Supporting Information

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SI Methods

Mice and Bacterial Strains. Six- to 8-wk-old wild-type (WT) C57BL/6, Toll-like receptor (TLR)$5^{-/-}$, $TLR2^{-/-}$, $MyD88^{-/-}$ (1), apoptosis-associated speck-like protein containing a caspase recruitment domain $(ASC)^{-/-}$ (2), caspase-$1/11^{-/-}$ and nucleotide oligomerization domain-like receptor (NLR) family, caspase activation recruitment domain domain-containing $(NLRC)4^{-/-}$ (3), and NOD-like receptor PYD-containing protein $(NLRP)3^{-/-}$ (4) mice were bred in our animal facilities at the Federal University of São Paulo. The *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) lethal toxin (LT)2 strains were obtained at the *Salmonella* Genetic Stock Center and included the flagellin-expressing SGSC 1412 strain (WT) and the flagellin-deficient SGCS 2157 strain (ΔFli). Both strains were cultured in Luria–Bertani (LB) broth for 12 h with aeration at 37 °C and stored in at −80 °C. Bacteria harvested at the exponential growth phase (OD_{600} of 0.55–0.75) were used for the experimental procedures.

Preparation of Mouse Macrophages. Bone marrow-derived macrophages (BMDMs) were obtained as described previously (5). Bone marrow progenitor cells were recovered from the mouse femur and plated in RPMI-1640 medium supplemented with 30 mL / 100 mL L929 supernatant containing macrophage-stimulating factor, 10 mL / 100 mL heat-inactivated FCS, 10 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/mL streptomycin and penicillin (Invitrogen). The cells were incubated at 37 °C, under 5% CO_2, for 7 d. Four days after i.p. treatment with starch solution (1%) (Sigma Aldrich), peritoneal macrophages (PMs) were obtained by peritoneal lavage. Macrophages were cultured overnight in RPMI that was supplemented with 3% heat inactivated FBS and antibiotics, and nonadherent cells were then removed by washing with warm RPMI medium.

Flagellins, Peptides, and Protein Transfection. Purified flagellins from *Bacillus subtilis* (FLA-BS) and *Salmonella* Typhimurium (FliC) were purchased from Invivogen. The recombinant *Salmonella* Typhimurium FliC flagellin and the HIV p24 protein were expressed in the *Escherichia coli* BL21 DE strain, and the recombinant proteins were purified by nickel affinity chromatography, as described previously (6, 7). VK210 peptide of *Plasmodium vivax* protein was purchased from GenScript. PMs (10^5 cells seeded in 96-well plates) were cultured at 37 °C under 5% CO_2 in RPMI-1640 medium that was supplemented with 3% heat-inactivated FBS, amino acids and antibiotics. The cells were cultured in the presence of purified flagellin from *B. subtilis* or *Salmonella* Typhimurium in its free form (FLA-BS or FliC) or inserted into N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) (FLA-BSDot or FliCDot) (Roche Diagnostics), a cationic lipid formulation that permits its delivery to cell cytosol (8). Protein concentration was calculated to maintain the same molarity. Therefore, 0.3, 1, or 2×10^{-7} M correspond to 1, 3, or 6 µg/mL flagellin from *B. subtilis* and 1.8, 5.4, or 10.8 µg/mL *Salmonella* Typhimurium FliC flagellin; VK210 peptide and p24 protein were also inserted into DOTAP at final concentrations of 0.38 µg/mL and 2.75 µg/mL, respectively, all corresponding to 10^{-7} M. DOTAP was used accordingly to the manufacturer's instructions. Briefly, purified protein is added to the DOTAP (5 µL of DOTAP to each 1 µg of protein) in the presence of Hepes-buffered saline. The mixture was gently homogenized for 1 min and incubated at room temperature for 15 min to form stable complexes generated spontaneously. After incubation, RPMI-1640 medium was added, and an aliquot of 200 µL (0.3, 1, or 2×10^{-7} M final concentration) was added to 10^5 macrophages and seeded into 96-well plates.

