Oxidative metabolism enables *Salmonella* evasion of the NLRP3 inflammasome

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Microbial infection triggers assembly of inflammasome complexes that promote caspase-1–dependent antimicrobial responses. Inflammasome assembly is mediated by members of the nucleotide binding domain leucine-rich repeat (NLR) protein family that respond to cytosolic bacterial products or disruption of cellular processes. Flagellin injected into host cells by invading *Salmonella* induces inflammasome activation through NLRC4, whereas NLRP3 is required for inflammasome activation in response to multiple stimuli, including microbial infection, tissue damage, and metabolic dysregulation, through mechanisms that remain poorly understood. During systemic infection, *Salmonella* avoids NLRC4 inflammasome activation by down-regulating flagellin expression. Macrophages exhibit delayed NLRP3 inflammasome activation after *Salmonella* infection, suggesting that *Salmonella* may evade or prevent the rapid activation of the NLRP3 inflammasome. We therefore screened a *Salmonella* Typhimurium transposon library to identify bacterial factors that limit NLRP3 inflammasome activation. Surprisingly, absence of the *Salmonella* TCA enzyme aconitase induced rapid NLRP3 inflammasome activation. This inflammasome activation correlated with elevated levels of bacterial citrate, and required mitochondrial reactive oxygen species and bacterial citrate synthase. Importantly, *Salmonella* lacking aconitase displayed NLRP3- and caspase-1/11–dependent attenuation of virulence, and induced elevated serum IL-18 in wild-type mice. Together, our data link *Salmonella* genes controlling oxidative metabolism to inflammasome activation and suggest that NLRP3 inflammasome evasion promotes systemic *Salmonella* virulence.

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Abbreviations used: BMDM, BM-derived macrophage; MCAT, mitochondrial catalase; MLN, mesenteric LN; NLR, nucleotide binding domain leucine-rich repeat; ROS, reactive oxygen species; SCV, *Salmonella*-containing vacuole; SPI-1, *Salmonella* pathogenicity island I; *Stm*, *Salmonella enterica* serovar Typhimurium; T3SS, type III secretion system.

Pattern recognition receptors (PRRs) that detect and respond to evolutionarily conserved microbial structures such as LPS or peptidoglycan, as well as pathogen-specific virulence activities, are critical for host immune defense (Medzhitov, 2007; Vance et al., 2009). To promote infection, microbial pathogens inject virulence factors into the cytosol of infected cells to disrupt or modulate critical host physiological processes (Cornelis, 2006). During this process, contamination of the target cell cytosol by microbial components triggers cytosolic PRRs of the nucleotide binding domain leucine-rich repeat (NLR) family (Lamkanfi and Dixit, 2009). Diverse NLRs respond to a variety of endogenous and exogenous signals associated with infection, tissue stress, or damage. For example, NLRC4 responds to microbial products such as bacterial flagellin or structurally related specialized secretion system components that are injected into the cytosol of infected cells during infection by bacterial pathogens including *Pseudomonas*, *Legionella*, and *Salmonella* spp.

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(Miao et al., 2006; Molofsky et al., 2006; Sutterwala et al., 2007). NLRs recruit pro–caspase-1 to multiprotein complexes termed inflammasomes, where pro–caspase-1 is processed and activated, leading to cleavage and secretion of caspase-1–dependent cytokines (Martinon et al., 2002, 2007), as well as pyroptosis, a caspase-1–dependent pro-inflammatory cell death (Bergsbaken et al., 2009).

Inflammasome activation and subsequent production of caspase-1–dependent cytokines is important for both innate and adaptive antimicrobial responses (Mariathasan and Monack, 2007), as IL-1 family cytokines released upon inflammasome activation promote neutrophil migration to infected tissues and drive T_H17 and T_H1 responses against mucosal pathogens (Chung et al., 2009; Ichinohe et al., 2009). How pathogens evade inflammasome activation, and whether persistent bacterial pathogens evade or suppress inflammasome activation to establish or maintain persistence remains poorly understood.

Salmonella enterica species cause a range of disease from severe gastroenteritis to persistent systemic infection (Bäumler et al., 1998). *Salmonella enterica* serovar Typhimurium (*Stm*) invades host cells by means of a type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island I (SPI-1; Lee, 1996; Collazo and Galán, 1997). *Salmonella* subsequently replicates within a *Salmonella*-containing vacuole (SCV) that is established and maintained by the activity of a second T3SS, encoded on a second pathogenicity island, SPI-2 (Cirillo et al., 1998; Hensel et al., 1998). Intestinal inflammation during *Stm* infection is triggered by NLRC4-dependent responses to *Stm* flagellin, accompanied by caspase-1–dependent cytokine secretion and pyroptosis (Franchi et al., 2012). Activity of a SPI-1 effector protein, SopE, also contributes to SPI-1–dependent inflammasome activation in intestinal epithelial cells (Müller et al., 2009). Within the inflamed intestine, specialized adaptations allow *Stm* to resist mucosal antimicrobial defenses (Raffatellu et al., 2009; Winter et al., 2010; Thiennimitr et al., 2011). However, flagellin expression is down-regulated at systemic sites (Cummings et al., 2005, 2006), and enforced flagellin expression enhances NLRC4 activation and bacterial clearance, indicating that inflammasome activation in response to bacterial flagellin is detrimental for *Stm* replication during systemic infection (Miao et al., 2010a; Stewart et al., 2011).

NLRP3 responds to a wide variety of structurally unrelated molecules and activities, including extracellular ATP, bacterial pore-forming proteins, bacterial nucleic acids, crystals, and unsaturated fatty acids (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Hornung et al., 2008; Wen et al., 2011). While ATP, crystals, and the *Yersinia* T3SS all induce rapid formation of an NLRP3 inflammasome that leads to caspase-1 activation within 1–2 h (Mariathasan et al., 2006; Martinon et al., 2006; Brodsky et al., 2010), *Stm* induces delayed activation of a noncanonical NLRP3 inflammasome 12–16 h after infection (Broz et al., 2010). This noncanonical NLRP3 inflammasome is independent of the activities of the SPI-1 T3SS and instead is regulated by caspase-11 and TLR4-dependent production of type I interferon (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012). We therefore hypothesized that *Stm* might evade or prevent rapid activation of a canonical NLRP3 inflammasome, and that this evasion might contribute to systemic bacterial virulence. Several bacterial and viral pathogens evade NLRP3 inflammasome activation (Taxman et al., 2010; Gregory et al., 2011), but whether *Salmonella* is capable of doing so is unknown.

To identify potential negative regulators of NLRP3 inflammasome activation, we generated and screened a transposon library of flagellin-deficient *Stm* mutants for elevated inflammasome activation in NLRC4-deficient BM-derived macrophages (BMDMs). Sequencing of candidate hits identified *acnB*, the gene encoding the TCA cycle enzyme aconitase, which converts citrate to isocitrate, as well as several other genes that had been previously isolated in a screen for *Salmonella* genes that are required for persistent *Salmonella* infection in vivo (Lawley et al., 2006). Intriguingly, isocitrate lyase (encoded by *aceA*), which generates glyoxylate from isocitrate in the glyoxylate cycle, contributes to persistent but not acute infection by *Salmonella* as well as *Mycobacterium tuberculosis* (McKinney et al., 2000; Fang et al., 2005).

To test the potential role of *Salmonella* TCA cycle metabolism in inflammasome modulation, we generated targeted deletions in *acnB* as well as genes encoding other TCA cycle enzymes. Notably, deletion of aconitase, isocitrate lyase, or isocitrate dehydrogenase (*icdA*), but not other TCA enzymes, induced rapid NLRP3-dependent inflammasome activation in *Stm*-infected macrophages, suggesting that activity of these enzymes limits NLRP3 inflammasome activation by intracellular *Salmonella*. Moreover, aconitase-deficient *Salmonella* exhibited a defect in acute systemic virulence after oral administration and were deficient in their ability to persist in a chronic infection. These findings define the first genes that mediate NLRP3 inflammasome evasion by *Salmonella* and suggest that inflammasome evasion contributes to persistence of bacterial pathogens. Our data further suggest that sensing of bacterial metabolites may provide an additional level of innate immune recognition, and that regulation of metabolite production by intracellular pathogens represents a pathogen immune evasion strategy.

RESULTS

Identification of *Salmonella* genes that modulate inflammasome activation

The NLRP3 inflammasome can respond to the pore-forming activities of diverse bacterial secretion systems (Brodsky et al., 2010; McCoy et al., 2010; Higa et al., 2013). *Salmonella* expresses two such systems, SPI-1 and SPI-2, yet paradoxically, in the absence of flagellin and NLRC4, NLRP3 inflammasome activation does not occur in BMDMs until 12–16 h after infection (Miao et al., 2006; Broz et al., 2010). We therefore considered the possibility that *Stm* might evade or inhibit rapid SPI-1– or SPI-2–dependent NLRP3 inflammasome activation and devised a screen to identify such modulators. We first generated a library of individual Tn*10d*::*Tet*-based

transposon insertions (Rappleye and Roth, 1997) in a flagellin-deficient (*fliCfljB*) strain of *Salmonella enterica* serovar Typhimurium (*Stm*). Candidate mutants were identified by increased LDH release relative to the *fliCfljB*-deficient parental strain 4 h after infection of immortalized *Nlrc4*$^{-/-}$ macrophages (Fig. 1 A). Candidate mutants were rescreened in triplicate in primary *Nlrc4*$^{-/-}$ BMDMs and a secondary screen was performed in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs to confirm that LDH release was indeed inflammasome-dependent (a representative group of five of initial candidate mutants is shown; Fig. 1 B). Candidate mutants were further tested for NLRC4-independent release of the caspase-1–dependent cytokine IL-1β (Fig. 1 C), and normal secretion of caspase-1–independent cytokines such as IL-6 or IL-12 (Fig. 1 D). Intriguingly, sequencing the transposon junction of a subset of candidate mutants identified four genes (*acnB*, *bcfB*, *rcsD*, and *melB*) that had previously been isolated in a genome-wide screen for genes involved in *Salmonella* persistence (Lawley et al., 2006), consistent with the possibility that modulating inflammasome activation might promote persistent infection.

