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Cutting Edge: Mutation of *Francisella tularensis mviN* Leads to Increased Macrophage Absent in Melanoma 2 Inflammasome Activation and a Loss of Virulence

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Abstract

The mechanisms by which the intracellular pathogen *Francisella tularensis* evades innate immunity are not well defined. We have identified a gene with homology to *Escherichia coli mviN*, a putative lipid II flippase, which *F. tularensis* uses to evade activation of innate immune pathways. Infection of mice with a *F. tularensis mviN* mutant resulted in improved survival and decreased bacterial burdens compared to infection with wild-type *F. tularensis*. The *mviN* mutant also induced increased AIM2 inflammasome-dependent IL-1β secretion and cytotoxicity in macrophages. The compromised *in vivo* virulence of the *mviN* mutant depended upon inflammasome activation, as caspase-1- and ASC-deficient mice did not exhibit preferential survival following infection. This study demonstrates that *mviN* limits *F. tularensis*-induced AIM2 inflammasome activation which is critical for its virulence *in vivo*.

The ability of *F. tularensis* to disrupt phagocyte function plays a key role in its virulence. Following phagocytosis, *F. tularensis* escapes the phagosome prior to phagosome/lysosome fusion and subsequently replicates within the cytosol (1,2). Intracellular survival of *F. tularensis* is also associated with its ability to inhibit NADPH oxidase activity as well as subsequent NF-κ B activation (3,4). In activated macrophages (Mφ) the escape of *F. tularensis* from the phagosome into the cytosol triggers activation of the AIM2 inflammasome (5–7), a multiprotein complex containing AIM2 (absent in melanoma 2; an interferon-inducible HIN-200 family member), ASC (apoptosis-associated speck-like protein containing a CARD) and the cysteine protease caspase-1 (8–11). Activation of the AIM2 inflammasome results in autocatalytic cleavage of caspase-1, resulting in the processing and secretion of IL-1β and IL-18. Inflammasome activation plays a crucial role in innate immune responses against *F.*

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tularensis, as AIM2-, caspase-1- and ASC-deficient mice have markedly increased bacterial burdens and mortality following infection with *F. tularensis* (5,6).

In this study we demonstrate that *F. tularensis* live vaccine strain (LVS) avoids efficient AIM2 inflammasome activation. We show that *mviN* is required to inhibit *F. tularensis*-induced caspase-1-dependent processing and secretion of IL-1β and IL-18 as well as to limit *F. tularensis*-mediated cytotoxicity. Mutation of *mviN* resulted in a marked change in the morphology of the bacterium and was required for full virulence of *F. tularensis* LVS in an *in vivo* model of infection.

Materials and Methods

Bacterial strains, plasmid construction and growth conditions

F. tularensis ssp. *holarctica* LVS was obtained from ATCC (ATCC 29684). The LVS strain containing a chromosomal mutation in *mviN* was transformed with the Tn*5* delivery plasmid pBB109 as previously described (12). Complementation of the *mviN*::Tn*5* strain with wild-type *mviN* containing the ribosomal binding site in the plasmid pBB103 was accomplished by cryotransformation as previously described (13). For *in vitro* studies LVS strains were grown on Difco cysteine heart agar supplemented with 9% SRBC for 48 h at 37° C. 25 μg/ml spectinomycin was added to plates for growth of the *mviN*::Tn*5* + *mviN* strain. For *in vivo* studies bacteria were grown overnight in modified Mueller-Hinton (MMH) broth (Becton Dickinson) supplemented with 1% (wt/vol) glucose, 0.025% ferric pyrophosphate, and 2% IsoVitaleX.

Scanning electron microscopy

Bacterial strains were grown in MMH broth for 6 h to an OD_{600} between 0.2 and 0.4. Samples were placed on silicon wafers and fixed in 2.5% glutaraldehyde and dehydrated using a standard graded ethanol series, with a final clearance in hexa-methyl-disilazane. Samples were coated with gold/palladium and viewed on a Hitachi S-4800 scanning electron microscope.

