

Role of Inflammasomes in Host Defense against *Citrobacter rodentium* Infection^{*[5]}

Received for publication, March 5, 2012. Published, JBC Papers in Press, March 29, 2012, DOI 10.1074/jbc.M112.358705

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Background: *Citrobacter rodentium* is an enteric bacterial pathogen of mouse intestinal tract.

Results: Mice lacking Nlrp3, Nlrc4, and caspase-1 are hypersusceptible to *C. rodentium*-induced gastrointestinal inflammation.

Conclusion: The Nlrp3 and Nlrc4 inflammasomes play a critical role in host defense against enteric infection caused by *C. rodentium*.

Significance: Our study establishes a critical role of inflammasomes in host defense against *Citrobacter rodentium* infection.

Citrobacter rodentium is an enteric bacterial pathogen of the mouse intestinal tract that triggers inflammatory responses resembling those of humans infected with enteropathogenic and enterohemorrhagic *Escherichia coli*. Inflammasome signaling is emerging as a central regulator of inflammatory and host responses to several pathogens, but the *in vivo* role of inflammasome signaling in host defense against *C. rodentium* has not been characterized. Here, we show that mice lacking the inflammasome components Nlrp3, Nlrc4, and caspase-1 were hypersusceptible to *C. rodentium*-induced gastrointestinal inflammation. This was due to defective interleukin (IL)-1 β and IL-18 production given that il-1 β ^{-/-} and il-18^{-/-} mice also suffered from increased bacterial burdens and exacerbated histopathology. *C. rodentium* specifically activated the Nlrp3 inflammasome in *in vitro*-infected macrophages independently of a functional bacterial type III secretion system. Thus, production of IL-1 β and IL-18 downstream of the Nlrp3 and Nlrc4 inflammasomes plays a critical role in host defense against enteric infections caused by *C. rodentium*.

Bacterial infections of the gastrointestinal tract represent a major cause of mortality worldwide and continue to threaten global health (1). Enteric bacterial pathogens such as enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli* are responsible for a large number of cases of diarrhea, which cause significant morbidity and mortality among infants and children in the developing world (2). Much of our knowledge on the

mechanisms by which the host immune system controls infection by enteric pathogens stems from experimental studies with *Citrobacter rodentium*, a naturally occurring pathogen that infects the rodent gastrointestinal tract (3). As with enterohemorrhagic and enteropathogenic *E. coli* in humans, *C. rodentium* invasion of murine colonic epithelial cells results in the formation of lesions and mucosal hyperplasia (4).

Both innate immune cells and T cells, along with *C. rodentium*-specific antibody responses, contribute to containment and eradication of *C. rodentium* infection (5). At the molecular level, extracellular immune surveillance by members of the Toll-like receptor family is known to play a major role in host defense against *C. rodentium* infection (6–8). In addition, the role of the intracellular NOD-like receptor family members NOD1 and NOD2 in protection against *C. rodentium* infection has recently been characterized. NOD1 and NOD2 promote clearance of *C. rodentium* infection by inducing enteric innate T helper type 17 (iT(H)17) and T(H)1 responses in the gastrointestinal tract (9, 10). Unlike Nod1 and Nod2, which regulate MAP kinase and NF- κ B signaling, a subset of NOD-like receptors that includes Nlrp3 and Nlrc4 controls innate immune responses by assembling inflammasomes. These multiprotein complexes are responsible for the activation of caspase-1 and subsequent production of bioactive interleukin IL-1 β and IL-18 (11). Notably, *C. rodentium* was recently shown to induce non-canonical activation of the Nlrp3 inflammasome in *in vitro*-infected macrophages (12). Here, we analyzed inflammasome activation during *in vivo* infection with *C. rodentium* and characterized the physiological role of inflammasome signaling in host defense against *C. rodentium*. Mice lacking the inflammasome components Nlrp3, Nlrc4, and caspase-1 were hypersusceptible to *C. rodentium*-induced gastrointestinal inflammation and failed to control *C. rodentium* as a consequence of defective production of IL-1 β and IL-18. Concurrently, il-1 β ^{-/-} and il-18^{-/-} mice suffered from increased bacterial burden and presented with more inflammatory lesions in the gut. Notably, *C. rodentium* specifically required Nlrp3, but not Nlrc4 or Aim2, to induce activation of caspase-1 and secretion

* This work was supported, in whole or in part, by National Institutes of Health Grant R01AR056296. This work was also supported by the American Lebanese Syrian Associated Charities (ALSAC) (to T.-D.K.).

[5] This article contains supplemental Figs. S1 and S2.

¹ These authors contributed equally to this work.

² Supported by a Gephardt fellowship.

³ Supported by Fund for Scientific Research-Flanders Grants 1.5.122.11N and 1.2.201.10N and European Union Marie-Curie Grant 256432.

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of IL-1 β from *in vitro*-infected macrophages. Moreover, the bacterial type III secretion system was dispensable for Nlrp3 inflammasome activation. In conclusion, we show that Nlrp3 and Nlrc4 inflammasome-mediated IL-1 β and IL-18 production is required for efficiently clearing *C. rodentium* infections in the gastrointestinal tract.

EXPERIMENTAL PROCEDURES

Mice—Nlrp3^{-/-} (13), Casp1^{-/-} (14), Nlrc4^{-/-} (15), Aim2^{-/-} (16), and il-1 β ^{-/-} (17) mice were all described before. il-18^{-/-} mice were purchased from Jackson Laboratory. Mice were housed in a pathogen-free facility, and all animal procedures followed protocols approved by the St. Jude Children's Research Hospital Committee on Use and Care of Animals.

Citrobacter Infection—*C. rodentium* (ATCC 51459) was grown in LB broth overnight with shaking at 37 °C. For *in vivo* experiments, mice were infected with 4 × 10⁹ cfu 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 or colon tissues were plated on MacConkey agar plates and incubated at 37 °C for 24 h.

For *in vitro* experiments, bone marrow-derived macrophages (BMDMs)⁵ were prepared as described before (18). Cells were stimulated with or without LPS (500 ng/ml) for 5 h and then infected at 20 multiplicity of infection WT and/or type III secretion system mutants *C. rodentium* (Δ escC and Δ escN) for 1 h. Then gentamycin (50 μ g/ml) was added to kill extracellular bacteria. After that, cells were cultured in CO₂ incubator at 37 °C for 19 h. The cell culture supernatants were collected for cytokine analysis. The cells were lysed for further Western blotting analysis.

Histological Analysis—Colons were fixed in 10% formalin and embedded in paraffin. Serial 5- μ m sections were cut and stained with hematoxylin and eosin (H&E). Bacteria in colon mucosa were stained by the Gram staining method. For immunostaining of macrophages and neutrophils, sections were immunostained with anti-F4/80 and anti-Gr-1 antibodies, respectively. Histological scores were evaluated blindly by a pathologist (Dr. Peter Vogel) at St. Jude Research Hospital. The parameters used for histological scoring included inflammation, edema, hyperplasia, and the extent of colonic damage.