Deletion of *Salmonella* aconitase, isocitrate lyase, or isocitrate dehydrogenase induces rapid NLRC4-independent NLRP3 inflammasome activation

Two genes initially identified by Lawley et al. (2006) in a genome-wide screen for genes required for long term persistence, *acnB* and *icdA*, encode the TCA cycle enzymes aconitase and isocitrate dehydrogenase, respectively. Aconitase converts citrate to isocitrate, and isocitrate dehydrogenase subsequently converts isocitrate to α-ketoglutarate. This suggested that the TCA cycle of intracellular *Salmonella* might be involved in modulating inflammasome activation of infected cells. Interestingly, isocitrate lyase, a component of the glyoxylate cycle pathway which converts isocitrate to glyoxylate, also contributes to persistent infection of multiple bacterial pathogens, including *Mycobacterium tuberculosis*, *Salmonella*, *Pseudomonas*, and *Burkholderia* species (McKinney et al., 2000; Fang et al., 2005; Lindsey et al., 2008; van Schaik et al., 2009). However, its potential role in modulating inflammasome activation is unknown. To further assess the role of TCA or glyoxylate cycle genes in modulation of inflammasome activation, we generated in-frame deletions in *icdA*, *sucAB*, *fumA*, *mdhA*, and *aceA*, which encode isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, fumarase, malate dehydrogenase, and isocitrate lyase, respectively (Fig. 2 A). Consistent with findings that the TCA cycle is dispensable for macrophage replication (Bowden et al., 2010), invasion and replication of *Stm* in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were unaffected by deletion of TCA cycle genes (Fig. 2 B). Each of these mutants was capable of growth in rich medium, although as expected, *icdA*, *sucAB*, and *mdhA* had growth defects in minimal medium with either glucose or glycerol as carbon sources (unpublished data). Intriguingly, infection of either B6 or *Nlrc4*$^{-/-}$ BMDMs with *acnB*, *icdA*, and *aceA* but not *sucAB*, *mdhA*, or *fumA* mutants resulted in greatly increased LDH release in comparison with BMDMs infected by the parental wild-type strain (Fig. 2 C). In contrast, *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages showed minimal levels of cytotoxicity in response to infection by *acnB*-, *icdA*-, and *aceA*-deficient bacteria, suggesting that the absence of *Stm* aconitase, isocitrate dehydrogenase, or isocitrate lyase induces NLRP3 inflammasome activation. Consistently, caspase-1 processing was observed in cell lysates after infection of B6 and *Nlrc4*$^{-/-}$ but not *Nlrp3*$^{-/-}$ BMDMs with *acnB*, *aceA*, or *icdA* mutants (Fig. 2 D). Moreover, *acnB*, *aceA*, or *icdA*

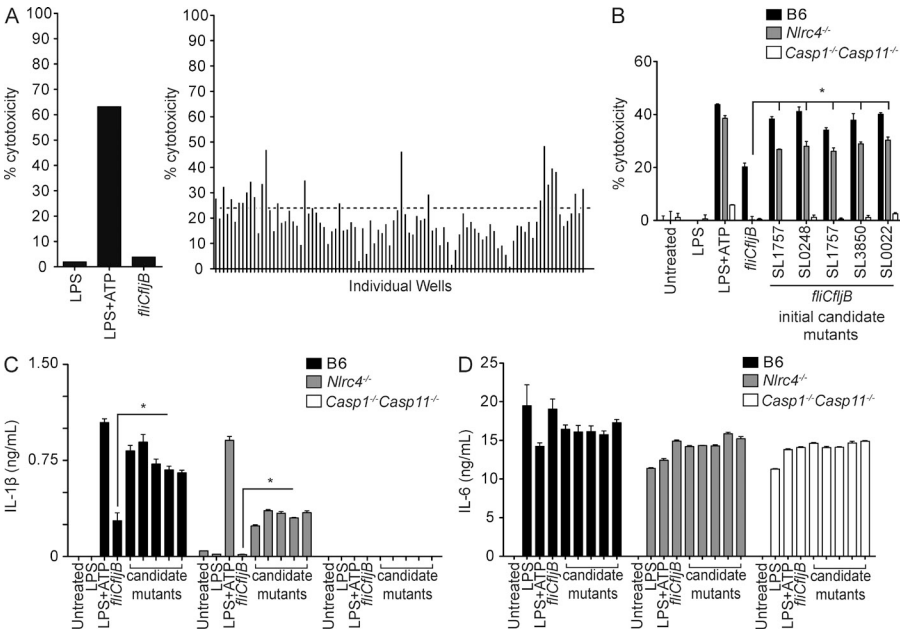

Figure 1. In vitro screen for bacterial mutants that fail to inhibit inflammasome activation. *fliCfljB* Tn10d::tet library was grown in 96-well format under SPI-1–inducing conditions and screened for ability to induce LDH release in immortalized *Nlrc4*$^{-/-}$ macrophages. (A) Data representative of plate from indicated screen. LPS+ATP is a positive control and *fliCfljB* parent strain is a negative control. (B) B6, *Nlrc4*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were infected in triplicate with initial bacterial mutants that exhibited elevated LDH release in primary screen. Supernatants were harvested 4 h after infection and assayed for LDH release as in A. (C) IL-1β released from B6, *Nlrc4*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs was measured by ELISA 4 h after infection. (D) IL-6 released from infected cells was measured 4 h after infection as a control cytokine. All experiments other than initial screen were performed in triplicate and are representative of at least 3 independent experiments. Error bars represent SEM. *, $P < 0.05$.

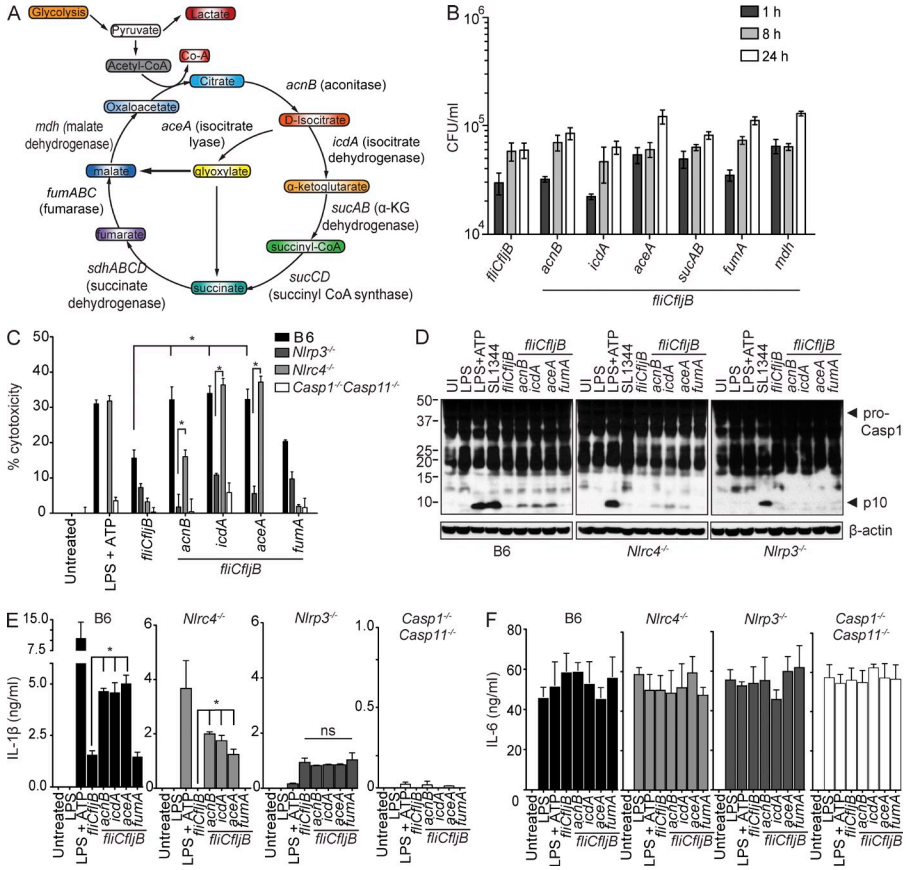

Figure 2. Deletion of *Salmonella* citrate or isocitrate TCA cycle enzymes induces NLRP3 inflammasome activation. (A) Schematic of *S.* Typhimurium TCA cycle. (B) *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ BMDMs were infected with *Stm* carrying targeted deletions in indicated TCA cycle genes, cells lysed, bacteria diluted, and intracellular bacterial CFUs were determined at 1, 8, and 24 h after infection. (C) Targeted mutations in specific core TCA cycle genes lead to NLRP3- and caspase-1/caspase-11–dependent cell death. LPS+ATP was used as a positive control for cell death, and *fliCfljB* was used as a negative control (parent strain). (D) Western blot for active caspase-1 (p10) was performed on lysates from B6, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$ BMDMs 3 h after infection with indicated bacterial mutant strains. β-Actin was blotted for as a loading control. Molecular mass markers in kD are indicated on the left. (E) Supernatants from B6, *Nlrc4*,$^{-/-}$ *Nlrp3*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs infected with TCA mutants for 4 h were analyzed for IL-1β measured by ELISA. (F) IL-6 released from infected cells was measured as a control cytokine. All data are representative of at least three independently performed experiments. UI, uninfected; B6, C57BL/6. Error bars represent SEM. *, $P < 0.05$.

mutants induced significantly elevated IL-1β secretion by B6 and *Nlrc4*$^{-/-}$ BMDMs, but not *Nlrp3*$^{-/-}$ BMDMs, suggesting that NLRP3 activation was responsible for the increased production of IL-1β (Fig. 2 E). Importantly, secretion of caspase-1–independent cytokines and expression of pro–IL-1β itself was unaffected by the *Stm* TCA cycle genes, indicating that deletion of *Stm* TCA cycle genes did not impact TLR signaling per se (Fig. 2 F). The residual IL-1β secretion observed after infection of *Nlrp3*$^{-/-}$ BMDMs is likely due to activation of the NLRC4 inflammasome by the SPI-1 inner rod protein PrgJ (Miao et al., 2010b), as *Nlrc4*$^{-/-}$*Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ BMDMs did not exhibit LDH release or IL-1β secretion (unpublished data).

