The Brucella effector protein TcpB induces degradation of inflammatory caspases and thereby subverts non-canonical inflammasome activation in macrophages

Received for publication, September 6, 2017, and in revised form, October 8, 2017. Published, Papers in Press, October 23, 2017, DOI 10.1074/jbc.M117.815878

Padmaja Jakka§1,2, Swapna Namani§1, Subathra Murugan§3, Nivedita Rai†, and Girish Radhakrishnan†4

From the §1Laboratory of Immunology and Microbial Pathogenesis, National Institute of Animal Biotechnology, Hyderabad, Telangana 500049, India and †3Graduate Studies, Manipal University, Manipal, Karnataka 576104, India

Edited by Luke O’Neill

In The Brucella effector protein TcpB induces degradation of inflammatory caspases and thereby subverts non-canonical inflammasome activation in macrophages.

The inflammasome contains intracellular receptors that recognize various pathogen-associated molecular patterns and play crucial roles in innate immune responses to invading pathogens. Non-canonical inflammasome activation is mediated by caspase-4/11, which recognizes intracellular LPS and promotes pyroptosis and secretion of proinflammatory cytokines. Brucella species are infectious intracellular pathogens that replicate in professional and non-professional phagocytic cells and subvert immune responses for chronic persistence in the host. The Brucella effector protein TcpB suppresses Toll-like receptor 2 (TLR2)– and TLR4–mediated innate immune responses by targeted degradation of the Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein. TcpB is a cell-permeable protein with multiple functions, and its intracellular targets other than TIR domain-containing adaptor protein remain unclear. Here, we report that TcpB induces ubiquitination and degradation of the inflammatory caspases 1, 4, and 11. Furthermore, in both mouse and human macrophages, TcpB attenuated LPS-induced non-canonical inflammasome activation and suppressed pyroptosis and secretion of IL-1α and IL-1β induced by intracellular LPS delivery. The intact TIR domain was essential for TcpB to subvert the non-canonical inflammasome activation as a TcpB(G158A) mutant failed to suppress pyroptotic cell death and inflammatory responses. Brucella-infected macrophages exhibited minimal pyroptosis but secreted IL-1β, which was suppressed by TcpB. We also demonstrated that TcpB protein can efficiently attenuate Salmonella enterica serovar Typhimurium–induced pyroptosis and proinflammatory cytokine secretion in macrophages. Because TcpB suppresses both TLR4- and caspase-4/11–mediated inflammation, TcpB might be a candidate target for developing drugs against LPS-induced septicemia.

Inflammasomes are essential components of innate immunity, which is the first line of defense against invading microorganisms (1). Inflammasomes comprise pathogen recognition receptors (PRRs)§ grouped into nucleotide-binding domain and leucine-rich-repeat (NLRC)-containing proteins; absent in melanoma 2-like receptors; or pyrin, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) adaptor molecule and caspase-1 (2, 3). NLR sensors various microbial or physiological stimuli that lead to the assembly of inflammasome complexes. This molecular assembly activates procaspase-1 into active caspase-1 enzyme, which in turn activates proinflammatory cytokines, viz. IL-1β and IL-18. Secretion of mature IL-1β and IL-18 activates various inflammatory responses, which confer protection against infectious agents (4). Activation of inflammasomes also leads to a lytic form of programmed cell death termed pyroptosis (5).

Recent studies identified a non-canonical form of inflammasome activation that represents a new paradigm in the understanding of the innate immune mechanism (6). In non-canonical inflammasome activation, murine caspase-11 and its human orthologue, caspase-4, serve as the receptor for lipopolysaccharide (LPS), which is derived from the intracellular bacterial pathogens (7). Upon binding to the LPS, caspase-4/11 undergoes oligomerization that leads to caspase-1–mediated maturation of IL-1β, IL-18, and IL-1α and pyroptosis. Activation of caspase-4/11 appears to be independent of other PPRs, including NLRP3, NLRP6, and NLRC4 (8, 9). However, a role of NLRP3 downstream of caspase-4/11 activation for maturation of IL-1β has been reported (10). The mechanism of caspase-4/11–mediated pyroptosis remains unclear. Recently, caspase-4/11–mediated cleavage of gasdermin D was reported to play an essential role in LPS-induced pyroptosis (11, 12).

