Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis

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Francisella tularensis **is a highly infectious gram-negative coccobacillus that causes the zoonosis tularemia. This bacterial pathogen causes a plague-like disease in humans after exposure to as few as 10 cells. Many of the mechanisms by which the innate immune system fights *Francisella* are unknown. Here we show that wild-type *Francisella*, which reach the cytosol, but not *Francisella* mutants that remain localized to the vacuole, induced a host defense response in macrophages, which is dependent on caspase-1 and the death-fold containing adaptor protein ASC. Caspase-1 and ASC signaling resulted in host cell death and the release of the proinflammatory cytokines interleukin (IL)-1β and IL-18. *F. tularensis*–infected caspase-1– and ASC-deficient mice showed markedly increased bacterial burdens and mortality as compared with wild-type mice, demonstrating a key role for caspase-1 and ASC in innate defense against infection by this pathogen.**

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The gram-negative coccobacillus, *Francisella tularensis*, is a facultative intracellular pathogen that causes disease in humans, rabbits, hares, and many rodents. Humans can contract an ulceroglandular form of tularemia through direct exposure to infected animals or from fly, mosquito, and tick bites. A pneumonic form of tularemia can be acquired by inhalation of as few as 10 organisms from infected material and the rate of mortality from untreated infections may be as high as 30%.

Most research on *F. tularensis* uses either the live vaccine strain (LVS), derived from a *F. tularensis* subspecies (ssp.) *holarctica* (type B) strain, or the *F. tularensis* ssp. *novicida* strain Utah 112 (U112). Both LVS and U112 are attenuated in humans but still cause disease in mice. These lethal infections in mice are similar to the human disease and are therefore established animal models of *Francisella* infection (1). The mechanisms behind cell-mediated protective responses against *Francisella* have been well described and require both B and T cells (2). Neutrophils, inducible NO synthase (iNOS), phagocyte oxidase (*phox*), and cytokines such as IFNγ, TNFα, and IL-12 are involved in innate immunity to *Francisella* infection (2, 3).

Relatively little is known about the molecular mechanisms of *Francisella* pathogenesis. *F. tularensis* can survive and replicate within amoebae and in the cytosol of macrophages. Several genes necessary for intracellular survival and virulence in mice have been identified, including *mglA*, *iglC*, *pdpD*, and *pdpA* (1, 4). MglA is a transcriptional activator that regulates the transcription of the virulence factors encoded by *iglC*, *pdpD*, and *pdpA* (5), which are located within an ∼30-kb pathogenicity island (4). The exact functions of these gene products are not known. *iglC* is required for *Francisella* escape from the phagolysosome to the cytosol and subsequent replication (6). Thus, cytosolic replication is necessary for *Francisella* virulence.

Because *Francisella* grows in the cytosol, it is critical that the macrophage has defenses in place to prevent the bacteria from reaching their niche, as well as to fight those that manage to reach the cytosol. The array of macrophage defenses that are localized to the phagosome limit the spread of *Francisella* to the cytosol (e.g., iNOS, phox). However, it is unknown how the macrophage fights intracytosolic *Francisella*. The inflammasome is a cytosolic complex of proteins that is activated by diverse stimuli including bacterial components (7). Caspases are cysteine proteases that can signal for host cell death and caspase-1 (casp-1) is a central component of the inflammasome. In

S. Mariathasan and D.S. Weiss contributed equally to this work.
The online version of this article contains supplemental material.

The Journal of Experimental Medicine

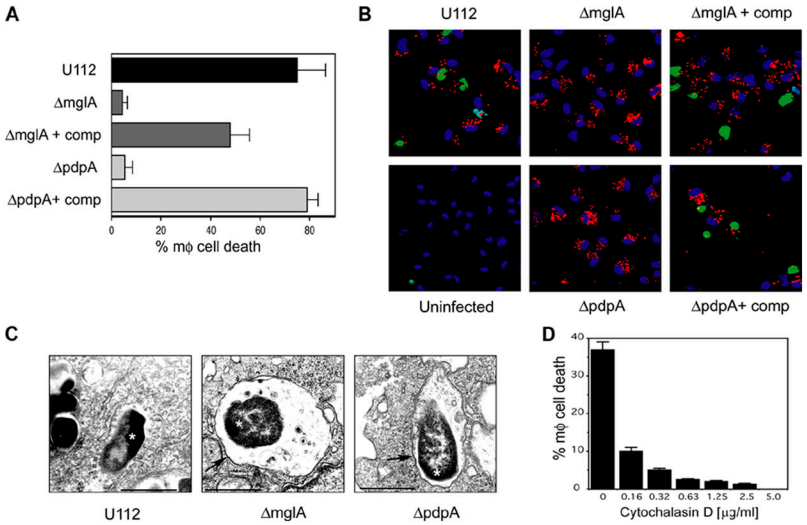

Figure 1. *Francisella*–induced macrophage death requires bacterial localization to the cytosol and the *mglA* and *pdpA* genes. Macrophages were infected with wild-type *Francisella* U112 (WT; multiplicity of infection [moi] 30), *ΔmglA* (moi 200), *ΔmglA + mglA* (moi 30), *ΔpdpA* (moi 200), *ΔpdpA + pdpA* (moi 30), and cell death was measured by (A) LDH release during the 8-h infection (% mϕ cell death) or (B) TUNEL. Macrophages were fixed 4 h after infection and stained with TUNEL (green), chicken anti-*Francisella* primary antibody, anti–chicken-alexa594 secondary antibody (red), and the TOTO-3 DNA stain (blue). (C) Macrophages were infected with WT, *ΔmglA*, or *ΔpdpA* for 6 h, fixed, and processed for transmission electron microscopy. Asterisks (*) denote intracellular bacteria and arrows point to phagosomal membranes. WT bacteria are not surrounded by a phagosomal membrane. Bar, 0.7 μm. (D) Macrophages were pretreated for 1 h with the indicated concentrations of cytochalasin D, washed, and infected with WT (moi 30) for 5 h followed by measurement of LDH release. Samples were performed in triplicate. Experiments were performed at least three times. Means and standard deviations from a representative experiment are shown.

