SUPPLEMENTAL MATERIAL

Broz et al., http://www.jem.org/cgi/content/full/jem.20100257/DC1

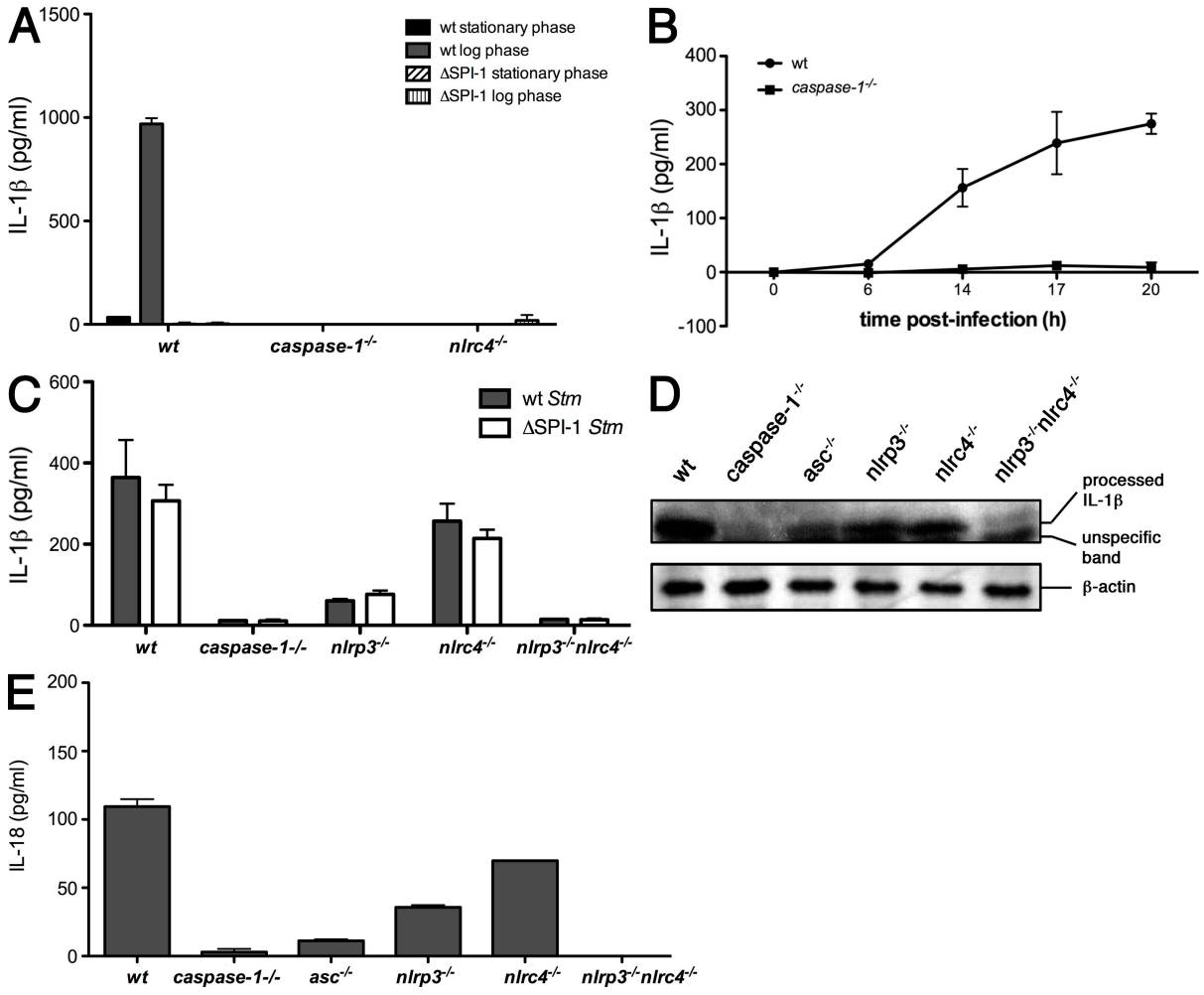

Figure S1. SPI-1–dependent and –independent inflammasome activation. (A) IL-1β released from WT, *caspase-1*$^{-/-}$, and *nlrc4*$^{-/-}$ BMDMs infected with WT or SPI-1 mutant *Stm* in different growth phases for 30 min. Macrophages were cultured in 0.1 µg/ml LPS for 16 h before infection to induce pro–IL-1β expression because the SPI-1 T3SS induces rapid cell death that prevents induction of pro–IL-1β expression. *Stm* was grown either overnight at 37°C (stationary phase) or subcultured 1/50 for 3 h at 37°C (logarithmic phase) to induce SPI-1 gene T3SS expression. (B) IL-1β released from WT and *caspase-1*$^{-/-}$ BMDMs infected with WT *Stm* grown to stationary phase. (C) IL-1β release from WT, *caspase-1*$^{-/-}$, *nlrp3*$^{-/-}$, *nlrc4*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs infected for 17 h with either WT *Stm* grown to stationary phase or isogenic SPI-1 mutant *Stm*. (D) Western blot analysis of IL-1β processing in culture supernatants from WT, *caspase-1*$^{-/-}$, *asc*$^{-/-}$, *nlrc4*$^{-/-}$, *nlrp3*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs infected with WT *Stm* for 17 h. Band intensities were determined with Image J1.33 software and are displayed as a percentage of the WT band intensity. Equal loading was controlled for by Western blotting for β-actin in the corresponding cell lysates. (E) IL-18 release from WT, *caspase-1*$^{-/-}$, *asc*$^{-/-}$, *nlrp3*$^{-/-}$, *nlrc4*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs infected with WT *Stm* for 17 h. Data are representative of two (A, C, D, and E) to three (B) independent experiments. Error bars represent the mean SD of triplicate wells.

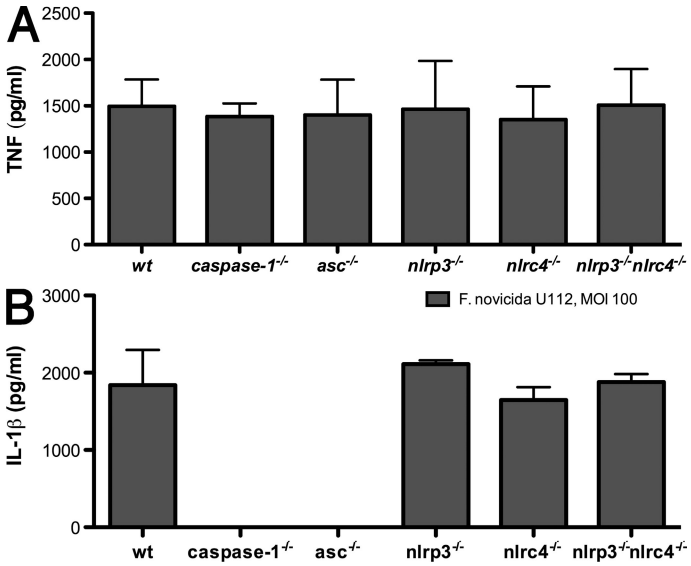

Figure S2. Macrophages release comparable levels of TNF when infected with *Stm* and respond normally to *F. novicida* infections. (A) TNF release from WT, *caspase-1*$^{-/-}$, *asc*$^{-/-}$, *nlrp3*$^{-/-}$, *nlrc4*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs infected with WT *Stm* for 17 h. (B) IL-1β release from WT, *caspase-1*$^{-/-}$, *asc*$^{-/-}$, *nlrp3*$^{-/-}$, *nlrc4*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs infected with *F. novicida* U112 for 5.5 h. Data are representative of two (A) and three (B) independent experiments. Error bars represent the mean SD of triplicate wells.