Salmonella aconitase, isocitrate lyase, or isocitrate dehydrogenase mutants induce activation of a canonical NLRP3 inflammasome

Intracellular bacteria can activate the AIM2 inflammasome via release of bacterial DNA into the cytosol (Sauer et al., 2010; Warren et al., 2010; Peng et al., 2011). However, *Aim2*$^{-/-}$ BMDMs had no defect in inflammasome activation in response to *acnB*, *icdA*, or *aceA* mutants, suggesting that AIM2 was not involved in this response (unpublished data). NLRP3 can participate in noncanonical inflammasome activation in response to Gram-negative bacteria by a mechanism involving caspase-11 (Kayagaki et al., 2011; Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012). However, *Casp11*$^{-/-}$ BMDMs had no defect in either LDH (Fig. 3 A) or IL-1β secretion (Fig. 3 B) in response to infection by *acnB*, *icdA*, or *aceA*, whereas *Casp1*$^{-/-}$ BMDMs were as defective as *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ BMDMs, indicating that these mutants activated a caspase-1– and NLRP3-dependent canonical inflammasome. Caspase-1–independent cytokines were unaltered in response to infection by TCA cycle mutants or in the absence of caspase-1 and/or 11, again indicating a lack of a direct role for *Stm* TCA cycle genes in modulating TLR signaling (Fig. 3 C).

NLRP3 inflammasome activation in response to *Salmonella* TCA cycle mutants requires the bacterial SPI-1 T3SS

As the NLRP3 inflammasome can be activated by the T3SS of other bacteria, we next sought to test whether the rapid NLRP3 inflammasome activation induced by *Stm* TCA mutants involved T3SS activity. The SPI-1 T3SS promotes bacterial invasion into non-phagocytic cells (Galán and Curtiss, 1989; Lee and Falkow, 1990; Galán, 2001), whereas the SPI-2 T3SS is up-regulated within the SCV and is required for intracellular replication within host macrophages (Cirillo et al., 1998; Hensel et al., 1998). Intriguingly, SipB, the integral membrane component of the SPI-1 translocon, but not SseC, the integral membrane component of the SPI-2 translocon, was required for LDH release and IL-1β secretion in response to infection by the TCA cycle mutants (Fig. 4, A and B).

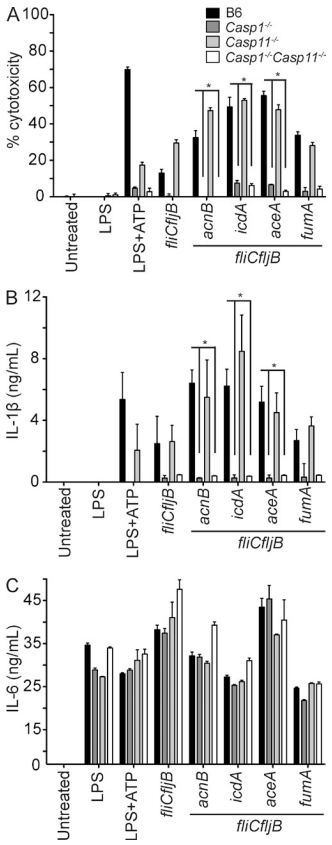

Figure 3. Deletion of bacterial TCA genes activates the canonical NLRP3 inflammasome. (A) B6, *Casp1*$^{-/-}$, *Casp11*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were infected with bacterial TCA cycle mutants, and cell death was measured 4 h after infection by LDH release assay. (B and C) Supernatants from infected cells were analyzed 4 h after infection by ELISA for IL-1β (B) and IL-6 (C). LPS+ATP was used as a positive control and *fliCfljB* (parent strain for indicated mutants) was used as a negative control. All experiments were performed three independent times in triplicate, and data from representative experiments are shown. Error bars represent SEM. *, $P < 0.05$.

Importantly, caspase-1–independent cytokines were unaffected by the presence or absence of SipB (Fig. 4 C). The defect in inflammasome activation caused by *sipB* mutation was not due to reduced numbers of intracellular bacteria, as infection with a fivefold higher dose of *sipB*-deficient bacteria was performed to ensure intracellular levels of WT and isogenic *sipB* mutant bacteria (unpublished data). The SPI-2 T3SS plays a role in a caspase-1/11–dependent *Salmonella*-induced late death (Monack et al., 2001; Broz et al., 2010). We therefore investigated the possibility that SPI-2 might play a role in later cell death induced by these TCA cycle mutants. No differences in cell death were detected between parental TCA cycle mutants and the isogenic *sseC* mutant strains over a 20-h infection time course, indicating that SPI-2 does not contribute to inflammasome activation by *Stm* TCA cycle mutants at either early or later time points (Fig. 4 D). Altogether, these data suggested that either a signal from the SPI-1 T3SS or the activity of the SPI-1 T3SS in combination with disruption of specific TCA cycle genes triggers the NLRP3 inflammasome in response to *Stm* infection. To distinguish between these possibilities, we generated *acnB*, *icdA*, and *aceA* mutant strains that also had combined deficiency in three SPI-1–secreted effector proteins, SopE, SopE2, and SopB. Deletion of these genes greatly reduces *Salmonella* invasion of epithelial cells but does not otherwise compromise the activity of the SPI-1 secretion system itself (Zhou et al., 2001; Bruno et al., 2009). Intriguingly, although each individual effector had a minimal effect on NLRP3 inflammasome activation, the combined additional deletion of *sopE*, *sopB*, and *sopE2* significantly reduced cytotoxicity and IL-1β secretion in response to infection by *acnB*, *icdA*, or *aceA* mutant *Salmonella* (Fig. 4, E and F). This was the case despite equivalent levels of bacterial uptake by macrophages as well as equivalent secretion of caspase-1–independent cytokines (Fig. 4 G). These data suggest that a combined signal from SPI-1–mediated disruption of host actin and a metabolic signal due to alteration of the *Stm* TCA cycle are responsible for inflammasome activation by the TCA cycle mutant bacterial strains. Interestingly, SopE triggers inflammasome activation in stromal cells and in macrophage cell lines independently of flagellin (Müller et al., 2009; Hoffmann et al., 2010), but the inflammasomes involved in this response have not been defined, and we did not observe a role for SopE alone in inflammasome activation in primary BMDMs.

Stm TCA cycle mutants trigger NLRP3 inflammasome activation through mitochondrial reactive oxygen species (ROS)

Multiple models have been proposed for how the NLRP3 inflammasome is activated in response to a large number of disparate stimuli (Latz et al., 2013). Along with potassium efflux, ROS is a key regulatory signal for NLRP3 inflammasome activation (Dostert et al., 2008; Hornung et al., 2009; Martinon, 2010; Zhou et al., 2011; Shimada et al., 2012). Mitochondrial ROS is reported to trigger NLRP3 inflammasome activation in response to uric acid and silica crystals (Martinon, 2010). Notably, both the *icdA* and *acnB* mutant strains induced significantly elevated levels of mitochondrial ROS production compared with the isogenic parental *fliCfljB* strain, as measured by MitoSOX staining of infected cells (Fig. 5, A and B). Recent studies have demonstrated that ROS itself is not the direct signal for NLRP3 inflammasome activation, and indeed, LPS treatment of BMDMs triggers significant levels of ROS but by itself is insufficient for robust inflammasome activation (Iyer et al., 2013; Muñoz-Planillo et al., 2013). We therefore asked whether this elevated mitochondrial ROS contributed to inflammasome activation during infection by *Stm* TCA mutants. Mitochondrial catalase (MCAT) transgenic mice overexpress human catalase targeted to the mitochondria and therefore generate lower levels of mitochondrial hydrogen peroxide than wild-type mice (Schriner et al., 2005; Lee et al., 2010; West et al., 2011). Intriguingly, MCAT BMDMs failed to induce elevated LDH or IL-1β secretion in response to infection by *acnB*, *icdA*, or *aceA* mutants compared with isogenic *fliCfljB Stm*, indicating that inflammasome activation by *Stm* TCA cycle mutants was

