The AIM2 inflammasome is critical for innate immunity against *Francisella tularensis*

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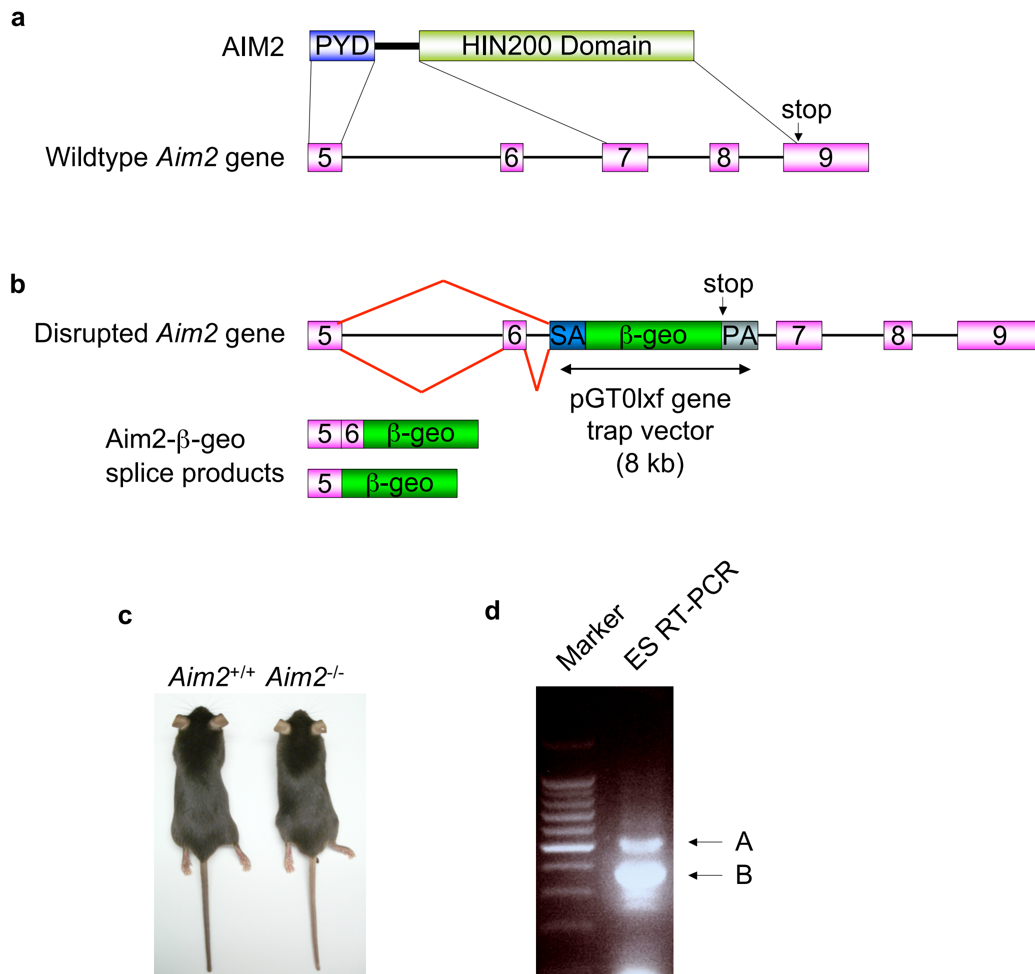
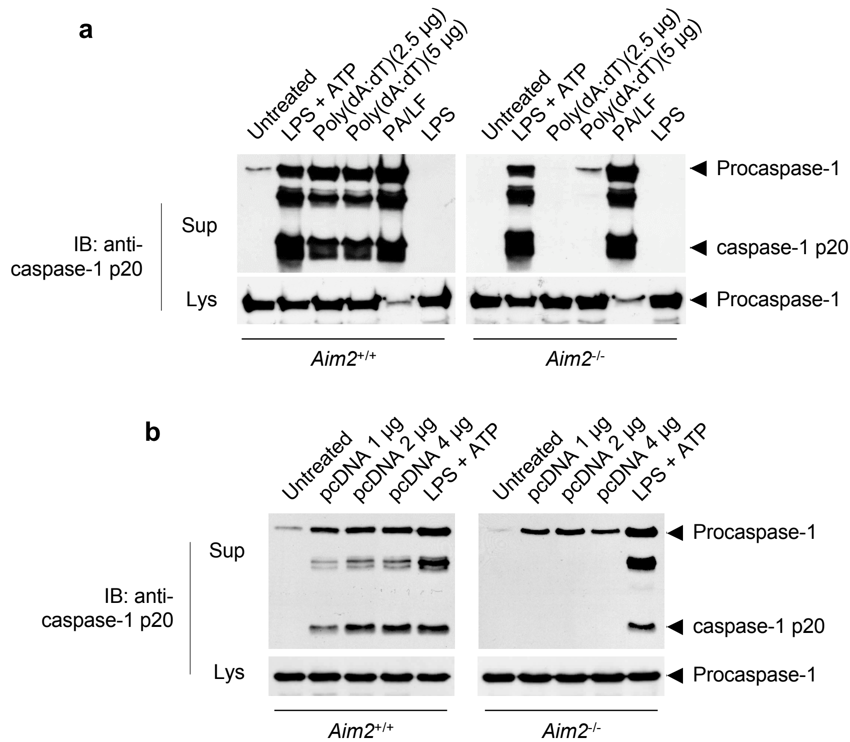
Supplementary Figure 1