Cytokine Analysis—Colon homogenates were prepared mechanically in PBS containing 1% Nonidet P-40 and complete protease inhibitor mixture (Roche Applied Science). The concentrations of mouse cytokines and chemokines in colon homogenates or cell supernatants were determined using multiplex ELISA (Millipore), IL-1 β ELISA kit (eBioscience), or IL-18 ELISA kit (MBL International).

Serum Antibody ELISA—Serum antibody analysis methods were modified as described previously (19). Briefly, heated-killed *C. rodentium* were coated in ELISA plates overnight. After wash, plates were blocked with 5% milk for 1 h. Serum

samples were assayed in triplicate and incubated for 2 h at room temperature. Then Ig isotypes were detected with peroxidase-conjugated goat-anti-mouse IgG and IgM (Jackson ImmunoResearch). After that, plates were developed with 3,3', 5,5'-tetramethylbenzidine (TMB) and read at A₄₅₀ after stopping reaction with 1 N H₂SO₄.

Flow Cytometry Analysis—Single cell suspensions were made from mouse spleen. Cells were stained with anti-CD11b (M1/70), CD11c (N418), Gr-1 (RB6-8C5), CD4 (RM4-5), and CD19 (6D5) (all from Biolegend) and CD8 (53-6.7) (from eBioscience). After staining, cells were analyzed on a FACSCalibur (BD Biosciences).

Western Blotting—For analysis of caspase-1 activation, tissue homogenate or BMDMs 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 rabbit antibody against caspase-1.

Statistics—Data are represented as mean \pm S.E. or S.D. Statistical significance was determined by Student's *t* test; *p* < 0.05 was considered statistically significant.

RESULTS

Deficiency in Nlrp3, Nlrc4, and Caspase-1 Increases Host Susceptibility to *C. rodentium* Infection—To examine the *in vivo* importance of inflammasome signaling in intestinal inflammation caused by *C. rodentium* infection, wild-type, Nlrp3^{-/-}, and Casp1^{-/-} mice were infected with *C. rodentium* at 4 × 10⁹ cfu/mouse. Clinical manifestation of colitis was monitored during a course of 3 weeks by monitoring changes in body weight and bacterial burden in the stool. Casp1^{-/-} mice were highly susceptible to *C. rodentium* infection as they lost significantly more body weight than wild-type mice from day 4 onward (Fig. 1A). Nlrp3^{-/-} mice initially presented with a milder phenotype that gradually worsened in the course of the 3rd week (Fig. 1A). To further understand the roles of inflammasome signaling in *C. rodentium* infection, cfu in feces of Nlrp3^{-/-} and Casp1^{-/-} mice were determined at days 10, 17, and 21 after infection. In agreement with the differential changes in body weight at day 10 after infection, *C. rodentium* counts were initially comparably elevated in wild-type and Nlrp3^{-/-} mice (~10⁷ cfu/g feces) (Fig. 1B). In contrast, cfu in Casp1^{-/-} mice were significantly higher at this time point (~10⁸ cfu/g feces). At day 17, bacterial burden in wild-type mice markedly dropped to 10³–10⁴ cfu/g feces, whereas cfu remained elevated in Nlrp3^{-/-} and Casp1^{-/-} mice (10⁶–10⁹ cfu/g feces). This suggests that wild-type mice successfully started clearing the *C. rodentium* infection at this time point, whereas defective inflammasome signaling in Nlrp3^{-/-} and Casp1^{-/-} mice hampered this process. To obtain additional evidence of increased susceptibility to *C. rodentium*-induced colitis in inflammasome-deficient mice, we examined the gross anatomy and histology of the colon at days 14 and 21 after infection. Shortening and thickening of the colon are hallmark features of *C. rodentium*-induced colitis (4). Consistent with the increased body weight loss and higher bacterial burden in inflammasome-deficient mice, colons of Casp1^{-/-} mice were significantly shorter and thicker than those of wild-type mice (Fig. 1, C and D). A tendency toward slightly shorter colons was observed in Nlrp3^{-/-} mice,

⁵ The abbreviations used are: BMDM, bone marrow-derived macrophage; T3SS, type 3 secretion system; MIP2, macrophage inflammatory protein 2; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1.

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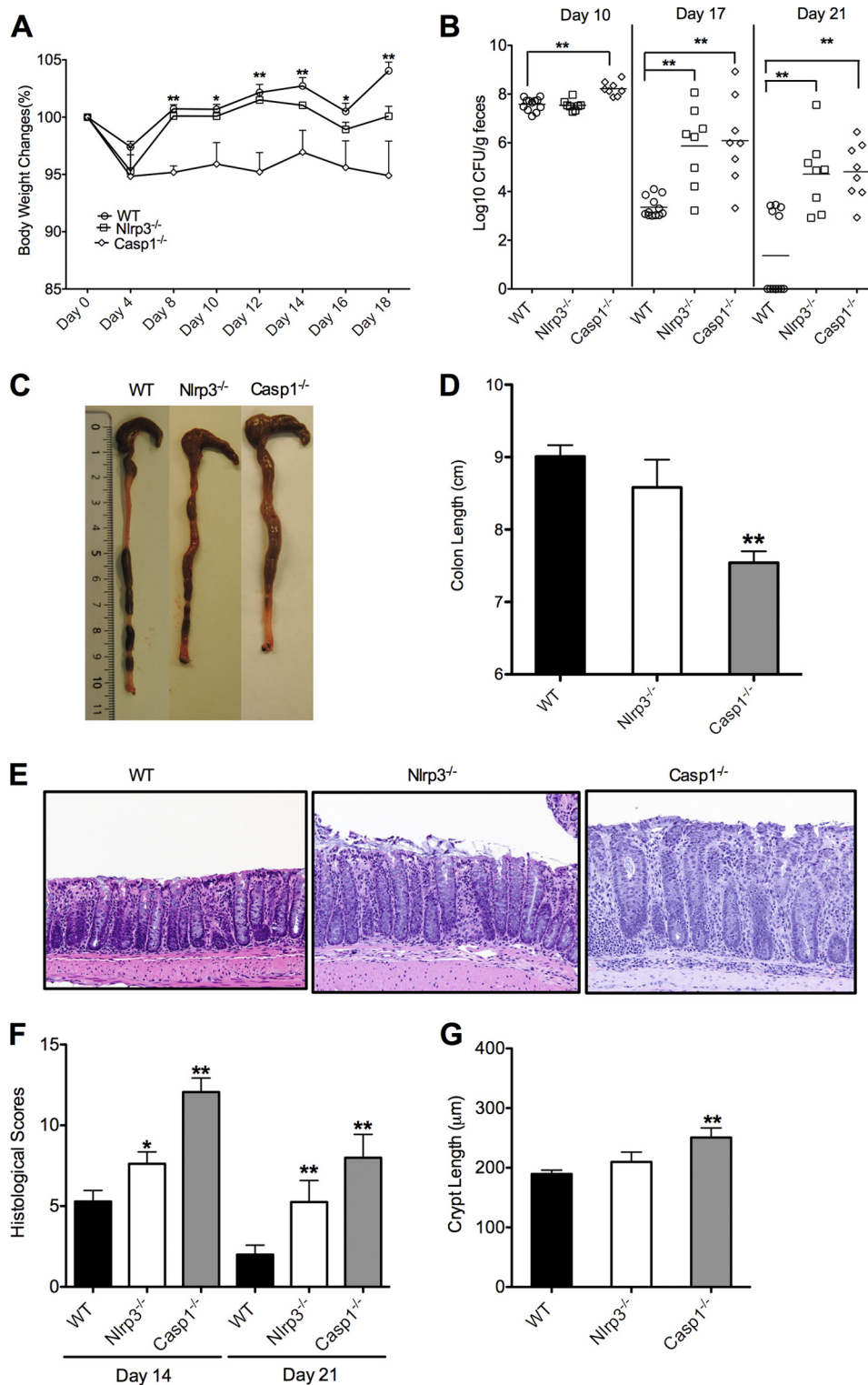