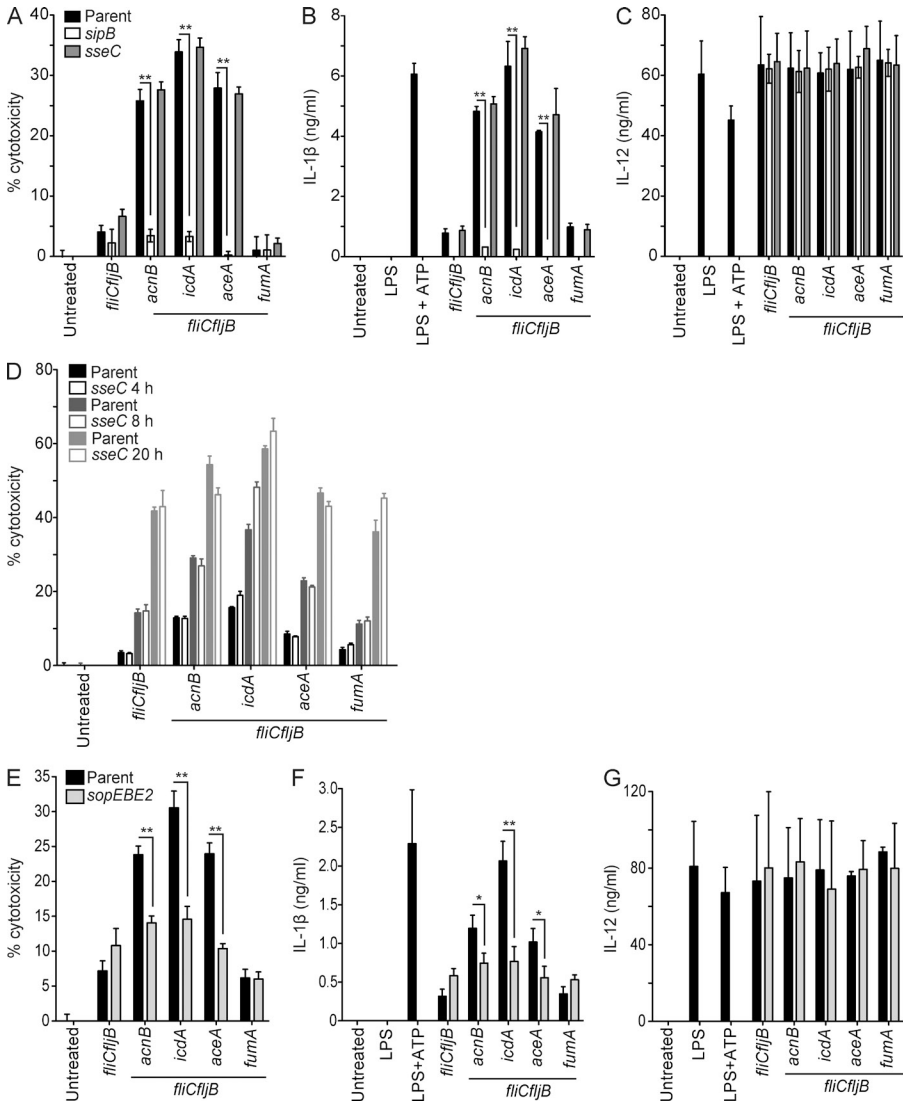

Figure 4. NLRP3 inflammasome activation in response to *Salmonella* TCA cycle mutants requires the bacterial SPI-1 T3SS. (A–C) Supernatants of *Nlrc4*$^{-/-}$ BMDMs were analyzed 4 h after infection with indicated bacterial strains for LDH release (A), IL-1β release (B), and IL-12 release (C). (D) *Nlrc4*$^{-/-}$ BMDMs were infected with indicated parental bacterial strains or isogenic *sseC* mutants and assayed 4, 8, and 20 h after infection for cytotoxicity by LDH release. (E–G) *Nlrc4*$^{-/-}$ BMDMs were infected with parental or *sopEBE2* mutant bacterial strains and supernatants were assayed 4 h after infection for cell death by LDH release (E), IL-1β release (F), and IL-12 release (G) by ELISA. All experiments were performed three independent times in triplicate and representative data are shown. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$.

limited by reduction of mitochondrial ROS (Fig. 5, C and D). Importantly, NLRP3 inflammasome activation was unaffected in response to LPS+ATP, indicating that BMDMs from MCAT mice do not have a global defect in NLRP3 inflammasome activation. Furthermore, production of the inflammasome-independent cytokine IL-12 and expression of NLRP3 protein were unaffected in MCAT BMDMs, demonstrating that the effect of MCAT on the NLRP3 inflammasome was related to activation and not due to reduced expression of inflammasome components (Fig. 5 E). In accordance with our previous observations in *Nlrp3*$^{-/-}$ BMDMs, residual inflammasome activation in MCAT cells was likely due to an NLRC4-dependent response to the PrgJ protein. To test this, and to rule out a possible developmental effect of catalase overexpression, we acutely depleted mitochondrial ROS by treating cells with the mitochondrial-specific ROS scavenger MitoQ after the cells were primed with LPS (James et al., 2007; Srinivasan et al., 2010). Notably, treatment of *Nlrc4*$^{-/-}$ BMDMs with MitoQ before infection significantly reduced release of LDH and IL-1β in response to *Stm* TCA cycle mutants but did not affect secretion of inflammasome-independent cytokines or the response to LPS+ATP (Fig. 5, F–H). Together, these data demonstrate a requirement for mitochondrial ROS in NLRP3 inflammasome activation triggered by *Stm* TCA cycle mutants.

NLRP3 inflammasome activation by *Stm* TCA cycle mutants requires bacterial citrate synthase

We next wanted to determine how deletion of TCA cycle genes involved in citrate or isocitrate metabolism might induce inflammasome activation. Accumulation of excess citrate in cells lacking aconitase or isocitrate dehydrogenase can inhibit phosphofructokinase, a rate-limiting enzyme of glycolysis (Underwood and Newsholme, 1965). Thus, deletion of aconitase or isocitrate dehydrogenase can result in growth inhibition coupled with export of excess citrate (Koziol et al., 2009; Baumgart et al., 2011). However, whether deficiency in these genes causes citrate accumulation in *Stm* has not been

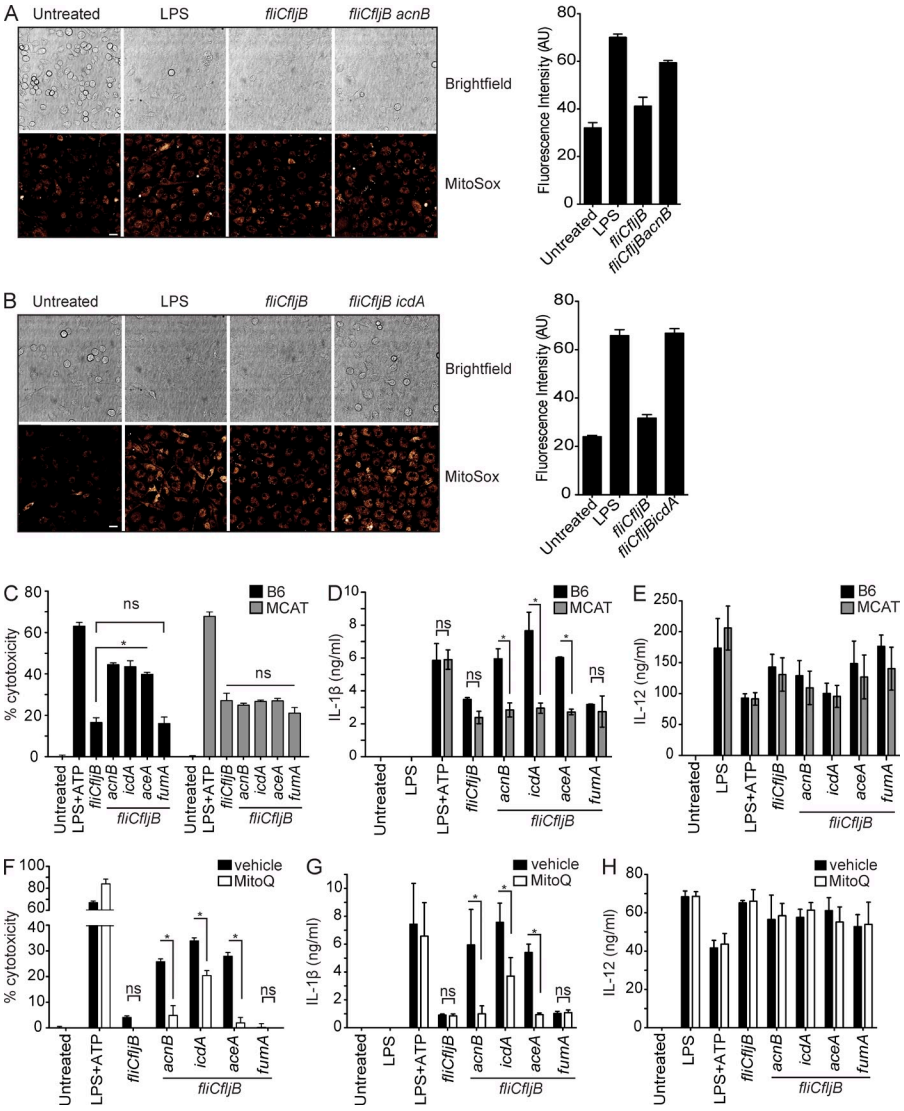

Figure 5. *Salmonella* TCA cycle mutants trigger NLRP3 inflammasome activation through mitochondrial ROS. (A and B) Production of mitochondrial superoxide in *Stm*-infected *Nlrc4*$^{-/-}$ BMDMs was assayed 4 h after infection with *acnB* (A) or *icdA* (B) strains. (C) B6 and MCAT BMDMs were infected with indicated bacterial strains, and cell death was assayed 4 h after infection. (D and E) IL-1β (D) and IL-12 (E) in supernatants of B6 and MCAT BMDMs infected as in C were measured by ELISA. (F) *Nlrc4*$^{-/-}$ BMDMs were pretreated with either vehicle control or 10 nM MitoQ 3 h before infection, and cell death was assayed 4 h after infection by measuring release of LDH in cell supernatants. (G and H) Supernatants of samples treated with vehicle control or MitoQ as in F were assayed for IL-1β (G) and IL-12 (H). *fliCfljB* is the parent strain background for all indicated bacterial mutants. All experiments were performed three independent times in triplicate and representative data are shown. Bars, 20 µm. Error bars represent SEM. *, $P < 0.05$.

