salmonella*, incubated for 2 h post-infection, fixed and stained with anti-*Salmonella* antibodies (blue) and anti-Lamp-1 mAbs (red). (B) MEFS from Syt VII +/+ and -/- mice were infected with GFP-*Salmonella*, incubated with IPTG for 6 h, fixed and stained with anti-Lamp-1 mAbs (red). Arrows point to intracellular bacteria in vacuoles containing Lamp-1.

**Figure S2. *Salmonella*-induced cytotoxicity.** Bone marrow macrophages from Syt VII +/+ or -/- mice were infected with *Salmonella* for 1 h at an MOI of 10, washed and further incubated for 2, 4 and 6 h prior to fixation and DAPI/TUNEL staining. (A, B) Representative images of DAPI and TUNEL-positive cells at 2, 4 and 6 h after infection of Syt VII +/+ (A) and -/- (B) macrophages. No TUNEL-positive cells were detected on macrophages not exposed to *Salmonella* (not shown). (C) The percent of TUNEL-positive macrophages increases gradually with time, but remains below 17% during a 6 h period. The data represents the mean ± SD of triplicates. No significant difference in the number of TUNEL-positive macrophages at the 3 time points was observed between Syt VII +/+ and -/- (P = 0.714, t test). (D) Apoptosis does not cause cell loss during the 6 h incubation period. The data represents the mean ± SD of triplicates. No
significant difference in the number of macrophages/10 microscopic fields at the 3 time points was observed between Syt VII +/+ and -/- (P = 0.404, t test).

**Figure S3. Syt VII deficiency does not alter endocytic traffic, lysosomal degradation and generation of NO and superoxide.** (A) Syt VII +/+ and -/- MEFs were infected with *Listeria monocytogenes* for 30 min and the number of intracellular bacteria was determined by a gentamicin- protection assay at various times post-infection. The data represents the mean ± SD of triplicates. (B) Syt VII +/+ and -/- MEFs were incubated with $^{125}$I-EGF and the rate of intracellular degradation was determined at various times after endocytosis. The data represents the mean ± SD of triplicates. (C) Syt VII +/+ and -/- bone marrow macrophages were incubated with *E. coli* and the number of viable bacteria was determined at various times post-internalization. The data represents the mean ± SD of triplicates. (D) Bone marrow macrophages were treated with LPS and IFNγ overnight and nitrite resulting from NO production was assayed. The data represents the mean ± SD of triplicates. (E, F) Bone marrow macrophages were infected with GFP-expressing *Salmonella* (green) and processed for detection of reactive oxygen products with DHE (red). (G-J) Bone marrow macrophages were incubated with zymosan and processed for detection of reactive oxygen products with DHE (G, H, red) or H$_2$DCFDA (I, J, green). The arrows point to extracellular zymosan particles, not associated with oxidation products.
Figure S4. Type III secretion systems of *Salmonella* and *Yersinia* permeabilize the plasma membrane of mammalian cells. (A) CHO cells were incubated with medium alone (0 h) or containing *Yersinia yopEH* or the type III secretion mutant *ysc* for 3 h (top panel) or *Salmonella* wild type SL1344 or the type III secretion mutant *invA* (lower panel) for 1 h at 37°C at an MOI=100, followed by exposure to 25 µg/ml ethidium bromide (red) and 5 µg/ml acridine orange (green) and image acquisition. Only bacteria expressing a functional type III secretion system allowed the entry of ethidium bromide (nuclear red stain). (B) CHO cells were incubated with *Salmonella* wild type SL1344, effectorless SB1304 or *invA* at an MOI=100 for 30 min or 1 h at 37°C, followed by exposure to ethidium bromide (red) and acridine orange (green). Wild type and non-effectorless strains caused significantly more entry of ethidium bromide (nuclear red stain) than the type III secretion mutant *invA*. (C) CHO cells were incubated with *Salmonella* wild type SL1344, effectorless SB1304 or *invA* at an MOI=100 for 2 h at 37°C, the supernatant was removed, the cells washed and lysed, and LDH activity was determined. The data represents the mean ± SD of quadruplicates. The similar, low levels of LDH released from cells exposed to all 3 strains of *Salmonella* (27.2%, 26.6% and 32.1%) indicate that no extensive cell disruption occurred during this period.

Figure S5. Model for the Syt VII-dependent, injury-triggered mechanism of intracellular killing. 1- bacteria approach cell in medium containing high [Ca^{2+}]; 2- bacteria enter cell; 3- bacteria reside intracellularly in a phagosome that
contains high $[\text{Ca}^{2+}]$; 4- type III secretion translocons form pores on the phagosome membrane, causing $\text{Ca}^{2+}$ flux into the cytosol; 5- elevation in $[\text{Ca}^{2+}]$ triggers Syt VII-dependent fusion of lysosomes with phagosomes; 6- bacteria are killed.
Supporting References