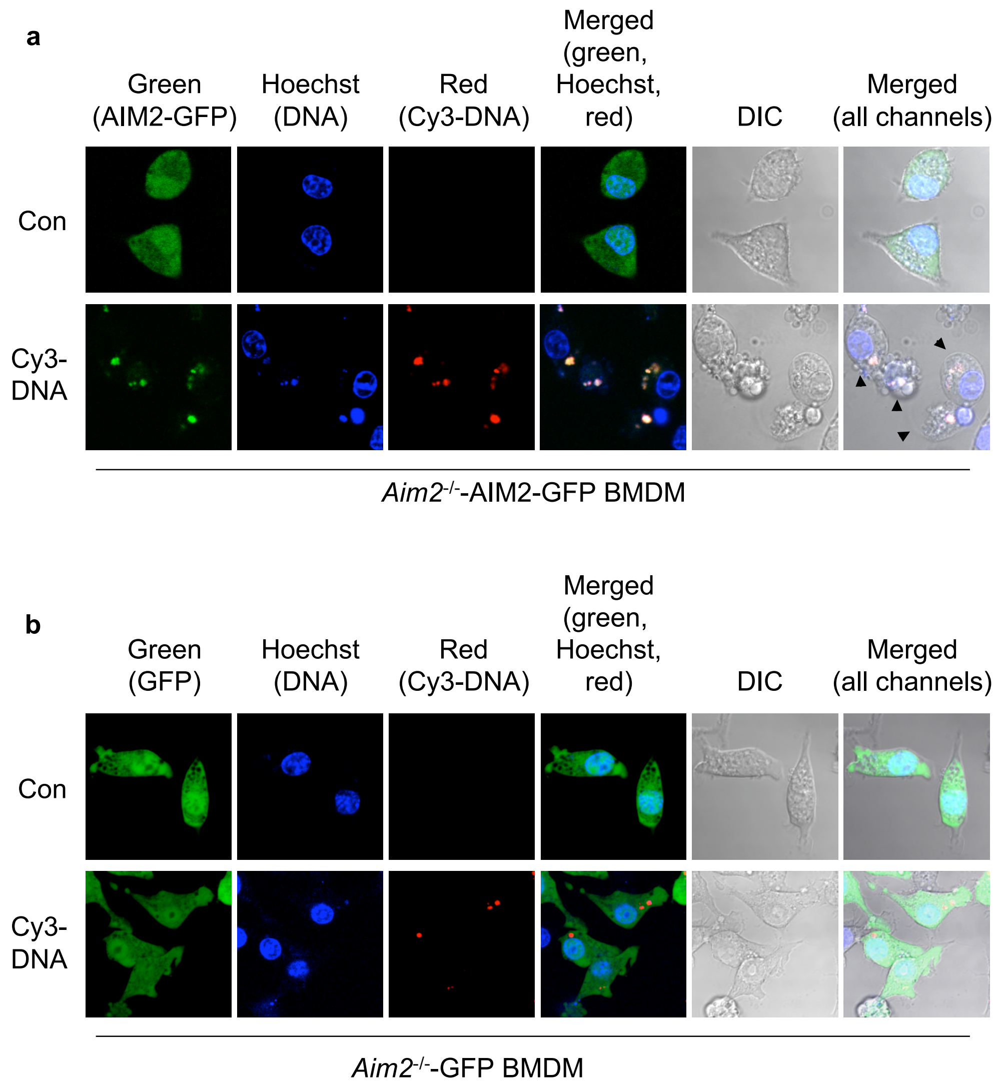


Supplementary Figure 1 Generation of AIM2-deficient mice.
(**a**) Schematic representation of the wildtype *Aim2* gene (lower diagram) showing its exons 5-9 (boxes) and the intronic regions (lines) between them. Exon 5 encodes the PYD, whereas exons 7-9 encode the HIN200 DNA binding domain of AIM2 protein (upper diagram). Exon 6 encodes the linker region between the PYD and HIN200 domains of AIM2. (**b**) Schematic representation of the pGT0lxf gene trap vector insertion into the intron following exon 6 of the *Aim2* gene. The splice acceptor (SA) of the pGT0lxf gene trap vector interrupts normal splicing of the *Aim2* gene allowing the downstream vector sequence to be transcribed. The gene trap cassette contains a combination of selection and reporter constructs (β-geo) and is followed by a polyadenylation (PA) signal, which causes a stop in translation. The insertion leads to the synthesis of a severely truncated fusion protein (AIM2-β-geo), which lacks the entire DNA binding HIN200 domain of AIM2 fused to b-geo reporter. (**c**) A photograph of one $Aim2^{+/+}$ and its $Aim2^{-/-}$ littermate showing normal phenotype of $Aim2^{-/-}$ mouse. (**d**) RT-PCR analysis of mRNA from the *Aim2*-genetraped ES cell clone (BayGenomics clone CSG445) with genetrap specific primers.



