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

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***Francisella tularensis*, the causative agent of tularemia, infects host macrophages, which triggers production of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18. We elucidate here how host macrophages recognize *F. tularensis* and elicit this proinflammatory response. Using mice deficient in the DNA-sensing inflammasome component AIM2, we demonstrate here that AIM2 is required for sensing *F. tularensis*. AIM2-deficient mice were extremely susceptible to *F. tularensis* infection, with greater mortality and bacterial burden than that of wild-type mice. Caspase-1 activation, IL-1β secretion and cell death were absent in *Aim2*$^{-/-}$ macrophages in response to *F. tularensis* infection or the presence of cytoplasmic DNA. Our study identifies AIM2 as a crucial sensor of *F. tularensis* infection and provides genetic proof of its critical role in host innate immunity to intracellular pathogens.**

The innate immune system is an evolutionary conserved first line of defense against invading organisms. It is now well established that the innate immune system uses molecular pattern recognition to detect infection and mount an immune response to eliminate the pathogen and infected cells. Specialized receptors collectively called pathogen- or pattern-recognition receptors (PRRs) are present on the cytoplasmic or endosomal membranes or in the cell cytosol[1,2]. PRRs sense highly conserved molecular components of invading organisms known as pathogen-associated molecular patterns.

The PRR AIM2 (absent in melanoma 2) recognizes cytoplasmic double-stranded DNA through its HIN-200 domain[3–6]. AIM2 assembles a large inflammasome complex with cytosolic DNA; the complex is visible by confocal microscopy[3]. The fully assembled AIM2 inflammasome then recruits and subsequently oligomerizes the caspase-1-activating adaptor protein ASC, which activates caspase-1, leading to production of the mature cytokines interleukin 18 (IL-18) and IL-1β and subsequent cell death[3,7,8].

Despite such advances in the understanding of how cytoplasmic DNA is recognized by the AIM2 inflammasome, little is known about the precise role of the AIM2 inflammasome in the innate immune defense against intracellular microbial and viral pathogens. Of particular interest is the facultative intracellular Gram-negative bacteria *Francisella tularensis,* the causative agent of tularemia. Invasion of the cytosol by *F. tularensis* leads to the production of type I interferon as well as activation of caspase-1 and cell death[9,10]. *F. tularensis* activates the interferon response by a mechanism that is independent of signaling by plasma-membrane or endosomal Toll-like receptors, cytosolic RNA helicases RIG-I or Mda5 or biosensors Nod1 or Nod2 but requires signaling by the IRF3 transcription factor[10,11]. It has been proposed that the host cell may sense cytosolic *F. tularensis* DNA via unidentified cytosolic DNA sensors, leading to the induction of type I interferons as well as activation of caspase-1 and cell-death pathways during *F. tularensis* infection[10,11].

We describe here the generation of AIM2-deficient mice to investigate whether AIM2 has an important role in the host proinflammatory innate immune response to *F. tularensis* specifically and to cytosolic DNA in general. Our results show that AIM2-deficient macrophages were defective in caspase-1 activation, IL-1β secretion and cell death in response to cytosolic DNA or infection with *F. tularensis.* Although the AIM2 deficiency did not affect the transcriptional type I interferon response to *F. tularensis* infection, AIM2-deficient mice were more susceptible to the lethal effect of *F. tularensis* than were wild-type mice. Our results thus suggest that the type I interferon response is not sufficient on its own for protection against *F. tularensis* infection and that the AIM2 inflammasome activity is required for full innate immunity to this pathogen. Our work also indicates that among all known PRRs, AIM2 is uniquely required for the proinflammatory innate immune response to *F. tularensis*.

RESULTS

AIM2 is important for caspase-1 activation by DNA

To investigate the precise role of AIM2 in the host innate immune defense against dangerous cytosolic DNA produced by intracellular viral and microbial pathogens, we generated AIM2-deficient mice by gene-trap technology[12,13] (**Supplementary Fig. 1a**,**b**). Immunoblot analysis of spleen and bone marrow samples from an *Aim2*$^{-/-}$ mouse

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and its *Aim2*$^{+/+}$ littermate showed the presence of full-length AIM2 protein in the *Aim2*$^{+/+}$ mouse but not in the *Aim2*$^{-/-}$ littermate (**Fig. 1a**, top), which indicated that insertion of the gene-trap vector in *Aim2* resulted in disruption of this gene. Heterozygous *Aim2*$^{+/-}$ mice had lower expression of AIM2 in the spleen than did *Aim2*$^{+/+}$ mice (**Fig. 1a**, bottom), which indicated that the expression of AIM2 was dependent on gene dose. No *Aim2*$^{-/-}$ mice had any obvious phenotypic abnormalities and all were morphologically indistinguishable from their *Aim2*$^{+/-}$ or wild-type littermates (**Supplementary Fig. 1c**), which indicated that AIM2 deficiency did not have any apparent adverse effects on mouse development.

In vitro studies with bone marrow–derived macrophages (BMDMs) from *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ littermates showed that liposome-delivered DNA activated caspase-1 and induced cell death in wild-type *Aim2*$^{+/+}$ BMDMs but not in *Aim2*$^{-/-}$ BMDMs (**Fig. 1b**,**c**), which indicated that AIM2 is indeed critical for sensing cytoplasmic DNA and for the activation of caspase-1. The AIM2 deficiency had no effect on caspase-1 activation by the PRR NLRP3 stimulus of lipopolysaccharide (LPS) plus ATP or LPS plus the pore-forming toxin nigericin[14] or the PRR NLRP1 stimulus of anthrax lethal factor[15] (**Fig. 1b** and **Supplementary Fig. 2a**,**b**). Reconstitution of the *Aim2*$^{-/-}$ macrophages with green fluorescent protein (GFP)-tagged AIM2 by retroviral transduction restored their responsiveness to transfected indocarbocyanine (Cy3)-labeled DNA, as shown by oligomerization of AIM2-GFP and the induction of pyroptotic cell death, which is dependent on caspase-1 activation (**Supplementary Fig. 3a**). There was no morphological evidence of pyroptotic death in the *Aim2*$^{-/-}$ macrophages expressing GFP alone (**Supplementary Fig. 3b**).

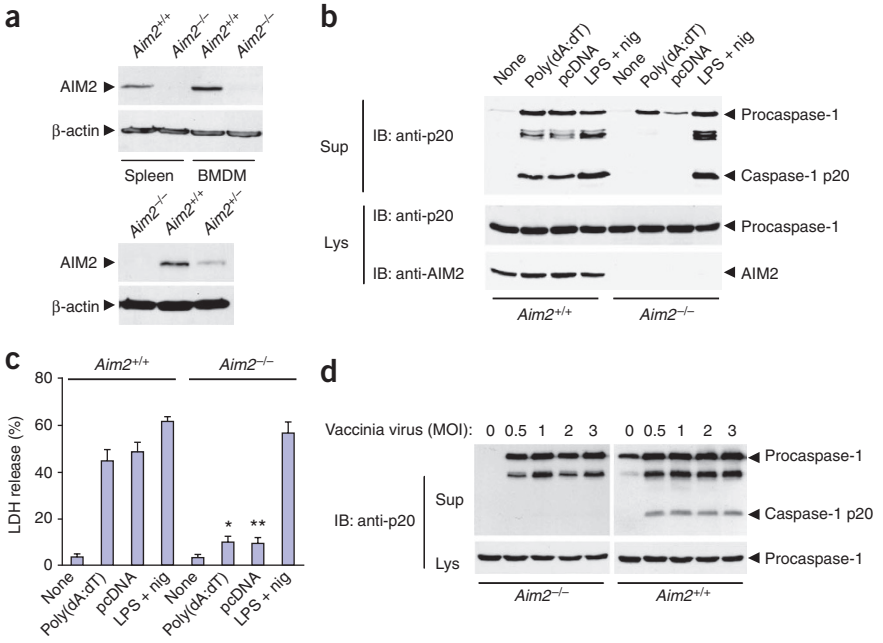

Figure 1 Disruption of mouse *Aim2* abolishes activation of the inflammasome by cytoplasmic DNA and vaccinia virus. (**a**) Immunoblot analysis of the expression of AIM2 in spleens and BMDMs from *Aim2*$^{+/+}$ and *Aim2*$^{-/-}$ littermates (top) and in spleens from *Aim2*$^{-/-}$, *Aim2*$^{+/+}$ and *Aim2*$^{+/-}$ mice (bottom), assessed with an antibody specific for mouse AIM2. β-actin serves as a loading control. (**b**) Immunoblot (IB) analysis of mouse procaspase-1, caspase-1 (p20 subunit) and/or AIM2 in culture supernatants (Sup) and lysates (Lys) of *Aim2*$^{+/+}$ and *Aim2*$^{-/-}$ BMDMs left untreated (None) or transfected with the synthetic DNA poly(dA:dT) or plasmid DNA (pcDNA), or treated for 5 h with LPS (500 ng/ml) followed by nigericin (2.5 μM) for 45 min (LPS + nig), assessed with monoclonal antibody to mouse caspase-1 p20 (anti-p20). (**c**) Release of LDH into culture supernatants of the BMDMs described in **b**, presented relative to the total cellular LDH content. $*P < 0.05$ and $**P < 0.01$, *Aim2*$^{+/+}$ versus *Aim2*$^{-/-}$ (Student's *t*-test). (**d**) Immunoblot analysis of mouse procaspase-1 and caspase-1 in culture supernatants and lysates of mouse *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ BMDMs infected for 18 h with vaccinia virus (multiplicity of infection (MOI), above lanes). Data are representative of at least three experiments (mean and s.d. in **c**).