investigated. Notably, profiling of TCA cycle metabolite levels in the *acnB*, *icdA*, *aceA*, *fumA*, and parental wild-type (*fliCfljB*) strains revealed that aconitase, isocitrate dehydrogenase, or isocitrate lyase mutants all exhibited elevated levels of citrate, although relative citrate levels were most substantially increased in *acnB* and *icdA* mutants (Fig. 6 A). To test the possibility that excess citrate might be responsible for NLRP3 inflammasome activation, we sought to eliminate citrate production through the TCA cycle in the *acnB*, *icdA*, or *aceA* mutant strains by additionally deleting *gltA*, which encodes citrate synthase. Intriguingly, deletion of citrate synthase significantly reduced cytotoxicity and release of IL-1β caused by *acnB* and *icdA* mutants (Fig. 6, B and C). Lack of citrate synthase did not reduce inflammasome activation caused by *aceA* (unpublished data), perhaps because deletion of *aceA* did not result in as high of an increase in citrate levels as the other two mutations. Together, these data suggest that excess buildup of bacterially derived citrate triggers inflammasome activation in response to intracellular *Salmonella*.

AcnB limits inflammasome-dependent antibacterial immune responses during acute infection

Systemic NLRC4 inflammasome activation in response to *Salmonella* flagellin promotes antibacterial immune defense and enhances in vivo bacterial clearance (Miao et al., 2010a). We therefore sought to test whether NLRP3 inflammasome activation triggered by altered *Stm* TCA cycle metabolism could also promote antibacterial defense in vivo. Aconitase-deficient *Stm* had no defect in virulence after i.p. infection of C57BL/6 mice and showed equivalent levels of spleen and liver colonization as parental *fliCfljB* bacteria (Fig. 7, A and B). Mice infected with *icdA* mutant bacteria had significantly reduced tissue burdens in the liver and spleen after i.p. infection, perhaps because of an overall reduced growth rate of the *icdA* mutant (unpublished data). We therefore used the *acnB* strain for subsequent studies. Although *acnB* deficiency did not result in loss of virulence after i.p. infection, *acnB* mutant *Stm* exhibited significantly reduced splenic tissue burden 6 d after intragastric inoculation compared with *fliCfljB*-infected

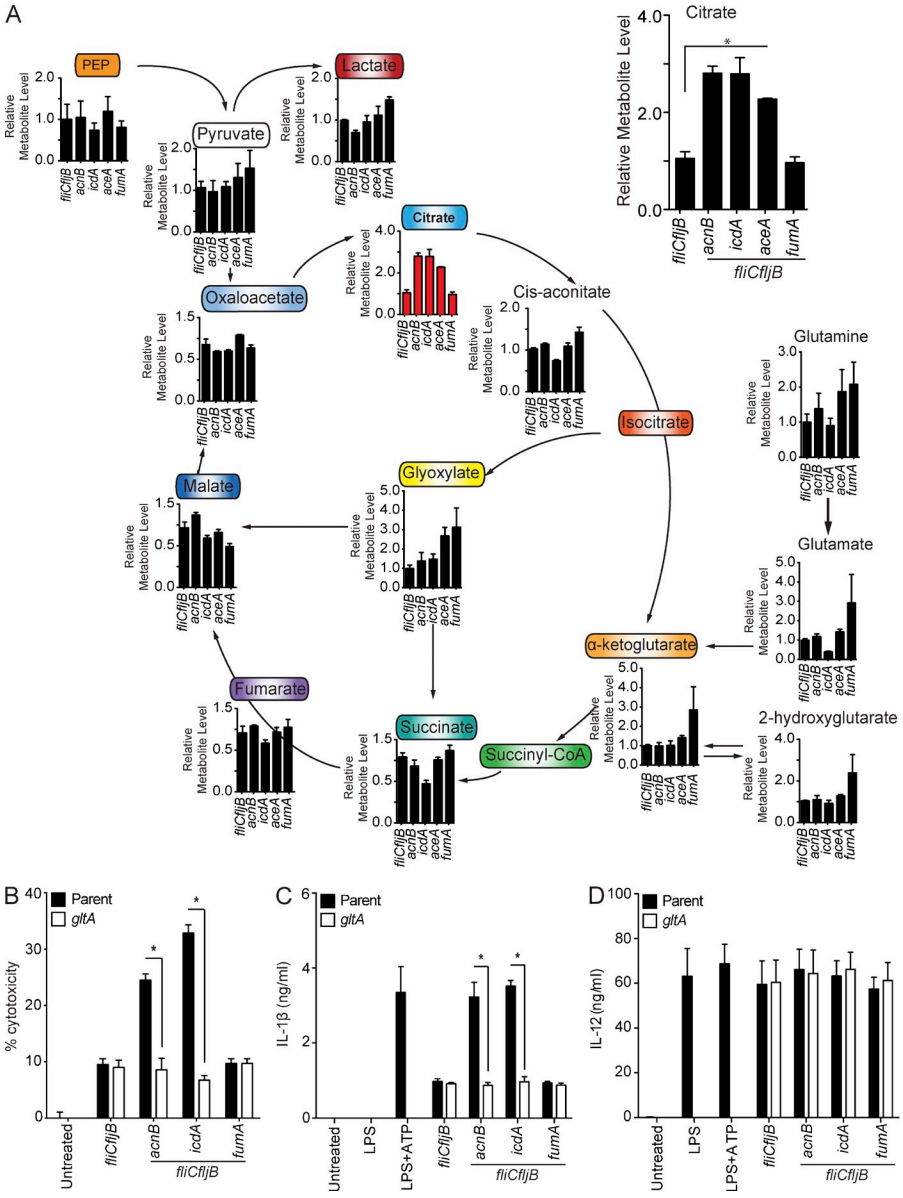

Figure 6. NLRP3 inflammasome activation by *Salmonella* aconitase and isocitrate dehydrogenase mutants correlates with excess bacterial citrate production and requires citrate synthase. (A) Bacterial metabolites from bacteria grown under SPI-I–inducing conditions were analyzed using GC-MS analysis. *Nlrc4*$^{-/-}$ BMDMs were infected with indicated bacterial mutants. (B–D) Supernatants were assayed 4 h after infection for levels of LDH release (B), IL-1β (C), and IL-12 (D). Error bars represent SEM. *fliCfljB* is the parent strain background for all indicated bacterial mutants. Experiments were performed with four independently grown bacterial cultures for each strain. *, $P < 0.05$.

mice (Fig. 7 C). Intriguingly, despite these lower burdens, serum IL-18 levels of *acnB*-infected C57BL/6 mice were significantly elevated in comparison with mice infected by *fliCfljB* Stm (Fig. 7 E), consistent with enhanced in vitro inflammasome responses against *acnB*-deficient *Stm*. Critically, the virulence defect of the *acnB* mutant was abrogated in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice, coinciding with significantly reduced serum IL-18 levels in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice infected with either bacterial strain. *Nlrp3*$^{-/-}$ mice also exhibited reduced serum IL-18 in response to the *acnB* mutant, and IL-18 production was not significantly different from *Nlrp3*$^{-/-}$ mice infected with *fliCfljB* bacteria, indicating that NLRP3 plays a functionally important in vivo role in IL-18 production in response to *acnB* infection (Fig. 7 F). Notably, *Nlrp3*$^{-/-}$ mice did not exhibit statistically significant differences in either CFU or serum IL-18 between the *acnB* and *fliCfljB* strains (Fig. 7, D and F). *Nlrp3*$^{-/-}$ mice also succumbed to *acnB* mutant infection in higher numbers than B6 mice, providing further support for the role of NLRP3 in inflammasome activation in vivo in the context of infection by aconitase-deficient *Stm*.