FIGURE 1. Deficiency in inflammasome components increases host susceptibility to *C. rodentium* infection. WT, Nlrp3^{-/-}, and Casp1^{-/-} mice were orally infected with *C. rodentium* at a dose of 4×10^9 cfu/mouse. **A**, body weight changes monitored until day 18. *, $p < 0.05$; **, $p < 0.01$ WT versus Casp1^{-/-} mice. **B**, *C. rodentium* load in feces measured by colony assay at days 10, 17, and 21. **C**, *C. rodentium*-infected mice were killed at days 14 and 21 to examine colons macroscopically. **C**, representative gross images of colons from *C. rodentium*-infected WT, Nlrp3^{-/-}, and Casp1^{-/-} mice at day 21. **D**, colon length at day 21. **E**, representative photomicrographs of H&E-stained colon sections at day 21. **F**, histological lesion scores at days 14 and 21. **G**, crypt length at day 21. *, $p < 0.05$; **, $p < 0.01$ versus WT. Data represent means \pm S.E. (error bars) (WT, $n = 12$; Nlrp3^{-/-}, $n = 8$; Casp1^{-/-}, $n = 8$) from three independent experiments.

although these differences failed to reach statistical significance (Fig. 1D). The delayed onset of body weight loss and increases in bacterial burden in Nlrp3^{-/-} mice (Fig. 1, A and B) might be explained by redundant mechanisms contributing to caspase-1

activation in response to infection with *C. rodentium*. Indeed, Nlrp3^{-/-} mice that were infected with *C. rodentium* as described above resembled Nlrp3^{-/-} mice in that *C. rodentium* counts in the stool were normal at day 10, but markedly

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increased at days 17 and 21 after infection (supplemental Fig. S1).

The clinical manifestation of increased colon inflammation in *Nlrp3*^{-/-} and *Casp1*^{-/-} mice was confirmed by histological analysis. H&E-stained colon sections of *C. rodentium*-infected *Nlrp3*^{-/-} and *Casp1*^{-/-} mice showed increased inflammation in the lamina propria along with elongated crypts and hyperplasia in inflammasome-deficient mice (Fig. 1E). Semiquantitative scoring of inflammation, edema, hyperplasia, and the extent of colonic damage confirmed that colitis severity was significantly higher in the *Nlrp3*^{-/-} and *Casp1*^{-/-} cohorts relative to the levels seen in wild-type mice (Fig. 1, F and G).

Increased Inflammatory Cytokine Production and Immune Invasion in Colons of *C. rodentium*-infected *Casp1*^{-/-} Mice—Cytokines and chemokines play central roles in shaping inflammatory and host defense responses against microbial pathogens. To gain a better understanding of the mechanisms contributing to increased colitis in the absence of a functional inflammasome, we measured the local production of a variety of inflammatory cytokines and chemokines in *C. rodentium*-infected wild-type and *Casp1*^{-/-} mice. Compared with wild-type mice, colon homogenates of *Casp1*^{-/-} mice contained markedly higher levels of proinflammatory cytokines and chemokines such as IL-6, MIP2, KC, and MCP-1 at both days 14 (Fig. 2A) and 21 (Fig. 2B) after infection. These results suggest that defective inflammasome activation leads to increased local production of proinflammatory cytokines and chemokines in the gastrointestinal tract of *C. rodentium*-infected animals.

To characterize the cell types contributing to production of these inflammatory mediators, we analyzed the immune cell populations migrating to the colon and secondary lymphoid tissues of *C. rodentium*-infected wild-type and *Casp1*^{-/-} mice. Immunohistochemical analysis of micrographs stained for F4/80 and Gr-1 showed a markedly increased infiltration of macrophages and neutrophils in colons of *Casp1*^{-/-} mice at day 21 after infection (Fig. 3A). This was associated with splenomegaly in *Casp1*^{-/-} mice as evidenced by the doubled splenic size and weight (supplemental Fig. S2, A and B) compared with wild-type mice at day 14 after infection. Analysis of splenic immune cell populations showed that both the total myeloid cell (CD11b⁺) population as well as the subpopulations of neutrophils (CD11b⁺Gr1⁺) and dendritic cells (CD11b⁺CD11c⁺) were significantly elevated in *Casp1*^{-/-} mice at day 14 (Fig. 3B). The elevated proinflammatory cytokine production and immune cell infiltration in *C. rodentium*-infected *Casp1*^{-/-} colons were accompanied by a markedly increased bacterial colonization of the gastrointestinal tract (Fig. 3C). *In situ* Gram staining of wild-type and *Casp1*^{-/-} colon sections confirmed an augmented bacterial presence in the mucosal tissue of *Casp1*^{-/-} colons relative to wild-type colons at day 14 after *C. rodentium* infection (Fig. 3D), suggesting that the increased inflammatory responses in *Casp1*^{-/-} colons may be due to the uncontrolled proliferation of *C. rodentium* in the absence of a functional inflammasome.

