THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 41, pp. 34474–34483, October 5, 2012

Toll or Interleukin-1 Receptor (TIR) Domain-containing Adaptor Inducing Interferon-β (TRIF)-mediated Caspase-11 Protease Production Integrates Toll-like Receptor 4 (TLR4) Protein- and Nlrp3 Inflammasome-mediated Host Defense against Enteropathogens*[S]

Received for publication, July 16, 2012, and in revised form, August 9, 2012 Published, JBC Papers in Press, August 16, 2012, DOI 10.1074/jbc.M112.401406

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Background: *C. rodentium* and *E. coli* induce noncanonical Nlrp3 inflammasome activation through caspase-11.
Results: TLR4-TRIF are important for caspase-11 expression, caspase-1 activation, and downstream IL-1β and IL-18 production.
Conclusion: TLR4-TRIF axis plays an important role in the up-regulation of caspase-11 and activation of noncanonical inflammasome.
Significance: Our study identifies novel molecules upstream of caspase-11 that are involved in activation of noncanonical inflammasome.

Enteric pathogens represent a major cause of morbidity and mortality worldwide. Toll-like receptor (TLR) and inflammasome signaling are critical for host responses against these pathogens, but how these pathways are integrated remains unclear. Here, we show that TLR4 and the TLR adaptor TRIF are required for inflammasome activation in macrophages infected with the enteric pathogens *Escherichia coli* and *Citrobacter rodentium*. In contrast, TLR4 and TRIF were dispensable for *Salmonella typhimurium*-induced caspase-1 activation. TRIF regulated expression of caspase-11, a caspase-1-related protease that is critical for *E. coli*- and *C. rodentium*-induced inflammasome activation, but dispensable for inflammasome activation by *S. typhimurium*. Thus, TLR4- and TRIF-induced caspase-11 synthesis is critical for noncanonical Nlrp3 inflammasome activation in macrophages infected with enteric pathogens.

Enteric pathogens such as enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli* are responsible for a large number of cases of diarrhea, which causes significant morbidity and mortality among infants and children in the developing world (1–3). Orchestration of an appropriate immune response against these bacterial pathogens is accomplished in part through their recognition by a limited number of germ line-encoded pattern recognition receptors expressed by immune and epithelial cells (4). Activation of members of the mammalian Toll-like receptor (TLR)[3] family at the cell surface and within endosomes triggers NF-κB activation through the adaptor proteins MyD88 and TRIF (5). NF-κB target genes include proinflammatory cytokines such as members of the interleukin (IL) family, the transcriptional up-regulation of which drives induction of host responses contributing to effective eradication of the bacterial pathogen (5). This is illustrated by the observation that mice with mutations in TLR4 are hyporesponsive to lipopolysaccharide (LPS)-induced cytokine production (6, 7) and that $Tlr4^{-/-}$ and $Myd88^{-/-}$ mice are highly resistant to *E. coli*-induced septic shock (8).

In addition to TLRs, intracellular pattern recognition receptors of the NOD-like receptor (NLR), HIN-200, and RIG-I-like protein families are increasingly recognized as critical sensors that detect conserved microbial components and endogenous danger-associated molecules in intracellular compartments (4, 9). A subset of NLR and HIN-200 proteins assembles inflammasomes, cytosolic multiprotein complexes that drive the proteolytic maturation of caspase-1, a proinflammatory protease whose activity is implicated in a variety of infectious and autoinflammatory diseases (10). Caspase-1 contributes to inflammatory responses by proteolytically maturing the proinflam-

* This work was supported, in whole or in part, by National Institutes of Health Grants AR056296, AI101935, and CA163507 (to T.-D. K.). This work was also supported by Marie-Curie Grant 256432 from the European Union, European Research Council Grant 281600, and the Fund for Scientific Research-Flanders Grants G030212N, 1.2.201.10.N.00, and 1.5.122.11.N.00 (to M. L.), and by the American Lebanese Syrian Associated Charities (ALSAC) (to T.-D. K.).
[S] This article contains supplemental Figs. 1–4.
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[3] The abbreviations used are: TLR, Toll-like receptor; m.o.i., multiplicity of infection; NLR, NOD-like receptor; TRIF, Toll or interleukin receptor (TIR) domain-containing adaptor-inducing interferon-β.

matory cytokines IL-1β and IL-18, and by initiating pyroptosis, a rapid proinflammatory cell death mode in infected myeloid cells (10). Genetic studies in mice indicate that at least four inflammasomes are assembled depending on the infectious agent or danger-associated molecule that is encountered (9, 10). Notably, the Nlrp3 inflammasome responds to a wide variety of insults, including the detection of bacterial mRNA in intracellular compartments (11, 12). Recently, the Nlrp3 inflammasome was demonstrated to be responsible for inflammasome-mediated IL-1β and IL-18 production in macrophages infected with the enteric pathogens *E. coli*, *Citrobacter rodentium*, and *Vibrio cholerae* (13). Moreover, the Nlrp3 inflammasome plays a critical role in induction of immune and host responses against *C. rodentium* infection *in vivo* (14). However, how TLR and inflammasome activation are integrated during infection with enteric bacterial pathogens remains unclear.

Here, we show that Nlrp3 inflammasome activation and caspase-1 processing in macrophages infected with the enteric pathogens *E. coli* and *C. rodentium* specifically required the TLR adaptor TRIF downstream of TLR4. In contrast, TLR4 and TRIF were dispensable for *Salmonella typhimurium*-induced caspase-1 activation, which proceeded through the Nlrc4 inflammasome. TLR4 and TRIF mediated synthesis of caspase-11, a protease that is critical for *E. coli*- and *C. rodentium*-induced inflammasome activation. Thus, we identified TRIF-induced caspase-11 production as a new immune pathway that integrates TLR4- and Nlrp3 inflammasome-mediated recognition of enteric pathogens in macrophages.

EXPERIMENTAL PROCEDURES

Mice—All mice were fully backcrossed to C57BL/6 and housed at St. Jude Children's Research Hospital and Ghent University in a specific pathogen-free animal care facility. *Tlr2*$^{-/-}$ (15), *Tlr4*$^{-/-}$ (16), *Tlr7*$^{-/-}$ (17), *Myd88*$^{-/-}$ (18), *Trif*$^{-/-}$ (19), and *Myd88*$^{-/-}$*Trif*$^{-/-}$ (19) mice were kindly provided by Dr. Akira (Osaka University) and have been described previously. *Caspase-11*$^{-/-}$ mice (13) were a kind gift of Dr. Vishva Dixit (Genentech). *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$, *Nod1*$^{-/-}$, *Nod2*$^{-/-}$, *Mavs*$^{-/-}$, *Nlrp6*$^{-/-}$, and *Nlrp12*$^{-/-}$ mice used in this study have all been described previously (20–29). Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital and Ghent University Committee on Use and Care of Animals.

Macrophage Culture and in Vitro Infections—Bone marrow-derived macrophages were prepared as described previously (14, 22, 30). Briefly, bone marrow cells were cultured in L cell-conditioned Iscove's modified Dulbecco's medium supplemented with 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin for 5 days to differentiate into macrophages. Bone marrow-derived macrophages were seeded in 6-well cell culture plates, stimulated with or without LPS (500 ng/ml) for 5 h, and infected with *E. coli*, *C. rodentium*, or *S. typhimurium* at the indicated multiplicity of infection (m.o.i.) for 24 h in a CO_2 incubator at 37 °C. 1 h after infection, gentamycin (50 μg/ml) was added to the culture medium. As a positive control, macrophages were stimulated with LPS (1 μg/ml) for 4 h, the last half-hour of which in the presence of ATP (5 mM).