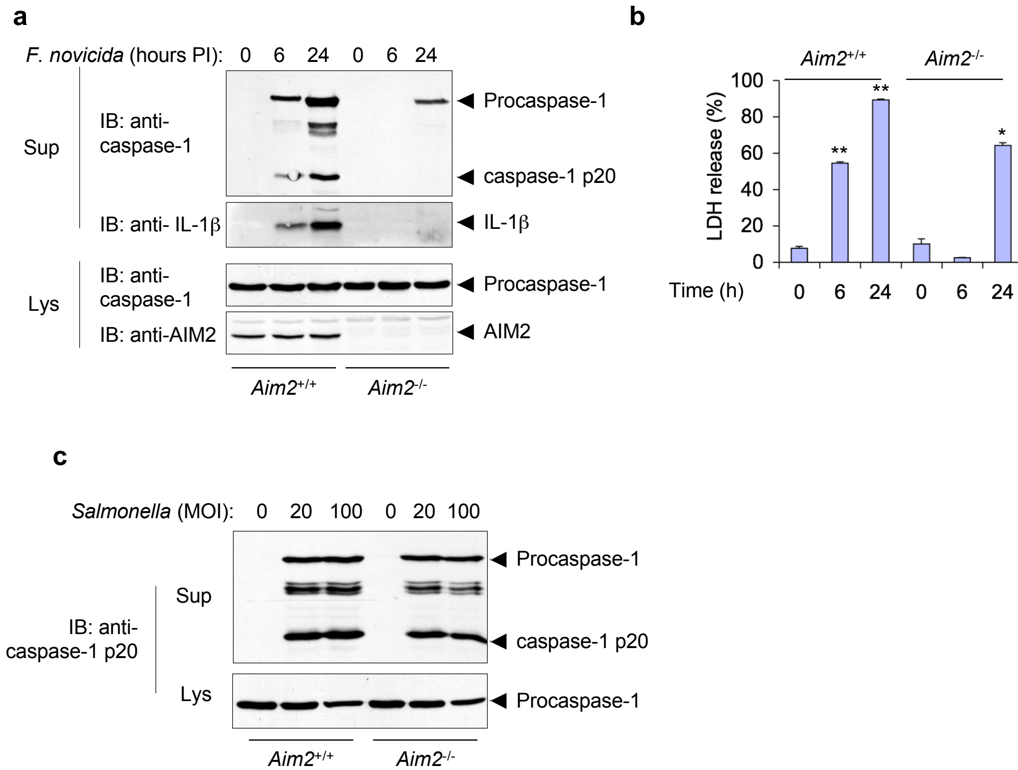
Supplementary Figure 2 AIM2 deficiency does not affect caspase-1 activation by Nlrp1 and Nlrp3 stimuli. (**a**) Immunoblots for mouse caspase-1 in culture supernatants (upper panels) and cell lysates (lower panels) of mouse *Aim2*$^{+/+}$ (left panels) or *Aim2*$^{-/-}$ (right panels) BMDMs, left untreated, or treated with LPS (500 ng/ml) for 5h followed with ATP (5 mM) for 45 min, or treated with anthrax toxin PA/LF for 3h, or transfected with 2.5 or 5 µg synthetic DNA poly(dA:dT) for 3h, as indicated. Note the activation of caspase-1 by LPS plus ATP (Nlrp3 stimulus) or anthrax toxin PA/LF (Nlrp1 stimulus) is not affected by AIM2 deficiency. (**b**) Immunoblots for mouse caspase-1 in culture supernatants (upper panels) and cell lysates (lower panels) of mouse *Aim2*$^{+/+}$ (left panels) or *Aim2*$^{-/-}$ (right panels) BMDMs, left untreated, or treated with LPS (500 ng/ml) for 5h followed with ATP (5 mM) for 45 min, or transfected with increasing amounts of pcDNA plasmid for 3h, as indicated. Sup, culture supernatants. Lys, Cell lysates

Supplementary Figure 3



Supplementary Figure 3 Reconstitution of *Aim2*$^{-/-}$-macrophages with AIM2-GFP restores their responsiveness to transfected DNA. Confocal live cell images of *Aim2*$^{-/-}$-AIM2-EGFP-N1 (**a**), or *Aim2*$^{-/-}$-EGFP-N1 (**b**) immortalized bone marrow macrophages following transfection with vehicle (control, upper panels) or Cy™3-labeled DNA (lower panels). The green (AIM2-GFP in **a**, or GFP in **b**), red (Cy™3-labeled DNA), gray (DIC), and blue (Hoechst stain, nucleus and DNA) channels, and the merged channels are indicated. The arrows (**a**, 6th panel from left in the lower row) indicate pyroptotic cells containing oligomerized AIM2-GFP-DNA complexes. Note the pyroptotic cell death features induced by the Cy™3-labeled DNA in *Aim2*$^{-/-}$-AIM2-EGFP-N1 macrophages (**a**, lower panels), but not in the *Aim2*$^{-/-}$-EGFP-N1 (**b,** lower panels**).** Con, control untransfected (Upper panels in **a** and **b**). Cy3-DNA, Cy3-DNA transfected (lower panels in **a** and **b**).