addition to its ability to signal for cell death, casp-1 processes the immature pro-inflammatory cytokines, pro–IL-1β and pro–IL-18, to their mature, active forms, IL-1β and IL-18, respectively. Several adaptor molecules, including ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and Ipaf, are capable of activating casp-1.

We show that macrophages have evolved a final line of defense against *Francisella* that reach the cytosol. Macrophages undergo cell death in response to cytosolic *Francisella*, which results in the loss of the bacteria's intracellular niche. This cell death is dependent on casp-1 and ASC, as is the release of IL-1β and IL-18. Ipaf, however, is not required for these processes. In vivo, *casp-1*$^{-/-}$ and *Asc*$^{-/-}$ mice, but not *Ipaf*$^{-/-}$ mice, are extremely sensitive to *Francisella* infection, highlighting their role in innate defense against this bacterial pathogen.

RESULTS AND DISCUSSION

The primary target of *F. tularensis* during human and animal infection is the macrophage (1). The bacterium escapes from the phagolysosome between 3 and 4 h postinfection (p.i.) and proliferates in the cytosol of macrophages (8, 9). We noticed that activated macrophages underwent rapid death after infection with *Francisella* as measured by lactate dehydrogenase (LDH) release (Fig. 1 A) and Tdt-mediated dUTP nick end labeling (TUNEL) staining (Fig. 1 B). The *Francisella* transcription factor MglA, and a gene under its regulation (5), *pdpA*, were required to induce macrophage death (Fig. 1, A and B). The lack of macrophage death observed with *mglA* and *pdpA* mutants could be rescued by complementing with the appropriate WT allele (Fig. 1, A and B). Interestingly, we found that in contrast to WT *F. tularensis*, *pdpA* and *mglA* mutants could not escape the phagosome and reach the cytosol (Fig. 1 C), although the mutants are taken up by macrophages as efficiently as WT (unpublished data). Both *mglA* and *pdpA* are required for intracellular bacterial replication (4, 10). Together, these data strongly suggest that survival and/or replication of *F. tularensis* within the cytosol of macrophages is tightly associated with the induction of host cell death. Consistent with these results, cytochalasin D, an inhibitor of actin polymerization and bacterial internalization, blocked death of macrophages exposed to *F. tularensis* in a dose-dependent manner (Fig. 1 D). Thus, we hypothesized that macrophage death in response to *F. tularensis* is dependent on the bacteria escaping the macrophage phagolysosome, which leads to sensing of cytosolic bacteria by the host and activation of a specific molecular cascade.

The inflammasome is a complex of proteins that is assembled in response to intracellular bacterial components (7). Casp-1, which is in the inflammasome, signals for cell death in response to many stimuli (11). We tested whether casp-1 is required for *Francisella*-induced macrophage death. Nearly 90% of *F. tularensis*–infected WT macrophages died by 5 h p.i. (Fig. 2 A). However, macrophages from *casp-1*$^{-/-}$ mice were highly resistant to *Francisella*-induced death 8 h p.i. (Fig. 2 A). This result was not unique to *F. tularensis* ssp. *novicida* U112 as *F. tularensis* ssp. *holarctica* LVS also induced death of WT macrophages but not casp-1–null macrophages (Fig. 2 B).

Casp-1 activation in infected macrophages involves the autocatalytic processing of the 45-kD zymogen form of

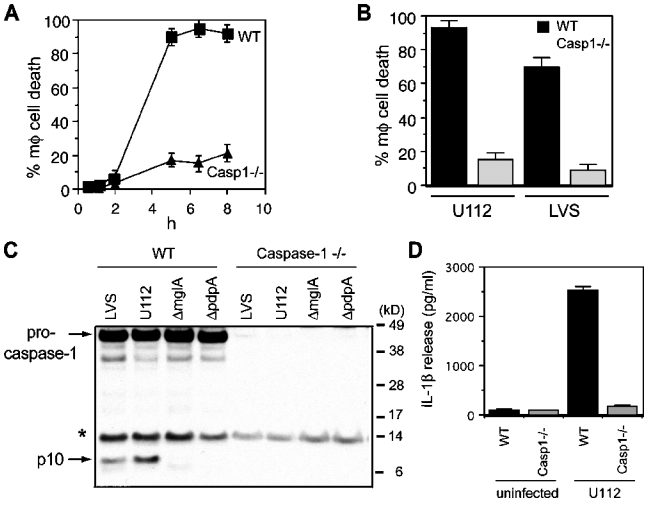

Figure 2. Casp-1 is essential for early *F. tularensis*–induced macrophage death. WT and *casp-1*$^{-/-}$ macrophages were infected (moi 30) with *F. tularensis* U112 (A and B) or LVS (B) for 8 h. Cell death was measured by LDH release into the culture supernatant (% mϕ cell death). (C) Macrophages were infected with LVS (moi 30), U112 (moi 30), *ΔmglA* (moi 200) or *ΔpdpA* (moi 200) and cell lysates were collected at 4 h and immunoblotted with antibodies against the p10 subunit of casp-1. The asterisk (*) denotes a nonspecific cross-reactive band. (D) Macrophages were infected with U112 (moi 30) and IL-1β release into the supernatant at 6 h was measured by ELISA. Samples were performed in triplicate. Experiments were performed at least three times. Means and standard deviations from a representative experiment are shown.