C. rodentium in Vivo Infection—*C. rodentium* (ATCC 51459) was grown in LB broth overnight with shaking at 37 °C. For *in vivo* experiments, mice were infected with 1×10^{10} c.f.u. by oral gavage. Food and water intake were stopped 8 h prior to infection and allowed to resume 1 h after infection. To determine bacterial counts, serial dilutions of homogenized feces were plated on MacConkey agar plates and incubated at 37 °C for 24 h.

Cytokine Analysis—Concentrations of mouse cytokines and chemokines in cell supernatants were determined using multiplex ELISA (Millipore), IL-1β ELISA (eBioscience), and IL-18 ELISA (MBL International).

Real-time Quantitative PCR—Total RNA was isolated using the TRIzol method (Invitrogen). First strand cDNA was generated from total RNA using High Capacity cDNA Reverse Transcription reagents (Applied Biosystems). cDNA samples were prepared in duplicate and subjected to real-time quantitative RT-PCR on an ABI Prism 7900 instrument using SYBR Green PCR Master Mix (Applied Biosystems) and normalized to the housekeeping gene *Gapdh*. The following primer pairs were used for quantitative RT-PCR analysis: mouse *Gapdh* forward, 5′-CGTCCCGTAGACAAAATGGT-3′ and reverse, 5′-TTGATGGCAACAATCTCCAC-3′; mouse *caspase-11* forward, 5′-ACGATGTGGTGGTGAAAGAGGAGC-3′ and reverse, 5′-TGTCTCGGTAGGACAAGTGATGTGG-3′; mouse *Nlrp3* forward, 5′-TGCAGAAGACTGACGTCTCC-3′ and reverse, 5′-CGTACAGGCAGTAGAACAGTTC-3′. Quantitative RT-PCR data were reported according to the standard curve method.

Western Blotting—For protein analysis of Nlrp3, caspase-1, and caspase-11, cell lysates were denatured with SDS plus 100 mM DTT and boiled for 5 min. SDS-PAGE-separated proteins were transferred to PVDF membranes and immunoblotted with primary antibodies against caspase-1, caspase-11, and Nlrp3 followed by secondary anti-rabbit HRP antibodies as described previously (23).

Statistics—GraphPad Prism 5.0 software was used for data analysis. Data are represented as mean ± S.E. or S.D. Statistical significance was determined by Student's *t* test; $p < 0.05$ was considered statistically significant.

RESULTS

Caspase-11 Is Required for C. rodentium- and E. coli-induced Caspase-1 Activation and Secretion of IL-1β and IL-18—C. rodentium is an enteropathogen of the murine gastrointestinal tract and triggers inflammatory responses resembling those of humans infected with enteropathogenic and enterohemorrhagic *E. coli* (31). Macrophages infected with these pathogens induce activation of the Nlrp3 inflammasome (13), and we recently described a critical role for this inflammasome in regulating host responses against *C. rodentium in vivo* (14). In agreement with previously published results from Kayagaki *et al.* (13), we show here that *C. rodentium*- and *E. coli*-induced caspase-1 activation was defective in *caspase-11*-deficient macrophages infected *in vitro* (Fig. 1, *A* and *B*). Interestingly, *S. typhimurium*-induced caspase-1 activation was intact in

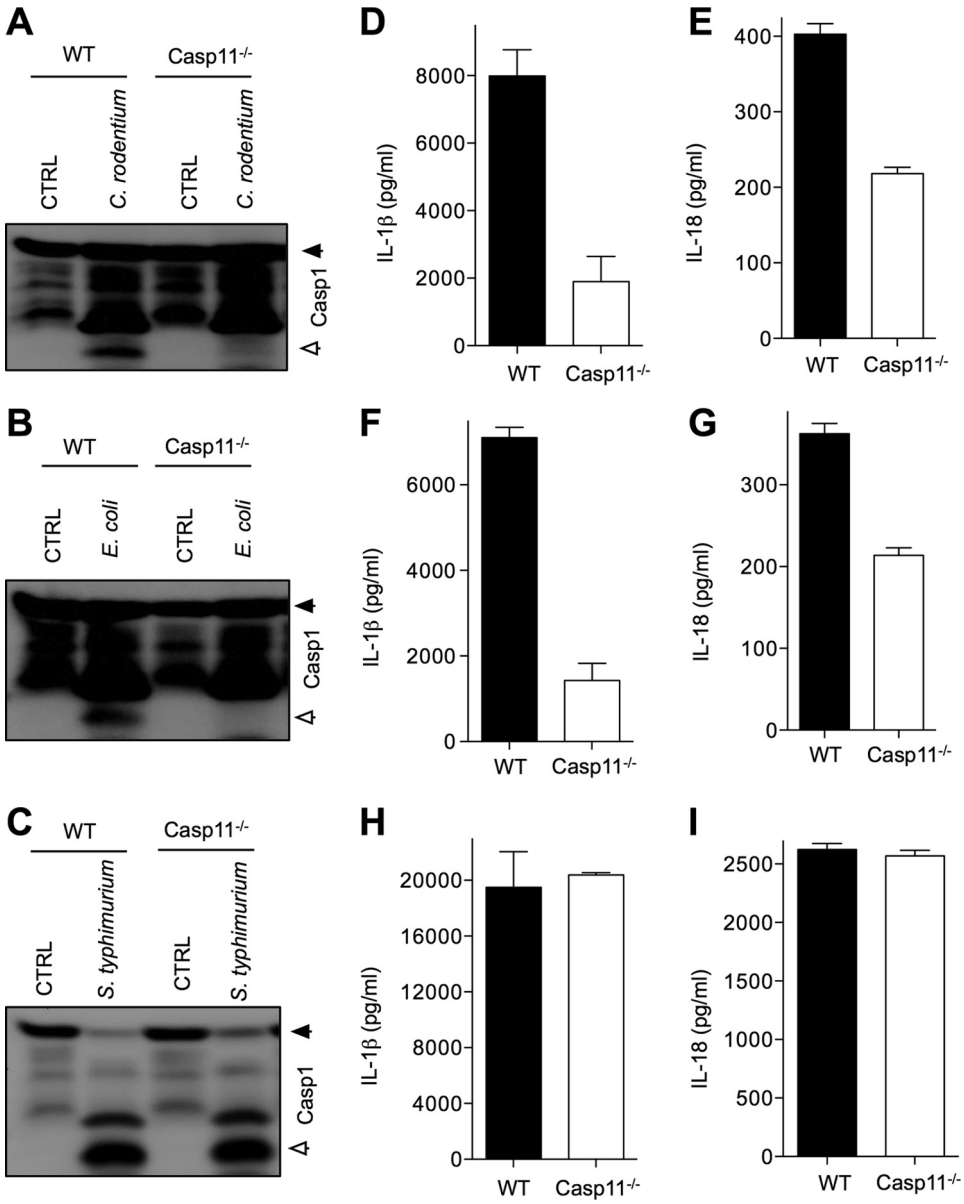

FIGURE 1. **Caspase-11 is required for inflammasome signaling in macrophages infected with *C. rodentium and E. coli*, but dispensable during *S. typhimurium* infection.** Bone marrow-derived macrophages from WT and *caspase-11*$^{-/-}$ mice were infected with *C. rodentium* (m.o.i. 20), *E. coli* (m.o.i. 20), and *S. typhimurium* (m.o.i. 5) as described under "Experimental Procedures." *A–C*, caspase-1 activation was determined by Western blotting of cell lysates. *D–I*, secreted IL-1β and IL-18 in supernatants of infected macrophages were quantified by ELISA. Data represent means ± S.E. (*error bars*) and are representative of three independent experiments.

caspase-11-deficient macrophages (Fig. 1*C*), suggesting a specific role for caspase-11 in noncanonical inflammasome activation during *C. rodentium* and *E. coli* infection, as described before. In addition to caspase-1 processing, secretion of mature IL-1β and IL-18 in response to *E. coli* and *C. rodentium* infection was dependent on caspase-11 (Fig. 1, *D–G*), whereas *S. typhimurium*-induced secretion of IL-1β and IL-18 was not (Fig. 1, *H* and *I*). The role of caspase-11 in regulating production of IL-1β and IL-18 was specific because production of the inflammasome-independent cytokines TNF-α and IL-6 was similar in WT and caspase-11 macrophages infected with *E. coli* and *C. rodentium* (supplemental Fig. 1).