Next we investigated the role of AIM2 in the innate immunity of macrophages to cytosolic DNA produced by infection with vaccinia virus. In contrast to wild-type *Aim2*$^{+/+}$ BMDMs, *Aim2*$^{-/-}$ BMDMs had no caspase-1 processing at 18 h after infection with vaccinia virus (**Fig. 1d**), which provides definitive proof that the AIM2 inflammasome is indeed critical for detection of vaccinia virus infection, as demonstrated before by experiments using knockdown by small interfering RNA[4].

AIM2 is important for caspase-1 activation by *F. tularensis*

F. tularensis is a highly infectious bacterium that replicates in the cytoplasm of infected cells[16,17], which leads to activation of an IRF3-dependent and Toll-like receptor–independent type I interferon response, as well as an NLRP3-independent but ASC-dependent inflammasome that causes cell death[9,10]. Such observations suggest that cytosolic DNA produced by *F. tularensis* during its escape from the phagosome might be the common ligand that activates both the type I interferon response and possibly the AIM2 inflammasome pathways. To test that possibility, we analyzed inflammasome activation and cell death by measuring the release of lactate dehydrogenase (LDH) from macrophages from wild-type and *Aim2*$^{-/-}$ mice after infection with *F. tularensis* subspecies *novicida* (called '*F. tularensis*' here). Indeed, processing of caspase-1, secretion of IL-1β and release of LDH were completely absent in *Aim2*$^{-/-}$ macrophages at 6 h after infection with *F. tularensis* (**Fig. 2a**,**b**), even at a high multiplicity of infection (**Fig. 2a**). Processing of caspase-1 and secretion of IL-1β were also absent in *Aim2*$^{-/-}$ macrophages at 24 h after infection, although there was LDH release at this time point (**Supplementary Fig. 4a**,**b**), probably due to caspase-1-independent cell death, as reported before for caspase-1-deficient macrophages[11]. In contrast, we found normal processing of caspase-1, secretion of IL-1β and release of LDH in wild-type macrophages (**Fig. 2a**,**b** and **Supplementary Fig. 4a**,**b**). Processing of caspase-1 in response to *F. tularensis* infection or transfected DNA was also unimpaired in heterozygous *Aim2*$^{+/-}$ macrophages, although it was less than that in wild-type macrophages because of the smaller amount of AIM2 in the *Aim2*$^{+/-}$ macrophages (**Supplementary Fig. 5a**,**b**). Consistent with a critical role for AIM2 in the pyroptotic cell-death pathway, morphological features of pyroptosis, including plasma membrane swelling and nuclear condensation, were very obvious in *F. tularensis*–infected *Aim2*$^{+/+}$ macrophages but not in *Aim2*$^{-/-}$ macrophages (**Fig. 2c**). Infection with *Salmonella typhimurium*, which specifically activates the NLRC4 (Ipaf) inflammasome[18], resulted in normal processing of caspase-1 in both wild-type and *Aim2*$^{-/-}$ macrophages (**Supplementary Fig. 4c**), which indicated that AIM2 is not

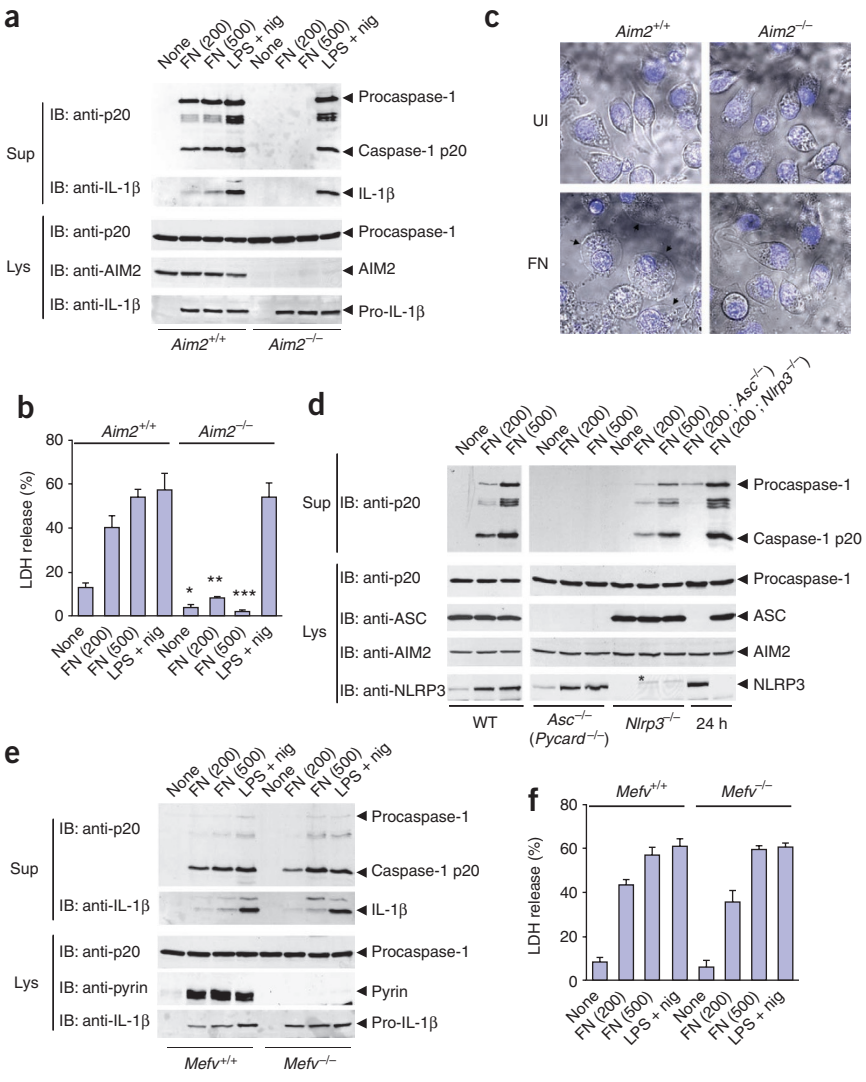

Figure 2 AIM2 is required for *F. tularensis*–induced activation of the inflammasome. (**a**) Immunoblot analysis of mouse procaspase-1, caspase-1, IL-1β, AIM2 and/or pro-IL-1β in culture supernatants and lysates of mouse *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ macrophages left untreated or infected for 6 h with *F. tularensis* (FN; MOI in parentheses above lanes) or treated with LPS and nigericin as described in **Figure 1b**. (**b**) Release of LDH into culture supernatants of the macrophages in **a**. $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$, *Aim2*$^{+/+}$ versus *Aim2*$^{-/-}$ (Student's *t*-test). (**c**) Confocal live-cell microscopy of *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ BMDMs left uninfected (UI) or infected for 6 h with *F. tularensis*; nuclei were stained with Hoechst stain (blue). Images are merged differential interference contrast and Hoechst channels. Original magnification, ×40. (**d**) Immunoblot analysis of mouse procaspase-1, caspase-1, ASC, AIM2 and/or NLRP3 in culture supernatants and lysates of mouse wild-type, ASC-deficient (*Pycard*$^{-/-}$; called '*Asc*$^{-/-}$' here) and *Nlrp3*$^{-/-}$ macrophages infected with *F. tularensis* for 6 h or for 24 h (far right; MOI in parentheses above lanes). (**e**) Immunoblot analysis of mouse procaspase-1, caspase-1, IL-1β, pyrin and/or pro-IL-1β in culture supernatants and lysates of mouse pyrin-deficient (*Mefv*$^{-/-}$) and pyrin-sufficient (*Mefv*$^{+/+}$) macrophages infected for 6 h with *F. tularensis* (MOI in parentheses above lanes) or treated with LPS and nigericin as described in **a**. (**f**) Release of LDH into culture supernatants of the macrophages in **e**. Data are representative of at least three experiments (mean and s.d. in **b**,**f**).

involved in the proinflammatory response to *S. typhimurium* infection. Consistent with published findings[10], the activation of caspase-1 by *F. tularensis* infection was dependent on ASC but not on NLRP3 (**Fig. 2d**). Together our results indicate that AIM2 is critical for the proinflammatory and cell-death responses to *F. tularensis* infection but is dispensable for these processes in response to *S. typhimurium* infection.

