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.
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, cytchalasin 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−/− macrophages (Fig. 2 B).

Casp-1 activation in infected macrophages involves the autolytic processing of the 45-kD zymogen form of
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 U112 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; 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 10⁵ 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 10⁴ or 10⁵ 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
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/AP-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 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 (% m 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 antianti 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.
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
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 adaptors 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 *mda* 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 (Food and Drug Administration, Rockville, MD). Bacteria were grown in media containing 10% fetal bovine serum, 50 U/mL penicillin, 50 mg/mL streptomycin, 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 infections.** Mice were injected intraperitoneally with 4% thioctic acid 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 penicillin, 50 μg/mL streptomycin, and 50 ng/mL 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% CO₂ for 2 h. Gentamicin (100 μg/mL) was then added 90 min after, which the cells were washed and incubated in media containing 1.25 μg/mL gentamicin. Where indicated, the appropriate concentrations of cytochalasin D (Sigma-Aldrich) were added to macrophages for 15 min at 37°C (5% CO₂) 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 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) 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 reaction products 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-alexap594 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 heilmannii LVS bacteria fixed in 4% paraformaldehyde. Coverslips were mounted over anaphase (Vector Laboratories) and sealed. The images were collected on a confocal laser scanning microscope (Carl Zeiss) attached to a confocal laser scanning microscope (Leica; Bio-Rad Laboratories) using LeicaTCS 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. Velocity 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 antisera 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 anti-quench (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 As−/− 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.

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