Treatments. To assess the involvement of different molecules in flagellin-induced cell death, PMs were cultured in the presence or absence of the pan-caspase inhibitor Q-VD-fmk (20 µM), the cathepsin B inhibitor N-(L-3-*trans*-propylcarbonyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester (CA074-Me) (25 µM), the cathepsin D inhibitor pepstatin A (25 µM), or the lysosomal acidification inhibitor bafilomycin A1 (20 nM). The inhibitors were added in culture 1.5 h before stimulation. For detection of IL-1β, BMDMs were primed with LPS (100 ng/mL) for 2 h, for induction of the immature forms of these cytokines. To assess the involvement of cathepsins in cytokine production, primed macrophages were treated or not with CA074-Me or pepstatin A (4, 20 or 100 µM) 1.5 h before stimulation. To induce apoptosis, cells were stimulated with actinomycin D (ActD) (5 µg/mL).

Measurement of IL-1β, IL-6, and IL-1α. IL-1β, IL-6, and IL-1α were measured in culture supernatant with ELISA kits from BD (OptEIA), following the manufacturer's instructions.

Ethidium Bromide and Acridine Orange Stain. Pore formation and cytotoxicity were assessed using ethidium bromide (EtBr) incorporation in combination with acridine orange (AO) (Sigma) staining as described previously (9). After 1.5–24 h of stimulation, the culture supernatant was removed, and a solution of PBS containing EtBr and AO (50 µg/mL; vol/vol) was then added. Living cells stained with AO. Only cells that had membrane pores or cells that had ruptured allowed the diffusion of EtBr into the cell cytosol. Images were acquired using an inverted fluorescence microscope with original magnification of 400×. The percentage of EtBr single-positive cells, representing the percentage of cell death, was analyzed using ImageJ software.

LDH Release. Cell lysis was quantified by evaluating the lactate dehydrogenase (LDH) activity that was released from stimulated cells. Supernatants were collected after 1–6 h of stimulation and assayed for LDH release using a LDH-release kit (Doles) following the manufacturer's instructions. The absorbance was measured at 510 nm, and the percentage of cell lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release-spontaneous release)] × 100, where spontaneous release is the amount of release from unstimulated macrophages or cells treated with empty DOTAP vesicles, and maximum release is the value obtained by lysis of macrophages with a solution of 0.1% Triton X-100.

3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide Assay. Cytotoxicity was quantified using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a tetrazole salt that is converted to insoluble formazan by the succinate dehydrogenase enzyme in mitochondria. The purple formazan product is impermeable to cell membranes. Therefore, this product accumulates within living cells (10). Initially, the supernatant was discarded and 100 µL of PBS containing 10% of a MTT stock solution at 5 mg/mL was added to the cell culture. Plates were incubated at 37 °C under 5% CO_2. Two hours after incubation, the supernatant was discarded, and 100 µL of an isopropanol/HCl solution was then added. After careful homogenization, this solution dissolved the formazan crystals. Absorbance was measured at 570 nm, and the number of viable cells was estimated by the percentage

above a positive viability control (nonstimulated macrophages or cells treated with empty DOTAP vesicles).

Western Blot. For the detection of activated caspase-3, BMDMs from C57BL/6, caspase-1/11$^{-/-}$, and NLRC4$^{-/-}$ mice were seeded into 24-well plates and stimulated with FLA-BSDot (6 μg/mL) or ActD (5 μg/mL). After 6 h, cells were harvested, washed once using ice-cold PBS, lysed directly in an SDS sample buffer [50 mM Tris·HCl (pH 6.8), 2% SDS, 10% glycerol, and 2.5% β-mercaptoethanol], and heated for 5 min. Samples were resolved under reducing conditions for 2.5 h at 75 V using SDS/polyacrylamide gels. Proteins were then transferred onto PVDF membranes for 1 h at 20 V using a semidry system. Blots were blocked for 1 h in TBS-T [10 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween] that contained 0.1% sodium azide and 5% nonfat dried milk. Blots were then probed using a monoclonal antibody against caspase-3 (1:500) (Cell Signaling) and a polyclonal antibody to β-actin (1:20,000) (Sigma) overnight. Reactions were detected using a suitable secondary antibody (1:1,000) conjugated to horseradish peroxidase (Santa Cruz) and visualized using an enhanced chemiluminescence solution (Pierce).