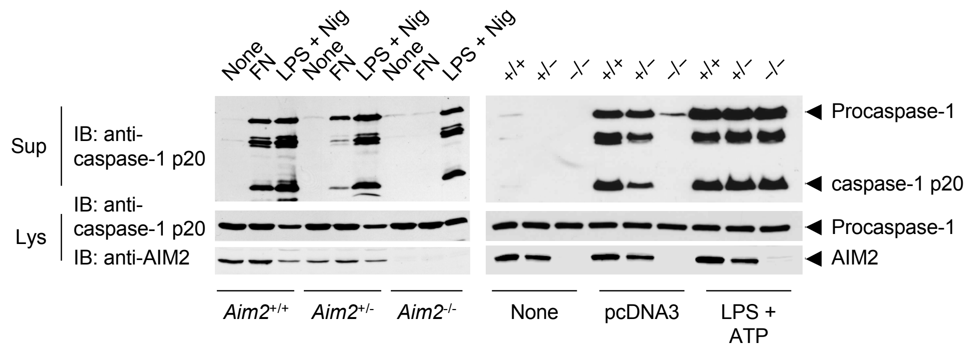
Supplementary Figure 4



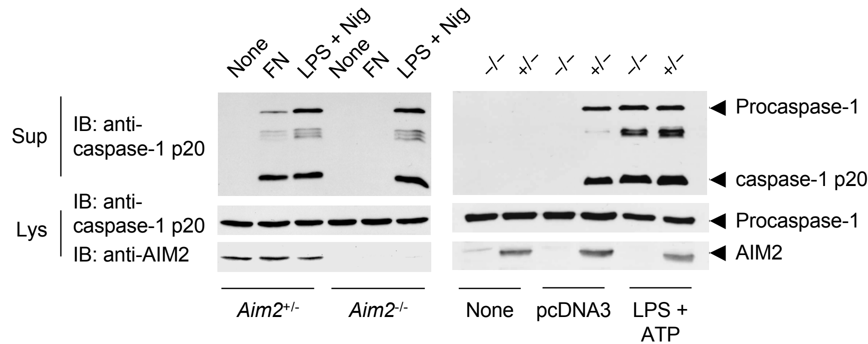
Supplementary Figure 4: Caspase-1 activation and IL-1β release require AIM2 after *Francisella*, but not *Salmonella* infection. **a,** Immunoblots for mouse caspase-1 and IL-1β in culture supernatants (upper two panels) of mouse $Aim2^{+/+}$ (1st to 3rd lanes) or $Aim2^{-/-}$ (4th to 6th lanes) BMDMs, 0, 6 or 24 hours post-infection (PI) with *F. novicida*, as indicated. The lower two panels show immunoblots for mouse caspase-1 and AIM2 in the corresponding cell lysates. Asterisks indicate significant differences between untreated and infected group (b, n = 3; *, $P<0.005$; **, $P<0.001$). **b**, Percentages of LDH release into the culture supernatants of the BMDMs described in (**a**) above. **c**, Immunoblot for mouse caspase-1 in culture supernatants (upper panel) or cell lysates of mouse $Aim2^{+/+}$ (1st to 3rd lanes) or $Aim2^{-/-}$ (4th to 6th lanes) BMDMs, infected with the indicated MOIs of *Salmonella* for 3 h, as indicated.