Role of NLRs and AIM2 in E. coli- and C. rodentium-induced Activation of Noncanonical Inflammasome Signaling—Both the recent report by Kayagaki *et al.* (13) and our results (Fig. 1) demonstrate that caspase-11 is required for activation of the Nlrp3 inflammasome upon *E. coli* and *C. rodentium* infection. However, the adaptor molecules regulating Nlrp3 inflammasome activation upstream of caspase-11 are not known. To determine whether additional NLRs were involved in activation of noncanonical Nlrp3 inflammasome upstream of caspase-11, we examined caspase-1 activation in macrophages deficient for various NLR receptors and the HIN-200 protein AIM2. As expected, both *C. rodentium*- and *E. coli*-induced caspase-1 activation and IL-1β production were dependent on NLRP3 and ASC, whereas *S. typhimurium*-induced caspase-1 activation was dependent on NLRC4 and ASC (Fig. 2). Similar results were observed for LPS+ATP-induced caspase-1 activation, which required NLRP3 and ASC (supplemental Fig. 2). However, none of the other molecules tested (AIM2, NLRC4, NOD1,

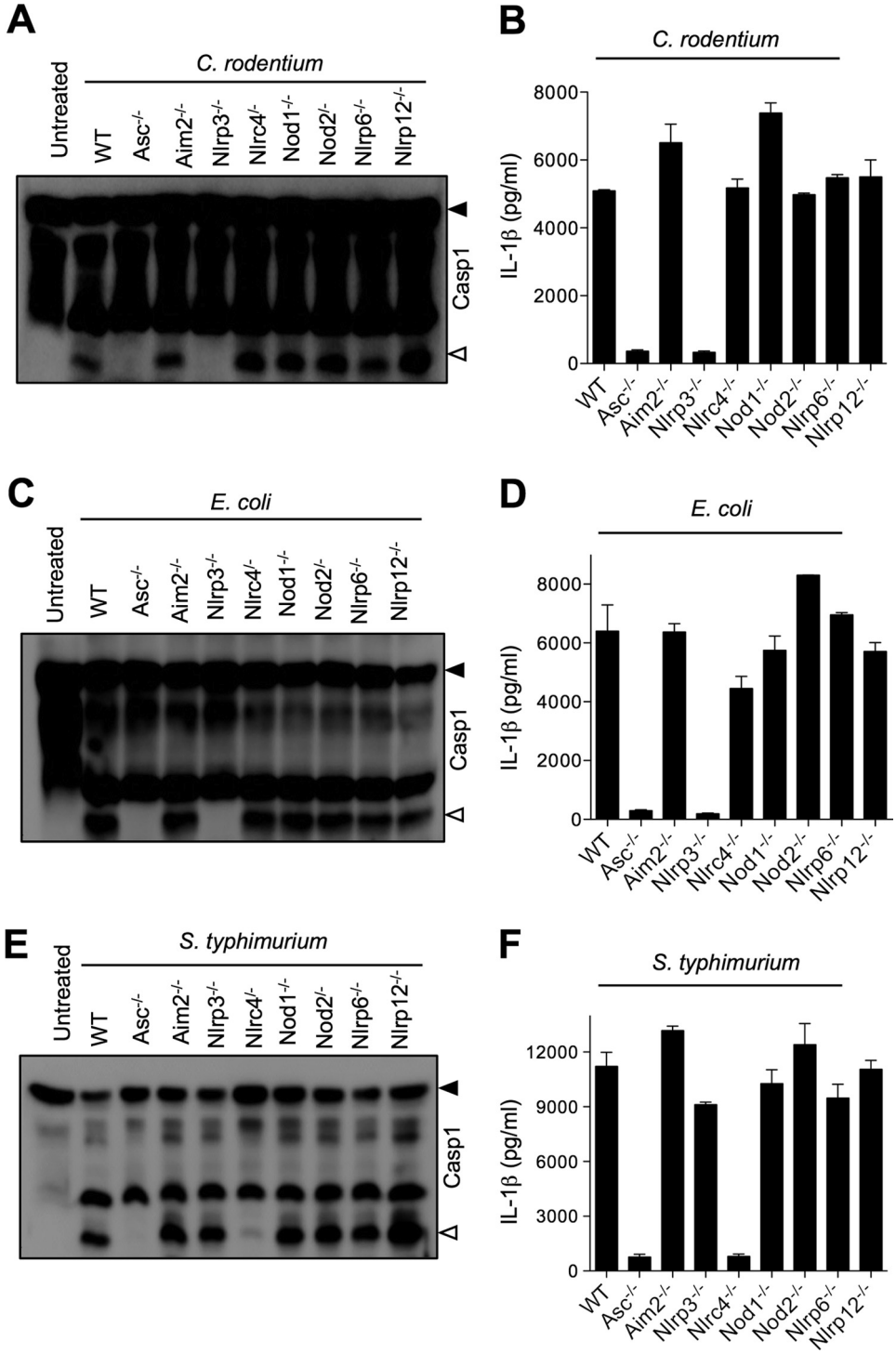

FIGURE 2. **Role of NLRs and adaptor molecules in caspase-1 activation and IL-1β secretion from macrophages infected with Gram-negative bacteria.** Bone marrow-derived macrophages from WT, *Asc*$^{-/-}$, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Nod1*$^{-/-}$, *Nod2*$^{-/-}$, *Nlrp6*$^{-/-}$, and *Nlrp12*$^{-/-}$ mice were infected with *C. rodentium* (m.o.i. 20), *E. coli* (m.o.i. 20), or *S. typhimurium* (m.o.i. 5) for 24 h as described under "Experimental Procedures." Caspase-1 activation was determined by Western blotting of cell lysates (*A*, *C*, and *E*), and secreted IL-1β in supernatants of infected macrophages was quantified by ELISA (*B*, *D*, and *F*). Data represent means ± S.E. (*error bars*) and are representative of at least three independent experiments.

NOD2, NLRP6, and NLRP12) was required for caspase-1 activation in *E. coli*- and *C. rodentium*-infected macrophages, suggesting that these cytoplasmic adaptors do not play an important role in noncanonical inflammasome activation.