Mice and F. tularensis LVS infections

The generation of ASC-, caspase-1-, NLRP3-, NLRC4- and AIM2-deficient mice have been described previously (6,14–16). The University of Iowa Institutional Animal Care and Use Committee approved all protocols used in this study. Mice (6–8 wk old) were injected i.p. withthe indicated dose of *F. tularensis* LVS or the *mviN* mutant. Mice were monitored every 12 h for lethality; mice found to be in a moribund state for more than 4 h were considered terminal and euthanized.

Mφ infections

Bone marrow-derived Mφ were generated as previously described (14). Unless indicated, Mφ were activated by stimulating with 50 ng/ml LPS from *E. coli* serotype 0111:B4 (Invivogen) for 3–6 h prior to infection. Mφ were infected with *F. tularensis* LVS or the *mviN* mutant at an MOI of 50:1 unless otherwise indicated. 9 h later, or at the indicated time, supernatants were collected and assayed for IL–1β , IL-18, TNFα and IL-6 by ELISA (14). Mφ cell death wasdetermined by measuring lactate dehydrogenase (LDH) release usinga cytotoxicity detection kit (Promega). Western blotting was performed as previously described (14).

Statistical analysis

Two-tailed Mann-Whitney U test were performed using Prism software. Values of $p < 0.05$ were considered statistically significant. Unless stated otherwise, determinations are expressed as the mean ± SEM.

Results and Discussion

MviN is required for F. tularensis LVS virulence in vivo

Peptidoglycan, a major component of bacterial cell walls, is composed of glycan chains that are cross-linked by peptide bridges. A lipid-linked peptidoglycan precursor (Lipid II) is generated in the cytoplasm by a series of highly conserved enzymatic steps. MviN was recently identified as being responsible for flipping the lipid II across the cytoplasmic membrane (17, 18); once on the periplasmic side, penicillin-binding proteins use lipid II to generate mature peptidoglycan. *F. tularensis* LVS possesses an *mviN* gene (FTL_1305) that shares homology to that of *E. coli* (30% identity, 54% similarity) as well as *Salmonella typhimurium* (28% identity, 52% similarity) and *Legionella pneumophila* (31% identity, 54% similarity), two pathogens that upon infecting Mφ can also activate caspase-1 (19–21). *mviN* is also highly conserved amongst other *F. tularensis* subspecies with homology to the *mviN* genes of *F. tularensis* ssp. *novicida* (98% identity 99% similarity) and *F. tularensis* ssp. *tularensis* (98% identity 99% similarity).

To determine if *mviN* is important for *F. tularensis* LVS virulence *in vivo*, we utilized an *mviN* mutant, *mviN*::Tn*5*, isolated from a *F. tularensis* LVS transposon library. WT mice were challenged i.p. with either LVS or the *mviN* mutant, and survival monitored. Infection with 3×10^4 CFU of LVS resulted in 100% mortality by day 6 (Fig. 1A). In contrast, all mice infected with 3×10^4 CFU of the *mviN* mutant survived until day 14 (Fig. 1A). Infection with 3×10^5 or 3×10^6 CFU of *mviN* mutant resulted in a 90% and 70% survival rate respectively (Fig. 1A). The increased survival of mice infected with the *mviN* mutant relative to wild-type LVS was also reflected in the 10–100,000 fold lower bacterial burdens in infected organs 3 d post-infection (Fig. 1B). Histology of liver 3 d after infection revealed fewer hepatic lesions in mice infected with the *mviN* mutant compared to LVS (Fig. 1C and D). Although fewer in number, the necropurulent foci seen in *mviN* mutant infected mice trended towards being larger than those of LVS infected mice (Fig. 1D). Similarly, histology of spleen 3 d after infection demonstrated occasional necropurulent foci in *mviN* mutant infected mice, whereas LVS infected mice had extensive coalescing necropurulent foci in the splenic red pulp that extended into the white pulp (Suppl. Fig. 1A and B). Collectively, these data suggest that the *F. tularensis* LVS *mviN* mutant was highly attenuated *in vivo*.