casp-1, as detected by Western blot by the appearance of the p10 cleavage product. We detected the p10 fragment of casp-1 in WT macrophages infected with *Francisella* for 4 h (Fig. 2 C) but not in infected *casp-1*$^{-/-}$ cells (Fig. 2 C). These results support our genetic data that casp-1 plays an important role in the induction of macrophage death by *F. tularensis*. In addition, processing of casp-1 did not occur in macrophages infected with *mglA* and *pdpA* mutants (Fig. 2 C), demonstrating that casp-1 activation is dependent on the presence of cytosolic bacteria (Fig. 1 C).

WT macrophages infected with *Francisella* U112 or LVS secreted IL-1β in a casp-1-dependent manner (Fig. 2 D; and unpublished data). These data indicate that casp-1 is essential not only for macrophage death, but is also required for secretion of IL-1β in response to cytosolic *F. tularensis*. In addition, WT macrophages that were preincubated with neutralizing antibodies against IL-1β and IL-18 were killed as efficiently as macrophages treated with control antibodies (unpublished data). Therefore, neither of the cytokines downstream of casp-1, IL-1β and IL-18, are involved in signaling for macrophage death.

The inflammasome contains distinct adapters, such as ASC and Ipaf, that are engaged in a stimulus-dependent manner (7, 12). We used ASC- and Ipaf-deficient macrophages to test whether these adapters are essential for macrophage death and casp-1 activation in response to *F. tularensis* infection. *Asc*$^{-/-}$ macrophages, similar to *casp-1*$^{-/-}$ macrophages, were resistant to *F. tularensis*–induced cell death as indicated by LDH release and TUNEL (Fig. 3, A and B), failed to process casp-1 (Fig. 3 C) and produced negligible IL-1β (Fig. 3 D) and IL-18 (Fig. 3 E). In contrast, *Ipaf*$^{-/-}$ macrophages were killed as efficiently as WT macrophages (Fig. 3, A and B) and processed casp-1 normally after *F. tularensis* infection (Fig. 3 C). These data identify ASC as the critical inflammasome adaptor for casp-1 activation and cell death in response to *F. tularensis* infection.

Despite defective casp-1 activation in *Asc*$^{-/-}$ macrophages infected with *F. tularensis*, other innate immune pathways activated by bacteria, such as NF-κB signaling, appeared normal. For example, IκBα was degraded in both WT and *Asc*$^{-/-}$ macrophages after infection with *F. tularensis* (Fig. 3 F) and secretion of the NF-κB–dependent cytokine TNFα was unaffected (Fig. 3 G). Phosphorylation of the mitogen-activated kinases ERK1 and ERK2 was also normal in *Asc*$^{-/-}$ macrophages (unpublished data). Therefore, ASC appears dispensable for normal NF-κB and ERK signaling in response to *F. tularensis* infection, but is essential for *Francisella*-induced macrophage death, casp-1 activation, and release of IL-1β and IL-18.

To assess the in vivo role of ASC and casp-1 in the innate immune response against *F. tularensis* infection, WT, *Asc*$^{-/-}$, *casp-1*$^{-/-}$, and *Ipaf*$^{-/-}$ mice were challenged subcutaneously with 10^5 CFU of *F. tularensis*, a dose that caused 65–75% mortality in WT mice. After infection, *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice (Fig. 4, A and C) succumbed to infection more rapidly than WT and *Ipaf*$^{-/-}$ mice (Fig. 4 E). Notably, *Asc*$^{-/-}$ mice died even more rapidly (~75% mortality on day 3) than *casp-1*$^{-/-}$ mice (~30% mortality on day 3). The increased susceptibility of *Asc*$^{-/-}$ mice was reflected in their 1,000–10,000-fold higher bacterial burdens in infected organs 1 d p.i. compared with WT, *casp-1*$^{-/-}$, and *Ipaf*$^{-/-}$ mice (Fig. 4, B and F and Fig. S1 for *casp-1*$^{-/-}$ available at http://www.jem.org/cgi/content/full/jem.20050977/DC1). The bacterial burden in organs of infected *casp-1*$^{-/-}$ mice, although similar to WT mice on day 1, was higher than in WT mice on day 2 and resembled the high burden seen in *Asc*$^{-/-}$ mice on day 1 (Fig. 4 D). This dramatic increase in bacterial levels was evidenced by staining the spleens from infected mice with an anti-*Francisella* antibody. High numbers of bacteria were distributed throughout the spleens of infected *Asc*$^{-/-}$ (day 1) and *casp-1*$^{-/-}$ (day 2) mice, whereas spleens from WT, *Ipaf*$^{-/-}$, and *casp-1*$^{-/-}$ mice (day 1) contained lower numbers of bacteria (Fig. 4 G). *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice inoculated with 10^4 or 10^3 CFU of *F. tularensis* harbored higher levels of bacteria than infected WT mice, demonstrating that the phenotypes of these knockout mice are not dependent on the inoculation dose (Fig. S2 available at http://www.jem.org/cgi/content/full/jem.20050977/DC1). Together, these data show that the ASC/casp-1 axis is required for innate host defense against *Francisella* infection in vivo.