TLR4 Is Specifically Required for Nlrp3 Inflammasome Activation in Macrophages Infected with C. rodentium and E. coli—Activation of the Nlrp3 inflammasome by extracellular ATP, pore-forming toxins and crystals requires NF-κB-mediated priming (32), but whether TLR-induced NF-κB activation mediates Nlrp3 inflammasome activation in response to enteropathogens is not known. Wild type, *Tlr2*$^{-/-}$, and *Tlr7*$^{-/-}$ macrophages infected with *C. rodentium* secreted normal levels of IL-1β and IL-18 in the culture medium, but secretion of these inflammasome-dependent cytokines was signifi-

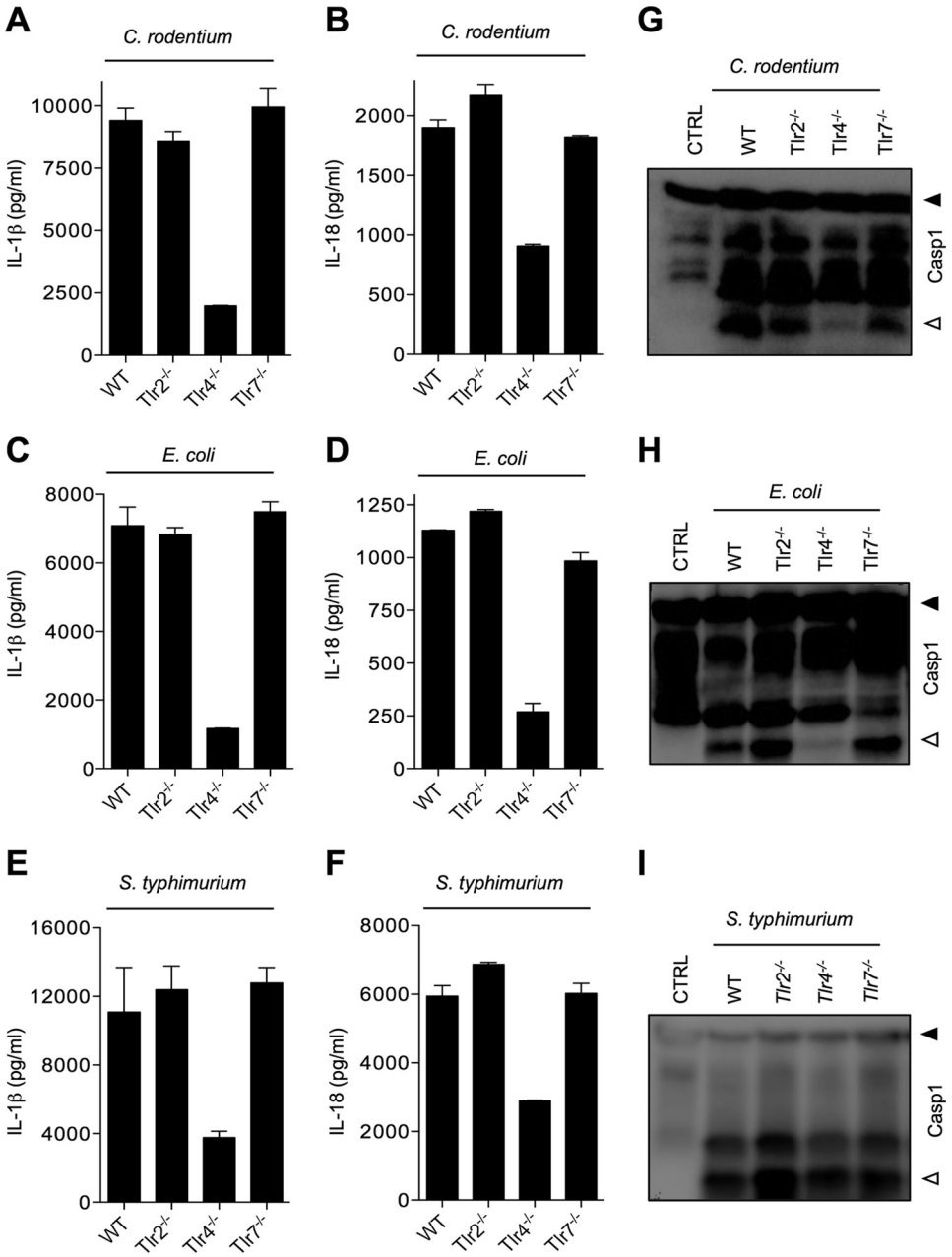

FIGURE 3. **Role of TLR4 in inflammasome activation and secretion of IL-1β and IL-18 by *C. rodentium*-, *E. coli*-, and *S. typhimurium*-infected macrophages.** WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *Tlr7*$^{-/-}$ bone marrow-derived macrophages were infected with *C. rodentium* (m.o.i. 20), *E. coli* (m.o.i. 20), and *S. typhimurium* (m.o.i. 5) for 24 h as described under "Experimental Procedures." Secreted IL-1β and IL-18 in supernatants of infected macrophages were quantified by ELISA (*A–F*), and caspase-1 activation was determined by Western blotting of cell lysates (*G–I*). Data represent means ± S.E. (*error bars*) and are representative of at least three independent experiments.

cantly attenuated in *TLR4*-deficient macrophages (Fig. 3, *A* and *B*). Concurrently, *E. coli*-induced IL-1β and IL-18 secretion were severely hampered in *Tlr4*$^{-/-}$ macrophages (Fig. 3, *C* and *D*). Notably, *Tlr4*$^{-/-}$ macrophages infected with *S. typhimurium* also secreted less IL-1β and IL-18 (Fig. 3, *E* and *F*). This suggests that TLR4 is required for production of proIL-1β and proIL-18 in macrophages infected with Gram-negative bacteria irrespective of the inflammasome involved. To examine directly the role of TLR signaling in inflammasome activation by *C. rodentium* and *E. coli*, we analyzed caspase-1 maturation in cell lysates of macrophages infected with these pathogens. Caspase-1 processing into the large catalytic subunit (p20) was observed in wild type, *Tlr2*$^{-/-}$, and *Tlr7*$^{-/-}$ macrophages infected with *C. rodentium* or *E. coli*, but not in *Tlr4*$^{-/-}$ macrophages (Fig. 3, *G* and *H*). Notably, *S. typhimurium*-induced caspase-1 activation was not affected in macrophages lacking TLR4 (Fig. 3*I*). These results suggest that in addition to its general role in inducing proIL-1β synthesis in macrophages infected with bacterial pathogens, TLR4 is specifically required for inflammasome activation by enteric bacterial pathogens.

TRIF Mediates Nlrp3 Inflammasome Activation by Enteropathogens—To examine further the mechanism by which TLR4 mediates Nlrp3 inflammasome activation in macrophages infected with enteropathogens, cells lacking the