Increased Lymphocyte Infiltration and Humoral Immunity in *C. rodentium*-infected *Casp1*^{-/-} Mice—T cell and humoral responses have previously been implicated in host defense against *C. rodentium* (19, 20). To investigate whether caspase-1

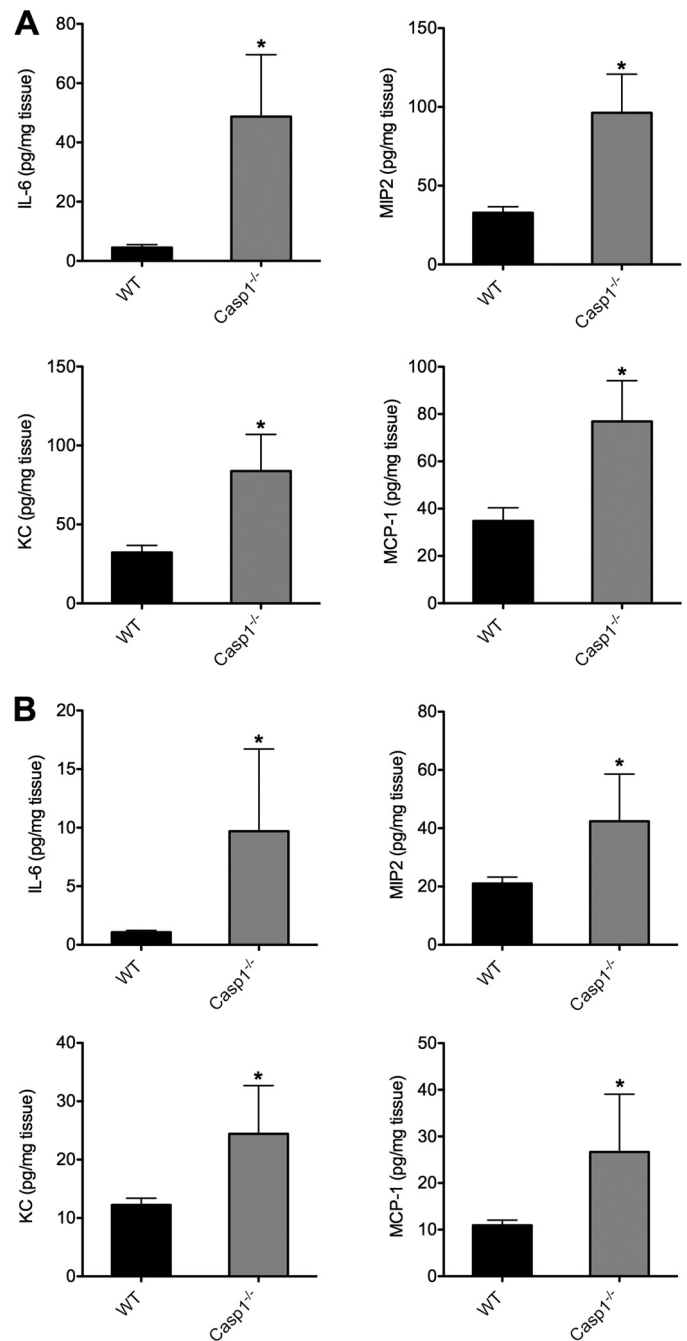


FIGURE 2. Defects in inflammasome activation are associated with increased inflammatory responses in the colon. WT and *Casp1*^{-/-} mice were orally infected with *C. rodentium* at a dose of 4×10^9 cfu/mouse. The infected mice were killed at days 14 and 21 to examine colons. At days 14 and 21 after infection, proteins were extracted from mouse colons, and the amounts of proinflammatory cytokines were measured. A, IL-6, MIP2, KC, and MCP-1 at 14 days after infection. B, IL-6, MIP2, KC, and MCP-1 at 21 days after infection. *, $p < 0.05$ versus WT. Data represent means \pm S.E. (error bars) (WT, $n = 12$; *Casp1*^{-/-}, $n = 8$) from two independent experiments.

deficiency affects *C. rodentium*-induced adaptive immune responses, we first determined CD4⁺, CD8⁺, and CD19⁺ cell numbers in the spleen of infected wild-type and *Casp1*^{-/-} mice at days 14 and 21 after infection. Whereas T and B lymphocyte numbers in the spleens were comparable in the two genotypes at early stages of infection (days 0 and 14), *Casp1*^{-/-} mice had significantly more CD8⁺ and CD19⁺ cells in the spleen at day