In agreement with our in vitro results, serum from infected *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice did not contain detectable levels of IL-18 (Fig. 4 H), consistent with the requirement of

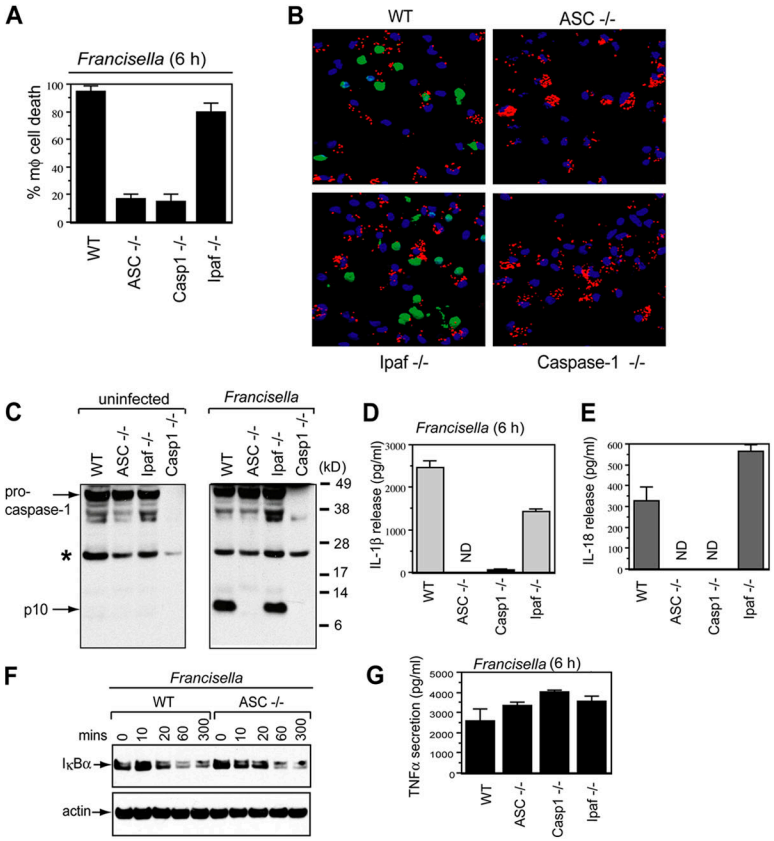

Figure 3. ASC is essential for *Francisella*–induced casp-1 activation and macrophage death. Macrophages from WT, *Asc*$^{-/-}$, *Ipaf*$^{-/-}$, or *casp-1*$^{-/-}$ mice were infected with WT *Francisella* U112 (moi 30) for 6 h. (A) Cell death was monitored by LDH release (% mϕ cell death). (B) 6 h p.i., macrophages were fixed and stained with TUNEL (green), chicken anti-*Francisella* primary antibody, anti–chicken-alexa594 secondary antibody (red), and TOTO-3 DNA stain (blue). (C) Cell lysates were immunoblotted with antibodies against the p10 subunit of casp-1. The asterisk (*) denotes a nonspecific cross-reactive band. (D and E) IL-1β and IL-18 release into the supernatant was measured by ELISA. (F) Analysis of IκB degradation in *Francisella*-infected macrophages. Macrophages were infected with WT U112 for 6 h. Cell lysates were analyzed by Western blot using anti-phospho-IκB antibody (S32; top) or antiactin antibody (bottom). (G) TNFα release into the supernatant was measured by ELISA. Western blot analyses were performed at least three times and representative blots are shown. ELISAs were performed in triplicate, means and standard deviations are shown.

ASC for casp-1 activation leading to IL-18 secretion. Serum IL-1β levels were below the limit of detection (unpublished data). The lack of detectable IL-18 in the serum of *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice suggested that the increased susceptibility of these mice could be due to deficiency in IL-18 and/or IL-1β, even though the latter was undetectable in the serum. To test the roles of these cytokines in the host response to *Francisella*, we treated WT mice with IL-18– and IL-1β–neutralizing antibodies, or with control antibodies, before infection. 2 d p.i., mice treated with IL-18– and IL-1β–neutralizing antibodies harbored more bacteria than mice treated with control antibodies (Fig. 4 I), demonstrating that IL-18 and IL-1β contribute to host defense against *Francisella*. However, the IL-18– and IL-1β–depleted mice did not contain as many bacteria as *casp-1*$^{-/-}$ mice, suggesting that the phenotype of *casp-1*$^{-/-}$ mice is due, only in part, to the deficiency in IL-18 and IL-1β. Inefficient macrophage death in the *casp-1*$^{-/-}$ mice may account for the rest of the phenotype observed in these mice. Therefore, macrophage death may be an important antibacterial defense mechanism. Taken together, these data show that the ASC/casp-1 axis is required for innate host defense against *Francisella* and that IL-18 and IL-1β play a role in host defense against *Francisella*, but that casp-1 likely has other functions in host defense that are independent of these cytokines.