The familial Mediterranean fever protein pyrin, which can also form an inflammasome complex with caspase-1 and ASC[19,20], might be involved in the activation of caspase-1 by *F. tularensis* in human macrophages[21]. To test this possibility in mouse macrophages, we infected peritoneal macrophages derived from wild-type or pyrin-deficient mice[22] with *F. tularensis* or stimulated them with the NLRP3 stimulus of LPS plus nigericin (**Fig. 2e**,**f**). Despite notable upregulation of pyrin protein in wild-type macrophages by these stimuli, there was no notable difference between wild-type and pyrin-deficient macrophages in the processing of caspase-1, secretion of IL-1β or release of LDH. These results indicate that pyrin does not have a role in the proinflammatory and cell-death responses to *F. tularensis* infection in mice or negatively regulate the activity of ASC or the inflammasome, as proposed before[22–24].

Mechanism of activation of AIM2 by *F. tularensis*

Engagement of AIM2 by cytoplasmic DNA leads to formation of the oligomeric ASC 'pyroptosome' (a macromolecular aggregate of ASC), which induces pyroptotic cell death by activating caspase-1 (refs. 3,8). To investigate whether *F. tularensis* infection induces AIM2-dependent formation of the ASC pyroptosome, we analyzed the presence of oligomeric ASC in cell pellets of *F. tularensis*–infected wild-type and *Aim2*$^{-/-}$ macrophages. As expected, *F. tularensis* infection induced the formation of ASC pyroptosomes in wild-type macrophages but not in *Aim2*$^{-/-}$ macrophages (**Fig. 3a**). Similarly, liposome-delivered cytoplasmic DNA induced the formation of ASC pyroptosomes in wild-type but not *Aim2*$^{-/-}$ macrophages (**Fig. 3a**). In contrast, LPS plus nigericin, which activates the NLRP3 inflammasome, induced the formation of ASC pyroptosomes in both wild-type and *Aim2*$^{-/-}$ macrophages (**Fig. 3a**). Collectively, our data indicate that *F. tularensis* infection induces pyroptotic cell death via AIM2-mediated formation of ASC pyroptosomes.

To gain further insight into the signaling pathway by which *F. tularensis* infection activates the AIM2 inflammasome, we investigated the role of cytoplasmic potassium efflux, which is critical for formation of the ASC pyroptosome and recruitment of caspase-1 to ASC oligomers[8]. Inhibition of potassium efflux by increasing the potassium concentration in the culture medium completely blocked the formation of ASC pyroptosomes, activation of caspase-1 and secretion of IL-1β induced by *F. tularensis* infection (**Fig. 3b**), which indicated that depletion of intracellular potassium is required for assembly of the AIM2 inflammasome and formation of the ASC

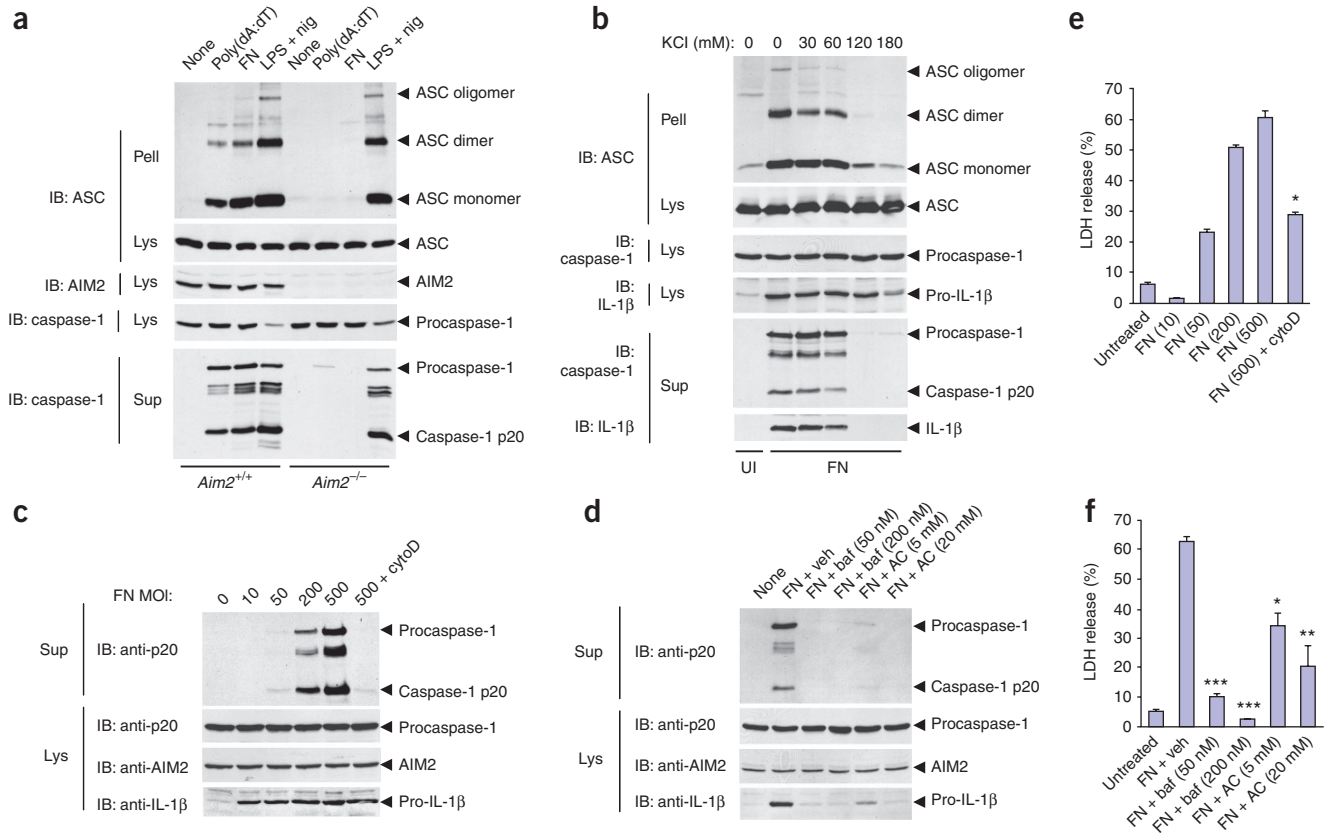

Figure 3 Role of the ASC pyroptosome, potassium depletion, actin polymerization and lysosomal acidification in activation of the AIM2 inflammasome by *F. tularensis*. (**a**) Immunoblot analysis of ASC pyroptosomes in *Aim2*$^{+/+}$ and *Aim2*$^{-/-}$ BMDMs transfected with poly(dA:dT), infected for 6 h with *F. tularensis* (MOI, 500), or treated with LPS and nigericin as described in **Figure 1b**; pellets of whole-cell lysates centrifuged at 3,800*g* followed by crosslinking with disuccinimidyl suberate (Pell), as well as cell lysates and culture supernatants, were hybridized with anti–mouse ASC, anti–mouse AIM2 and anti-caspase-1. (**b**) Immunoblot analysis of ASC pyroptosomes in *Aim2*$^{+/+}$ BMDMs left uninfected or infected for 6 h with *F. tularensis* (MOI, 500) in the presence of increasing concentrations of KCl in the culture medium, then fractionated and analyzed as in **a**. (**c**,**d**) Immunoblot analysis of mouse procaspase-1, caspase-1, AIM2 and/or pro-IL-1β in culture supernatants and lysates of *Aim2*$^{+/+}$ BMDMs infected for 6 h with *F. tularensis* (MOI, above lanes) in the presence (+ cytoD) or absence of cytochalasin D (**c**) or infected for 6 h with the *F. tularensis* (MOI, 250) in the presence of vehicle (+ veh), bafilomycin (+ baf) or NH_4Cl (+ AC; **d**). (**e**,**f**) Release of LDH into culture supernatants of the BMDMs in **c** (**e**) and **d** (**f**). (**e**) $^*P < 0.001$, with versus without cytochalasin D (Student's *t*-test); (**f**) $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, *F. tularensis* versus vehicle (Student's *t*-test). Data are representative of two (**a**–**d**) or three (**e**,**f**) experiments (mean and s.d. in **e**,**f**).