DNA Fragmentation. PMs (5×10^5 cells per well) were either treated or untreated with Q-VD-fmk (20 μM) for 1 h and stimulated with ActD (5 μg/mL) for ~12 h or stimulated with empty DOTAP or FLA-BSDot for 3 h. After stimulation, samples were collected and centrifuged at 200 × *g* at 4 °C for 5 min. Both the supernatant and adherent cells were collected and resuspended in 70% ethanol for fixation. Once fixed, the suspensions were washed twice with PBS and centrifuged at 400 × *g* at 4 °C for 5 min. A DNA extraction buffer was added during the second wash. Cells were incubated at room temperature (RT) for 5 min, centrifuged at 400 × *g* at 4 °C for 5 min, and resuspended in a DNA staining solution that contained propidium iodide (PI). RNase was then added to each sample, and the cells were incubated at RT for 30 min in the dark. Samples were analyzed by flow cytometry to determine PI incorporation.

Caspase-7 and Caspase-9 Activity Fluorimetric Assay. The activity of caspase-7 and caspase-9 was evaluated as described previously (11). Briefly, cell-free extracts were obtained from macrophages cultures stimulated with ActD (5 μg/mL) or with FLA-BSDot. Cells were washed with PBS and detached using 2 mM trypsin in PBS (Gibco). Four million cells were washed with sterile PBS by centrifugation (250 × *g* for 5min at 4 °C), and cell pellet was lysed in 50 μL of ice-cold lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 10 μg/mL leupeptin, 5 μg/mL aprotinin, and 100 μM PMSF]. Cell lysates were incubated on dry ice for 1 min followed by 10 min incubation at 4 °C, and supernatants were harvested after centrifugation at 15,000 × *g* for 15min at 4 °C. Fifty microliters of lysates were plated on a 96-well flat-bottom microplate containing 100 μL of caspase-7 (Ac-VDVAD-AFC; Kamiya Biomedical) or caspase-9 (Ac-LEHD-AFC; Kamiya Biomedical) fluorigenic substrates at 20 μM final concentration. Pan-caspase inhibitor z-VAD (R&D) and caspase-9 inhibitor LEHD-FMK (Kamiya Biomedical) were used in duplicates samples as controls of caspases activity. Samples were read on a spectrofluorometer (SpectroMax, GEMINI XS; Molecular Devices) at 40-nm excitation and 505-nm emitted light. Results represent relative fluorescence units.

Microbicidal Activity. PMs from C57BL/6 and caspase-1/11$^{-/-}$ mice were incubated at 3×10^5 cells per well in a 48-well plate and were either infected with the *Salmonella* Typhimurium WT 1412 strain or the flagellin-deficient 2157 strain at a multiplicity of infection of 5:1 at 37 °C, as described previously (12). The plates containing infected macrophages were centrifuged at 290 × *g* for 10 min to allow the same rate of ingestion of the two *Salmonella* Typhimurium strains by the macrophages. After 60 min, the cultures were washed and incubated with RPMI-1640 medium supplemented with 100 μg/mL gentamicin to eliminate any extracellular bacterial growth. After 1.5 h of incubation, the medium containing gentamicin was replaced with RPMI-1640 medium supplemented with 10 μg/mL gentamicin in the presence or absence of FliCDot (6 μg/mL). After 1–6 h of infection, cells were lysed with sterile water for 10 min, diluted in LB medium, and plated to determine the bacterial colony growth. The number of colony-forming units (CFUs) represents the number of bacteria present in macrophages before cell lysis. To evaluate in vivo microbicidal activity, C57BL/6 and TLR5$^{-/-}$ mice were infected i.p. with either WT or flagellin-deficient *Salmonella* Typhimurium strains (5×10^3 bacteria per mouse). Mice were i.p.-inoculated or not with purified *Salmonella* Typhimurium FliC flagellin (5 μg per mouse), 3 h after infection. At 6 h of infection, peritoneal lavages were obtained and plated to determine the CFU numbers recovered from the peritoneal cavity.

Recombinant Caspase-1 and Cathepsin B Activity Assay. The activity of recombinant activated caspase-1 (Sigma) and cathepsin B (Sigma) was measured using fluorogenic substrates in a spectrofluorimeter F-7000 (Hitachi). Caspase-1 (0.1 units per reaction) was assayed in a 50 mM Hepes buffer (pH 7.4) with DTT (5 mM) using the substrate Abz-YVADNQ-EDDnp (10 μM). Cathepsin B (10 nM) was assayed in a 100 mM sodium acetate buffer (pH 5.5) with DTT (5 mM) using the fluorogenic substrate Z-FR-MCA (100 μM) (Sigma). The enzymes were preincubated for 5 min with the specific cathepsin B inhibitor CA074-Me (ranging from 1 nM to 100 μM), at 37 °C, before the substrate addition. The fluorescence was measured for 20 min and the slope was represented in relative fluorescence units. The assays were performed in triplicates.