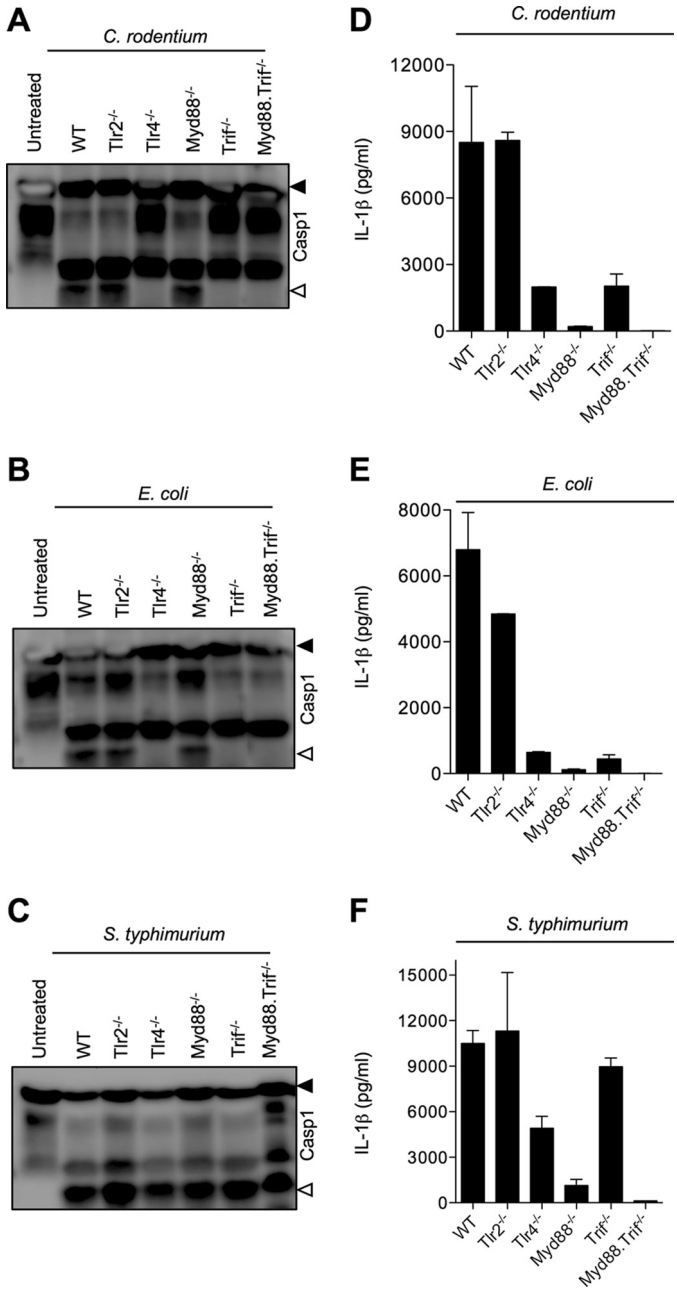

FIGURE 4. **TRIF is critical for *C. rodentium*- and *E. coli*-induced inflammasome activation.** WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages were infected with *C. rodentium* (m.o.i. 20), *E. coli* (m.o.i. 20), or *S. typhimurium* (m.o.i. 5) as described under "Experimental Procedures." Caspase-1 activation was determined by Western blotting of cell lysates (*A–C*), and secreted IL-1β in supernatants of infected macrophages was quantified by ELISA (*D–F*). Data represent means ± S.E.(*error bars*) and are representative of at least three independent experiments.

TLR adaptors MyD88 (*MyD88*$^{-/-}$), TRIF (*Trif*$^{-/-}$), or both MyD88 and TRIF (*MyD88*$^{-/-}$*Trif*$^{-/-}$) were infected with *C. rodentium* and *E. coli* as before. *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ macrophages were included in these experiments as positive and negative controls, respectively. *C. rodentium*-induced caspase-1 activation proceeded normally in *MyD88*$^{-/-}$ macrophages but was abrogated in macrophages lacking TRIF alone or in combination with MyD88 (Fig. 4*A*). Similarly, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, and *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages failed to activate caspase-1 when infected with *E. coli*, whereas wild type, *Tlr2*$^{-/-}$, and *MyD88*$^{-/-}$ macrophages responded with robust caspase-1 processing (Fig. 4*B*). Unlike these enteropathogens, *S. typhimurium*-induced caspase-1 activation proceeded unabated in macrophages lacking TRIF (Fig. 4*C*). Together with our previous results, this suggests TLR4- and TRIF-mediated signaling to be specifically required for enteropathogen-induced inflammasome activation. In agreement with these caspase-1-processing data, induction of pyroptotic cell death was also reduced in *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ macrophages compared with WT macrophages infected with *C. rodentium* or *E. coli*, respectively (supplemental Fig. 3). In contrast, pyroptosis in *Myd88*$^{-/-}$ macrophages was induced to levels comparable with those of WT macrophages infected with *C. rodentium* or *E. coli* as determined by lactate dehydrogenase release assay (supplemental Fig. 3).

Despite being dispensable for *C. rodentium*- and *E. coli*-induced caspase-1 activation, MyD88 was required for IL-1β secretion from macrophages infected with these pathogens (Fig. 4, *D* and *E*). Notably, IL-1β secretion was also reduced in *Trif*$^{-/-}$ macrophages, but its production was completely blunted in cells lacking MyD88 (Fig. 4, *D* and *E*). In agreement with a general role in proIL-1β synthesis, MyD88 was also required for *S. typhimurium*-induced IL-1β secretion (Fig. 4*F*). The modestly higher level of secreted IL-1β in *Tlr4*$^{-/-}$ cells relative to *MyD88*$^{-/-}$ macrophages suggests that MyD88 may contribute to NF-κB-driven production of proIL-1β downstream of multiple TLRs during Gram-negative infections. Unlike MyD88, TRIF was specifically required for secretion of IL-1β from macrophages infected with *C. rodentium* or *E. coli* (Fig. 4, *D* and *E*), and not in cells infected with *S. typhimurium* (Fig. 4*F*). To address further how TRIF regulates IL-1β expression, we determined IL-1β mRNA levels in WT and *Trif*$^{-/-}$ macrophages infected with *C. rodentium* or *E. coli*. IL-1β mRNA synthesis partially depended on TRIF, whereas MyD88 was critical for enteropathogen-induced IL-1β mRNA production (supplemental Fig. 4). Altogether, these results suggest MyD88 to be critical for proIL-1β production in macrophages infected with Gram-negative bacteria, whereas TRIF specifically controls enteropathogen-induced inflammasome activation and partially regulates IL-1β transcription.

TLR4 and TRIF Are Required for Caspase-11 Synthesis and Activation—Activation of the Nlrp3 inflammasome in *C. rodentium*- and *E. coli*-infected macrophages was previously shown to rely on caspase-11 (13). In agreement, we found caspase-1 maturation and IL-1β production to be significantly hampered in *caspase-11*-deficient cells infected with *C. rodentium* or *E. coli* (Fig. 1). Having established that caspase-11 is required for inflammasome activation by *C. rodentium* and *E. coli*, we explored the hypothesis that TLR4 and TRIF may specifically regulate enteropathogen-induced inflammasome activation by modulating the expression of caspase-11. Relative to levels in *C. rodentium*- and *E. coli*-infected wild type and *TLR2*-deficient macrophages, caspase-11 production in cells lacking MyD88 was largely normal (Fig. 5, *A* and *B*). In contrast, *caspase-11* mRNA induction was markedly reduced in *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, and *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages infected with *C.*