pyroptosome. Activation of caspase-1 and cell death by *F. tularensis* infection was blocked by the actin-oligomerization inhibitor cytochalasin D and the endosomal-acidification inhibitors bafilomycin A and NH_4Cl (**Fig. 3c–f**), which indicated that bacterial internalization and lysosomal acidification are required for recognition of *F. tularensis* infection and activation of the AIM2 inflammasome. Inhibition of *F. tularensis*–induced caspase-1 activation by bafilomycin was not due to inhibition of bacterial uptake or replication, as we found more intracellular bacteria in bafilomycin-treated cells than in untreated control cells at 3 h after infection (**Supplementary Fig. 6**). The greater bacterial count in bafilomycin-treated cells is consistent with published observations linking phagosomal acidification to the killing and degradation of phagocytosed bacteria[25].

IRF3 is important for AIM2 activation by *F. tularensis*

Activation of the inflammasome by *F. tularensis* infection requires an intact type I interferon response for efficient inflammasome activation[10]. Consistent with that, *F. tularensis* infection of *Irf3*$^{-/-}$ macrophages, which are defective in the secretion of type I interferons in response to cytosolic DNA, resulted in less-efficient activation of the AIM2 inflammasome than that in wild-type macrophages (**Fig. 4a**). In contrast, liposome-delivered DNA induced similar activation of the AIM2 inflammasome in both wild-type and *Irf3*$^{-/-}$ macrophages (**Fig. 4a**). We observed similar expression of AIM2 in wild-type and *Irf3*$^{-/-}$ macrophages (**Fig. 4a**), which ruled out the possibility that *Irf3*$^{-/-}$ macrophages have lower expression of AIM2. To determine if type I interferon signaling through its receptor, IFNAR1, could restore AIM2 activation induced by *F. tularensis* infection in the *Irf3*$^{-/-}$ macrophages, we treated these macrophages with interferon-β (IFN-β) at the time of infection and assayed caspase-1 activation at 6 h after infection. Simultaneous treatment of *Irf3*$^{-/-}$ macrophages with both IFN-β and *F. tularensis* restored *F. tularensis*–induced caspase-1 activation in these cells (**Fig. 4b**). Concomitant treatment with IFN-β at the time of infection slightly enhanced *F. tularensis*–induced activation of caspase-1 in wild-type *Irf3*$^{+/+}$ macrophages (**Fig. 4b**). Restoration of *F. tularensis*–induced activation of caspase-1 in *Irf3*$^{-/-}$ macrophages required concurrent treatment with IFN-β and *F. tularensis*, as we observed no restoration when we treated cells with IFN-β at 2 h after infection (**Supplementary Fig. 7a**, left). We observed no activation of caspase-1 after IFN-β treatment alone (**Supplementary Fig. 7a**, right).

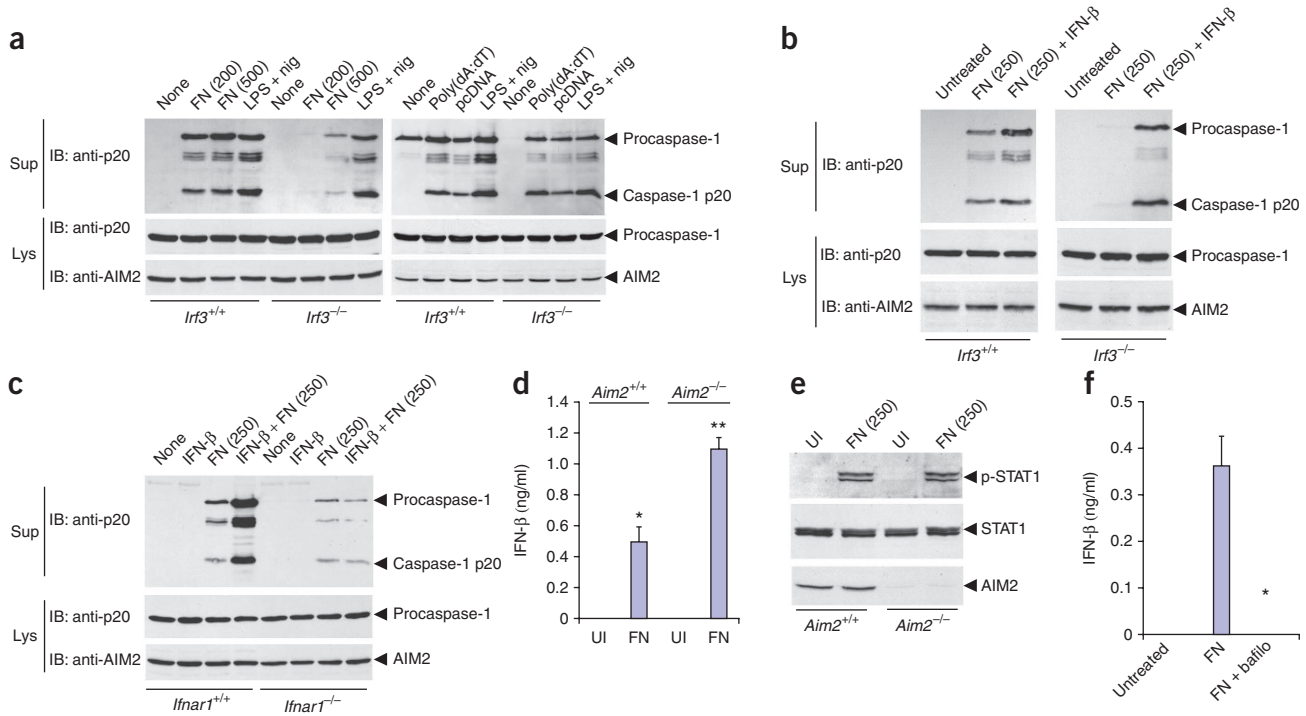

Figure 4 IRF3 signaling is required for activation of the AIM2 inflammasome by *F. tularensis* but not by liposome-delivered DNA. (**a**,**b**) Immunoblot analysis of mouse procaspase-1, caspase-1 and/or AIM2 in culture supernatants and lysates of mouse *Irf3*$^{-/-}$ and *Irf3*$^{+/+}$ macrophages infected for 6 h with *F. tularensis* (MOI, in parentheses above lanes), treated with LPS and nigericin as described in **Figure 1b**, or transfected with poly(dA:dT) (**a**), or infected with *F. tularensis* (MOI, 250) in the presence or absence of IFN-β (**b**). (**c**) Immunoblot analysis of mouse procaspase-1, caspase-1 and/or AIM2 in culture supernatants and lysates of *Ifnar1*$^{-/-}$ and *Ifnar1*$^{+/+}$ macrophages left untreated or treated for 2 h with IFN-β alone or followed by infection for 6 h with *F. tularensis* (MOI, in parentheses above lanes). (**d**) Enzyme-linked immunosorbent assay of IFN-β in culture supernatants of *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ macrophages left uninfected or infected for 6 h with *F. tularensis* (MOI, 250). $^{*}P < 0.05$ and $^{**}P < 0.005$ (Student's *t*-test). (**e**) Immunoblot analysis of mouse STAT1 phosphorylated at Tyr701 (p-STAT1), total STAT1 and AIM2 in lysates of mouse *Aim2*$^{-/-}$ and *Aim2*$^{+/+}$ macrophages left uninfected or infected with *F. tularensis* (MOI, 250). (**f**) Enzyme-linked immunosorbent assay of IFN-β in culture supernatants of *Aim2*$^{+/+}$ macrophages left untreated or infected for 6 h with *F. tularensis* (MOI, 250) in the presence (+ bafilo) or absence of bafilomycin (50 nM). $^{*}P < 0.01$ (Student's *t*-test). Data are representative of two (**a**,**c**) or three (**b**,**d**–**f**) experiments (mean and s.d. in **d**,**f**).