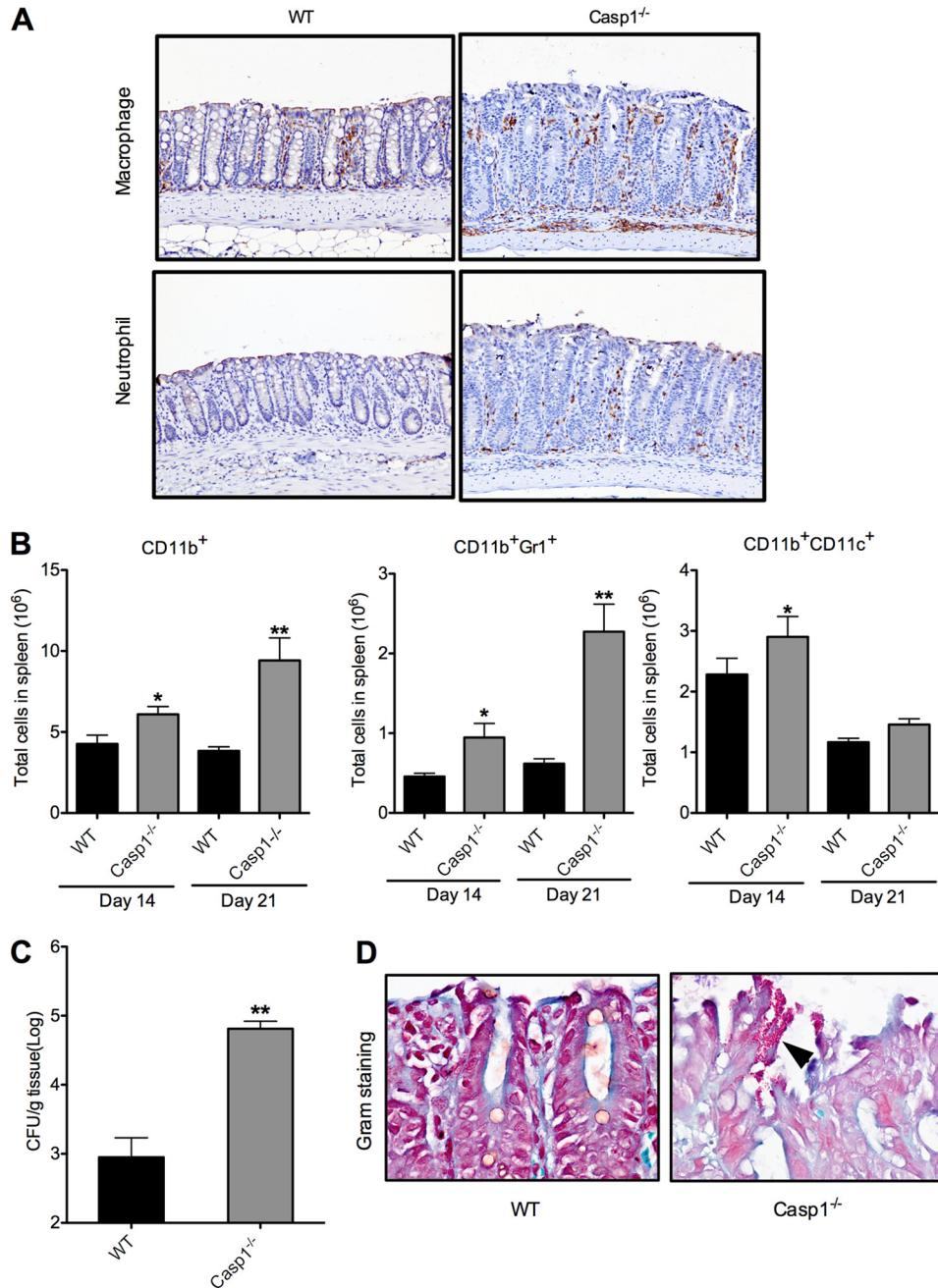


FIGURE 3. Increased inflammatory cell infiltration correlates with high colonic bacterial burdens in inflammasome-deficient mice after *C. rodentium* infection. WT and Casp1^{-/-} mice were orally infected with *C. rodentium* at a dose of 4×10^9 cfu/mouse. At days 14 and 21, mice were killed. *A*, photomicrographs of macrophage (F4/80) and neutrophil (Gr-1) immunostained colon sections collected at day 21 after *C. rodentium* infection. *B*, spleens collected at day 14 (WT, $n = 8$; Casp1^{-/-}, $n = 7$) and day 21 (WT, $n = 10$; Casp1^{-/-}, $n = 6$). Splenocytes were analyzed for myeloid cells (CD11b⁺), neutrophils (CD11b⁺Gr1⁺), and dendritic cells (CD11b⁺CD11c⁺) by flow cytometry. *, $p < 0.05$; **, $p < 0.01$ versus WT. Data represent means \pm S.E. (error bars) from two independent experiments. *C*, *C. rodentium* burdens in colon tissues of WT ($n = 8$) and Casp1^{-/-} ($n = 8$) mice at 21 days after infection. *, $p < 0.05$; **, $p < 0.01$ versus WT. Data represent means \pm S.E. of two independent experiments. *D*, representative photomicrographs of bacterial Gram staining of colons from infected WT and Casp1^{-/-} mice at day 14. Arrowheads point to Gram-negative bacteria colonies on colon mucosa.

21 after infection (Fig. 4, A–C), suggesting that defective inflammasome activation is associated with enhanced adaptive immune responses. Concurrently, serum IgG and IgM levels were also higher in *C. rodentium*-infected Casp1^{-/-} mice than the levels detected in circulation of wild-type mice (Fig. 4, D and E).

IL-1 β and IL-18 Production Downstream of Inflammasomes Is Critical for Controlling *C. rodentium* Infection—To confirm that the host responds to infection with *C. rodentium* by acti-

vating inflammasomes in the gastrointestinal tract, we monitored caspase-1 maturation by Western blotting in colon samples of wild-type (C57BL/6) mice that had been infected orally with *C. rodentium* for 14 days. As expected, procaspase-1 was matured into a 20-kDa fragment in colon samples of *C. rodentium*-infected wild-type mice, but not in colon samples of uninfected animals (Fig. 5A). The antibody failed to detect immunoreactive bands in lysates of Casp1^{-/-} mice, demonstrating the specificity of these results (data not shown).

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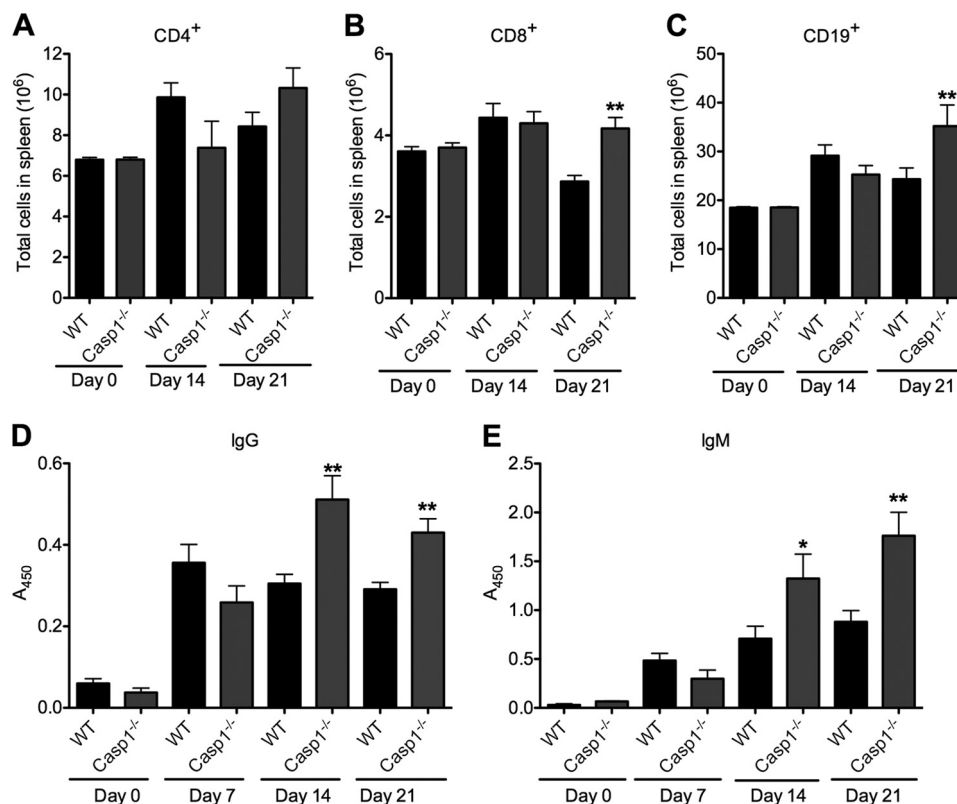


FIGURE 4. Increased adaptive immune responses in caspase-1-deficient mice infected with *C. rodentium*. A–C, WT and Casp1^{-/-} mice were orally infected with *C. rodentium* at a dose of 4×10^9 cfu/mouse. Mice were killed at days 0 (WT, $n = 3$; Casp1^{-/-}, $n = 3$), 7 (WT, $n = 10$; Casp1^{-/-}, $n = 7$), 14 (WT, $n = 8$; Casp1^{-/-}, $n = 7$), and 21 (WT, $n = 10$; Casp1^{-/-}, $n = 6$). Splenic lymphoid cells were analyzed by flow cytometry in splenic CD4⁺ cells (A), splenic CD8⁺ cells (B), and splenic CD19⁺ cells (C). D and E, serum IgG and IgM production were measured by ELISA. *, $p < 0.05$; **, $p < 0.01$ versus WT. Data represent means \pm S.E. (error bars) from two independent experiments.