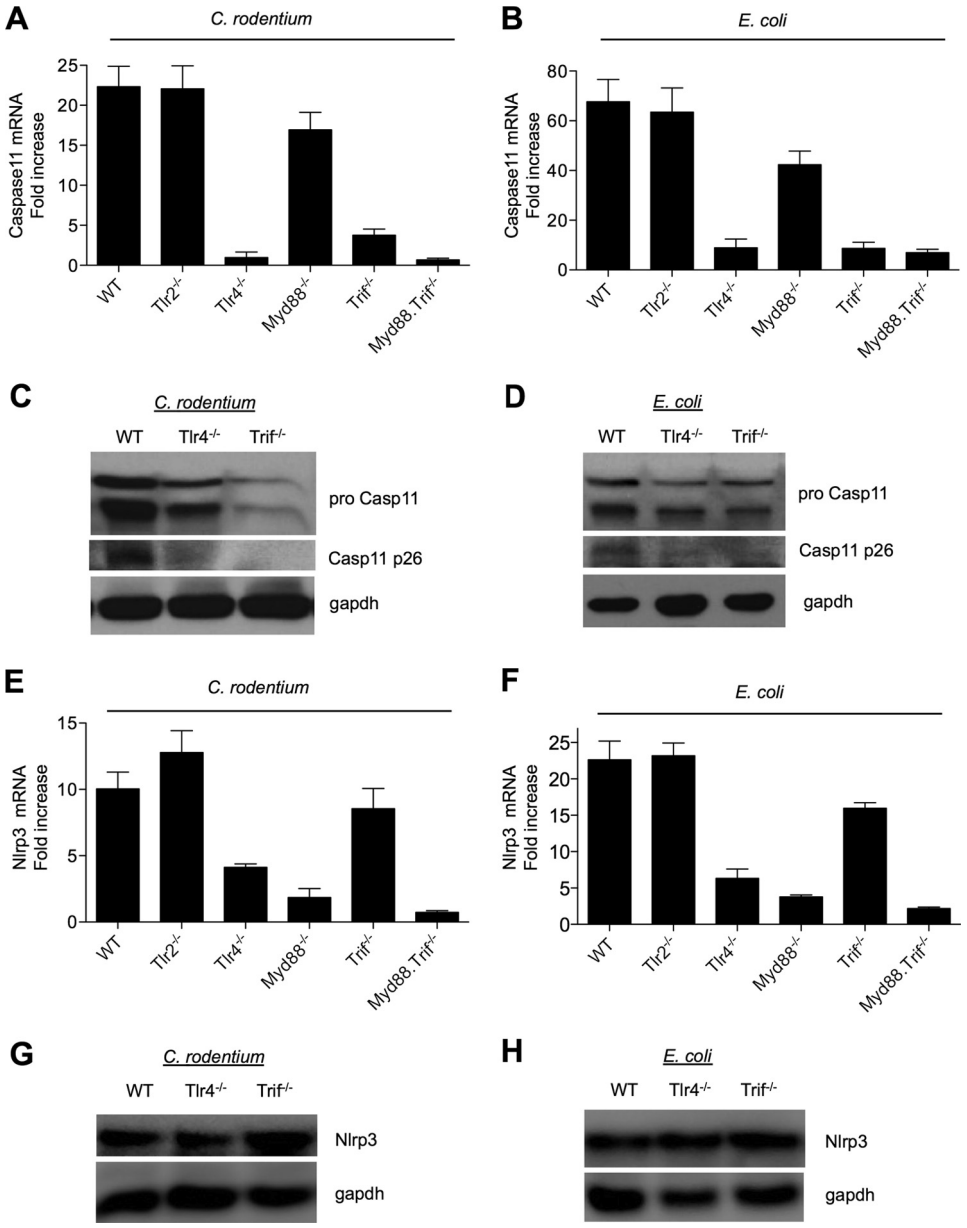

FIGURE 5. **The TLR4-TRIF axis regulates caspase-11 expression.** *A*, *B*, *E*, and *F*, WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages were infected with *C. rodentium* and *E. coli*. RNA was extracted 6 h after infection, and mRNA expression of caspase-11 and Nlrp3 was determined as described under "Experimental Procedures." RNA expression were normalized to the expression of GAPDH and then depicted as -fold increase. *C*, *D*, *G*, and *H*, cell lysates of WT, *Tlr4*$^{-/-}$, and *Trif*$^{-/-}$ macrophages infected with *C. rodentium* or *E. coli* for 24 h were analyzed for caspase-11 and Nlrp3 expression by Western blotting. Data represent means ± S.E. (*error bars*) and are representative of at least three independent experiments.

rodentium or *E. coli* (Fig. 5, *A* and B), indicating that TLR4 and TRIF are required for caspase-11 production. In agreement, procaspase-11 protein levels and caspase-11 processing in *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ macrophages that have been infected with *C. rodentium* or *E. coli* were significantly reduced relative to infected WT cells (Fig. 5, *C* and *D*). Notably, TLR4 and MyD88, but not TRIF, were required for efficient induction of Nlrp3 transcripts in response to these enteropathogens (Fig. 5, *C* and *D*) although Nlrp3 protein levels remained relatively stable in infected WT, *Tlr4*$^{-/-}$, and *Trif*$^{-/-}$ macrophages (Fig. 5, *G* and *H*). Together, these results suggest that TLR4 and TRIF modulate enteropathogen-induced inflammasome activation by promoting caspase-11 expression and activation.

Role of TRIF, MyD88, and Caspase-11 during C. rodentium Infection in Vivo—Previous studies showed that mice deficient in the TLR adaptor MyD88 are highly susceptible to *C. rodentium* infection (33). We showed here that the TLR4-TRIF signaling axis is important for caspase-11 expression and Nlrp3 inflammasome activation during *E. coli* and *C. rodentium* infection of macrophages *in vitro*. To determine whether TLR4 and TRIF were important for *C. rodentium* infection *in vivo*, mice deficient in *TLR4*, *TRIF*, *MyD88*, or both *TRIF* and *MyD88* were infected orally with *C. rodentium*. Mice lacking only *TRIF* or *MyD88* survived, whereas mice doubly deficient for *MyD88* and *TRIF* were highly susceptible to infection and all succumbed by day 8 (Fig. 6*A*). These results suggest that TRIF and MyD88 play