AcnB contributes to persistent *Salmonella* infection in vivo

Given the observation that isocitrate lyase contributes to persistence of various bacterial pathogens (McKinney et al., 2000; Fang et al., 2005; Lindsey et al., 2008; van Schaik et al., 2009), we sought to test the role of aconitase in promoting *Salmonella* persistence. *Stm* infection of B6 mice results in acute lethal systemic infection due to an inactivating mutation in *Nramp1* in B6 mice (Vidal et al., 1995). *Nramp1* encodes a lysosomal transporter that exports divalent metal cations from the SCV and limits *Salmonella* intracellular replication (Jabado

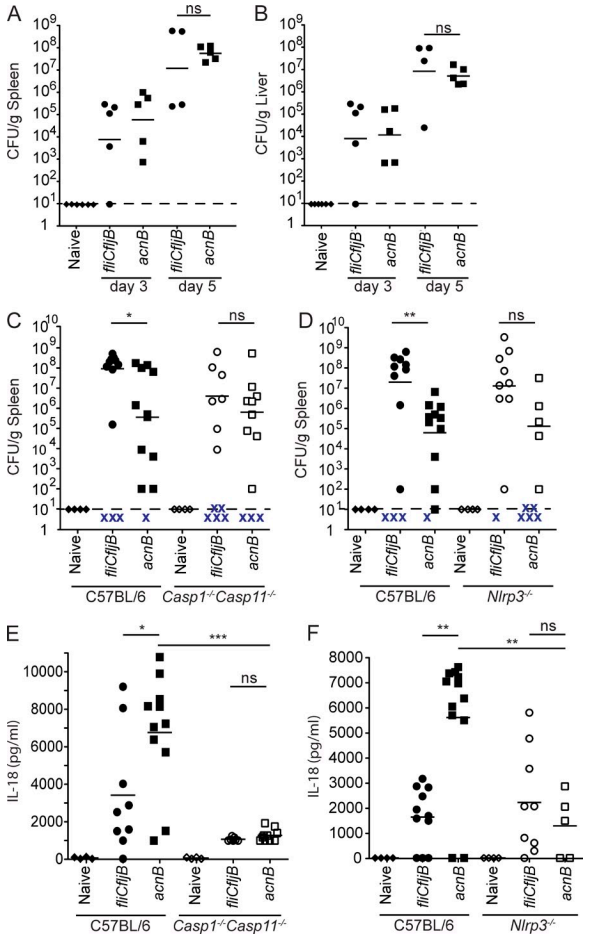

Figure 7. Deletion of bacterial TCA cycle aconitase leads to a decrease in bacterial virulence. C57BL/6 mice were infected i.p. with 5×10^2 CFU. (A and B) Spleens (A) and livers (B) were harvested on days 3 and 5, and CFUs were enumerated by plating dilutions of tissue homogenates on selective plates. (C–F) C57BL/6 and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice were infected with 2×10^7 CFU orally, and 7 d after infection, spleens were harvested for bacterial enumeration (C) and serum for IL-18 ELISAs (E). C57BL/6 and *Nlrp3*$^{-/-}$ mice were infected with 2×10^7 CFU orally and 7 d after infection spleens were harvested for bacterial enumeration (D) and serum for IL-18 ELISAs (F). Mice that succumbed to infection before are designated with an X. Dashed lines indicate the limit of detection. Mann-Whitney *U* test and unpaired two-tailed Student's *t* test were used to determine statistical significance for mouse CFU and cytokine data, respectively. Bars represent geometric means for CFU, and arithmetic mean for cytokine data. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of two independently performed experiments (A and B) or pooled data from two to three independent experiments (C–F).

et al., 2000; Forbes and Gros, 2001). To test the role of AcnB in chronic *Stm* infection, we used 129S6/SvE (129) mice which express functional NRAMP1 protein (Vidal et al., 1995). i.p. infection of 129 mice with 1,000 CFU WT *Stm* results in a chronic infection in 129 mice (Monack et al., 2004). Importantly, under these conditions, we observed similar bacterial burdens in the mesenteric LNs (MLNs), spleens, and livers of parental (*fliCfljB*)- or *acnB*-infected mice in the early stages of persistent infection at days 7 and 21 after infection (Fig. 8, A–C). However, at later times after infection (day 60), bacterial burdens in the spleens of *acnB*-infected mice were significantly lower compared with mice infected with isogenic aconitase-sufficient bacteria, although bacterial numbers in the livers were not significantly different at this time (Fig. 8, B and C). In competitive infection in which 129S6/SvE mice were infected with equal numbers of *fliCfljB* and *acnB*-deficient *Stm*, *acnB*-deficient bacteria had a significant competitive disadvantage at both 7 and 21 d after infection in the spleens and livers but not in the MLNs (Fig. 8 D). Finally, 129S6/SvE mice survived an elevated infectious dose (10,000 CFU i.p.) of *acnB*-deficient *Stm* but succumbed over 2–3 wk to infection by isogenic *acnB*-sufficient bacteria (Fig. 8 E). Interestingly, day 7 bacterial burdens in 129S6/SvE mice infected with the higher dose were similar in spleens and livers, although bacterial numbers were statistically lower in the spleens of *acnB*-infected mice (Fig. 8 F). There was no difference in the bacterial burdens at this time point in the livers between infected mouse groups (Fig. 8 G). Importantly, similar to our findings in oral infection of B6 mice, *acnB*-infected mice exhibited significantly higher levels of serum IL-18 compared with *fliCfljB*-infected animals, whereas IL-6 levels were equivalent (Fig. 8, H and I). Altogether, these data indicate that infection with acnB-deficient *Salmonella* triggers inflammasome activation in vivo.

DISCUSSION

NLRs detect exogenous and endogenous molecules that serve as indicators of infection or tissue stress. Together with the NAIP proteins, NLRC4 detects the cytosolic presence of bacterial flagellin and inner rod proteins of bacterial T3SSs, leading to rapid inflammasome activation and pyroptosis (Kofoed and Vance, 2011; Zhao et al., 2011). Down-regulation of flagellin expression or inactivating the genes encoding flagellin enables pathogens to evade NLRC4 detection (Chain et al., 2004; Cummings et al., 2005; Miao et al., 2010a). Pathogens also use active mechanisms of inflammasome suppression, either by down-regulating NLRC4 or by targeting other host pathways, such as autophagy, that regulate inflammasome activation (Higa et al., 2013; Perez-Lopez et al., 2013). The pathogenic *Yersinia* species possess several distinct effector-mediated mechanisms for inflammasome modulation (Brodsky et al., 2010; LaRock and Cookson, 2012). The activation of the AIM2 inflammasome in response to *Mycobacteria* is blocked by the ESX system of virulent mycobacteria (Shah et al., 2013). Collectively, these studies indicate that inflammasome activation is a target of bacterial immune evasion strategies. However, the mechanisms by which bacterial pathogens evade NLRP3 inflammasome activation are not well understood.

The NLRP3 inflammasome responds to a wide variety of structurally and chemically unrelated signals, and, along with NLRC4, contributes to host defense against *Stm* infection (Broz et al., 2010). NLRP3 induces delayed inflammasome activation in response to *Stm* independently of the SPI-1 and

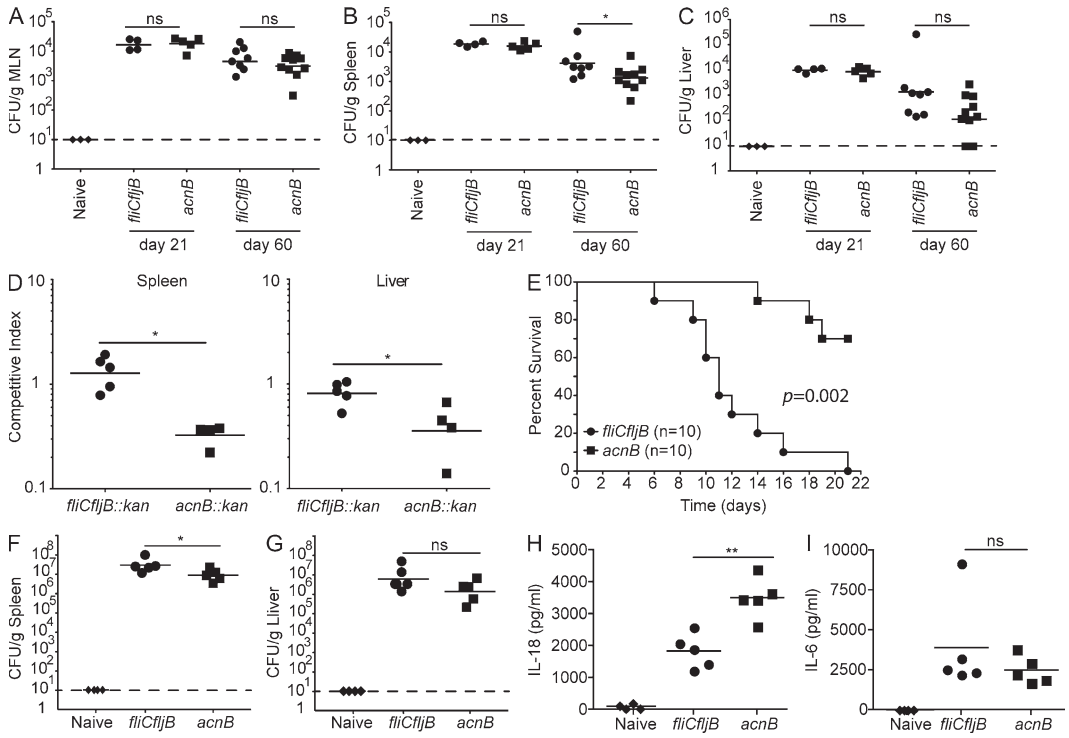

Figure 8. Deletion of bacterial TCA cycle aconitase leads to a defect in bacterial persistence. (A–C) 129S6/SvE mice were infected with 10^3 CFU of bacteria i.p., and MLNs (A), spleens (B), and livers (C) were harvested on days 21 and 60, and CFUs were enumerated by plating dilutions of tissue homogenates. (D) 129S6/SvE mice were co-infected with 10^3 CFU of each indicated bacterial strain i.p., and MLNs, spleens, and livers were harvested on day 21 and competitive index (CI) was determined by plating tissue homogenates on different selective plates. (E) 129S6/SvE mice were infected i.p. with 10^4 CFU of *fliClfljB* or isogenic *acnB* mutant bacterial strains and survival of the mice was monitored over 21 d. (F and G) 129S6/SvE mice were infected with 10^4 CFU *fliCfljB* or isogenic *acnB* mutant bacteria, and 7 d after infection CFU per gram of spleens (F) or liver (G) was determined by plating tissue homogenates. (H and I) Serum levels of IL-18 (H) and IL-6 (I) from mice were assayed at day 7 after infection. Dashed lines indicate limit of detection. Mann-Whitney *U* test and unpaired two-tailed Student's *t* test were used to determine statistical significance for mouse CFU and cytokine data, respectively. Bars represent geometric means for CFU and arithmetic mean for cytokine data. *, $P < 0.05$; **, $P < 0.01$. Data are pooled from two independently performed experiments, except competitive index and survival curve studies which indicate individual experiments.