Inflammasomes are responsible for the proteolytic maturation and secretion of bioactive IL-1 β and IL-18 (11). To examine whether the increased susceptibility and elevated cytokine production in Casp1^{-/-} mice could be attributed to defective production of IL-1 β and IL-18, we measured colonic levels of these inflammasome-dependent cytokines. Wild-type mice produced high levels of IL-18 at day 14 after infection (Fig. 5B), whereas IL-1 β production in the colon was not easily detected (data not shown). Unlike wild-type mice, IL-18 levels were markedly reduced in colons of *C. rodentium*-infected Casp1^{-/-} mice, whereas Nlrp3^{-/-} mice had intermediate levels of IL-18 (Fig. 5B), suggesting a critical role for this cytokine in regulating susceptibility to *C. rodentium*.

To examine the role of IL-18 in additional detail, wild-type and il-18^{-/-} mice were infected with *C. rodentium* to determine bacterial burden and histological lesions as described above. At day 14 after infection, *C. rodentium* counts and histological lesions were significantly higher in il-18^{-/-} mice than in wild-type mice (Fig. 5, C and D), confirming the role of IL-18 in host defense against *C. rodentium*. We next examined the potential role of IL-1 β in *C. rodentium* infection. Similar to il-18^{-/-} mice, mice lacking IL-1 β had significantly higher *C. rodentium* cfu in the stool (Fig. 5E) and markedly more colonic histological lesions than the control group (Fig. 5F). Analysis of H&E-stained colon sections of *C. rodentium*-infected WT, il-1 β ^{-/-}, and il-18^{-/-} mice confirmed that mice lacking either of the inflammasome-dependent cytokines displayed increased his-

topathology and hyperplasia (Fig. 5G). These data support the notion that both IL-1 β and IL-18 production downstream of inflammasomes regulates replication of *C. rodentium* in the colon.

C. rodentium Type III Secretion System Is Dispensable for Nlrp3 Inflammasome Activation in Macrophages—*C. rodentium* infection of BMDMs was recently shown to induce non-canonical caspase-1 activation downstream of Nlrp3 and caspase-11 (12). In agreement, *C. rodentium* induced caspase-1 maturation in wild-type BMDMs, but not in macrophages lacking Nlrp3 (Fig. 6A). In contrast, the Nlr4 and AIM2 inflammasomes were dispensable for *C. rodentium*-induced caspase-1 activation because caspase-1 processing was not affected in Nlr4^{-/-} and Aim2^{-/-} macrophages (Fig. 6A). Concurrently, significant production of IL-1 β (Fig. 6, B and C) and IL-18 (Fig. 6, D and E) was observed in wild-type, Nlr4^{-/-}, and Aim2^{-/-} macrophages, but not in Nlrp3^{-/-} and Casp1^{-/-} BMDMs. Together, these results suggest that *C. rodentium* activates caspase-1 specifically through the Nlrp3 inflammasome in infected macrophages.

Effector proteins secreted through type III secretion systems (T3SS) are critical for bacterial virulence. For instance, *C. rodentium* T3SS mediates translocation of multiple effector proteins that are required for bacterial virulence in mice (21, 22). To examine whether *C. rodentium* T3SS is implicated in inflammasome activation, BMDMs were infected with wild-type *C. rodentium* or the Δ escC and Δ escN mutants lacking essential structural components that render the T3SS inactive (22). Notably, BMDMs infected with wild-type *C. rodentium*