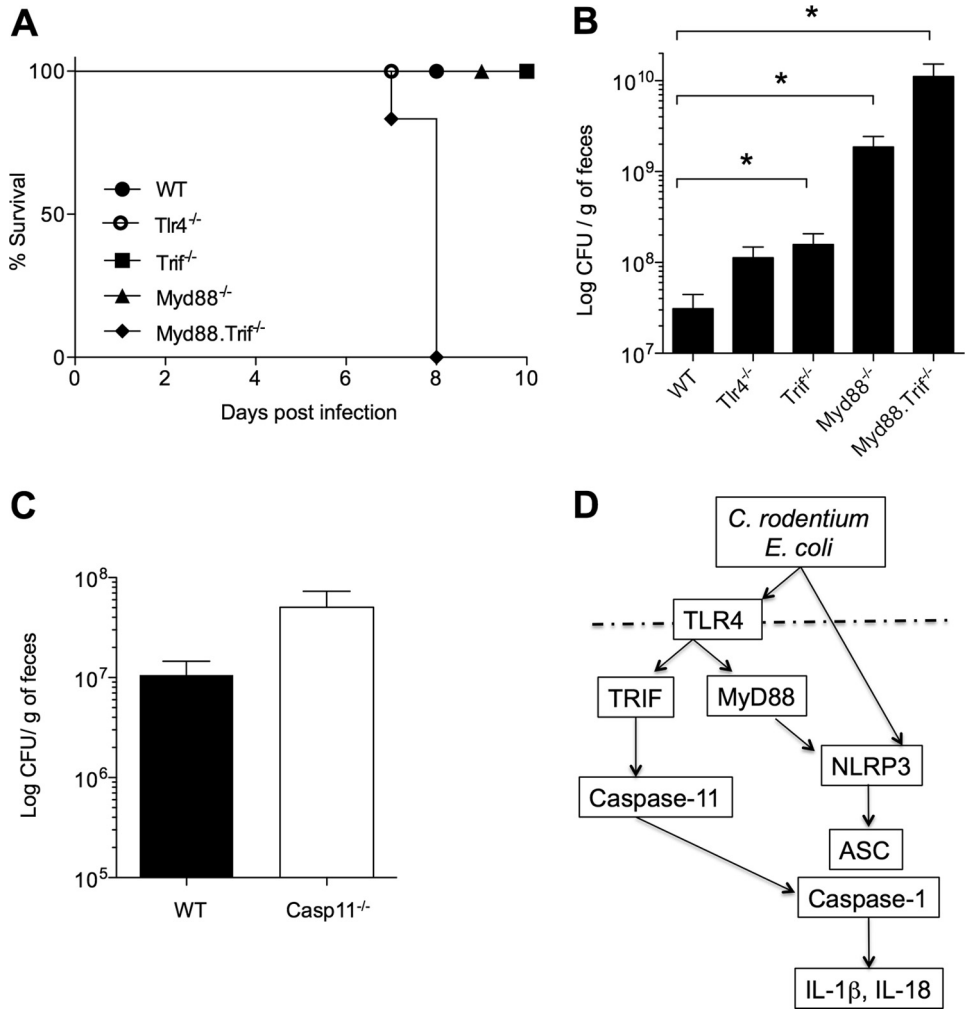

FIGURE 6. **Roles of TLR4-TRIF and caspase-11 during *C. rodentium* infection *in vivo*.** *A*, survival of WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice infected with 1×10^{10} *C. rodentium* by oral gavage. *B* and *C*, bacterial burden in fecal pellets of infected mice determined at day 7 after infection. *D*, working model for TLR4-/TRIF-mediated regulation of caspase-11 expression and noncanonical inflammasome activation in *C. rodentium*- and *E. coli*-infected macrophages.

redundant roles during *C. rodentium* infection and that deletion of both markedly affected host responses against enteropathogen infection. Although *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice showed no difference in survival compared with WT mice, bacterial counts in feces were increased, albeit only modestly in *Trif*$^{-/-}$ mice (Fig. 6*B*). As expected, bacterial burdens in *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice were more elevated than in animals lacking *MyD88* or *TRIF* alone (Fig. 6*B*), suggesting that these mice succumbed to infection consequent to uncontrollable bacterial replication. The response of *C. rodentium*-infected *caspase-11*$^{-/-}$ mice resembled that of *Trif*$^{-/-}$ mice in that they also had nearly normal (statistically not significant) bacterial numbers (Fig. 6*C*), and none of the infected *caspase-11*$^{-/-}$ mice succumbed to infection (data not shown). These results suggest that unlike in *in vitro*-infected macrophages, TRIF and caspase-11 play relatively modest roles during *C. rodentium* infection *in vivo*.

DISCUSSION

Kayagaki *et al.* (13) previously described a critical role for caspase-11 in Nlrp3 inflammasome activation and IL-1β secretion in macrophages infected with the enteropathogens *C. rodentium, E. coli*, and *V. cholerae*. We extended these results to an *in vivo* setting by demonstrating that the Nlrp3 inflammasome contributed importantly to host defense against *C. rodentium* because mice lacking *Nlrp3* or doubly deficient for the inflammasome proteases *caspase-1* and *-11* were hypersusceptible to *C. rodentium* infection (14). However, insight into the mechanisms controlling caspase-11-mediated inflammasome activation during infections with enteropathogens in infected macrophages and the relative importance of caspase-11 during *C. rodentium* infection *in vivo* was lacking.

Here, we provided genetic and biochemical evidence implicating TLR4 and TRIF in regulating expression of caspase-11 and activation of the Nlrp3 inflammasome in response to infection with enteropathogens such as *E. coli* and *C. rodentium*. Notably, our results revealed differential roles for MyD88 and TRIF in modulating enteropathogen-induced Nlrp3 inflammasome activation and IL-1β secretion, suggesting a mechanistic model in which these TLR adaptors integrate TLR and inflammasome responses through nonredundant mechanisms (Fig. 6*D*). Indeed, the TLR4-TRIF signaling axis was critical for

up-regulation of procaspase-11 and its processing during noncanonical activation of the Nlrp3 inflammasome by *C. rodentium* and *E. coli*. Caspase-11 activation may result from "spontaneous" autocatalytic processing in *cis* or *trans* when a certain concentration threshold has been surpassed (34). More likely, however, caspase-11 activation may require its recruitment in a multiprotein complex that induces proximity-induced oligomerization and activation-promoting conformational changes in caspase-11 zymogens, as described for activation of other initiator caspases (35, 36) In this model, autocatalytic maturation may function to subsequently stabilize activity of assembled caspase homo- and heterodimers.

Our results implicate TRIF signaling in regulating caspase-11 production and activation, but further analysis is required to clarify the molecular mechanism by which the TLR4-TRIF axis regulates caspase-11 expression. TRIF is known to regulate IRF3/7-mediated type I interferon production and has been implicated in delayed NF-κB activation (37, 38). Type I interferon signaling was recently confirmed to contribute to caspase-11 synthesis (39), but the role of delayed NF-κB signaling remains to be examined. Notably, the TLR4-TRIF signaling axis appears to be specifically required during enteropathogen infections, as TRIF and caspase-11 were dispensable for inflammasome activation by *S. typhimurium*. In contrast, MyD88 mediated transcriptional up-regulation of *NLRP3* and *proIL-1β*, but was dispensable for caspase-11 synthesis during *E. coli* and *C. rodentium* infection. Moreover, MyD88 was required for proIL-1β production in response to enteropathogens and other stimuli alike, including LPS+ATP and *S. typhimurium* infection. Interestingly, macrophages that were stimulated with LPS or infected with *S. typhimurium* both responded with inducing caspase-11 expression (data not shown), similar to cells infected with *C. rodentium* or *E. coli*. However, why caspase-11 is critical for inflammasome activation during *C. rodentium* and *E. coli* infections, but not in response to *Salmonella* or LPS+ATP treatment requires further investigation. Relative to *C. rodentium* and *E. coli*, LPS+ATP and *S. typhimurium* potently induce inflammasome activation in a short time frame (*i.e.* $<$3 h). One possibility may therefore be that relatively fast inflammasome activation may bypass the requirement for caspase-11, whereas stimuli that trigger inflammasome activation at later time points (as during *E. coli* and *C. rodentium* infection) may proceed through caspase-11. In support of this hypothesis is the observation that a mutant strain of *S. typhimurium* that lacks flagellin (*S. typhimurium* flib/c) fails to activate the Nlrc4 inflammasome in the first hours after infection, but instead required caspase-11 to activate the Nlrp3 inflammasome upon prolonged infection ($>$12 h) (data not shown). An alternative explanation for the differential requirement for caspase-11 is that *C. rodentium* and *E. coli* produce yet unknown molecules that trigger caspase-11 activation, which are absent during LPS+ATP stimulation and during the initial phases of *S. typhimurium* infection. Additional work is needed to dissect these mechanistic models further.

We previously demonstrated a critical role for the Nlrp3 inflammasome in host defense against *C. rodentium in vivo* (14). Similarly, *MyD88*-deficient mice are highly susceptible to *C. rodentium* infection (33). Notably, susceptibility increased further when both TRIF and MyD88 signaling were abrogated simultaneously. In addition to increased bacterial counts in the stool of *C. rodentium*-infected $Myd88^{-/-}Trif^{-/-}$ mice, this was reflected by a 100% mortality rate. Unlike $Myd88^{-/-}Trif^{-/-}$ mice, however, we found that $Trif^{-/-}$ mice displayed only slightly increased bacterial burdens in the stool without any associated morbidity or mortality. Also, $caspase\text{-}11^{-/-}$ mice presented with few signs of increased disease progression, suggesting that noncanonical inflammasome signaling plays a relatively minor role during *in vivo* infection with *C. rodentium*. Because Nlrp3 signaling is important for host defense against *C. rodentium in vivo* (14) and given that caspase-11 is critical for Nlrp3 inflammasome activation in *C. rodentium*-infected macrophages, this suggests that the role of noncanonical inflammasome signaling in *C. rodentium*-infected mice might be mitigated by canonical Nlrp3 activation in other cell types. Further analysis is required to examine the mechanism of enteropathogen-induced Nlrp3 inflammasome activation in additional cell types that may contribute to host responses *in vivo* such as epithelial cells, fibroblasts, and other hematopoietic cell types.