The higher bacterial burdens in *Asc*$^{-/-}$ mice as compared with *casp-1*$^{-/-}$ mice on day 1 is consistent with the mouse survival data, and suggests that there might be subtle differences between *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice with respect to their innate immune response to *Francisella* infection. Intriguingly, ASC-null macrophages infected in vitro with *F. tularensis* for 24 h exhibited significantly less cell death than *casp-1*$^{-/-}$ macrophages (Fig. 5). These observations suggest that ASC-dependent casp-1 activation is critical for macrophage death during the early stages of infection (Fig. 3 A, 6 h). However, at later stages, ASC is essential for cell death in a casp-1–independent manner (Fig. 5). The casp-1–independent pathway may involve additional roles of ASC in the intrinsic mitochondrial pathway to apoptosis through a Bax

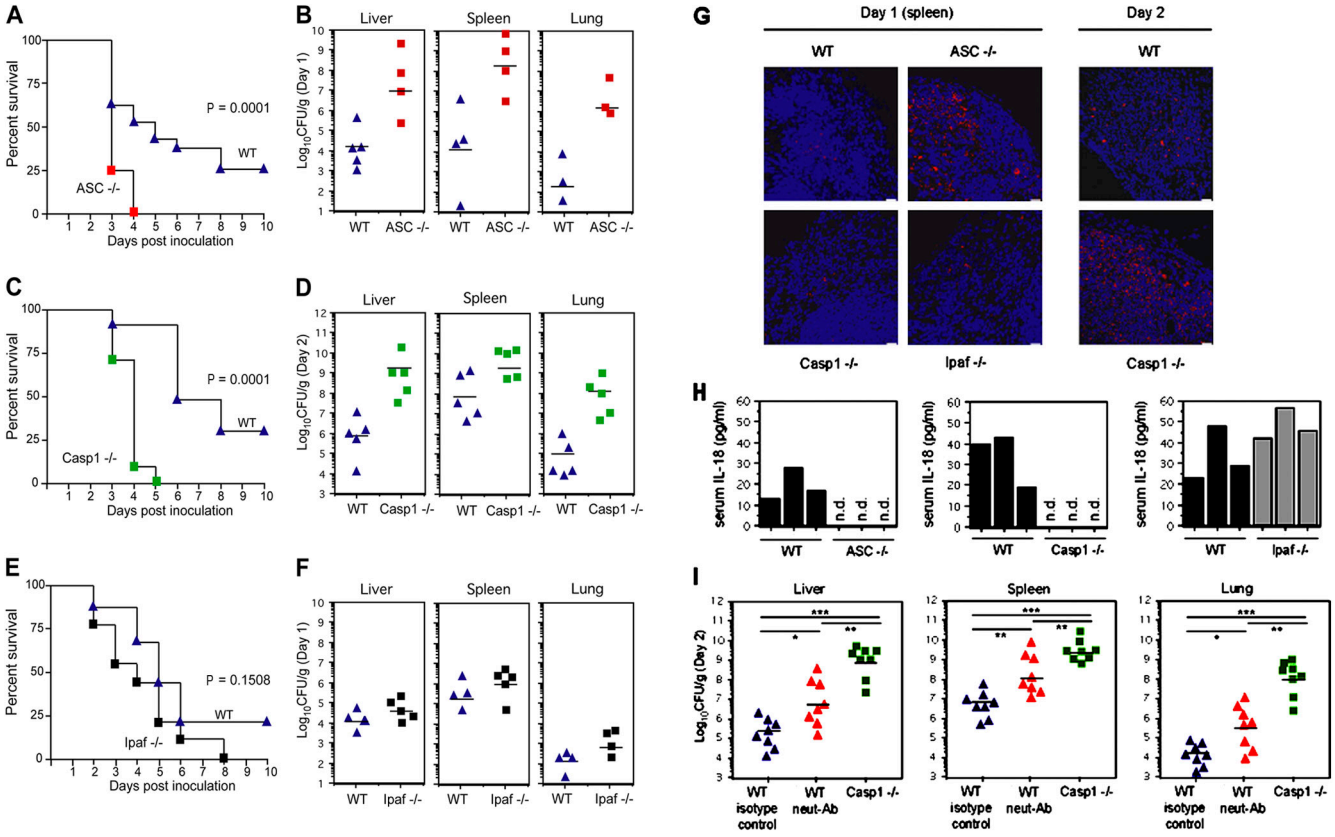

Figure 4. ASC and casp-1 are essential for host defense against *F. tularensis* in vivo. (A, C, and E) Mice were injected subcutaneously with WT *Francisella* U112 (1.5×10^5 CFU) and their survival was monitored (*Asc*$^{-/-}$ $n = 22$, *Asc*$^{+/+}$ $n = 23$; *casp-1*$^{-/-}$ $n = 10$, *casp-1*$^{+/+}$ $n = 10$; *Ipaf*$^{-/-}$ $n = 9$, *Ipaf*$^{+/+}$ $n = 9$) or (B, D, and F) tissues were recovered 1 or 2 d p.i., homogenized, and dilutions plated on bacterial media for enumeration of CFU. Bacterial counts on day 1 from the liver, spleen, and lung of *Asc*$^{-/-}$ mice were significantly higher compared with WT mice ($P = 0.0317$, 0.0471, and 0.0159, respectively). Day 2 counts from *casp-1*$^{-/-}$ mice were also significantly higher compared with WT mice (liver; $P = 0.0079$; spleen, $P = 0.0317$; lung, $P = 0.0079$). (G) Spleen sections were labeled with anti-*Francisella* antibody (red) and TOTO-3 (blue) to stain all nuclei. The image is a projection of an 8-μm *z*-stack collected through the 60× objective on a confocal microscope. Bar, 20 μm. (H) Levels of IL-18 in the serum of mice 1 d p.i. were determined by ELISA. (I) WT mice were intraperitoneally injected with neutralizing IL-18 and IL-1β antibodies ($n = 8$) or control antibodies ($n = 8$). 30 min later, antibody-treated mice, as well as untreated *casp-1*$^{-/-}$ mice ($n = 8$), were subcutaneously injected with 10^5 CFU of strain U112. 2 d p.i., tissues were collected, homogenized, and dilutions were plated on bacterial media to count CFU. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. All experiments were performed at least three times.