Supplementary Figure 5

a

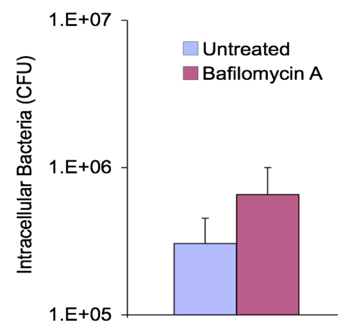


b



Supplementary Figure 5 Effect of Aim2 heterozygosity on caspase-1 activation by *Francisella* infection or transfected DNA. (**a)** Immunoblots for mouse caspase-1 in culture supernatants of macrophages derived from littermates (siblings) generated from interbreeding of heterozygous *Aim2*$^{+/–}$ parents, infected with the indicated *F. novicida* (MOI 250) or treated with LPS (500 ng/ml) for 5h followed with nigericin (2.5 μM) for 45 min, as indicated (upper left panels), or transfected with pcDNA3 (4 μg) for 6 h, or treated with LPS (500 ng/ml) for 5h followed with ATP (5 mM) for 45 min as indicated (upper right panels). (**b**) Immunoblots for mouse caspase-1 in culture supernatants of macrophages derived from *Aim2*$^{–/–}$ and *Aim2*$^{+/–}$ littermates (siblings) generated from interbreeding of a homozygous *Aim2*$^{–/–}$ with its heterozygous *Aim2*$^{+/–}$ sibling, infected with *F. novicida* (MOI 250) or treated with LPS (500 ng/ml) for 5h followed with nigericin (2.5 μM) for 45 min, as indicated (upper left panels), or transfected with pcDNA3 (4 μg) for 6 h, or treated with LPS (500 ng/ml) for 5h followed with ATP (5 mM) for 45 min as indicated (upper right panels). Similar results were obtained with macrophages derived from two additional *Aim2*$^{–/–}$ and *Aim2*$^{+/–}$ littermates (siblings) from the same progeny of this cross between the *Aim2*$^{–/–}$ X *Aim2*$^{+/–}$ siblings in **b** (not shown). The lower panels in **a** and **b** show immunoblots for mouse caspase-1 and AIM2 in the corresponding cell lysates.

Supplementary Figure 6



Supplementary Figure 6 Intracellular growth of *F. novicida* in *Aim2*$^{-/-}$ BMDMs treated with bafilomycin. *F. novicida* were inoculated at an MOI of ~50 into untreated or bafilomycin-treated *Aim2*$^{-/-}$ BMDMs. After 1 h incubation at 37° C and 5 % CO2, gentamycin (50 µg/ml) was added to the medium to eliminate extracellular bacteria. The macrophage cells were lysed with 0.2 % deoxycholate at 3 h post-infection, and the lysate was plated on Cystine Heart Agar plates and incubated at 37° C for 24 h, and CFU were enumerated.

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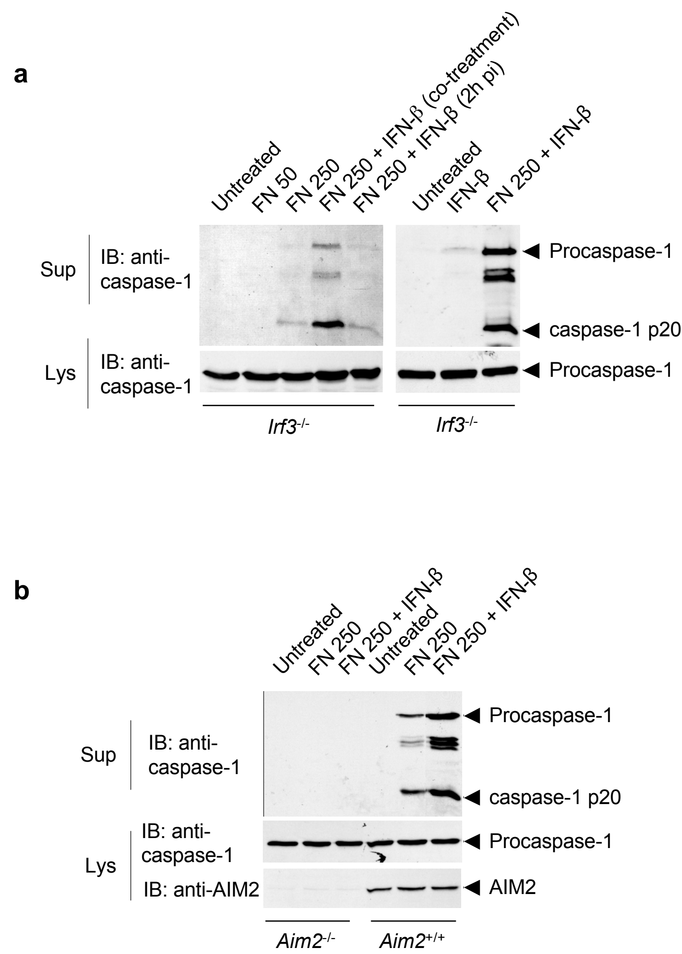
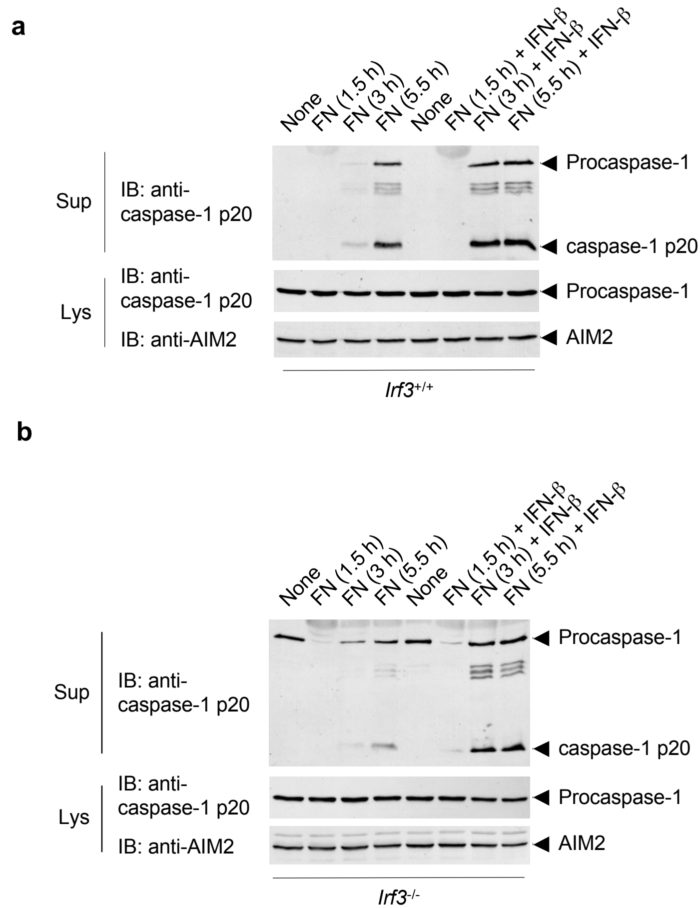
Supplementary Figure 7