MviN is dispensable for F. tularensis LVS growth in vitro

Mutant *E. coli* strains lacking functional *mviN* fail to grow and undergo lysis (17,18); similarly, *mviN* was essential for the viability of *Sinorhizobium meliloti* and *Burkholderia pseudomallei* (22,23). However, the *F. tularensis* LVS *mviN* mutant grew normally in broth (Suppl. Fig. 2A), consistent with the findings that *mviN* mutations in *S. typhimurium* and mutations of *mviN* homologs in *Bacillus subtilis* do not exhibit defective growth (24,25). These results suggest that *mviN* is dispensable for the growth of *F. tularensis* LVS and it is possible that LVS may possess redundant pathways that compensate for the lack of *mviN*.

Since Mφ support *Francisella* replication in the host during natural infection, we examined the intracellular replication of the *mviN* mutant *in vitro*. Both LVS and the *mviN* mutant replicated with similar kinetics within unprimed Mφ (Suppl. Fig. 2B), suggesting that the attenuation of infection by the *mviN* mutant in mice was not simply due to an inability of the intracellular organism to replicate in this cell type.

Although we did not detect a defect in bacterial replication of the *mviN* mutant *in vitro*, scanning electron microscopy revealed a striking change in cellular morphology of the *mviN* mutant; *mviN* mutants appeared round and lacked the pleomorphic structure typical of LVS (Suppl. Fig. 2C). These morphological results suggest that *mviN* serves an important role in the synthesis of *Francisella* cell wall structures, although additional studies are required assess the full impact of *mviN* on *F. tularensis* peptidoglycan synthesis.

MviN restricts Francisella-induced caspase-1 activation

Since *Francisella* induces cell death in primed Mφ in a caspase-1-dependent manner (5), we tested whether *mviN* was necessary for *Francisella*-mediated cytotoxicity. LPS-primed WT Mφ infected with LVS underwent celllysis with 25% cytotoxicity 12 h after infection. However, infection of LPS-primed WT Mφ infected with the *mviN* mutant resulted in more extensive cell death compared to LVS, with all cells lysed at 12 h (Fig. 2A). Complementation of the *mviN* mutant induced Mφ death at levels similar to wild-type LVS (Fig. 2B) confirming the role of *mviN* in this process. The Mφ death induced by the *mviN* mutant was dependent on caspase-1, as caspase-1-deficient Mφ infected with the *mviN* mutant had a delayed cell death compared to WT Mφ (Suppl. Fig. 3A). We also examined the intracellular replication of the *mviN* mutant within LPS-primed Mφ and found decreased growth of the *mviN* mutant compared to LVS at 24 h (Suppl. Fig. 3B) consistent with the increased Mφ death induced by the *mviN* mutant.

In addition to its role in mediating *F. tularensis*-induced Mφ death, caspase-1 is required for the processing and secretion of IL-1β and IL-18 by infected cells (5). The *mviN* mutant induced greater secretion of IL-1β and IL-18 from LPS-primed Mφ compared to LVS (Fig. 2C–E), at all multiplicities of infection tested (Supp. Fig. 3C). Mutant bacteria complemented with *mviN* failed to induce increased IL-1β and IL-18 secretion (Fig. 2D, E). In contrast to the augmented IL-1β and IL-18 release, the secretion of IL-6 and TNFα by unprimed Mφ infected with *F. tularensis* LVS was unaffected by the absence of *mviN* (Fig. 2F, G).