Statistical Analysis. Experimental groups were always performed in duplicate or triplicate. Data are presented as the mean values ± SEM. Statistical analysis was performed using one-way or two-way ANOVA, followed by Bonferroni test. Differences between experimental groups were considered significant for $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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Fig. S1. Only cytosolic flagellin induces IL-1β secretion and macrophage lysis. (*A*) IL-1β levels determined in culture supernatants from PMs derived from C57BL/6 (WT) mice stimulated with 1, 3, or 6 μg/mL of purified flagellin from *B. subtilis* in its free form (FLA-BS) or inserted into DOTAP (FLA-BSDot) after 6 h using an ELISA kit. Numbers represent the means ± SEM ($n = 4$). Data are shown as the means of two independent experiments. (*B*) IL-6 levels determined in culture supernatants from nonprimed stimulated PMs after 6 h using an ELISA kit. Numbers represent the means ± SEM ($n = 2$). Data are representative of two independent experiments. (*C*) LDH levels quantified in culture supernatants from nonprimed stimulated PMs after 6 h using a LDH kit. Numbers represent the mean ± SEM ($n = 4$). Data are shown as the means of two independent experiments. (*D*) Cell viability evaluated in nonprimed stimulated PMs cultures at 6 h poststimulation using a MTT assay. Numbers represent the means ± SEM ($n = 4$). Data are shown as the means of two independent experiments. (*E*) Cytotoxicity was assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining in nonprimed stimulated PMs. Numbers represent the means ± SEM of at least 10 images per treatment. Data are representative of multiple independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control group. $^{\iota\iota}P < 0.01$; $^{\iota\iota\iota}P < 0.001$ compared with other concentrations of stimuli in the same group.