SPI-2 T3SSs via a noncanonical pathway involving a TLR4–TRIF–IFN signaling pathway that requires caspase-11 (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012). However, whether *Stm* might evade rapid T3SS-dependent activation of a canonical NLRP3-mediated inflammasome has not been previously examined.

In this work, we sought to identify *Stm* genes that modulate NLRP3 inflammasome activation during infection. Four of the genes identified in our screen (*acnB*, *bcfB*, *rcsD*, and *melB*) were previously found in a genome-wide screen for *Salmonella* persistence genes (Lawley et al., 2006), consistent with the possibility that modulating inflammasome activation might promote long-term systemic infection. *bcfB* encodes a fimbrial chaperone, *rcsD* encodes a member of a two-component system previously found to contribute to persistent infection and regulate resistance to antimicrobial peptide responses (Detweiler et al., 2003; Erickson and Detweiler, 2006), whereas *melB* encodes a symporter of melibiose and monovalent cations. *acnB* encodes the enzyme aconitase, which mediates conversion of citrate to isocitrate as a key step in the TCA cycle. Interestingly, several other TCA cycle enzymes contribute to persistent infection by *Salmonella*, as well as several other bacterial pathogens (McKinney et al., 2000; Fang et al., 2005; Lindsey et al., 2008; van Schaik et al., 2009).

Here, we demonstrate that *Stm* mutants deficient in aconitase or isocitrate dehydrogenase, but not other TCA cycle enzymes, induce rapid canonical NLRP3 inflammasome activation in BMDMs. Notably, this inflammasome activation correlated with elevated levels of bacterial citrate and was abrogated upon deletion of citrate synthase (*gltA*), suggesting a role for citrate in the triggering of the NLRP3 inflammasome. Surprisingly, this inflammasome activation was dependent on both the genes encoding bacterial citrate synthase and the SPI-1 effector proteins that mediate *Stm* invasion. These findings suggest that the innate immune system can detect intracellular bacteria not only through sensing of bacterial proteins and virulence activities but also by combining sensing of bacterial metabolites with sensing of virulence activity.

Whether regulating levels of citrate production by *acnB* and *icdA* during infection is an active mechanism of evading inflammasome activation is not clear, as these enzymes normally function in a fundamental biosynthetic pathway. However,

acnB is not essential for either intramacrophage replication or virulence in the context of certain routes of in vivo infection. Intriguingly, the *Salmonella* genome contains two aconitase enzymes, but deletion of *acnB* alone is sufficient to mediate inflammasome activation. Thus dynamic regulation of AcnB may function in vivo to promote immune evasion.

Altered bacterial citrate production may lead to altered production of other bacterial metabolites, which may themselves be the proximal triggers of NLRP3 activation. For example, mitochondrial cardiolipin was recently reported to be a direct ligand for NLRP3 (Iyer et al., 2013). Cardiolipin is also a constituent phospholipid of bacterial membranes, and cardiolipin metabolism may be altered as a consequence of TCA cycle dysregulation. Future studies will determine whether infected macrophages respond directly to elevated cytoplasmic levels of citrate or whether other bacterial metabolites are involved. Signatures of bacterial viability, termed vita-PAMPs or PAMPs per vita produced by metabolically active microbes, have been proposed to constitute an additional level of innate immune sensing, together with the classical pathogen associated molecular patterns or PAMPs as originally formulated by Janeway (1989; Vance et al., 2009; Sander et al., 2011). Bacterial mRNA (Kanneganti et al., 2006; Sander et al., 2011) and cyclic dinucleotides (Woodward et al., 2010) function as vita-PAMPs during bacterial infection. Our data suggest that bacterial metabolites potentially serve as another class of vita-PAMP that signals inflammasome activation.

What is the mechanism by which aconitase or isocitrate dehydrogenase-deficient *Stm* induces rapid activation of the NLRP3 inflammasome? This inflammasome activation is unlikely to be due to lysis of bacteria and cytosolic release of bacterial nucleic acids as it is independent of AIM2. It is also dependent solely on caspase-1, indicating that it does not mobilize a noncanonical caspase-11 inflammasome. Interestingly, both *acnB* and *icdA* mutants induced elevated levels of mitochondrial ROS production from infected macrophages, and limiting mROS production by either genetic or pharmacological methods eliminated inflammasome activation in response to TCA mutant *Stm*. Our data therefore suggest a link between core metabolic processes of intracellular bacteria and mitochondria in regulating inflammasome activation.

The precise role and sources of ROS in inflammasome activation in response to different stimuli remains mysterious (Latz et al., 2013). ROS plays a role both in the activation of the inflammasome complex itself, as well as in up-regulation of NLRP3 inflammasome components (Bauernfeind et al., 2011). Notably, NLRP3 inflammasome activation appears to mobilize different cellular sources of ROS for different stimuli, as LPS+ATP induces NLRP3 inflammasome activation through a pathway involving NOX2 (encoded by $gp91^{phox}$) rather than mitochondrial ROS (Moore and MacKenzie, 2009; Martinon, 2010). Previous studies have demonstrated a requirement for mitochondrial ROS in antimicrobial killing downstream of TLR signaling during *Salmonella* infection (West et al., 2011). Our studies now provide a link between mitochondrial ROS and NLRP3 inflammasome activation in response to alteration of *Salmonella* citrate metabolism. These findings suggest that although the *Salmonella* TCA cycle is dispensable for intracellular replication, it may enable *Salmonella* to evade the NLRP3 inflammasome by limiting the production of bacterial citrate.

Although *icdA* and *acnB* were not required for intracellular replication of *Stm* in vitro, these mutants were attenuated during in vivo infection; *icdA* was attenuated after i.p. infection of $Nramp^S$ B6 mice, although it is not currently clear whether this relates to a metabolic requirement for *icdA* during in vivo infection. Interestingly, although *acnB* was dispensable for i.p. infection of C57BL/6 mice, *acnB*-deficient bacteria were significantly attenuated after oral infection of B6 mice. *acnB* mutant bacteria also had a defect during persistent infection in $Nramp1^R$ 129S6/SvE mice in both single and competitive infection, indicating that absence of *acnB* impacts in vivo bacterial virulence. Notably, despite having reduced bacterial tissue burdens, mice infected with *acnB*-deficient Stm displayed significantly higher levels of serum IL-18 than mice infected with the parental *acnB*-sufficient bacteria, indicating that *Salmonella* lacking *acnB* induce elevated inflammasome activation in vivo. Indeed, $Casp1^{-/-}Casp11^{-/-}$ and $Nlrp3^{-/-}$ mice showed significantly reduced production of IL-18 in response to *acnB Stm*.

Altogether, our data indicate that *acnB*-deficient *Stm* trigger inflammasome-dependent immune responses that contribute to antibacterial immune defense. *Stm* can limit adaptive immune responses through a variety of mechanisms including the induction of host nitric oxide as well as production of bacterial L-asparaginase (Schwacha et al., 1998; Kullas et al., 2012). As inflammasome activation leads to release of IL-1β and IL-18, which contribute to T_H1 and T_H17 responses (Ghayur et al., 1997; Xu et al., 1998; Chung et al., 2009), infection with TCA cycle mutants may overcome these bacterial inhibitory mechanisms to prime *Stm*-specific adaptive responses that promote control of bacterial infection. A longstanding challenge of antibacterial vaccine design has been to produce attenuated live vaccines that retain immunogenicity (Wang et al., 2013). Interestingly, commonly used adjuvants that contain alum in human vaccine preparations trigger the NLRP3 inflammasome for efficient priming of T cell responses (Eisenbarth et al., 2008). Thus, in addition to revealing a novel aspect of interactions between intracellular bacterial pathogens and host macrophages, our studies may provide a strategy to induce robust adaptive cellular immune responses by using strains of *Salmonella* that trigger robust NLRP3 inflammasome activation in vivo.

To our knowledge, this work describes the first identification of *Salmonella* genes that modulate NLRP3 inflammasome activation, and the first demonstration that bacterial TCA cycle enzymes impact activation of the NLRP3 inflammasome. Our data further provide evidence that *Stm* lacking the glyoxylate shunt enzyme isocitrate lyase also induce NLRP3 inflammasome activation in infected macrophages. Given the requirement of isocitrate lyase for persistence of bacterial pathogens such as *M. tuberculosis*, *Pseudomonas*, *Burkholderia*,

and *Salmonella* (McKinney et al., 2000; Fang et al., 2005; Lindsey et al., 2008; van Schaik et al., 2009) and the overlap between the genes found in our inflammasome screen and the genes found in the *Salmonella* persistence screen, our data raise the likelihood that modulation of inflammasome activation may be important for long-term bacterial persistence as well as in acute systemic infection, and that targeting such bacterial factors may provide a broadly applicable strategy to enhance antibacterial immune defense.