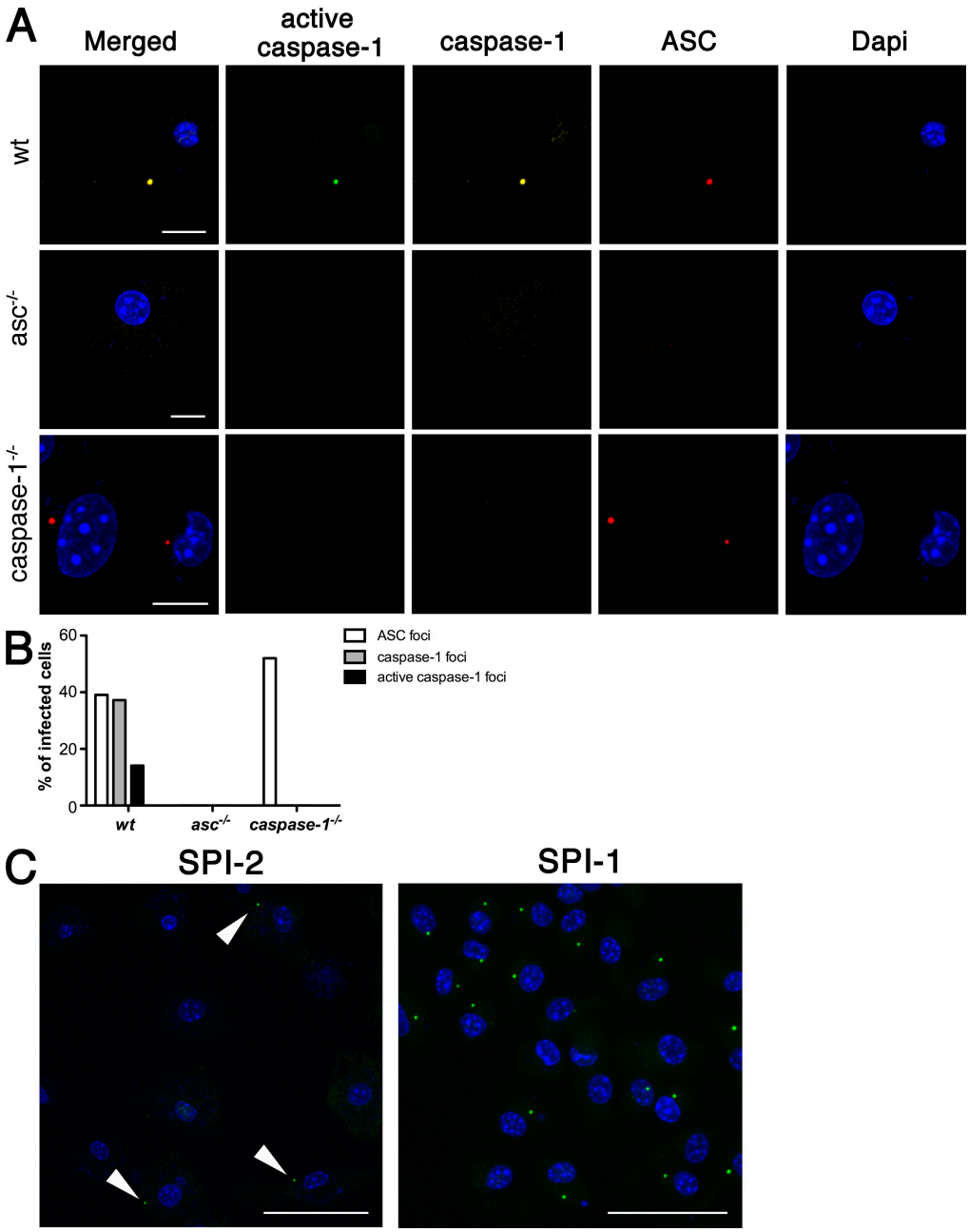

Figure S3. ASC foci are formed during Stm SPI-1–mediated inflammasome activation. (A) Fluorescence microscopy of WT, *asc*$^{-/-}$, and *caspase-1*$^{-/-}$ BMDMs infected with WT *Stm* expressing the SPI-1 T3SS for 1.5 h, and then stained for active caspase-1 (with FAM-YVAD-FMK), caspase-1, ASC, and DNA (with DAPI). (B) Percentage of infected cells from A containing foci of ASC, caspase-1, and active caspase-1. Images in A were acquired at 63× magnification. Cell counts in B, with 500 cells per sample, were determined at 40× magnification. Representative images and counts from two independent experiments are shown. (C) Low magnification images of WT macrophages infected with *Stm* visualizing the extent of ASC focus formation during SPI-2– and SPI-1–mediated inflammasome activation. Arrowheads indicate ASC foci formed during SPI-2–mediated inflammasome activation. Images in C were acquired at 40× magnification and are representative of at least five independent experiments. Bars, 50 µm.

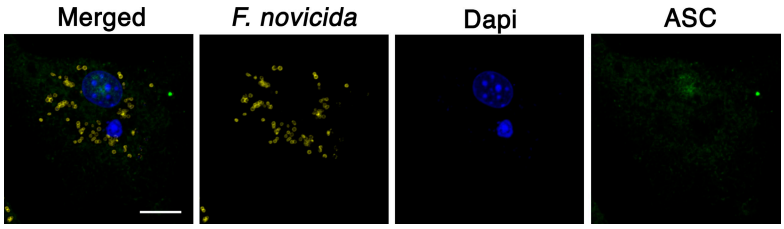

Figure S4. ASC foci are also formed during *F. novicida*–mediated inflammasome activation. Fluorescence microscopy of WT BMDMs infected with *F. novicida* U112 at an MOI of 100 for 7 h and stained for *F. novicida*, DNA (with DAPI), and ASC. Macrophages were pretreated with 0.1 µg/ml LPS for 16 h. Images were acquired at 63× magnification. Images are representative of four independent experiments. Bar, 10 µm.