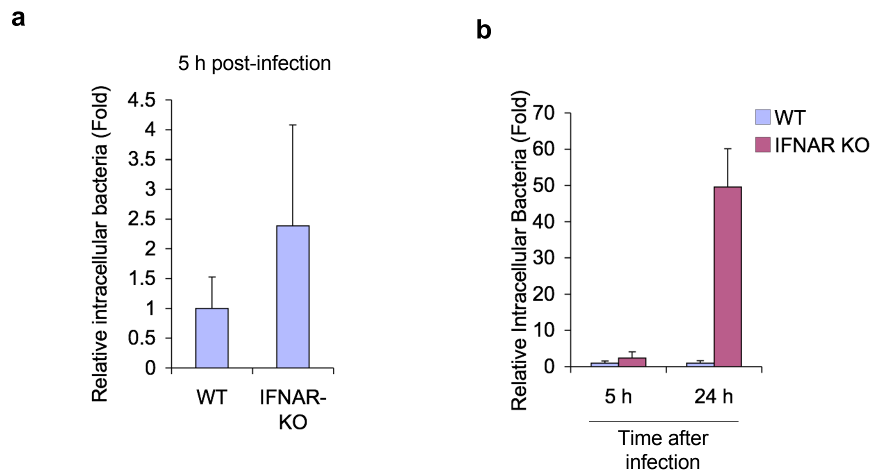
Supplementary Figure 7 *Francisella*-induced caspase-1 activation in *Irf3*$^{-/-}$ macrophages requires priming with IFN-β. (a and b) Immunoblots for mouse caspase-1 in culture supernatants of mouse *Irf3*$^{-/-}$ (a, upper panels), or *Aim2*$^{+/+}$ or *Aim2*$^{-/-}$ (b, upper panel) BMDMs, infected with the indicated MOIs of *F. novicida*, in the presence or absence of IFN-β (250 U/ml), as indicated. The lower panels show immunoblots for mouse caspase-1 and AIM2 in the corresponding cell lysates.

Supplementary Figure 8



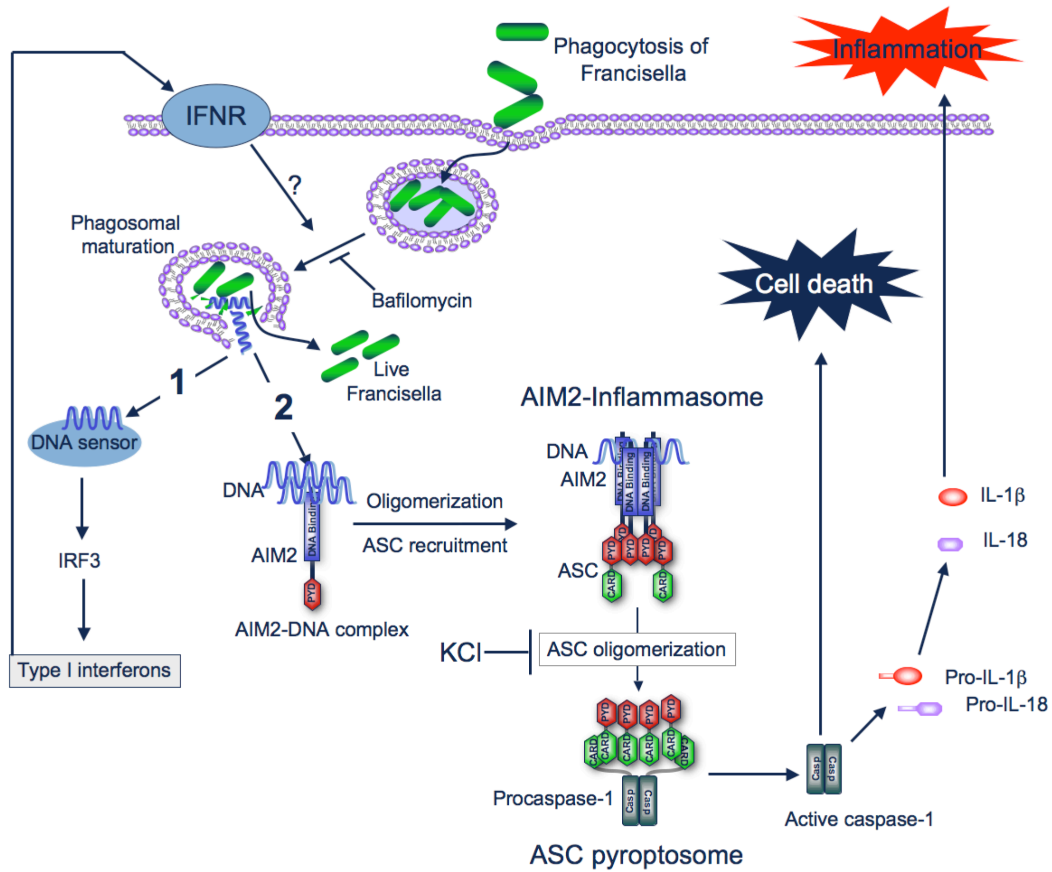
Supplementary Figure 8: Time course analysis of the effect of IFN-β priming on AIM2 inflammasome activation by *Francisella.* (**a** and **b**) Immunoblots for mouse caspase-1 in culture supernatants of mouse *Irf3*$^{+/+}$ (a, upper panel) or *Irf3*$^{-/-}$ (b, upper panel) BMDMs, untreated or treated with IFN-β for 2 h followed by infection with of *F. novicida* (MOI 250) for the indicated periods of time. The lower panels show immunoblots for mouse caspase-1 and AIM2 in the corresponding cell lysates.