MATERIALS AND METHODS

Bacterial strains and infection conditions. Targeted deletion strains used in this study were made on the SL1344 strain background of *S. enterica* Typhimurium in which both subunits of flagellin, *fliC and fljB*, had been deleted through clean deletion of both genes (*fliCfljB*) or clean deletion of *fliC* and a kanamycin cassette insertion into *fljB* (*fliCfljB::kan*) using standard methods (Datsenko and Wanner, 2000). When necessary, clean deletions were generated using the FRT recombinase (Datsenko and Wanner, 2000). Isogenic mutants were made on the *fliCfljB* strain background: *fliCfljBacnB::kan*, *fliCfljBicdA::kan*, *fliCfljBaceA::kan*, *fliCfljBfumA::kan*, *fliCfljBsipB::cat*, *fliCfljBacnB::kansipB::cat*, *fliCfljBicdA::kansipB::cat*, *fliCfljBaceA::kansipB::cat*, *fliCfljBfumA::kansipB::cat*, *fliCfljBsseC::kan*, *fliCfljBsseCacnB::kan*, *fliCfljBsseCicdA::kan*, *fliCfljBsseCaceA::kan*, and *fliCfljBsseCfumA::kan*. TCA cycle mutations were moved into a *fliCfljBsopEBE2::cat* background. The *sopEBE2*::*cat* mutant strain was generated by sequential lambda *red*-mediated deletion of *sopB* and *sopE2*, followed by generation of an unmarked deletion of *sopE* by use of P22 lysate from SL1344 sopE::pSW245 (Lopez et al., 2012; provided by S. Winter and A. Baumler, University of California, Davis, Davis, CA). Bacteria were routinely grown at 37°C. For infection of cultured cells, bacteria were grown shaken overnight at 37°C in LB medium. Bacteria were diluted in LB containing 300 mM NaCl. Bacteria were grown standing for 3 h to induce SPI-1 expression (Lee and Falkow, 1990). For mouse infections, bacteria were grown overnight with aeration at 37°C and diluted in PBS.

Genomic DNA isolation, PCR, and transposon insertion sequencing. Random transposition of Tn*10*d::*Tet* into Salmonella chromosome was achieved by use of a modified low-specificity transposase and T-POP (Rappleye and Roth, 1997; provided by E. Kofoid and J. Roth, University of California, Davis, Davis, CA). Genomic DNA was isolated from individual transposon mutants by phenol-chloroform extraction. A nested random priming method (O'Toole and Kolter, 1998) was used to determine the location of transposon insertion with the first round of PCR using a TetA primer (5′-ACCTTTGGTCACCAACGCTTTTCC-3′) together with a random primer (5′-GTTTCCCAGTCACGATCNNNNNNNNN-3′) at low stringency, followed by a second higher stringency PCR using a universal primer (5′-GACAAGATGTGTATCCACCTTACC-3′) together with a primer containing the 5′ defined sequence of the random primer (5′-GTTTCCCAGTCACGATC-3′). PCR reactions were purified and sequenced using the universal primer.

Mice and macrophage infections. C57BL/6 (B6) mice were from NCI and 129S6/SvE mice were from Taconic. The following knockout mice used in these studies were on the B6 background and have been previously described: *Casp1*$^{-/-}$*Casp11*$^{-/-}$ (Kuida et al., 1995), *Asc*$^{-/-}$ (Sutterwala et al., 2006), *Nlrc4*$^{-/-}$ (Lara-Tejero et al., 2006), *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ (Case et al., 2009), *Nlrp3*$^{-/-}$ (Sutterwala et al., 2006), *Casp1*$^{-/-}$ (Case et al., 2013), and *Casp11*$^{-/-}$ (Wang et al., 1998; provided by T. Horng and J. Yuan, Harvard University, Boston, MA). Animals were maintained in accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee (IACUC). BM cells were grown at 37°C in a humidified incubator in DMEM containing Hepes, 10% FCS, and 30% L929 supernatant for 7–8 d. Differentiated BMDMs were replated into 24-, 48-, or 96-well dishes 16–20 h before infection. MCAT BM was provided by A. Philip West and Gerald Shadel (Yale University, New Haven, CT). Bacterial strains described above were harvested, washed three times with DMEM, resuspended, and added to the cells at an MOI of 20:1. Bacteria were spun onto the cells at 1,000 RPM for 5 min, and the infected cells placed in a humidified tissue culture incubator at 37°C for 1 h. Gentamicin was added to the cells 1 h after infection to a final concentration of 100 µg/ml, and the cells placed in the incubator until harvesting. 50 ng/ml LPS used in indicated experiments was *E. coli* O55:B5 (Sigma-Aldrich). 2.5 mM ATP (Sigma-Aldrich) used in indicated experiments remained in wells for 4 h after addition. For experiments using MitoQ, BMDMs were pretreated with 50 ng/ml LPS 4 h before infection for the indicated conditions. 10 µM MitoQ (provided by N. Avadhani and S. Srinavasan, University of Pennsylvania, Philadelphia, PA) or vehicle control (DMSO) was added to cells 1 h after LPS treatment and remained for the duration of the experiment.

Cell death assays. BMDMs were seeded into 96-well plates at a density of 7×10^4 cells per well. The next day, culture medium was replaced with fresh DMEM. Cells were infected as described above and supernatants harvested at 4 h after infection. Lactate dehydrogenase release was quantified using the Cytotox96 Assay kit (Takara Bio Inc.) according to the manufacturer's instructions.

Cytokine production. BMDMs were seeded into 48-well plates at a density of 1.5×10^5 cells per well. Cells were pretreated with *E. coli* LPS (Sigma-Aldrich) for 3 h before bacterial infection as described above, and supernatants were harvested 4 h after infection. Release of proinflammatory cytokines was measured by enzyme-linked immunosorbent assay (ELISA) using capture and detection antibodies against IL-1β (eBioscience), IL-6 (BD), and IL-12 (BD).

Western blotting and antibodies. BMDMs were seeded into 24-well plates at a density of 3×10^5 cells per well and infected with bacteria as described above. 3 h after infection, cells were lysed in 20 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM EDTA. Lysates were mixed with protein loading buffer, boiled, centrifuged, and 20% of the total cell lysate loaded onto 4–12% NuPAGE gels (Invitrogen). Proteins were transferred to PVDF membrane (Millipore) and blotted with rabbit anti–mouse caspase-1 antibody (Santa Cruz Biotechnology, Inc.). Secondary antibody was goat anti–rabbit HRP (Jackson ImmunoResearch Laboratories, Inc.). The membrane was exposed on radiographical film using SuperSignal West Pico kit (Thermo Fisher Scientific).

Measurement of mitochondrial superoxide. Mitochondrial superoxide (O_2^-) was measured by using the mitochondrial O_2^- indicator MitoSOX red (molecular probes; Invitrogen). In brief, *Nlrc4*$^{-/-}$ BMDMs were grown as described above and seeded into 35-mm plastic Petri dishes with glass coverslip bottoms. Infections were performed as described above. 1 µg LPS was added to indicated plate 6 h before imaging. BMDMs were loaded with 5 µM MitoSOX Red for 30 min. Residual dye was removed by washing and dishes were mounted in an open perfusion microincubator and imaged by confocal microscopy. Confocal images were obtained using an inverted confocal microscope (SP5-2; Leica) at 561 nm excitation with a 63× oil objective. Images were analyzed by masking the perinuclear region of the cells and the mean MitoSOX red fluorescence was quantified using Application Suite software (Leica).

Metabolite analysis. Bacterial cultures were shaken overnight at 37°C in LB medium. Bacteria were diluted in LB containing 300 mM NaCl. Bacteria were grown standing to OD 0.5. Bacteria were immediately transferred to cold conical tubes and spun down at 4,000 rpm for 5 min at 4°C. Bacterial pellets were washed with 0.8% saline solution and then pelleted. Metabolites were extracted with ice-cold 80% methanol and centrifuged for 10 min at 4°C. D_{27}-myristic acid was used as an internal reference standard. TCA metabolites were reduced with sodium borodeuteride and deuterated standards added as previously described (Mamer et al., 2013). Dried samples were resuspended in 30 µl anhydrous pyridine and added to GC-MS autoinjector vials containing 70 µl *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide

derivatization reagent. The samples were incubated at 70°C for 1 h, after which aliquots of 1 µl were injected for analysis. GC-MS data were collected on a 5975C series GC/MSD system (Agilent Technologies) operating in election ionization mode (70 eV) and selected ion monitoring. Quantified metabolites were normalized relative to cell number as described previously (Mamer et al., 2013).

Mouse infections. Mice were housed in accordance with National Institutes of Health (NIH)– and University of Pennsylvania–approved guidelines, and all studies involving mice were performed in accordance with approved University of Pennsylvania IACUC protocols and procedures. 8–10-wk-old age- and sex-matched mice were i.p. infected with 5×10^2 bacteria for C57BL/6 mice, and 10^3 or 10^4 bacteria for 129S6/SvE mice. For oral infections, C57BL/6 mice, or isogenic *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice were fasted overnight and intragastrically inoculated with 2×10^7 bacteria. For single infection experiments, unmarked *fliCfljB*, *acnB::kan*, or *icdA::kan* were used. For competition infections, 10^3 unmarked *fliCfljB* with 10^3 *fliCfljB::kan*, or 10^3 unmarked *fliCfljB* with 10^3 marked *fliCfljBacnB::kan* were mixed 1:1 and injected i.p. Mice were sacrificed, and the tissues and sera were harvested at the indicated times after infection. Bacterial load was determined by plating dilutions of tissue homogenates on appropriate selective plates. For competitive index experiments, identical dilutions of homogenates were plated on both streptomycin and kanamycin plates, and CI was calculated in accordance with previously described methods (Beuzón and Holden, 2001).

Statistical analysis. Kaplan-Meier curves were used to plot survival of infected mice. Plotting of data and statistical analysis was performed using Prism 5.0 (GraphPad Software). Statistical significance—indicated in respective figure legends—was determined by unpaired two-tailed Student's *t* test or Mann-Whitney *U* test, as indicated.

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