Caspase-1 activation is a two-step process culminating in the autocatalytic processing of the45-kD pro-caspase-1 to generate two subunits, p20 and p10. In the first step, treatment with an agent such as LPS not only results in the generation of pro-IL-1β but also primes the inflammasome for subsequent activation (26). In the case of AIM2 inflammasome activation, phagosomal escape of *F. tularensis* into the Mφ cytoplasm, likely with concurrent release of bacterial DNA into the cytosol, serves as the second signal (6). The *mviN* mutant did not enhance IL-1β secretion by bypassing the need for a priming step, as neither wild-type LVS nor the *mviN* mutant was capable of inducing IL-1β secretion from unprimed Mφ (Suppl. Fig. 3D). Whereas primed Mφ infected with LVS activated caspase-1by 9 h post infection, as judged by Westernblot detection of the p10 cleavage product (Fig. 2H), those infected with the *mviN* mutant induced caspase-1 activation more rapidly (by 6 h post infection) and to a greater extent compared to wild-type LVS (Fig. 2H). Taken together, these data suggest that *F. tularensis* LVS expression of *mviN* was required to limit caspase-1 activation and subsequent cytotoxicity and secretion of IL-1β and IL-18; and although the *mviN* mutant was able to replicate within unprimed Mφ *in vitro*, attenuation of infection by the *mviN* mutant *in vivo* was most probably due to increased death of *F. tularensis*-infected Mφ and increased proinflammatory cytokine production.

MviN limits Francisella-induced AIM2 inflammasome activation

In addition to AIM2, the NLR family members NLRP3 and NLRC4 can activate caspase-1 in response to bacterial pathogens (27). To determine if NLRP3 or NLRC4 was responsible for the increased IL-1β secretion induced by infection with the *mviN* mutant, Mφ from WT, caspase-1-/-, ASC-/-, NLRP3-/- and NLRC4-/- mice were infected with either LVS or the

mviN mutant (Fig. 3A). Secretion of IL-1β in response both to wild-type LVS and to the *mviN* mutant was dependent on caspase-1 and ASC but independent of NLRP3 and NLRC4 (Fig. 3A). In contrast, caspase-1 activation and IL-1β secretion in response to infection both with wild-type LVS and with the *mviN* mutant were completely dependent on AIM2 (Fig. 3B, C). As expected, AIM2-deficient Mφ were fully capable of secreting IL-1β in response to the NLRP3 agonist alum (Fig. 3B). Taken together, these data demonstrate that the augmented caspase-1 activation induced by the *mviN* mutant did not reflect the *mviN* mutant engaging a different inflammasome pathway, such as NLRP3 or NLRC4, but rather increasing activation of the AIM2-dependent pathway. Consistent with our *in vitro* findings, ASC-/- and caspase-1-/- mice infected i.p. with the *mviN* mutant succumbed to infection at similar rates as did mice infected with wild-type LVS (Fig. 3D, E). In contrast to WT mice, no difference in bacterial burdens was observed in the spleen and liver of caspase-1-/- mice infected with either LVS or the *mviN* mutant (Supp. Fig. 4). Similarly, no difference in bacterial burdens was observed in the liver of ASC-/- mice (Supp. Fig. 4). Although ASC-/-spleens did have statistically lower burdens of the *mviN* mutant compared to LVS (Supp. Fig. 4) this difference was dramatically smaller than that observed in WT mice (3 fold vs 150,000 fold respectively). These observations suggest that the attenuation of survival of the *mviN* mutant *in vivo* was due to its increased activation of the AIM2 inflammasome.

Given the importance of the AIM2 inflammasome in host defense against *F. tularensis* (6), it is not unexpected that a successful pathogen such as *F. tularensis* would evolve mechanisms to limit activation of this important innate immune pathway. Our results indicate that *mviN* was a critical for virulence of *F. tularensis* by virtue of its ability to restrict inflammasome activation. In fact, two genes have been identified in the related organism *F. novicida*, FTT_0748 and FTT_0584, that also inhibit Mφ cytotoxicity and IL-1β secretion (28). Although the role of *F. tularensis* LVS genes FTL_1364 and FTL_1327, homologues of FTT_0748 and FTT_0584 respectively, remain to be elucidated, they do not appear to compensate for a deficiency in *mviN*. It appears that *Francisella* possesses multiple genes that contribute to evading inflammasome activation, raising the possibility that strains that elicit more severe systemic disease, such as the type A strain *F. tularensis* ssp. *tularensis*, may also more efficiently evade AIM2 inflammasome activation.