and caspase-9–dependent pathway (13, 14), and may correspond to the late cell death observed by Lai et al. involving caspase-3 and -9 (15). *Francisella*-induced macrophage death may share features of the cell death induced by other bacteria, including *Listeria*, *Mycobacteria*, and *Salmonella* (16). For example, *Listeria* induces a macrophage death that is dependent on the expression of the listeriolysin O toxin and type I interferon–dependent signaling, which correlates with the presence of cytosolic bacteria (17, 18). Whether *Francisella*, which also replicates in the cytosol, induces type I interferon–dependent signaling and the possible contribution of this pathway to *Francisella*-induced macrophage death remains to be determined.

Our results identify a critical role for ASC and casp-1 in the innate immune response against infection by the bacterial pathogen *Francisella*. *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice are extremely susceptible to *Francisella* infection, highlighting the importance of this axis in host defense. We further show that ASC and casp-1 are required for macrophage death and that this response specifically correlates with bacterial survival and replication in the cytosol. This suggests that the host cell senses the cytosolic presence of *Francisella*, perhaps through intracellular detector molecules of the NBS-LRR (nucleotide-binding site and leucine-rich repeat) family of proteins. Nod2, a NOD-LRR subfamily member that recognizes bacterial peptidoglycan (19), signaling is important for innate and adaptive immunity in the intestinal tract in response to *Listeria* (20). We found that Nod2 was dispensable for detection of intracellular *F. tularensis* leading to cell death, suggesting that an unidentified sensor(s) is involved (unpublished data). Interestingly, the intracellular bacterial pathogens *Shigella* and *Salmonella* induce macrophage death

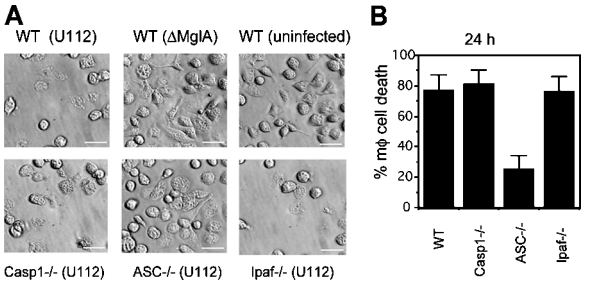

Figure 5. ASC is required for two stages of macrophage death in response to *Francisella*. Macrophages from WT, *Asc*$^{-/-}$, *casp-1*$^{-/-}$, or *Ipaf*$^{-/-}$ mice were infected with *F. tularensis* U112 (moi 30) for 24 h and (A) imaged by differential interference contrast microscopy or (B) assayed for macrophage death by LDH release (% mϕ cell death). Samples were performed in triplicate. Experiments were performed at least three times. Means and standard deviations from a representative experiment are shown.

through casp-1 (16). For these enteric pathogens, Ipaf is essential for sensing and transducing the signal for casp-1 activation and macrophage death, whereas ASC plays a minor role (12; and unpublished data). In contrast, ASC, but not Ipaf, is essential for casp-1 activation in response to *Francisella*. These observations suggest that the host possesses a complex cytosolic network to detect and respond to intracellular infection and that inflammasome adapters have the ability to discriminate between different types of pathogenic bacteria. Given the many sensor proteins (NALPs/PANs/PYPAFs) known to signal through ASC (21), it is likely that ASC represents a major inflammasome adaptor and therefore mediates resistance to a broad range of intracellular pathogens in addition to *Francisella*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. WT *F. tularensis* ssp. *novicida*, strain U112 and isogenic strains carrying mutations in *mglA* and *pdpA* were described previously (4, 10). The LVS strain was obtained from K. Elkins (Food and Drug Administration, Rockville, MD). Bacteria were grown overnight with aeration in modified Mueller Hinton broth (Difco Laboratories) supplemented with 0.025% ferric pyrophosphate and IsoVitaleX (Becton Dickinson). The bacteria were washed and resuspended in PBS to the appropriate concentration.

Mice and *F. tularensis* infections. The generation of *Asc*- and *Ipaf*-deficient mice has been described previously (12). These knockout mice were backcrossed to C57BL/6 mice at least eight times (Genentech, Inc.). *casp-1*$^{-/-}$ mice were received from BASF (22) on a mixed C57BL/6 × 129Sv background and then backcrossed another seven times to C57BL/6 mice (B. Raupach, Max Planck Institute, Berlin, Germany). All mice were between 8 and 12 wk of age. Age-matched C57BL/6 (Jackson ImmunoResearch Laboratories) served as WT mice with *casp-1*$^{-/-}$ mice and age-matched littermates served as WT mice with *Asc*$^{-/-}$ and *Ipaf*$^{-/-}$ mice. All mice were kept under specific pathogen-free conditions in filter-top cages at Stanford 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. Mice were inoculated with the indicated dose of strain U112 subcutaneously in a 0.05-ml vol. The mice were monitored for signs of illness and lethality twice daily for 20 d for the survival study. Spleen, liver, and lung were harvested 1 or 2 d p.i., homogenized, and dilutions were plated on supplemented Mueller Hinton agar plates to determine CFU/g tissue. Mice were bled retroorbitally at the time of euthanization for collection of serum. For the antibody-depletion experiment, WT mice were injected intraperitoneally with a mixture of 500 μg of the anti–mouse IL-18–neutralizing antibody (Peprotech; gift from A. Zychlinsky, Max Planck Institute, Berlin, Germany) and 500 μg of the anti–mouse IL-1β–neutralizing antibody (eBioscience), or a mixture of isotype-matched control antibodies, in a volume of 700 μl sterile, endotoxin-free PBS. 30 min later, mice were injected subcutaneously with 10^5 CFU of strain U112. Organs were collected 2 d p.i. and CFU/g tissue was determined as described before.