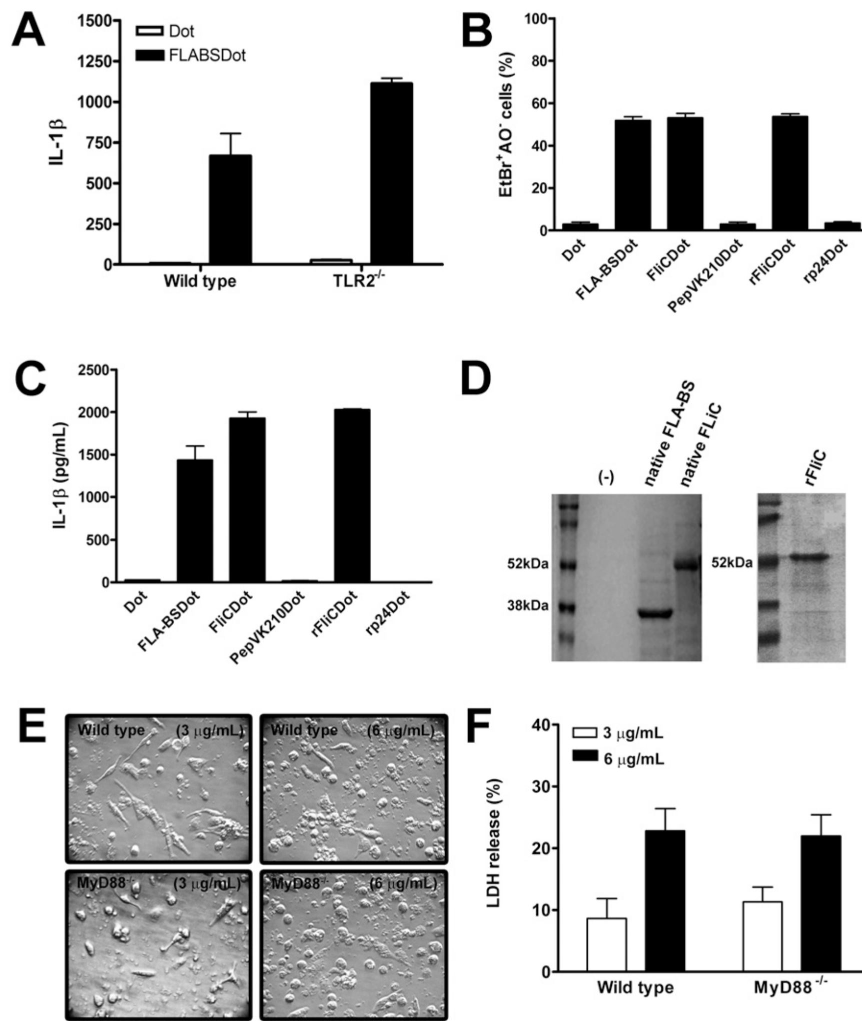

Fig. S2. Cytosolic flagellin induces MyD88-independent cell lysis and TLR2-independent IL-1β secretion. (*A*) IL-1β levels determined in culture supernatants from PMs derived from WT and TLR2-deficient mice stimulated with 3 μg/mL purified flagellin from *B. subtilis* inserted into DOTAP (FLA-BSDot) after 6 h using an ELISA kit. Numbers represent the means ± SEM ($n = 2$). (*B*) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO and EtBr staining after 6 h in WT PMs cultures stimulated with native purified flagellin from *B. subtilis* (3 μg/mL) or *Salmonella* Typhimurium (5.4 μg/mL), with unrelated peptide from CS protein of *P. vivax* (0.38 μg/mL) or with recombinant flagellin from *Salmonella* Typhimurium (5.4 μg/mL) or p24 protein (2.75 μg/mL) inserted into DOTAP (FLA-BSDot, FLiCDot, PepVK210Dot, rFLiCDot, and rp24Dot, respectively). Data are presented as means ± SEM of at least seven images, with an original magnification of 400 × per treatment. Data are representative of two independent experiments. (*C*) IL-1β levels determined in culture supernatants derived from PMs after 6 h using an ELISA kit. All data are presented as means ± SEM ($n = 2$). Data are representative of two independent experiments. (*D*) SDS/PAGE gel for native flagellin from *B. subtilis* or *Salmonella* Typhimurium (*Left*) and recombinant flagellin from *Salmonella* Typhimurium (*Right*). (*E*) Representative light field micrographs of FLA-BSDot–stimulated C57BL/6 (WT) and MYD88-deficient PMs at 6 h. All micrographs represent an original magnification of 400× . (*F*) LDH levels determined in culture supernatants from FLA-BSDot-stimulated WT and MYD88-deficient PMs after 6 h using a LDH kit. Numbers represent the means ± SEM ($n = 4$). Data are shown as the means of two independent experiments.

Fig. S3. Cell death induced by cytosolic flagellin occurs in the absence of NLRC4/ASC-caspase-1/11 axis. (*A*) IL-1β levels determined in culture supernatants from PMs derived from C57BL/6 (WT) or caspase-1/11, Asc, and Nlrc4-deficient mice stimulated with 6 μg/mL purified flagellin from *B. subtilis* inserted into DOTAP (FLA-BSDot), after 6 h using an ELISA kit. Numbers represent the means ± SEM ($n = 2$). ***$P < 0.001$ compared with knockout groups. (*B*) LDH levels determined in culture supernatants from nonprimed PMs by LDH kit. Numbers represent the means ± SEM ($n = 2$). (*C*) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining. All data are presented as means ± SEM of at least seven images per treatment. **$P <$ 0.01 FLA-BSDot/Nlrc4$^{-/-}$ compared with the WT group; ***$P < 0.001$ FLA-BSDot/caspase-1/11$^{-/-}$ group compared with WT group. All data are representative of four (C57BL/6 vs. caspase-1/11$^{-/-}$) or two independent experiments. (*D*) LDH levels determined in culture supernatants from LPS-primed BMDMs derived from WT, caspase-1/11, and Nlrc4-deficient mice stimulated with 6 μg/mL FLA-BS or FLA-BSDot, at 6 h using a LDH kit. (*E*) IL-1β levels determined in culture supernatants from LPS-primed BMDMs. All data are presented as means ± SEM ($n = 2$). *$P < 0.05$ FLA-BSDot/Nlrc4$^{-/-}$ compared with the WT group; ***$P < 0.001$ compared with the knockout groups.