It is postulated that acidification of the Mφ phagosome results in lysis of some ingested *F. tularensis* with the subsequent release of bacterial DNA (6). This DNA enters the cytoplasm, possibly through *F. tularensis*-induced phagosomal membrane damage, and activates the AIM2 inflammasome, resulting in the pyroptotic death of the Mφ along with processing and secretion of IL-1β and IL-18. The aberrant morphology of the *mviN* mutant may leave the bacteria more susceptible to lysis, with increased release of bacterial DNA. Alternatively, bacteriolysis may be occurring within the cytosol once the bacteria have escaped the phagosome. Similar to our findings here, a recent study by Sauer et al. identified an *lmo2473* mutant of *Listeria monocytogenes* that induces hyperactivation of the AIM2 inflammasome following bacteriolysis in the macrophage cytosol (29). In conclusion, our data demonstrate that *mviN* played a critical role in limiting *F. tularensis* LVS-induced AIM2 inflammasome activation and in doing so subverted an important innate immune defense pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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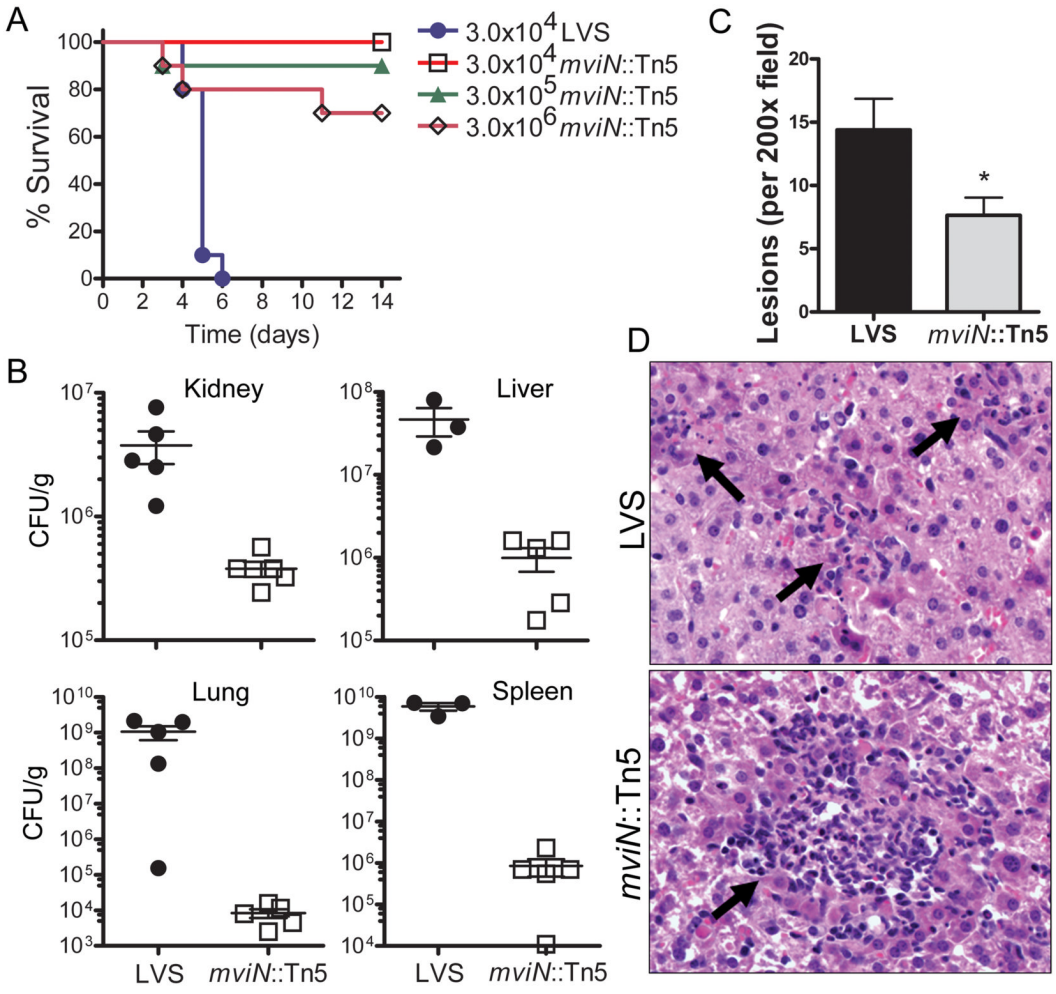