Macrophage infections. Mice were injected intraperitoneally with 4% thioglycollate and macrophages were collected by peritoneal lavage 4 d later. 2×10^6 cells were plated in a six-well dish and nonadherent cells were removed after 2 h. Adherent macrophages were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml^{-1} penicillin, 50 μg/ml^{-1} streptomycin, and 50 ng/ml^{-1} LPS. *Francisella* strains were incubated with 10% normal horse serum for 15 min at 37°C before infection. Macrophages were washed with media that lacked antibiotics, bacteria were added, and plates were spun for 15 min at 850 *g*. Cells were incubated at 37°C, 5% CO_2 for 2 h. Gentamicin (100 μg/ml) was then added for 90 min, after which the cells were washed and incubated in media containing 10 μg/ml gentamicin. Where indicated, the appropriate concentrations of cytochalasin D (Sigma-Aldrich) were added to macrophages for 15 min at 37°C (5% CO_2) before bacterial infection and washed out before incubation with bacteria. Neutralizing IL-1β and IL-18 antibodies (50 μg/ml of each) were added to macrophages 1 h before infection and were present for the 6-h infection.

Western blotting. Macrophages infected with bacteria were lysed in 1% NP-40 lysis buffer (50 mM Tris buffer, pH 7.4, 150 mM NaCl, 1% NP-40) supplemented with complete protease inhibitor cocktail (Roche) and 2 mM dithiothreitol. Lysates were resolved in 4–12% Tris-glycine gradient gels (Invitrogen) and transferred to nitrocellulose (Invitrogen) by electroblotting. For immunoblotting, rabbit anti–casp-1 (sc-514; Santa Cruz Biotechnology, Inc.) was used.

Macrophage death assays. Macrophages were seeded in 96-well plates at 5×10^4 cells per well for cytotoxicity assays or in 24-well plates with coverslips at 2.5×10^5 per well for TUNEL stainings and incubated overnight with 50 ng/ml^{-1} LPS. Before infection, the medium was replaced with phenol red-free RPMI 1640 medium. Cells were infected with the indicated multiplicities of infection. Cultures were supplemented with gentamicin (50 μg/ml^{-1}) after 2 h to kill extracellular bacteria. Cell death was quantified with the CytoTox96 LDH-release kit (Promega) at the indicated times. In brief, the LDH-release assay is a colorimetric test that measures the amount of LDH, a cytosolic enzyme, which is released into the supernatant of untreated cells, lysed cells, or the experimental sample. Percent cell death is calculated measuring the OD_{490} of each sample and using the formula: [(experimental cells − untreated cells)/(lysed cells − untreated cells)] × 100%. TUNEL reactions were performed on infected cells fixed to coverslips as described for the in situ Cell Death Detection Kit for Fluorescein (Boehringer). Bacteria were stained with chicken anti-*Francisella* polyclonal antiserum (1/5,000 dilution) followed by anti–chicken-alexa594 antibody (Invitrogen) and TOTO-3 (Invitrogen) to stain host cell nuclei. The anti-*Francisella* antibody used in this study was generated by injecting chickens (Aves Laboratories) with ~10^9 of *Francisella holarctica* LVS bacteria fixed in 4% paraformaldehyde. Coverslips were mounted over antiquench (Vector Laboratories) and sealed. The images were collected on a Optiphot-2 microscope (Nikon) attached to a confocal laser scanning microscope (MRC1024; Bio-Rad Laboratories) using LaserSharp software (Bio-Rad Laboratories). The laser lines on the krypton/argon laser were 488 nm (alexa488), 568 nm (alexa594), and 647 nm (alexa660 and TOTO-3). The numerical aperture was 0.75 on the 60× oil objective. Volocity 2.0 was used for image analysis and all images were based on maximum intensity projection.

Cytokine measurements. Culture supernatants from infected macrophages or serum from infected mice were assayed for IL-1β or TNFα (R&D Systems) or IL-18 (MBL International Corporation) by ELISA.

Histology and immunohistochemistry. For histological examinations of tissue sections, spleens were fixed in 10% buffered neutral formalin, embedded in paraffin, and serially sectioned (7–9 μm). Some sections were stained with hematoxylin and eosin. For immunohistochemistry, spleen sections were incubated with anti-*Francisella* polyclonal chicken antiserum in PBS containing 3% BSA and 0.2% saponin. Tissue sections were then incubated with anti–chicken-Alexa594 antibody (Invitrogen) and TOTO-3 (Invitrogen) to stain host cell nuclei. Coverslips were mounted over antiquench (Vector Laboratories) and sealed.