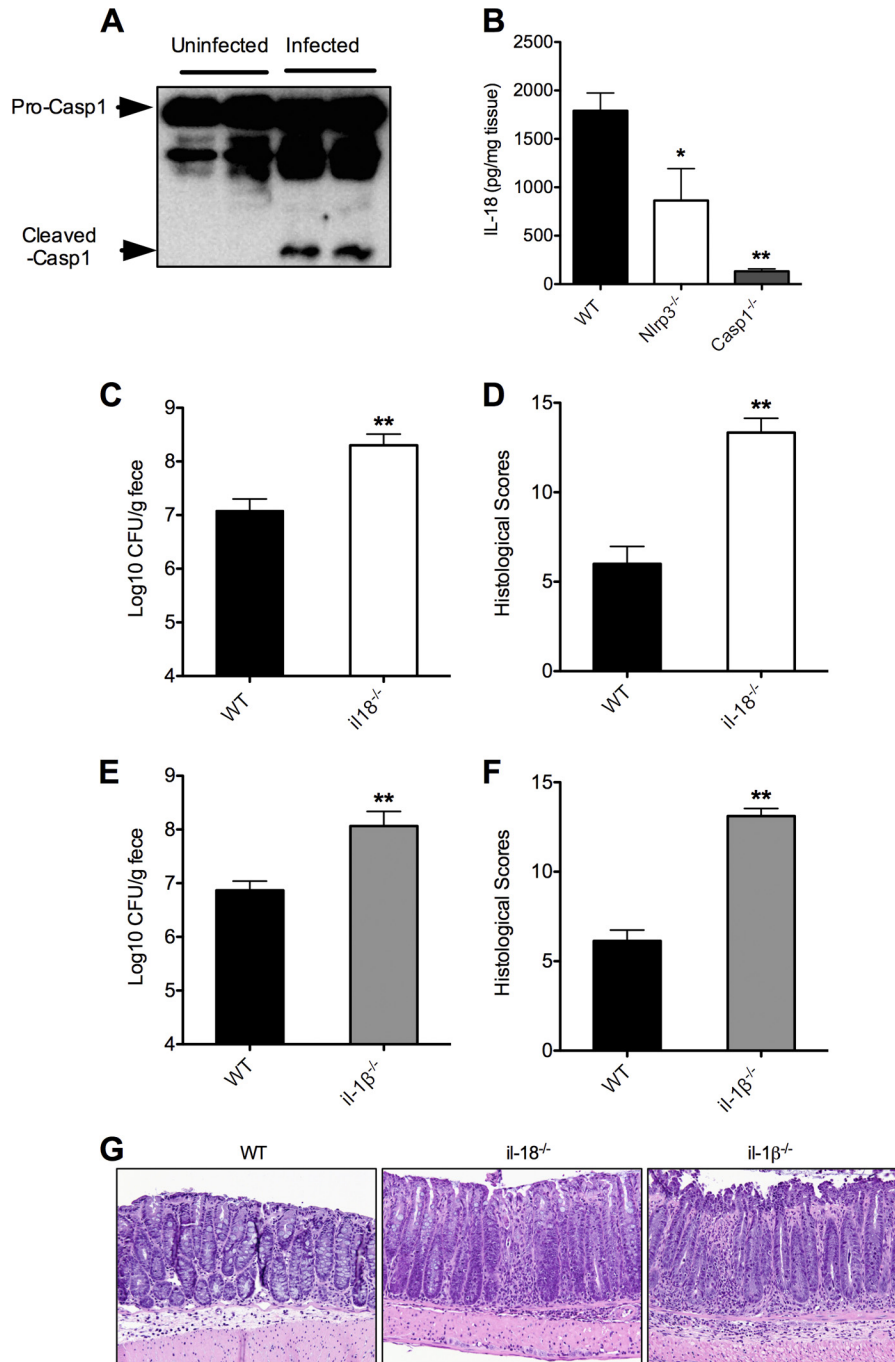


FIGURE 5. IL-1 β and IL-18 production in the downstream of inflammasomes is critical for controlling *C. rodentium* infection. A and B, WT, Nlrp3^{-/-}, and Casp1^{-/-} mice were infected with *C. rodentium* for 14 days. Proteins were extracted from colon. Caspase-1 activation was determined by Western blotting (A), and colonic IL-18 levels were measured by ELISA (B). Data represent means \pm S.E. (error bars) (WT, $n = 8$; Nlrp3^{-/-}, $n = 5$; Casp1^{-/-}, $n = 7$) from two independent experiments. *, $p < 0.05$; **, $p < 0.01$ versus WT. C and D, WT ($n = 10$) and il-18^{-/-} ($n = 7$) mice were infected with *C. rodentium*. *C. rodentium* counts (cfu) in stool (C) and colonic histology scores (D) were determined at day 14 after infection. Data represent means \pm S.E. (error bars) from two independent experiments. **, $p < 0.01$ versus WT. E and F, WT ($n = 10$) and il-1 β ^{-/-} ($n = 9$) mice were infected with *C. rodentium*. *C. rodentium* counts (cfu) in stool (E) and colonic histology scores (F) were determined at day 14 after infection. Data represent means \pm S.E. (error bars) from two independent experiments. **, $p < 0.01$ versus WT. G, representative photomicrographs of H&E-stained colon sections of *C. rodentium*-infected WT, il-18^{-/-}, and il-1 β ^{-/-} mice at day 14.

secreted levels of IL-1 β (Fig. 7, A and B) and IL-18 (Fig. 7 C, and D) in the culture supernatants similar to those infected with the Δ escC and Δ escN mutants. In agreement, caspase-1 processing in BMDMs infected with the mutant bacterial strains was similar to the levels seen in BMDMs infected with wild-type *C. rodentium* (Fig. 7E). These results suggest that *C. rodentium* activates the Nlrp3 inflammasome independently of T3SS effectors, raising the

possibility that the Nlrp3 inflammasome may detect *C. rodentium* indirectly through some other non-type III bacterial component (e.g. LPS, flagellin, or peptidoglycan).

DISCUSSION

Enteric bacterial pathogens represent a major public health problem in the developing world because of the significant

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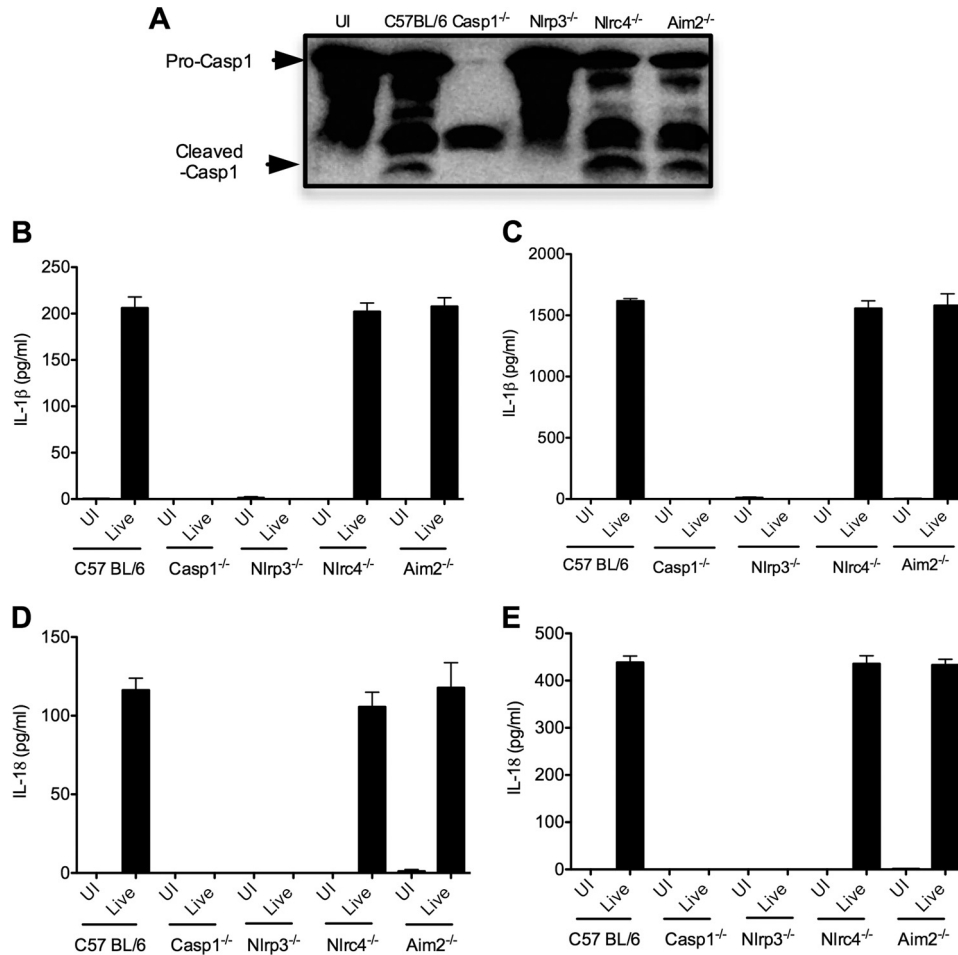


FIGURE 6. *C. rodentium* induces caspase-1 activation through Nlrp3 inflammasome in cultured macrophages. Bone marrow-derived macrophages from WT, Casp1^{-/-}, Nlrp3^{-/-}, Nlrc4^{-/-}, and Aim2^{-/-} mice were primed with or without LPS for 5 h and then infected with *C. rodentium* at 20 multiplicity of infection as described under "Experimental Procedures." *A*, cells were lysed, and proteins were used to determine caspase-1 activation by Western blotting. *B–E*, cell culture supernatants were collected, and the cytokines IL-1 β and IL-18 were measured by ELISA: IL-1 β levels without LPS priming (*B*), IL-1 β levels with LPS priming (*C*), IL-18 levels without LPS priming (*D*), and IL-18 levels with LPS priming (*E*). Data represent means \pm S.D. (error bars) of triplicate wells from three independent experiments. *UI*, uninfected; *Live*, live bacteria.