Figure 1.

F. tularensis LVS *mviN* mutant is attenuated *in vivo*. (A) WT (n=10 per group) mice were injected i.p. with the indicated amount of *F. tularensis* LVS (LVS) or the *mviN* mutant (*mviN*::Tn*5*) and survival monitored. Data are representative of 5 independent experiments at the 3×10^4 infective dose, each involving a minimum of 5 mice per group. (B) 3 d p.i. with 3×10^4 CFU of LVS or the *mviN* mutant organs were harvested, homogenized and dilutions plated for enumeration of CFU (n=3–5 mice per group). (C) Number of lesions per 200 × field of H&E-stained liver sections from WT mice 3 d p.i. with either LVS or the *mviN* mutant were scored; n=5 mice per group (3 random fields were examined per mouse); *, $p = 0.016$. (D) Representative H&E-stained sections of liver from WT mice 3 d p.i. with either *F. tularensis* LVS or the *mviN* mutant. Arrows indicate necropurulent foci.

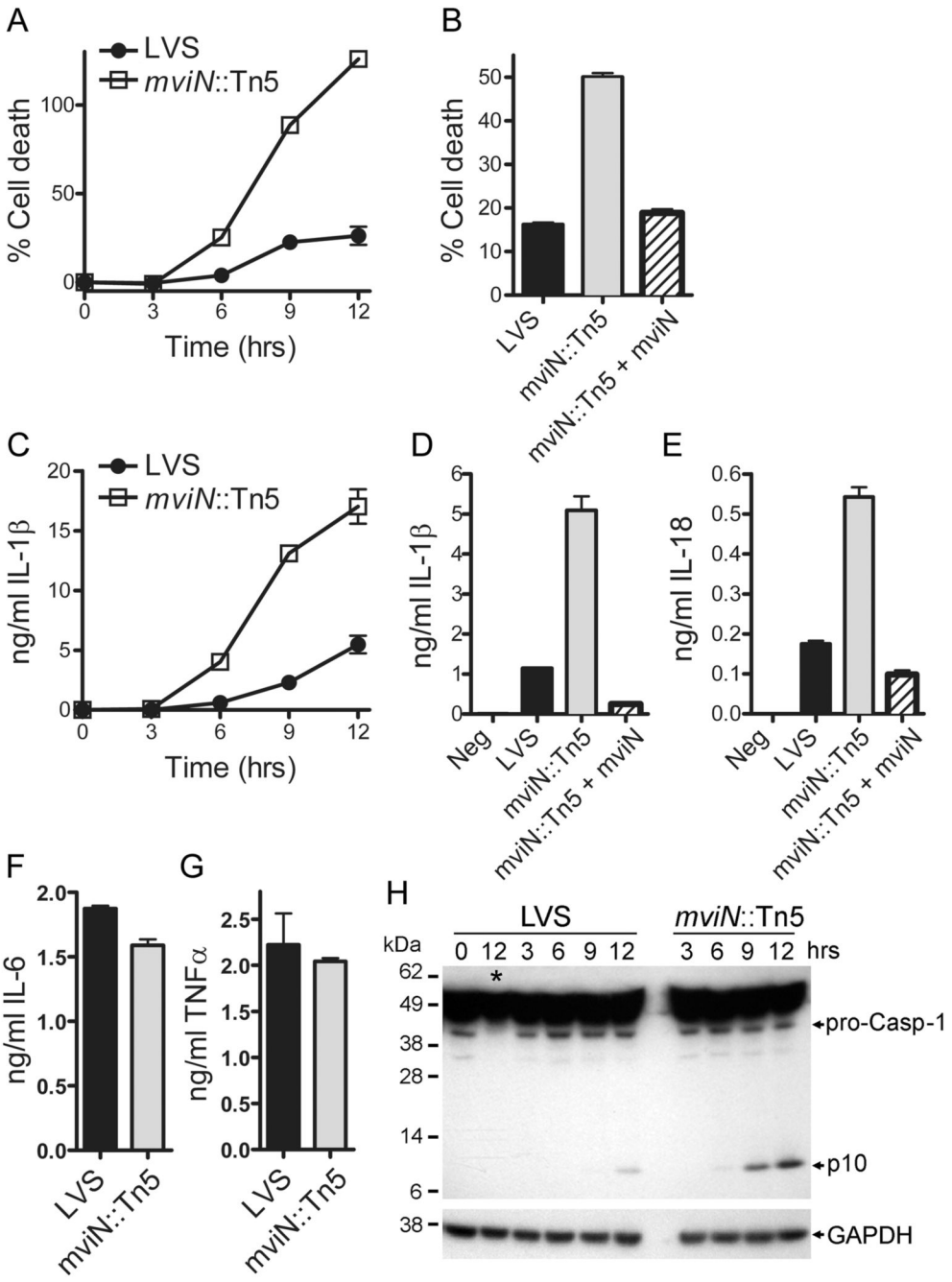

Figure 2.

Mutation of *F. tularensis* LVS *mviN* results in enhanced Mφ cytotoxicity and caspase-1 activation. (A–E) LPS-primed WT Mφ were infected with *F. tularensis* LVS (LVS), the *mviN* mutant (*mviN*::Tn*5*) or the complemented *mviN* mutant (*mviN*::Tn*5* + *mviN*) at an MOI of 50:1. Supernatants were collected 9 h after infection (B, D, and E) or as indicated (A and C). Cytotoxicity was measured by LDH release and expressed as a percentage of LDH release by Triton X-100 detergent (A and B). IL-1β secreted into supernatants was measured by ELISA (C-E). (F, G) Unprimed WT Mφ were infected with LVS or the *mviN* mutant for 9 h; IL-6 and TNFα released into the supernatant was measured by ELISA. (H) Lysates from LPS-primed WT and caspase-1-deficient Mφ (lane with caspase-1-/- Mφ lysate is marked *) infected with

LVS or the *mviN* mutant (50:1 MOI) for the indicated times were immunoblotted with antibodies against the p10 subunit of caspase-1 or GAPDH. Results are representative of two (B, F, G) and three (A, C-E, H) separate experiments.