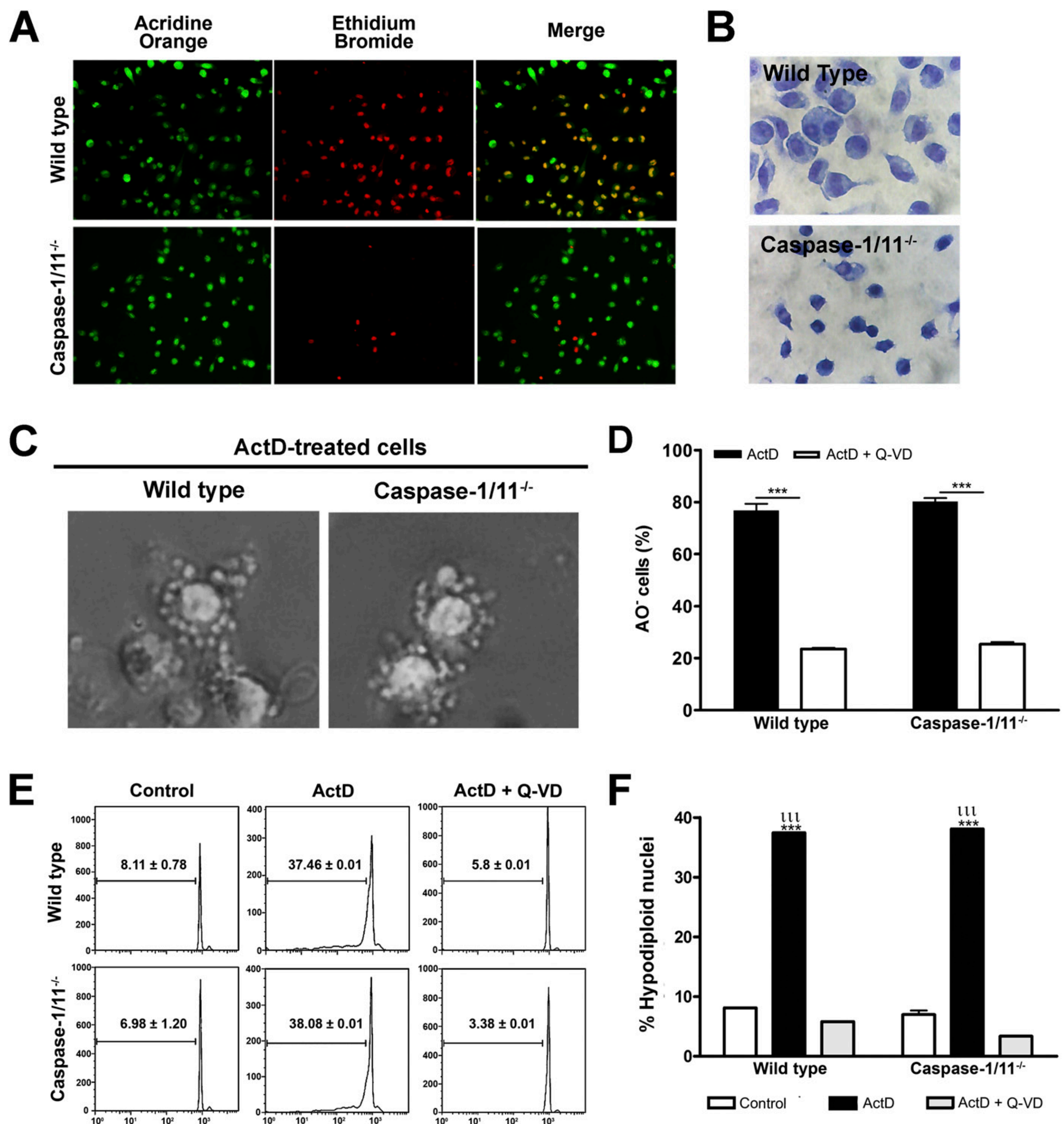

Fig. S4. Caspase-1/11–independent cell death induced by cytosolic flagellin displays apoptotic characteristics. (*A*) Pore-formation assay by double staining with EtBr and AO performed in FLA-BSDot–stimulated PMs (3 μg/mL). AO-stained PMs (green) show total viable cells in each field, and EtBr (red) indicates permeabilized PMs. At right are merged images. Micrographs represent an original magnification of 400×. (*B*) FLA-BSDot-stimulated PMs were stained with Giemsa to assess cell morphology. (*C*) Representative light field micrographs of ActD-stimulated PMs (5 μg/mL) derived from C57BL/6 (WT) or caspase-1/11–deficient mice at 12 h. Micrographs represent an original magnification of 400×. (*D*) Cytotoxicity assessed as the percentage of AO-negative cells in fluorescence micrographs according to AO/EtBr staining of ActD-stimulated WT and caspase-1/11–deficient PMs, at 12 h. Cells were either treated or untreated with Q-VD-fmk (20 μM) 1 h before stimulation. Data are presented as means ± SEM (n = 10). (*E* and *F*) Percentage of hypodiploid nuclei evaluated by flow cytometry according to PI incorporation in ActD-treated cells. ***$P < 0.001$ compared with the pretreated group; ιιι$P < 0.001$ compared with the control group.