Additionally, concurrent treatment of *Aim2*$^{-/-}$ macrophages with IFN-β and *F. tularensis* did not restore *F. tularensis*–induced caspase-1 activation in these cells (**Supplementary Fig. 7b**), which indicated that IFN-β is not sufficient for *F. tularensis*–induced caspase-1 activation in the absence of AIM2.

To further investigate the role of type I interferons in the mechanism of activation of AIM2 by *F. tularensis* infection, we primed wild-type and *Irf3*$^{-/-}$ macrophages with IFN-β 2 h before infection with *F. tularensis* and measured caspase-1 activation at various times after infection (**Supplementary Fig. 8**). In the absence of IFN-β priming, caspase-1 processing in wild-type macrophages was barely detectable at 3 h after infection. In contrast, we observed maximum caspase-1 processing at 3 h after infection in IFN-β-primed wild-type macrophages. Although unprimed *Irf3*$^{-/-}$ macrophages showed less-efficient caspase-1 activation than that of wild-type macrophages at 3 h and 5 h after infection, they showed caspase-1 activation similar to that of wild-type macrophages after IFN-β priming. These results indicate that priming of macrophages with IFN-β accelerates and enhances activation of the AIM2 inflammasome by *F. tularensis* infection not only in IRF3-deficient macrophages but also in wild-type macrophages. They also suggest that the delay in caspase-1 activation observed in unprimed wild-type macrophages might have been due to the lack of type I interferons in the initial stages of *F. tularensis* infection. Like IRF3-deficient macrophages, IFNAR1-deficient macrophages were also defective in activation of caspase-1 in response to *F. tularensis* infection[10] (**Fig. 4c**). However, this defect was not corrected by IFN-β priming, which indicated that signaling by IFNAR1 is required for efficient activation of the AIM2 inflammasome by *F. tularensis* infection.

The impaired AIM2 inflammasome activation in *Ifnar1*$^{-/-}$ macrophages was not caused by a lack of intracellular bacterial replication. On the contrary, the number of intracellular *F. tularensis* was notably higher in *Ifnar1*$^{-/-}$ macrophages than in wild-type macrophages at 5 h after infection and reached to almost 50-fold higher at 24 h after infection (**Supplementary Fig. 9**). These results are consistent with a published report[10]. Collectively, these observations indicate that type I interferon signaling acts upstream of the AIM2 inflammasome, perhaps to enhance the killing and lysis of *F. tularensis* in the phagosome to generate the cytosolic DNA that activates the AIM2 inflammasome (model, **Supplementary Fig. 10**, and discussed below).

AIM2 is not critical for type I interferon production

To rule out the possibility that the defective activation of the inflammasome in *Aim2*$^{-/-}$ macrophages was due to defective type I interferon production or signaling, we quantified secreted IFN-β in culture media of wild-type and *Aim2*$^{-/-}$ macrophages infected with *F. tularensis* and also determined the status of phosphorylation of the transcription factor STAT1 in lysates of these cells (**Fig. 4d**,**e**).

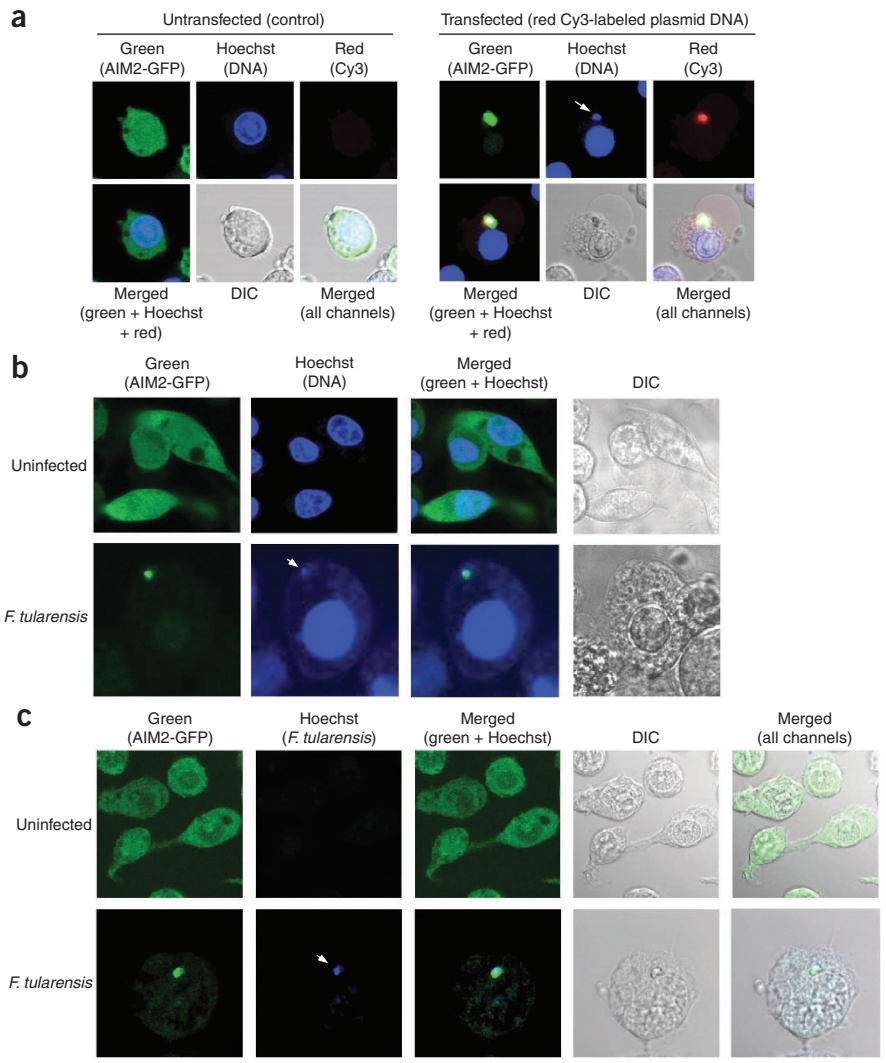

Figure 5 Cytoplasmic DNA secreted by *F. tularensis* induces AIM2 oligomerization. (**a**) Confocal live-cell microscopy of *Nlrp3*$^{-/-}$-AIM2-GFP-N1 BMDMs left untransfected or transfected with Cy3-labeled DNA. Arrow (right) indicates staining of clustered cytoplasmic DNA with the DNA-specific blue Hoechst dye. (**b**) Confocal live-cell microscopy of *Nlrp3*$^{-/-}$-AIM2-GFP-N1 BMDMs left uninfected or infected for 6 h with *F. tularensis* and then stained with Hoechst dye. Arrow (bottom row) indicates staining of the AIM2-GFP cluster with the DNA-specific blue Hoechst dye. (**c**) Confocal live-cell microscopy of *Nlrp3*$^{-/-}$-AIM2-GFP-N1 BMDMs left uninfected or infected for 6 h with Hoechst-labeled *F. tularensis* and then fixed on coverslips. Arrow (bottom row) indicates the Hoechst-labeled *F. tularensis* cytoplasmic DNA. Additional images, **Supplementary Figure 11**. DIC, differential interference contrast. Original magnification, ×40. Data are representative of at least three (**a**,**b**) or two (**c**) experiments.

Our results showed that IFN-β production was actually higher in *Aim2*$^{-/-}$ macrophages than in wild-type macrophages in response to *F. tularensis* infection (**Fig. 4d**). However, there was no substantial difference between *F. tularensis*–infected wild-type and *Aim2*$^{-/-}$ macrophages in STAT1 phosphorylation (**Fig. 4e**). These results indicate that AIM2 is not critical for type I interferon production or signaling and that AIM2 deficiency does not negatively affect the type I interferon response to infection with *F. tularensis*. In fact, AIM2 deficiency seemed to enhance IFN-β production in response to such infection. That result is consistent with the observation that small interfering RNA–mediated knockdown of AIM2 potentiates IFN-β production in response to transfected cytosolic DNA[4]. The production of IFN-β induced by *F. tularensis* infection was completely blocked by bafilomycin A (**Fig. 4f**), which indicated that lysosomal acidification is not only required for activation of the AIM2 inflammasome but also necessary for *F. tularensis*–induced production of type I interferon.