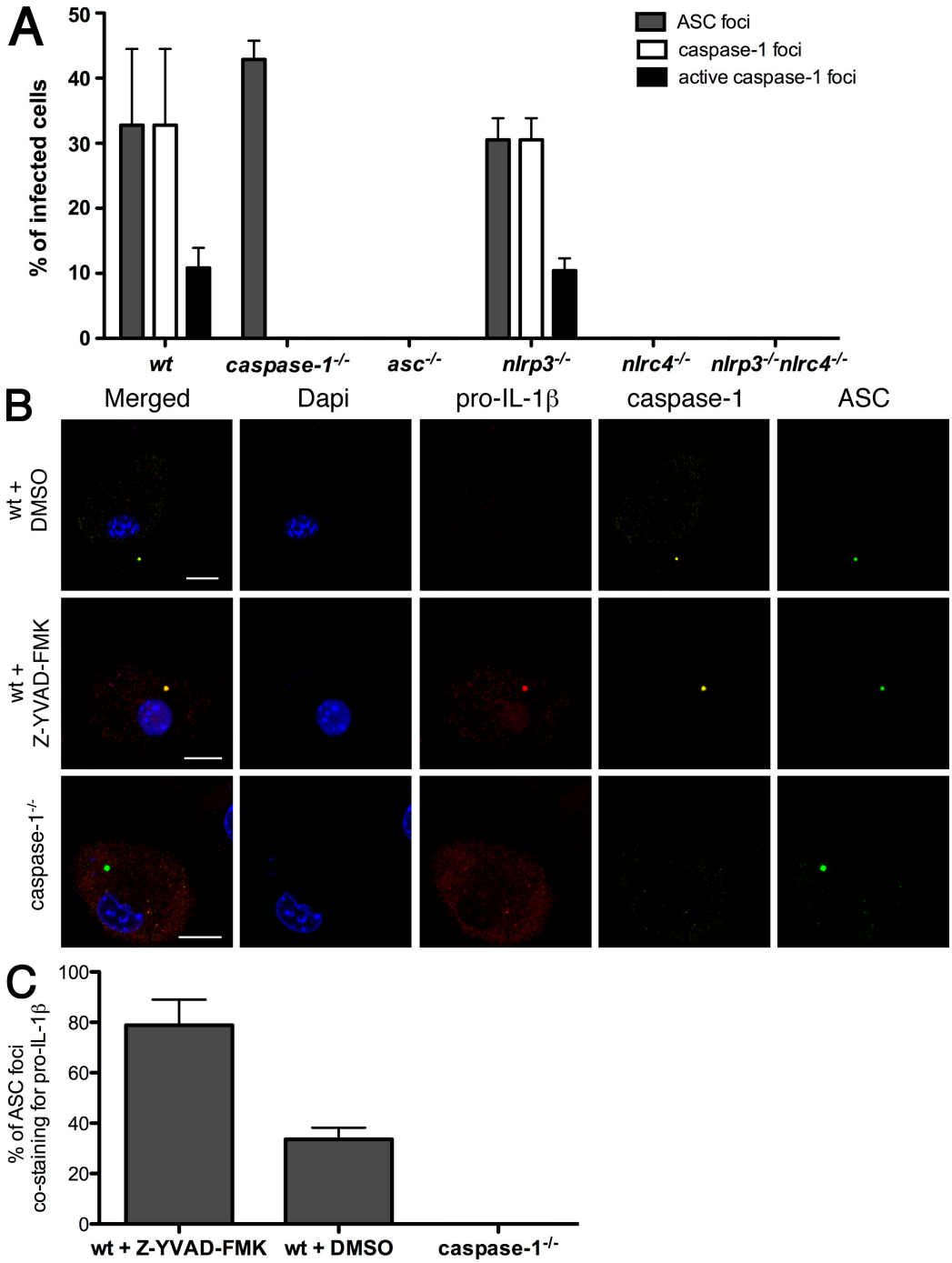

Figure S5. Characterization of ASC foci formed during SPI-1–mediated inflammasome activation. (A) WT, *caspase-1*$^{-/-}$, *asc*$^{-/-}$, *nlrp3*$^{-/-}$, *nlrc4*$^{-/-}$, and *nlrp3*$^{-/-}$*nlrc4*$^{-/-}$ BMDMs were infected with WT *Stm* expressing the SPI-1 T3SS for 1.5 h. Bars indicate the percentage of cells containing a focus positive for ASC, caspase-1, or active caspase-1. Numbers represent two independent fluorescence microscopy experiments, with at least 500 cells counted in each experiment. Cell counts were determined at 40× magnification. (B) Fluorescence microscopy of WT and *caspase-1*$^{-/-}$ BMDMs infected with WT *Stm* expressing the SPI-1 T3SS for 1.5 h. Cells were stained for DNA (with DAPI), pro-IL-1β, caspase-1, and ASC. Where indicated, WT BMDMs were infected in the presence of the caspase-1 inhibitor Z-YVAD-FMK or DMSO (vehicle control). (C) The percentage of ASC foci in B that costained for pro-IL-1β. Numbers are representative of two independent experiments. A mean of 50 ASC foci were counted in each experiment. Images and cell counts were acquired at 63× magnification. Error bars in A and C represent the mean SD of duplicate experiments. Bars, 10 µm.