morbidity and mortality associated with severe diarrhea in infants and children (2). Previous studies demonstrated that innate immune cells, T cell responses, and antibody production all contribute to containment and eradication of enteric infections (5). For example, extracellular immune surveillance by members of the Toll-like receptor family was shown to be critical for host defense against *C. rodentium* infection (6–8). More recently, the role of the intracellular NOD-like receptor family members NOD1 and NOD2 in protection against *C. rodentium* infection has been demonstrated (9, 10). However, the importance of inflammasome signaling in protection against enteric infections caused by *C. rodentium* has not been described. Our data demonstrated that mice lacking the inflammasome components Nlrp3, Nlrc4, and caspase-1 are hypersusceptible to *C. rodentium*-induced gastrointestinal inflammation because of their failure to produce normal levels of IL-1 β and IL-18. Defective inflammasome-mediated cytokine production was associated with increased destruction of the colonic epithelium, higher bacterial burden, and elevated immune cell infiltration and production of proinflammatory cytokines and chemokines. The importance of IL-1 β and IL-18 in this process was confirmed by the observation that il-1 β ^{-/-}

and il-18^{-/-} mice suffered from increased bacterial burden and colon pathology. In cultured macrophages, *C. rodentium* activated caspase-1 and induced secretion of IL-1 β and IL-18 through noncanonical activation of the Nlrp3 inflammasome. Recent work showed that available caspase-1-deficient mouse lines fail to express caspase-11 and implicated both inflammatory caspases in *C. rodentium*-induced IL-1 β and IL-18 secretion from infected macrophages (12). Our results indicated that the bacterial T3SS and the Nlrc4 inflammasome were dispensable for *C. rodentium*-induced caspase-1 activation in macrophages. However, given the importance of Nlrc4 in *in vivo* resistance to *C. rodentium* infection, the Nlrc4 inflammasome may nevertheless contribute to caspase-1 activation and inflammasome-dependent cytokine secretion in other cell types. Alternatively, the complex microbial context of the gastrointestinal tract may provide an explanation. Damage to the epithelial layer due to colonic infection with *C. rodentium* may allow opportunistic infections with commensal bacteria. Thus, during *in vivo* infections with *C. rodentium*, host inflammasome activation may not solely depend on *C. rodentium*, but multiple other bacterial species. Consequently, both the Nlrp3 and Nlrc4 inflammasomes may be activated during *C. rodentium*

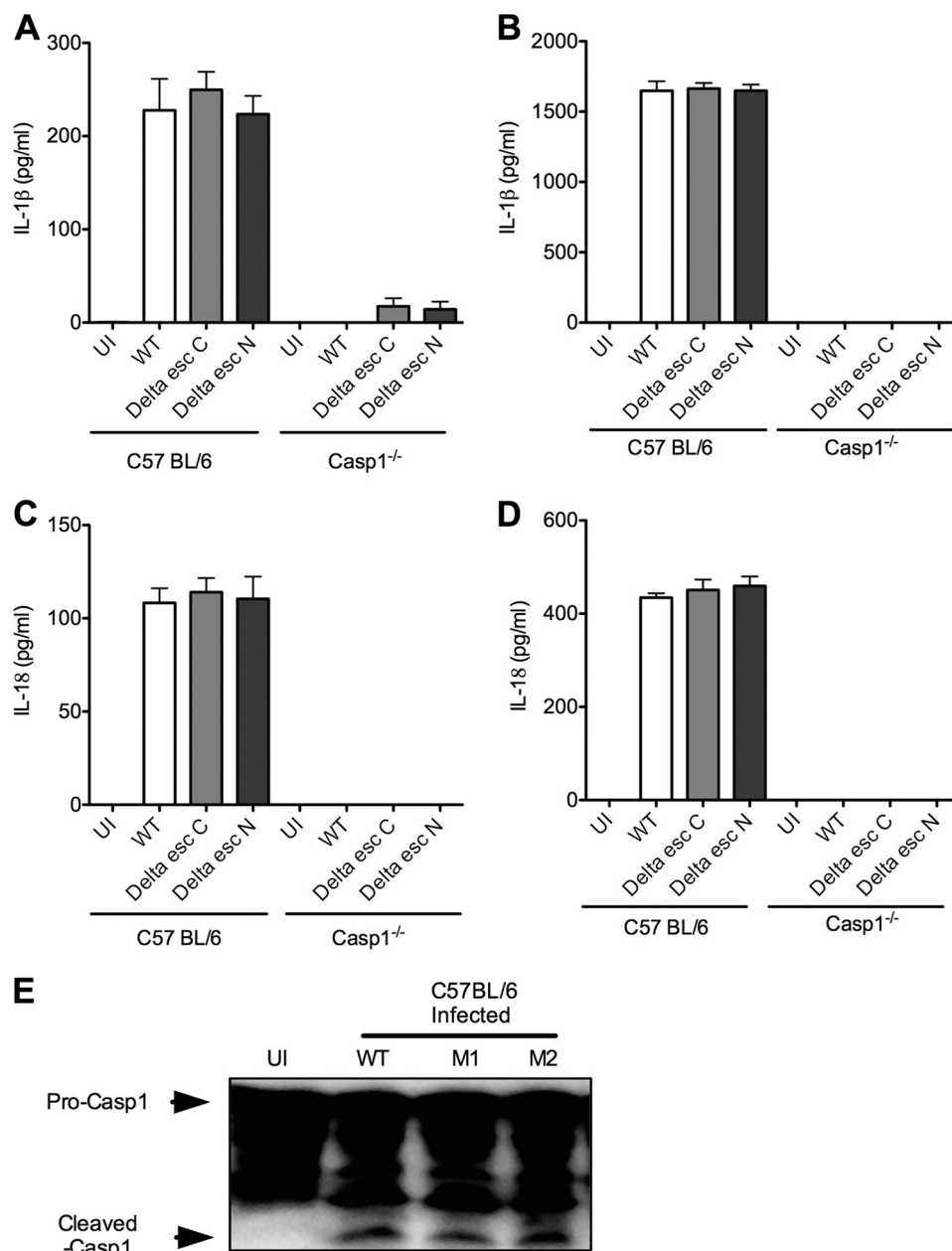


FIGURE 7. *C. rodentium* type III secretion system is dispensable for inflammasome activation in infected macrophages. BMDMs from WT and Casp1^{-/-} mice were primed with or without LPS for 5 h and then infected with *C. rodentium*, Δ escC (Mutant 1, M1) and Δ escN (Mutant 2, M2) at 20 multiplicity of infection as described under "Experimental Procedures." The cytokine IL-1 β and IL-18 in culture supernatants were measured by ELISA. A, IL-1 β levels without LPS priming. B, IL-1 β levels with LPS priming. C, IL-18 levels without LPS priming. D, IL-18 levels with LPS priming. E, Western blotting analysis of caspase-1 activation in cell lysates. Data represent means \pm S.D. (error bars) of triplicate wells from three independent experiments.

tium infection *in vivo*. Regardless, our results suggest that therapies aimed at modulating inflammasome activation may provide novel approaches for controlling inflammation and infections of the gastrointestinal tract.

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

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