F. tularensis DNA induces AIM2 oligomerization

Cytoplasmic DNA activates AIM2 by directly binding to its HIN-200 domain, which leads to its oligomerization. We visualized this process in live cells expressing GFP-tagged AIM2 and Cy3-labeled DNA[3] (**Fig. 5a**). Notably, we also visualized the cytosolic DNA bound to AIM2 in the oligomeric AIM2-DNA complex with the DNA-specific fluorescent stain Hoechst 33342 (**Fig. 5a**). To formally demonstrate that AIM2 senses cytosolic DNA produced by *F. tularensis* infection, we infected *Nlrp3*$^{-/-}$-AIM2-GFP-N1 macrophages (an *Nlrp3*$^{-/-}$ cell line stably expressing AIM2 tagged with GFP at the C terminus)[3] with *F. tularensis* and subsequently stained the infected cells with Hoechst dye. We used *Nlrp3*$^{-/-}$-AIM2-GFP-N1 macrophages in these experiments to rule out the possibility of involvement of NLRP3 in the response to cytosolic DNA or *F. tularensis* infection. AIM2 was evenly distributed in the cytoplasm and nuclei of uninfected cells (**Fig. 5b**). Infection of cells with *F. tularensis* resulted in AIM2 oligomerization (**Fig. 5b** and **Supplementary Fig. 11**), as shown by the clustering of AIM2-GFP. These AIM2-GFP clusters were also visible with the vital DNA-specific Hoechst stain, which indicated that AIM2 is clustered by binding to cytosolic DNA. The infected macrophages that contained the AIM2-DNA clusters showed distinct features of pyroptotic cell death.

To provide direct evidence that the cytosolic DNA responsible for the clustering of AIM2-GFP in those cells was derived from *F. tularensis*, we prestained *F. tularensis* DNA with Hoechst stain before using this *F. tularensis* to infect *Nlrp3*$^{-/-}$-AIM2-GFP-N1 macrophages. As expected, infection of these cells with the Hoechst-labeled *F. tularensis* resulted in clustering of AIM2-GFP around the Hoechst-labeled DNA (**Fig. 5c** and **Supplementary Fig. 11b**). Because only *F. tularensis* DNA was labeled with Hoechst stain, these results provide direct evidence that *F. tularensis* DNA is responsible for oligomerizing AIM2. Collectively, our results indicate that infection of macrophages with *F. tularensis* generates cytosolic DNA that specifically binds to AIM2 and induces AIM2 oligomerization, which leads to the activation of caspase-1 and cell death (model, **Supplementary Fig. 10**).

Innate immunity to *F. tularensis* infection requires AIM2

The results presented above indicated that AIM2 is important for the proinflammatory and cell-death responses to *F. tularensis* infection *in vitro* by a mechanism that involves the recognition of

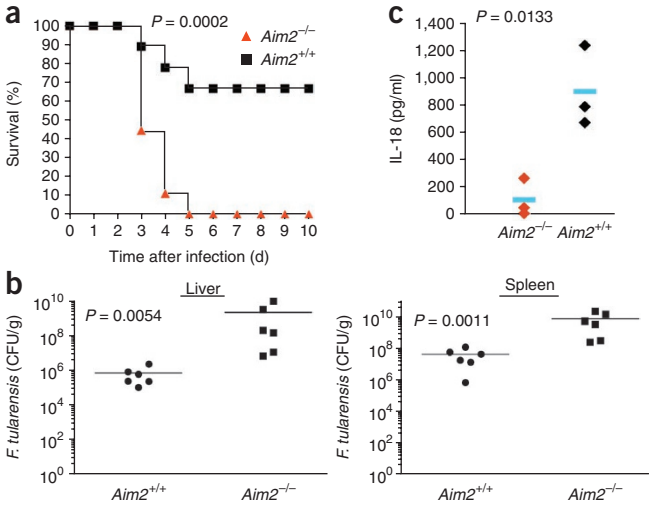

Figure 6 The AIM2 inflammasome is critical for innate immunity to *F. tularensis* infection. (**a**) Survival of $Aim2^{+/+}$ mice ($n = 9$) and $Aim2^{-/-}$ mice ($n = 9$) injected subcutaneously with *F. tularensis* (1.5×10^5 colony-forming units (CFU)) and monitored over a period of 3 weeks; 66% of $Aim2^{+/+}$ survived beyond 3 weeks after infection. (**b**) Bacterial burden in livers and spleens obtained from mice 48 h after subcutaneous infection with *F. tularensis* (1.5×10^5 CFU); organs were homogenized and dilutions were plated on cystine heart agar plates. (**c**) Enzyme-linked immunosorbent assay of IL-18 in serum from $Aim2^{+/+}$ mice ($n = 3$) and $Aim2^{-/-}$ mice ($n = 3$) at 1 d after subcutaneous infection with *F. tularensis*. In **b**,**c**, each symbol represents an individual mouse; small horizontal lines indicate the mean. *P* values, Kaplan-Meier log-rank test (**a**) or Student's *t*-test (**b**,**c**). Data are representative of two experiments.

cytoplasmic DNA produced by this intracellular pathogen. To assess the precise role of AIM2 in the host innate immune defense against *F. tularensis* infection, we infected wild-type and $Aim2^{-/-}$ mice subcutaneously with live *F. tularensis*. This resulted in much higher rate of mortality in $Aim2^{-/-}$ mice than in wild-type mice (**Fig. 6a**). The mortality rate of $Aim2^{-/-}$ mice on day 5 was 100%, compared with just 33% for wild-type mice. The remaining wild-type mice survived beyond 20 d after infection. Consistent with those results, the bacterial burdens in tissues of $Aim2^{-/-}$ mice were much higher than those in wild-type mice at 48 h after infection (**Fig. 6b**). These results indicate that the AIM2 inflammasome has a crucial role in the host defense against *F. tularensis* infection, probably by decreasing the bacterial burden in tissues, thereby preventing systemic infection. Consistent with the critical role of AIM2 in the production of caspase-1-generated cytokines, the serum concentration of IL-18 was much higher in wild-type mice than in $Aim2^{-/-}$ mice at 24 h after infection (**Fig. 6c**). Together these results indicate that the lack of AIM2 inflammasome activity increases the virulence of *F. tularensis* in mice because of defective activation of caspase-1, which is critical for the production of the caspase-1-generated cytokines such as IL-1β and IL-18 and the induction of pyroptotic death of the infected macrophages.

DISCUSSION

Biochemical evidence has linked AIM2 to the recognition of and innate immune response to cytosolic DNA[3–6]. The recognition of cytosolic DNA by AIM2 activates the nontranscriptional inflammatory caspase-1 pathway in macrophages, which leads to production of the potent inflammatory cytokines IL-1β and IL-18 and cell death. We have now provided genetic evidence that AIM2 is a critical PRR uniquely involved in the recognition and innate immune defense against infection with the potentially lethal intracellular pathogen *F. tularensis*.

Several proteins of the NLR family are involved in the activation of the inflammatory caspase-1 pathway in macrophages in response to pathogenic infection or products[26–28]. For example, NLRC4 (Ipaf) is a PRR involved in the detection of intracellular infection with *S. typhimurium* and activation of the caspase-1 pathway in response to this pathogen[18]. *S. typhimurium* flagellin is the suggested molecular component responsible for NLRC4 activation[29,30]. Another PRR, NLRP1, is involved in caspase-1 activation in response to stimulation with anthrax lethal toxin[15]. In contrast, NLRP3 (cryopyrin) is involved in caspase-1 activation after infection with a broad range of bacterial, viral or fungal pathogens and in response to various stress stimuli[28,31]. Nevertheless, the mechanism by which NLRP3 recognizes such a broad range of pathogens and stimuli is still unclear.

None of the NLRs mentioned above or other known NLRs, however, are involved in sensing *F. tularensis* infection. Although macrophages from AIM2-deficient mice are not defective in sensing NLRC4, NLRP1 and NLRP3 stimuli, as shown by their normal caspase-1 activation response to *S. typhimurium*, anthrax lethal toxin, or LPS plus ATP or nigericin, or monosodium urate, they were clearly unable to activate caspase-1 in response to *F. tularensis* infection. This suggests that *F. tularensis* may have developed mechanisms to evade detection of their non–nucleic acid pathogen-associated molecular patterns by the host cell inflammasome components such as NLRC4, NLRP1 or NLRP3 and probably by other as-yet-uncharacterized inflammasomes. Nevertheless, several studies have suggested that *F. tularensis* infection activates Toll-like receptor 2 signaling via the adaptor MyD88, leading to transcription factor NF-κB–dependent transcriptional induction of proinflammatory cytokines[32–34]. However, this response does not seem to be sufficient for activation of the NLRP3 inflammasome, because $Aim2^{-/-}$ macrophages were completely defective in caspase-1 activation in response to infection with *F. tularensis*.