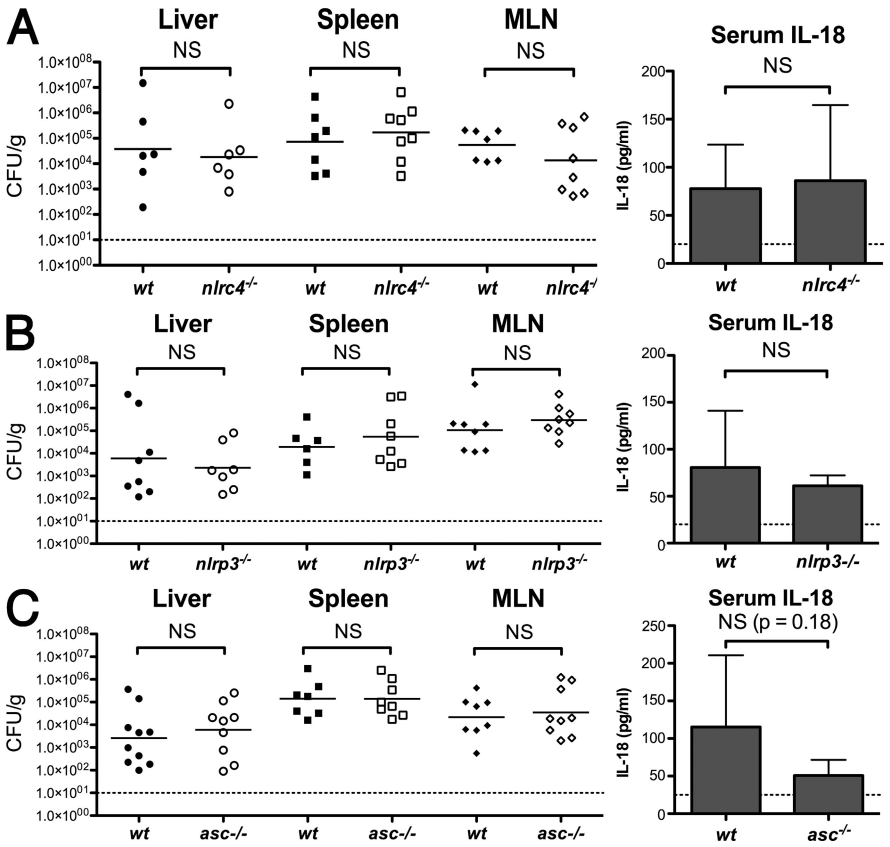

Figure S6. Infections of WT mice and single deficiencies in NLRC4, NLRP3, and ASC with *Stm*. Bacterial loads and serum IL-18 levels in WT and *nlrc4*$^{-/-}$ (A), WT and *nlrp3*$^{-/-}$ (B), and WT and *asc*$^{-/-}$ mice (C), infected with WT *Stm*. Mice were challenged orally with 2.4 × 10^7 CFU of WT *Stm*. Liver, spleen, and mesenteric lymph nodes (MLN) were collected at day 5 after infection. Organ homogenates were diluted and plated to determine cfu per gram of tissue. Blood was collected at day 4 after infection and serum IL-18 was determined by ELISA. Results are representative of two independent experiments, each done with groups of six to eight mice of each genotype. Horizontal bars represent the mean bacterial load. Error bars represent standard deviation. Statistical significance was determined using the unpaired Mann-Whitney *U* test. NS, no statistical significance.