In conclusion, we showed here that TRIF and MyD88 signaling downstream of TLR4 differentially regulate Nlrp3 inflammasome activation and IL-1β secretion in macrophages infected with enteropathogens. The TLR4-TRIF axis regulated noncanonical inflammasome activation by promoting transcription induction of procaspase-11 expression and procaspase-11 processing, whereas the TLR4-MyD88 pathway controlled transcriptional up-regulation of Nlrp3 and proIL-1β. These results highlight the nonredundant roles of MyD88 and TRIF in integrating TLR- and inflammasome activation during enteropathogen infection.

Acknowledgments—We thank Anthony Coyle, Ethan Grant, John Bertin (Millennium Pharmaceuticals), Shizuo Akira (Osaka University), Vishva Dixit (Genentech), Gabriel Nuñez (University of Michigan), and Richard Flavell (Yale University) for a generous supply of mutant mice.

REFERENCES

1. Kaper, J. B., Nataro, J. P., and Mobley, H. L. (2004) Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* **2,** 123–140
2. DuPont, H. L. (2009) Clinical practice: bacterial diarrhea. *N. Engl. J. Med.* **361,** 1560–1569
3. Kappelman, M. D., Rifas-Shiman, S. L., Porter, C. Q., Ollendorf, D. A., Sandler, R. S., Galanko, J. A., and Finkelstein, J. A. (2008) Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. *Gastroenterology* **135,** 1907–1913
4. Takeuchi, O., and Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell* **140,** 805–820
5. Kawai, T., and Akira, S. (2006) TLR signaling. *Cell Death Differ.* **13,** 816–825
6. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282,** 2085–2088
7. Qureshi, S. T., Larivière, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (*Tlr4*) *J. Exp. Med.* **189,** 615–625

8. Roger, T., Froidevaux, C., Le Roy, D., Reymond, M. K., Chanson, A. L., Mauri, D., Burns, K., Riederer, B. M., Akira, S., and Calandra, T. (2009) Protection from lethal Gram-negative bacterial sepsis by targeting Toll-like receptor 4. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 2348–2352
9. Kanneganti, T. D., Lamkanfi, M., and Núñez, G. (2007) Intracellular NOD-like receptors in host defense and disease. *Immunity* **27,** 549–559
10. Lamkanfi, M., Walle, L. V., and Kanneganti, T. D. (2011) Deregulated inflammasome signaling in disease. *Immunol. Rev.* **243,** 163–173
11. Kanneganti, T. D., Body-Malapel, M., Amer, A., Park, J. H., Whitfield, J., Franchi, L., Taraporewala, Z. F., Miller, D., Patton, J. T., Inohara, N., and Núñez, G. (2006) Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* **281,** 36560–36568
12. Sander, L. E., Davis, M. J., Boekschoten, M. V., Amsen, D., Dascher, C. C., Ryffel, B., Swanson, J. A., Müller, M., and Blander, J. M. (2011) Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* **474,** 385–389
13. Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., Zhang, J., Lee, W. P., Roose-Girma, M., and Dixit, V. M. (2011) Non-canonical inflammasome activation targets caspase-11. *Nature* **479,** 117–121
14. Liu, Z., Zaki, M. H., Vogel, P., Gurung, P., Finlay, B. B., Deng, W., Lamkanfi, M., and Kanneganti, T. D. (2012) Role of inflammasomes in host defense against *Citrobacter rodentium* infection. *J. Biol. Chem.* **287,** 16955–16964
15. Takeuchi, O., Hoshino, K., and Akira, S. (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* **165,** 5392–5396
16. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* **162,** 3749–3752
17. Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002) Small antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* **3,** 196–200
18. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9,** 143–150
19. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301,** 640–643
20. Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T. D., Ozören, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., and Núñez, G. (2006) Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in salmonella-infected macrophages. *Nat. Immunol.* **7,** 576–582
21. Jones, J. W., Kayagaki, N., Broz, P., Henry, T., Newton, K., O'Rourke, K., Chan, S., Dong, J., Qu, Y., Roose-Girma, M., Dixit, V. M., and Monack, D. M. (2010) Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis*. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 9771–9776
22. Kanneganti, T. D., Lamkanfi, M., Kim, Y. G., Chen, G., Park, J. H., Franchi, L., Vandenabeele, P., and Núñez, G. (2007) Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* **26,** 433–443
23. Kanneganti, T. D., Ozören, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., Akira, S., and Núñez, G. (2006) Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* **440,** 233–236
24. Kobayashi, K., Inohara, N., Hernandez, L. D., Galán, J. E., Núñez, G., Janeway, C. A., Medzhitov, R., and Flavell, R. A. (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* **416,** 194–199
25. Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nuñez, G., and Flavell, R. A. (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* **307,** 731–734
26. Lamkanfi, M., Malireddi, R. K., and Kanneganti, T. D. (2009) Fungal zymosan and mannan activate the cryopyrin inflammasome. *J. Biol. Chem.* **284,** 20574–20581
27. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Su, Q., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nuñez, G., and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* **4,** 702–707
28. Anand, P. K., Malireddi, R. K., Lukens, J. R., Vogel, P., Bertin, J., Lamkanfi, M., and Kanneganti, T. D. (2012) NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature* **488,** 389–393
29. Zaki, M. H., Vogel, P., Malireddi, R. K., Body-Malapel, M., Anand, P. K., Bertin, J., Green, D. R., Lamkanfi, M., and Kanneganti, T. D. (2011) The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* **20,** 649–660
30. Malireddi, R. K., Ippagunta, S., Lamkanfi, M., and Kanneganti, T. D. (2010) Cutting edge: proteolytic inactivation of poly(ADP-ribose) polymerase 1 by the Nlrp3 and Nlrc4 inflammasomes. *J. Immunol.* **185,** 3127–3130
31. Mundy, R., MacDonald, T. T., Dougan, G., Frankel, G., and Wiles, S. (2005) *Citrobacter rodentium* of mice and man. *Cell. Microbiol.* **7,** 1697–1706
32. Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgerald, K. A., Hornung, V., and Latz, E. (2009) Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* **183,** 787–791
33. Gibson, D. L., Ma, C., Bergstrom, K. S., Huang, J. T., Man, C., and Vallance, B. A. (2008) MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during *Citrobacter rodentium*-induced colitis. *Cell. Microbiol.* **10,** 618–631
34. Kang, S. J., Wang, S., Kuida, K., and Yuan, J. (2002) Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. *Cell Death Differ.* **9,** 1115–1125
35. Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., Ricci, J. E., Edris, W. A., Sutherlin, D. P., Green, D. R., and Salvesen, G. S. (2003) A unified model for apical caspase activation. *Mol. Cell* **11,** 529–541
36. Salvesen, G. S., and Dixit, V. M. (1999) Caspase activation: the induced-proximity model. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 10964–10967
37. Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Mühlradt, P. F., Sato, S., Hoshino, K., and Akira, S. (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **167,** 5887–5894
38. Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* **4,** 499–511
39. Rathinam, V. A., Vanaja, S. K., Waggoner, L., Sokolovska, A., Becker, C., Stuart, L. M., Leong, J. M., and Fitzgerald, K. A. (2012) TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by Gram-negative bacteria. *Cell* **150,** 606–619