Phagosomal disruption occurs during the escape of *F. tularensis* from the phagosome into the cytosol[35–37]. This may be necessary for the activation of caspase-1 and production of type I interferon induced by *F. tularensis*, because mutant strains that cannot disrupt the phagosome and escape into the cytosol are unable to induce type I interferons or activate caspase-1 (refs. 10,38). Despite published observations of activation of the NLRP3 inflammasome by phagosomal disruption[26,39,40], phagosomal disruption during *F. tularensis* escape did not lead to activation of the NLRP3 inflammasome, as shown by the absence of caspase-1 activation in *F. tularensis*–infected AIM2-deficient macrophages. This defect in caspase-1 activation cannot be attributed to a defect in NLRP3 signaling, because these macrophages had normal amounts of caspase-1 activation in response to specific NLRP3 stimuli such as LPS plus ATP or nigericin. Additionally, this defect is probably not due to a specific defect in *F. tularensis*–induced phagosomal disruption, as infection of the AIM2-deficient macrophages with *F. tularensis* led to even more production of type I interferon than that of wild-type macrophages, which indicates normal phagosomal disruption and escape. Together these observations suggest that phagosomal disruption generally does not induce activation of the NLRP3 inflammasome and it is likely that a more specific stimulus or additional stimuli are required. Alternatively, *F. tularensis* might have the means to inhibit activation of the NLRP3 inflammasome during its escape from the phagosome.

Activation of the AIM2 inflammasome requires direct interaction of AIM2 with DNA[3]. This interaction leads to oligomerization of AIM2 and the formation of a large AIM2-DNA complex visible

by confocal microscopy[3]. Our results have shown that infection of macrophages with *F. tularensis* also induced the formation of a large AIM2-DNA complex, which indicates that *F. tularensis* infection delivers DNA into the cytosol that is then recognized by AIM2. The exact mechanism by which *F. tularensis* delivers its DNA into the cytosol is unclear at present. Nevertheless, our results suggest that the DNA that activates AIM2, and probably type I interferon signaling, is produced by breakdown and digestion of killed *F. tularensis* by phagosomal enzymes. In support of that conclusion, bafilomycin, which can prevent the killing and degradation of bacteria in the phagosome by inhibiting the activity of vacuolar-type ATPases that mediate lumen acidification[25,41], completely inhibited the activation of the AIM2 inflammasome and production of IFN-β induced by *F. tularensis* infection in wild-type macrophages. Given that bafilomycin does not prevent *F. tularensis* phagosomal permeabilization and escape into the cytosol[35], phagosomal escape by live *F. tularensis* and its replication in the cytosol is probably not the signal that activates AIM2 or the type I interferon response. Instead, the killing and degradation of *F. tularensis* and the subsequent phagosomal disruption triggered by the escape of live *F. tularensis* is probably responsible for the delivery of *F. tularensis* DNA into the cytosol for recognition by AIM2 and type I interferon signaling pathways. As a consequence, we expect that more virulent strains of *F. tularensis* such as *F. tularensis tularensis*, which infects humans, must have evolved mechanisms that protect them from phagosomal lysis, perhaps by altering the rate of acidification and maturation of their phagosomes[36]. Therefore, these strains would be detected less efficiently by AIM2 because of less phagosomal release of killed bacterial DNA into the cytosol and thus would have a better chance of intracellular replication and a greater ability to cause systemic infection.

Signaling by IFN-β through its receptor, IFNAR1, is required for efficient caspase-1 activation by *F. tularensis*[10,26]. IFNAR1- or IRF3-deficient macrophages, which have impaired IFN-β production, showed less efficient activation of caspase-1 by *F. tularensis* than did wild-type macrophages[10,26]. Our data showed that the lower caspase-1 activation by *F. tularensis* in $Irf3^{-/-}$ or $Ifnar1^{-/-}$ macrophages was not due to lower AIM2 expression, because these macrophages had AIM2 expression similar to that of wild-type macrophages. Additionally, $Irf3^{-/-}$ and $Ifnar1^{-/-}$ macrophages[42] show normal caspase-1 activation in response to transfected DNA, which rules out the possibility that IFN-β signaling is required for transcriptional induction of a host factor critical for caspase-1 activation by the AIM2 inflammasome. Although *F. tularensis* and transfected DNA both activate the AIM2 inflammasome, IFN-β signaling might be required for efficient killing of *F. tularensis* in the phagosome and/or phagosomal permeablization and release of *F. tularensis* DNA into the cytosol. Indeed, $Ifnar1^{-/-}$ macrophages contained more bacteria at 5–24 h after infection than did wild-type macrophages, probably because of defective phagosomal bactericidal activity of the $Ifnar1^{-/-}$ macrophages. Consequently, these macrophages might produce less cytosolic DNA, which could provide a rationale for the observed defective AIM2 inflammasome activation in these macrophages.

On the basis of the information presently available about the life cycle of *F. tularensis* in macrophages[10,26,43] and our own data, we propose a two-step mechanism by which infection of macrophages with *F. tularensis* activates the AIM2 inflammasome. Shortly after *F. tularensis* enters the phagosome, the phagosome is rapidly acidified[17]. Acidification causes lysis of some of the ingested bacteria and release of bacterial DNA into the lumen of the phagosome. During phagosomal escape of live *F. tularensis,* which occurs as early as 1 h after infection[16,17], the phagosome is ruptured, which releases both live *F. tularensis* and undigested DNA of killed *F. tularensis* into the cytosol. This amount of cytosolic DNA might not be sufficient for AIM2 activation but is probably sufficient for the activation of an as-yet-unknown DNA sensor, which in turn activates IRF3, leading to the production of type I interferons such as IFN-β. Bafilomycin treatment completely inhibited *F. tularensis*–induced production of IFN-β, which supports this scenario. IFN-β binds to IFNAR1, resulting in the activation of this signaling pathway. This initial IFN-β signaling process acts like a positive feedback loop, perhaps to increase phagosomal acidification and/or the bactericidal activity of the phagosomal enzymes, thereby enhancing the release of more DNA from killed *F. tularensis* into the cytosol. This is supported by the observations that prior priming of wild-type or $Irf3^{-/-}$ macrophages before infection with *F. tularensis* or infection of these macrophages with *F. tularensis* together with IFN-β cotreatment accelerates and enhances AIM2 inflammasome activation, whereas inhibiting phagosomal acidification results in much less activation of the AIM2 inflammasome. In the second step of this process, the higher concentration of cytosolic DNA, as a result of IFN-β-induced phagosomal disruption, leads to full AIM2 activation through the induction of its oligomerization. The oligomerized AIM2-DNA complex serves as a molecular platform for the recruitment of ASC and facilitate its oligomerization into the large ASC pyroptosome[8]. Consistent with that, infection with *F. tularensis* or transfection with cytosolic DNA induced formation of the ASC pyroptosome in wild-type but not $Aim2^{-/-}$ macrophages and was completely blocked by ~120–180 mM extracellular KCl. It is likely that the ASC pyroptosome then activates procaspase-1, as described before[8], leading to pyroptotic cell death and production of the proinflammatory cytokines IL-1β and IL-18.