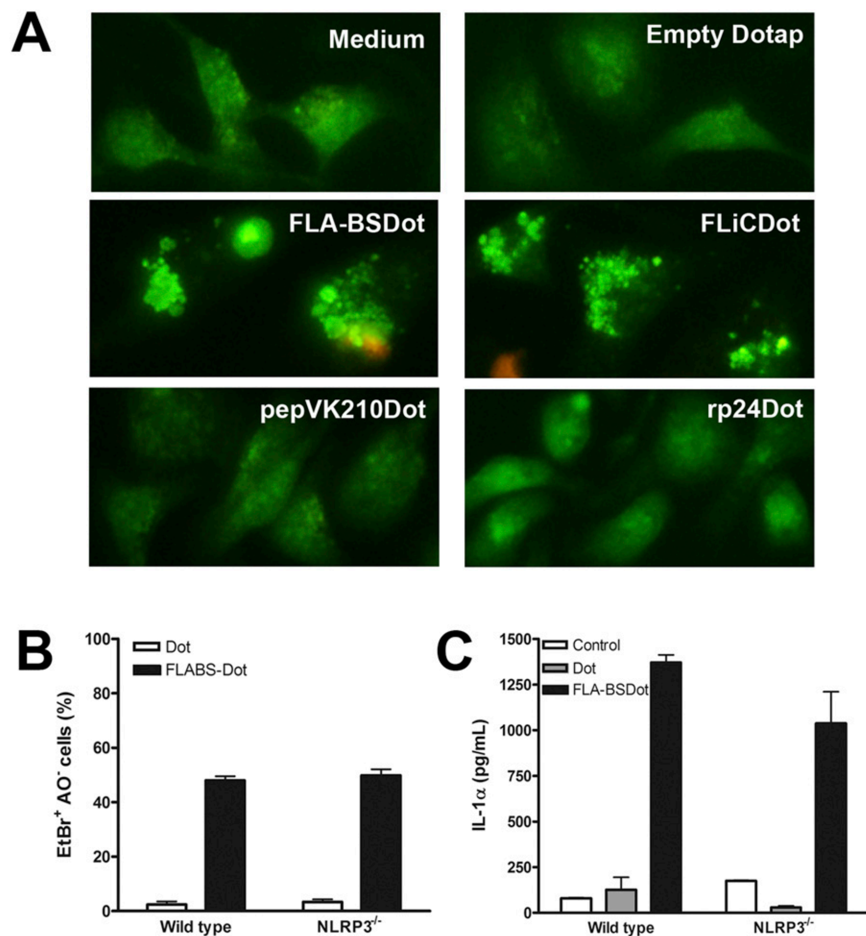

Fig. S5. Only cytosolic flagellin induces lysosomal acidification and macrophage death that is independent of NLRP3. (*A*) Representative fluorescence micrographs of AO-stained PMs derived from C57BL/6 (WT) mice stimulated with flagellin from *B. subtilis* (3 μg/mL) or *Salmonella* Typhimurium (5.4 μg/mL), with nonrelated peptide from CS protein of *P. vivax* (0.38 μg/mL) or with p24 protein (2.75 μg/mL) inserted into DOTAP (FLA-BSDot, FLiCDot, PepVK210Dot, and rp24Dot, respectively). The high cytoplasmic green fluorescence in PMs cytosol is an indicative of lysosomal acidification. Micrographs represent an original magnification of 400×. (*B*) Cytotoxicity assessed as the percentage of AO-negative cells in fluorescence micrographs according to AO/EtBr staining of FLA-BSDot–stimulated WT and NLRP3-deficient PMs, at 6 h. Data are presented as means ± SEM ($n = 6$). (*C*) IL-1α levels were determined in culture supernatants from PMs derived from C57BL/6 (WT) or NLRP3-deficient mice stimulated with 3 μg/mL of FLA-BSDot, after 6 h using an ELISA kit. Numbers represent the mean ± SEM ($n = 2$).