Supplementary Figure 9



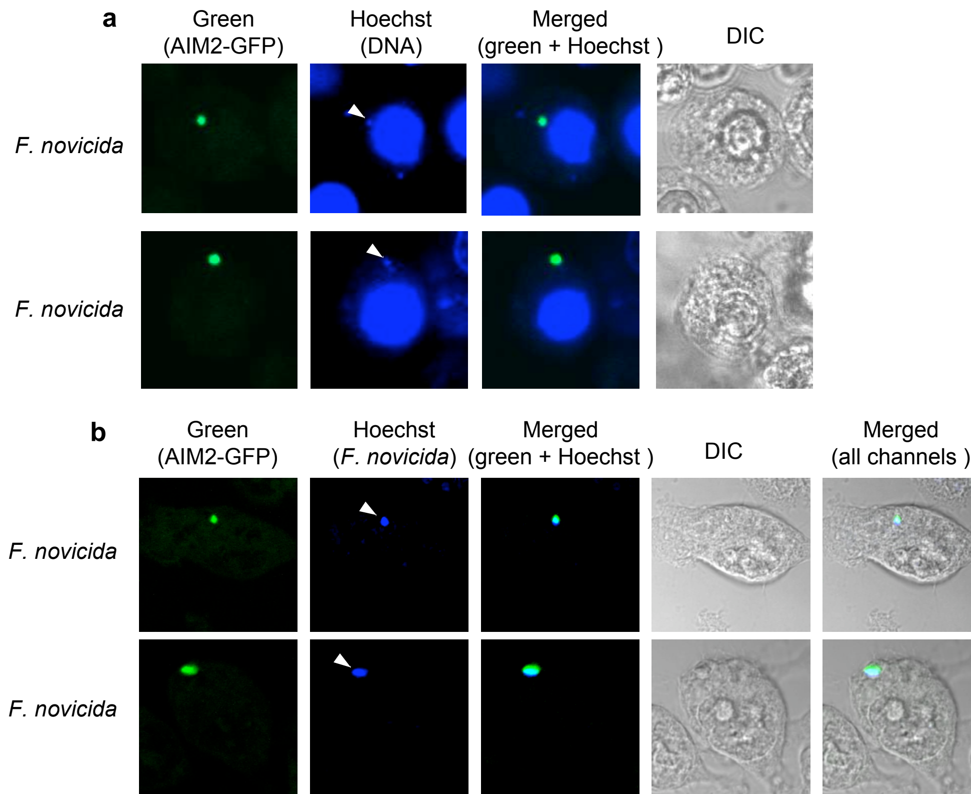
Supplementary Figure 9 Intracellular growth of *F. novicida* in WT and *Ifnar1*$^{-/-}$ BMDMs at 5 h (**a**) and 24h (**b**) post-infection. *F. novicida* were inoculated at an MOI of ~50 into WT or *Ifnar1*$^{-/-}$ BMDMs. After 1 h incubation at 37° C and 5 % CO2, gentamycin (50 µg/ml) was added to the medium to eliminate extracellular bacteria. The macrophage cells were lysed with 0.2 % deoxycholate at 3 h post-infection, and the lysate was plated on Cystine Heart Agar plates and incubated at 37° C for 5h or 24 h, and CFU were enumerated.