The phenotype of *F. tularensis*–challenged $Aim2^{-/-}$ mice provides further support for the idea that AIM2 is critical for sensing *F. tularensis* infection and activation of the ASC–caspase-1 pathway not only in isolated macrophages but also in a whole-animal model. Indeed, this phenotype is reminiscent of the phenotype of *F. tularensis*–challenged caspase-1-deficient or ASC-deficient mice[9]. In these mouse strains, as well as in $Aim2^{-/-}$ mice, *F. tularensis* infection is associated with more death due to a greater bacterial burden and systemic infection[9]. The greater bacterial burden in these mice is most probably due to less death of *F. tularensis*–infected macrophage and less proinflammatory cytokine production. *F. tularensis*–induced macrophage death shares features of the pyroptotic cell death induced by other pathogenic bacteria[9]. However, *F. tularensis*–induced macrophage death is dependent on AIM2 as well as caspase-1 and ASC[9], which provides further proof that AIM2, ASC and caspase-1 function in the same signaling pathway that recognizes *F. tularensis* infection. In conclusion, our data have provided genetic and biochemical evidence that activation of the AIM2 inflammasome represents a crucial innate immune defense against *F. tularensis* infection. Future studies with the AIM2-deficient mice should clarify its role in the innate immune response to other intracellular microbial and viral pathogens as well as its involvement in nucleic acid–dependant autoimmune diseases such as systemic lupus erythematosus.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

T.F.-A. conceived of this project and supervised the generation of AIM2-deficient mice and, together with J.-W.Y., did most of the experiments and analyzed and interpreted the data; C.J. and L.H. did the vaccinia virus experiments; L.E. provided advice and the vaccinia virus and supervised the vaccinia studies; L.S. and S.K. did the *in vivo F. tularensis* infection studies; C.P.L. and E.M. generated AIM2 gene-trap chimeric mice; J.W., P.D. and M.M. provided technical assistance with genotyping and immunoblot analysis; and E.S.A. directed the entire project and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of AIM2-deficient mice. AIM2-deficient mice were generated by the gene-trap method[13] from an embryonic stem cell clone obtained from the International Gene Trap Consortium[12]. All mice were used according to protocols approved by Institutional Animal Care and Use Committee, Thomas Jefferson University. Details are in the **Supplementary Methods**.

Antibodies and reagents. Polyclonal antibody to AIM2 was raised in rabbits (Invitrogen) against a mixture of recombinant mouse and human AIM2 proteins prepared in the Alnemri laboratory. This antibody can detect endogenous mouse and human AIM2 proteins. Polyclonal antibody to mouse pyrin was raised in rabbits (Invitrogen) against a truncated recombinant mouse pyrin protein (residues 1–349) prepared in the Alnemri laboratory. Monoclonal antibody 3ZD (anti-IL-1β) was obtained from the National Cancer Institute Preclinical Repository, Biological Resources Branch. Other antibodies have been described[8,20,31]: polyclonal antibody to mouse NLRP3 pyrin domain (from the Alnemri laboratory), polyclonal antibody to mouse ASC (from J. Sagara) and monoclonal antibody to mouse caspase-1 p20 (from J. Yuan). Antibody to STAT1 phosphorylated at Tyr701 (9171S) and antibody to mouse STAT1 (9172) were from Cell Signaling. ATP, nigericin, poly(dA:dT) sodium salt, bafilomycin A and cytochalasin D were from Sigma-Aldrich. Ultrapure LPS was from Invivogen. Anthrax lethal factor and protective antigen were from List Biological Laboratories. Disuccinimidyl suberate was from Thermo Fisher Scientific. The CytoTox 96 LDH-release assay kit was from Promega.

Macrophage cell culture and stimulation. Mouse bone marrow cells were isolated from mouse femurs in sterile DMEM and were cultured in six-well plates for 5–7 d in DMEM (GIBCO) supplemented with 10% (vol/vol) medium conditioned by L929 mouse fibroblasts, 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin (15140; Gibco). Macrophages were infected for 18 h with vaccinia virus (WR strain) at various MOI values. In some experiments, differentiated macrophages were treated for 5 h with Ultrapure LPS (500 ng/ml) in serum-free Opti-MEM I medium, followed by ATP (5 mM) or nigericin (2.5 μM). Macrophages were treated with anthrax lethal toxin (protective antigen and lethal factor) at a concentration of 5 μg/ml.

Infection of macrophages with *F. tularensis*. Wild-type *F. tularensis* (U112) was obtained from D.M. Monack and was grown overnight with shaking in tryptic soy broth (Difco Laboratories) supplemented with 0.2% (wt/vol) cysteine as described[10]. For analysis of the effect of AIM2 deficiency on *F. tularensis*–induced activation of caspase-1, $Aim2^{-/-}$ and $Aim2^{+/+}$ macrophages were seeded in six-well plates at a density of 2×10^6 cells per well in Opti-MEM I serum-free medium and were allowed to attach for 2 h. Cells were infected for 6 h with *F. tularensis* (at various MOI values) in 2 ml Opti-MEM I medium as described[10]. Culture supernatants and cells were separated and then processed for immunoblot analysis as described below. In the experiments involving IFN-β priming, cells were pretreated with 250 U/ml of mouse IFN-β at 2 h before infection. Cells were then infected with *F. tularensis*. After 1 h, gentamycin (100 μg/ml) was added, followed by incubation for 30 min. Cells were then washed with PBS and were further incubated in Opti-MEM containing a low concentration of gentamycin (2.5 μg/ml) for various periods of time until they were collected.

Immunoblot analysis. Proteins were precipitated from cell culture supernatants by the addition of an equal volume of methanol and 0.25 volumes of chloroform as described[3,40]. Samples were separated by 12.5% SDS-PAGE and were transferred onto nitrocellulose membranes. Blots were probed with rat monoclonal antibody to mouse caspase-1 p20 or monoclonal antibody to IL-1β (3ZD). Total cell lysates were mixed with SDS sample buffer and separated by 12.5% SDS-PAGE and then were analyzed by immunoblot with the appropriate antibodies as described above.

Assay of ASC pyroptosome formation in macrophages. These experiments were done essentially as described[44,45] (additional description, **Supplementary Methods**).

Infection of mice with *F. tularensis*. All mice were kept in specific pathogen–free conditions in filter-top cages at Thomas Jefferson University and experimental studies were in accordance with the Institutional Animal Care and Use Guidelines. Mice were provided with sterile water and food *ad libitum*. Pairs of $Aim2^{-/-}$ *and* $Aim2^{+/+}$ littermates 8–10 weeks of age were inoculated subcutaneously with the appropriate dose of *F. tularensis* strain U112 in a volume of 0.05 ml. Each pair of mice were siblings derived from the same parents. Mice were monitored for signs of distress and/or illness and lethality three times daily for 3 weeks for the survival study. For the determination of bacterial burden in mouse tissues, six pairs of $Aim2^{-/-}$ *and* $Aim2^{+/+}$ littermates (siblings) 10–12 weeks of age were inoculated subcutaneously with the appropriate dose of *F. tularensis* strain U112 in a volume of 0.05 ml. Spleens and livers were collected 48 h after infection and homogenized, and dilutions were plated on cystine heart agar plates and incubated for 24 h at 37 °C; CFU were counted and are presented as CFU per gram of tissue.

LDH-release assay. Cell culture supernatants and cell pellets from treated macrophages were collected at the end of treatment and LDH activity was assayed with the CytoTox 96 LDH-release kit as described by the manufacturer's protocols (Promega).

Confocal microscopy. In experiments with Cy3-labeled plasmid DNA, $Nlrp3^{-/-}$-AIM2-GFP-N1 or $Aim2^{-/-}$-AIM2-GFP-N1 macrophages were seeded on 35-mm glass-bottomed culture dishes (Mat Tek) and were allowed to attach for 24 h. The next day cells, were transfected for 2–3 h with Cy3-labeled plasmid DNA (0.5 μg/dish) using Lipofectamine 2000 and then were stained for 30 min with Hoechst 33342. Cells were then observed with a Zeiss LSM 510 Meta confocal microscope at the Kimmel Cancer Center core facility. In *F. tularensis* infection experiments, $Nlrp3^{-/-}$-AIM2-GFP-N1 macrophages seeded on 35-mm glass-bottomed culture dishes were infected for 6 h with unstained or Hoechst-stained *F. tularensis* (MOI, 200). Cells infected with unstained *F. tularensis* were then stained with Hoechst 33342 stain; cells infected with Hoechst-stained *F. tularensis* were not stained after infection. Cells were observed with a Zeiss LSM 510 Meta confocal microscope. GFP (green) was excited with a 488-nm argon laser; Hoechst 33342 (blue) was excited with a 405-nm diode laser; and Cy3 (red) was excited with a 543-nm He-Ne laser. The stable $Nlrp3^{-/-}$-AIM2-GFP-N1 or $Aim2^{-/-}$-AIM2-GFP-N1 cell lines were generated by retroviral transduction, followed by sorting with Flow Cytometry at the Kimmel Cancer Center core facility, as described[3].

Statistics. Samples were analyzed with Student's *t*-test.

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