Transmission electron microscopy. Infected macrophages on coverslips were fixed in a solution of 2.5% gluteraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.3, for 1 h at 0°C and were stained with 0.25% uranyl acetate in 0.1 M sodium acetate, pH 6.3. Samples were dehydrated through a graded series of alcohol and propylene oxide, infiltrated with 100% Epon, and polymerized at 60°C for 24 h. Serial sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (model 201c; Philips Electronic Instruments).

Statistical analysis. Statistical significance was calculated using the Mann-Whitney U test for bacterial colonization experiments and the Chi square test for mouse survival experiments.

Online supplemental material. Fig. S1 shows that the spleen, liver, and lung tissue bacterial counts from *casp-1*$^{-/-}$ mice infected with *F. tularensis* are the same as those from wild-type mice 1 day after subcutaneous inoculation. Fig. S2 shows that similar to the results seen with a high dose of *F. tularensis* (Fig. 4), mice infected with lower doses of *F. tularensis* still resulted in increased colonization in *Asc*$^{-/-}$ and *casp-1*$^{-/-}$ mice compared to wild-type mice. Online supplemental materials are available at http://www.jem.org/cgi/content/full/jem.20050977/DC1.

We thank K. Elkins for sending bacterial strains; K. Newton and E. Joyce for critical reading of the manuscript; N. Ghori for electron microscopy help; I. Bilis and H. Mathew for assistance with animal experiments.

This work was supported by National Institutes of Health grants PO1AI063302 and U-19AI057229 awarded to D.M. Monack and a fellowship from the Giannini Family Foundation awarded to D.S. Weiss.

The authors have no conflicting financial interests.

Submitted: 13 May 2005
Accepted: 12 September 2005

REFERENCES

1. Sjostedt, A. 2003. Virulence determinants and protective antigens of *Francisella tularensis*. *Curr. Opin. Microbiol.* 6:66–71.
2. Elkins, K.L., S.C. Cowley, and C.M. Bosio. 2003. Innate and adaptive immune responses to an intracellular bacterium, Francisella tularensis live vaccine strain. *Microbes Infect.* 5:135–142.
3. Lindgren, H., S. Stenmark, W. Chen, A. Tarnvik, and A. Sjostedt. 2004. Distinct roles of reactive nitrogen and oxygen species to control infection with the facultative intracellular bacterium *Francisella tularensis*. *Infect. Immun.* 72:7172–7182.
4. Nano, F.E., N. Zhang, S.C. Cowley, K.E. Klose, K.K. Cheung, M.J. Roberts, J.S. Ludu, G.W. Letendre, A.I. Meierovics, G. Stephens, and K.L. Elkins. 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J. Bacteriol.* 186:6430–6436.
5. Lauriano, C.M., J.R. Barker, S.S. Yoon, F.E. Nano, B.P. Arulanandam, D.J. Hassett, and K.E. Klose. 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. *Proc. Natl. Acad. Sci. USA.* 101:4246–4249.
6. Lindgren, H., I. Golovliov, V. Baranov, R.K. Ernst, M. Telepnev, and A. Sjostedt. 2004. Factors affecting the escape of *Francisella tularensis* from the phagolysosome. *J. Med. Microbiol.* 53:953–958.
7. Martinon, F., and J. Tschopp. 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell.* 117:561–574.
8. Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt. 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. *Infect. Immun.* 71:5940–5950.
9. Clemens, D.L., B.Y. Lee, and M.A. Horwitz. 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect. Immun.* 72:3204–3217.
10. Baron, G.S., and F.E. Nano. 1998. MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. *Mol. Microbiol.* 29: 247–259.
11. Riedl, S.J., and Y. Shi. 2004. Molecular mechanisms of caspase regulation during apoptosis. *Nat. Rev. Mol. Cell Biol.* 5:897–907.
12. Mariathasan, S., K. Newton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-Girma, S. Erickson, and V.M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature.* 430:213–218.
13. Ohtsuka, T., H. Ryu, Y.A. Minamishima, S. Macip, J. Sagara, K.I. Nakayama, S.A. Aaronson, and S.W. Lee. 2004. ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat. Cell Biol.* 6:121–128.
14. McConnell, B.B., and P.M. Vertino. 2004. TMS1/ASC: the cancer connection. *Apoptosis.* 9:5–18.
15. Lai, X.H., and A. Sjostedt. 2003. Delineation of the molecular mechanisms of *Francisella tularensis*-induced apoptosis in murine macrophages. *Infect. Immun.* 71:4642–4646.
16. Navarre, W.W., and A. Zychlinsky. 2000. Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies. *Cell. Microbiol.* 2:265–273.
17. Stockinger, S., T. Materna, D. Stoiber, L. Bayr, R. Steinborn, T. Kolbe, H. Unger, T. Chakraborty, D.E. Levy, M. Muller, and T. Decker. 2002. Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes. *J. Immunol.* 169:6522–6529.
18. Stockinger, S., B. Reutterer, B. Schaljo, C. Schellack, S. Brunner, T. Materna, M. Yamamoto, S. Akira, T. Taniguchi, P.J. Murray, et al. 2004. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. *J. Immunol.* 173:7416–7425.
19. Inohara, N., M. Chamaillard, C. McDonald, and G. Nunez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 74:355–383.
20. Kobayashi, K.S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R.A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science.* 307: 731–734.
21. Reed, J.C., K.S. Doctor, and A. Godzik. 2004. The domains of apoptosis: a genomics perspective. *Sci. STKE.* 2004:239:re9.
22. Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, et al. 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell.* 80:401–411.