Supplementary Figure 10



Supplementary Figure 10 A model depicting how *Francisella* infection leads to the activation of the AIM2 inflammasome**.** See discussion for details.

Supplementary Figure 11



Supplementary Figure 11 *Francisella* DNA and AIM2 co-localize in *Francisella*-infected cells. (**a**) Enlarged confocal live cell images of *Nlrp3*$^{-/-}$-AIM2-EGFP-N1 BMDMs infected with *F. novicida* for 6 h and then stained with Hoechst stain before microscopy. The white arrows in the blue channel indicate staining of AIM2-GFP clusters with the DNA-specific blue Hoechst stain. (**b**) Enlarged confocal cell images of *Nlrp3*$^{-/-}$-AIM2-EGFP-N1 BMDM infected with Hoechst-labeled *F. novicida* for 6 h and then fixed on coverslips before confocal microscopy. The white arrow in the blue channel (lower panels) indicates the Hoechst-labeled *Francisella* cytoplasmic DNA.

SUPPLEMENTARY METHODS

Generation of AIM2 deficient mice. AIM2-deficient mice were generated by the gene trap method [13], from an ES cell clone obtained from the International Gene Trap Consortium [12]. All mice were used in experiments following protocols approved by Institutional Animal Care and Use Committee, Thomas Jefferson University. To identify ES cell clone(s) containing a gene trap disrupted mouse *Aim2* gene, we used the open reading frame of mouse *Aim2* to search the NCBI mouse gene trap sequences database (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090) with the BLAST program. This search identified one ES clone (gb|DX812645.1|) that contains the pGT0lxf gene trap vector inserted in the *Aim2* gene resulting in a transcriptional fusion product containing *Aim2* exon 5, which encodes the initiator methionine fused to ß-galactosidase/neomycin reporter, ß-geo. This *Aim2* gene trapped ES cell clone (BayGenomics clone CSG445) was obtained from the Mutant Mouse Regional Resource Centers and further verified in our lab by RT-PCR and DNA sequencing. RT-PCR analysis of mRNA from this ES clone showed that these cells express two *Aim2* transcriptional fusion products (**Supplementary Fig. 1d**). Sequence analysis of these products revealed that the minor longer splice isoform represent a fusion of *Aim2* exons 5 and 6 fused to ß-geo, whereas the major shorter splice isoform represent an alternative splice variant of *Aim2* exon 5 fused to ß-geo (**Supplementary Fig. 1b**). Both AIM2-geo fusion products thus lack the entire C-terminal oligonucleotide/oligosaccharide-binding domain (HIN200 domain) (**Supplementary Fig. 1a**). Based on these

results PCR analysis of the intronic region following *Aim2* exon 6 was performed to verify the integration of the gene trap vector in this region. This analysis revealed the gene trap vector has inserted in the intron following *Aim2* exon 6. Chimeric mice were produced by injection of the ES clone into C57BL/6 female host blastocysts by our Transgenic and Gene Targeting Facility at Kimmel Cancer Center and the resulting chimeric mice were outbred to C57BL/6 females. Three chimeric male mice transmitted the *Aim2* gene trap allele through their germ line and were used in subsequent outbreeding with C57BL/6J females to produce F1 heterozygous (*Aim2*$^{+/-}$) pups. F1 heterozygous (*Aim2*$^{+/-}$) mice were interbred to produce F2 homozygous *Aim2*$^{-/-}$ *and Aim2*$^{+/+}$ littermates. The lack of AIM2 expression in the *Aim2*$^{-/-}$ was confirmed by immunoblot analysis with an anti-mouse AIM2 polyclonal antibody.

Assay of ASC pyroptosome formation in macrophages. These experiments were performed essentially as described recently [44]. Macrophages were seeded in 6-well plates (2 x 10^6 cells/well) and then infected with *F. novicida* in 1 ml of OPTI-MEM® I medium for 6 hours in the absence or presence of different concentrations of KCl. In some experiments macrophages were transfected with poly(dA:dT) for 6h or treated with vehicle or LPS for 5h, followed by nigercin (2.5 µM) for 45 min in SF OPTI-MEM®I. The culture supernatants were collected and used for immunoblot analysis of secreted caspase-1 p20 as described above. The cells were pelleted by centrifugation and lysed in 0.5 ml buffer containing 20 mM Hepes-KOH, pH7.5, 150 mM KCl, 1 % NP40, 0.1 mM PMSF and protease

inhibitor cocktail on ice by syringing 10 X. The cell lysates were centrifuged at 6000 rpm at 4°C for 10 min. The pellets were washed 2 X with PBS and then re-suspended in 500 µl PBS. The re-suspended pellets were cross-linked with fresh disuccinimidyl suberate (DSS, 2 mM) for 30 min as described before [45], and then pelleted by centrifugation at 6000 rpm for 10 min. The cross-linked pellets were resuspended in 30 µl SDS sample buffer and fractionated on 12 % SDS-polyacrylamide gel followed by immunobloting with anti-mouse ASC antibody.

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