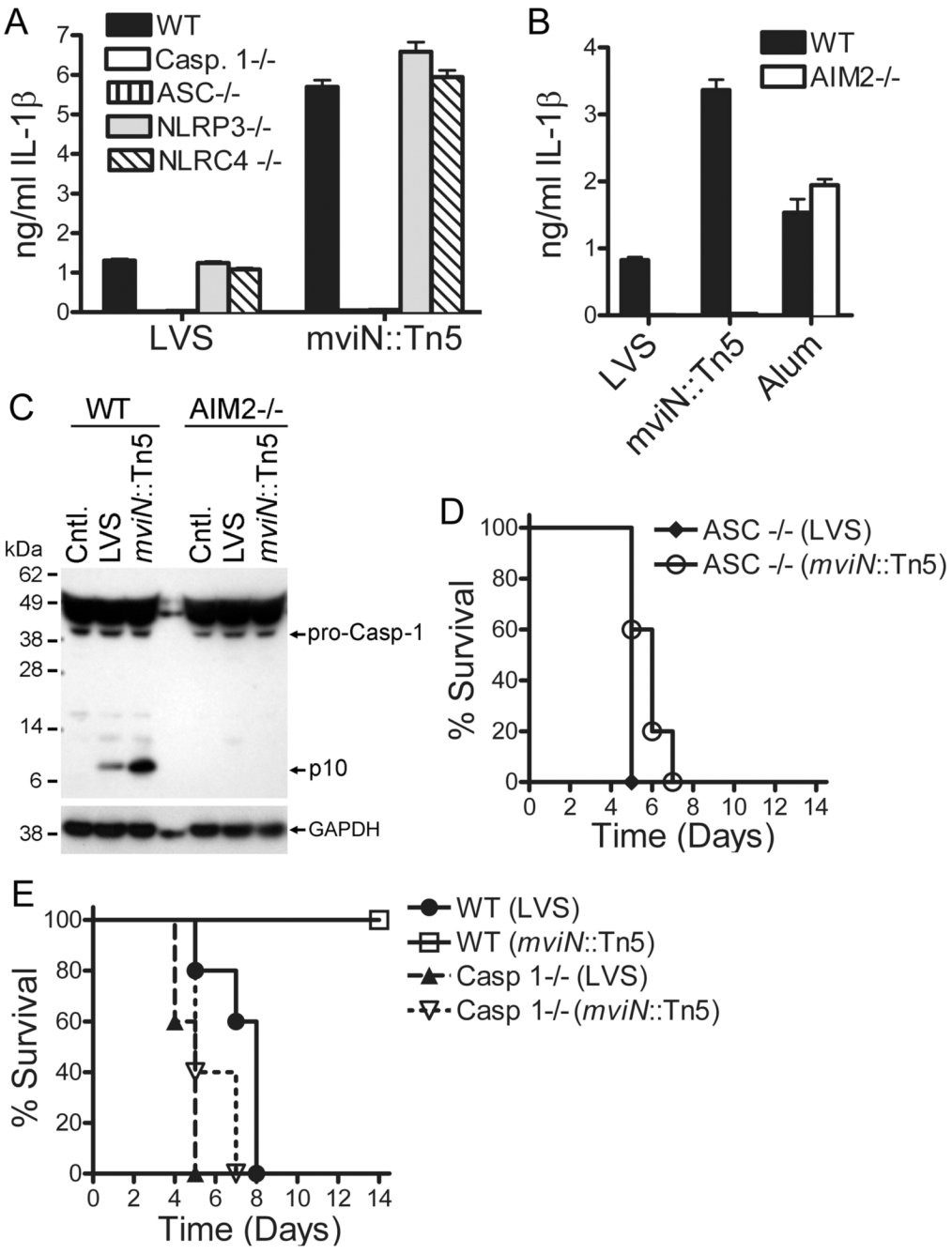

Figure 3.

MviN limits *Francisella*-induced AIM2 inflammasome activation. (A, B) LPS-primed Mφ from WT, caspase-1-, ASC-, NLRP3-, NLRC4, and AIM2-deficient mice were infected with either *F. tularensis* LVS (LVS) or the *mviN* mutant (*mviN*::Tn5) at a 50:1 MOI, or challenged with Alum (500 μg/ml). Supernatants were collected at 9 h and IL-1β release measured by ELISA. Results are representative of two (A) and five (B) separate experiments. (C) Lysates from LPS-primed WT and AIM2-deficient Mφ infected with LVS or the *mviN* mutant for 9 h were immunoblotted with antibodies against the p10 subunit of caspase-1 or GAPDH. Results are representative of two separate experiments. (D, E) WT, caspase-1- or ASC-deficient mice

(n=5 per group) were infected i.p. with 1×10^5 CFUs of LVS or the *mviN* mutant and survival monitored.