Activation of inflammasomes by intracellular pathogens or physiological stress leads to secretion of proinflammatory cytokines and pyroptosis. This process accomplishes two types of

5 The abbreviations used are: PRR, pathogen recognition receptor; NLR, nucleotide-binding domain and leucine-rich-repeat-containing protein; TIR, Toll-like receptor; TcpB, TIR domain-containing protein from Brucella; PFA, paraformaldehyde; TIRAP, TIR domain-containing adaptor protein; Tcp, Toll/interleukin-1 receptor; Xα-Gal, 5-bromo-4-chloro-3-indoly α-D-galactoside; MBP, maltose-binding protein; CARD, caspase recruitment domain; BMDMs, bone marrow-derived macrophages; LDH, lactate dehydrogenase; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; SD, synthetic defined; RIPA, radioimmune precipitation assay; m.o.i., multiplicity of infection; AD, activation domain; BD, binding domain.
host defense mechanisms, viz., induction of inflammation that activates various antimicrobial responses and pyroptosis that leads to elimination of infected or damaged cells (2). In addition, caspase-11 was reported to promote fusion of Legionella pneumophila-containing vacuoles with lysosomes by modulating the actin cytoskeleton through cofilin (13). Pathogenic microorganisms have developed various strategies to subvert the host defense mechanisms to facilitate their persistence in the host. Previous studies have established diverse microbial strategies to suppress or evade Toll-like receptor (TLR)–mediated host innate immune responses (14–17). However, the molecular mechanisms by which intracellular pathogens subvert inflammasome activation remain unclear. A recent study demonstrated that the intracellular bacterial pathogen Shigella inhibits caspase-4–mediated inflammasome activation (18). The effector protein OspC3 of Shigella interacts with the p19 subunit of caspase-4 and prevents heterodimerization of caspase-4–p19 and caspase-4–p10 that is required for activation of caspase-4 and subsequent pyroptosis of epithelial cells to decrease the bacterial burden (18). Similarly, the effector protein NleF of enteropathogenic Escherichia coli binds to the catalytic domain of caspase-4, which inhibits its catalytic activity and processing of IL-18. This resulted in the attenuation of caspase-4/11–IL-18–mediated innate immune responses in the gut (19).

Brucella are infectious intracellular pathogens found in a wide range of mammals, including humans, that cause abortion, infertility, and undulant fever (20). Brucella establish a replication niche in macrophages and subvert the host’s innate and adaptive immune responses (21, 22). The Brucella effector TcpB is a TIR domain-containing protein that is encoded by all known species of Brucella. TcpB harbors a phosphoinositide phosphate-binding domain at the N terminus and a TIR suppresses caspase-11–mediated secretion of IL-1 in inflammasome signaling. Furthermore, we show that TcpB that attenuated LPS- or motes ubiquitination and degradation of caspases 1, 4, and 11 for the first time that TcpB interacts with human caspase-4 and proinflammatory cytokine secretion mediated by TLR2 and -4 (16, 23, 24). TcpB induces targeted ubiquitination and degradation of the TLR2/4 adaptor protein TIRAP to suppress TLR signaling (23, 24). TcpB also modulates the dynamics of host microtubule polymerization by acting as the microtubule stabilization factor, which is correlated with its TLR suppression properties (25). TcpB exhibits cell permeability, and the internalized TcpB could efficiently inhibit LPS-induced NF-κB activation (26). In this study, we demonstrate for the first time that TcpB interacts with human caspase-4 and promotes ubiquitination and degradation of caspases 1, 4, and 11 that attenuated LPS– or Salmonella–induced non-canonical inflammasome signaling. Furthermore, we show that TcpB suppresses caspase-11–mediated secretion of IL-1β in Brucella-infected macrophages. Our experimental data indicate that TcpB attenuates non-canonical inflammasome–mediated pyroptosis and inflammatory responses in mouse and human macrophages.

**Results**

**Brucella effector protein TcpB interacts with caspase-4**

TcpB interacted with human caspase-4 in a high-throughput yeast two-hybrid screening using the Mate and Plate technique. AH109 yeast strain harboring a TcpB-DNA-binding domain fusion was mated with a library of Y187 yeast strain carrying human cDNAs fused with the activation domain. The plasmids harboring the human cDNA were rescued from the blue colored diploid yeast colonies that were growing on quadruple amino acid dropout medium containing X-α-gal. Subsequent sequencing of isolated plasmids indicated caspase-4 as one of the interacting partners of TcpB. We also examined the interaction of TcpB with other caspases by yeast two-hybrid analysis. TcpB interacted with caspase-11, which is the murine orthologue of human caspase-4 (Fig. 1A). Our yeast two-hybrid analysis did not indicate interaction between TcpB and CARD-deficient caspase-4 or caspase-3 (Fig. 1A). Next, we analyzed the interaction of TcpB(G158A), which harbors a point mutation in the BB-loop region of the TIR domain (23). TcpB(G158A) interacted with both caspase-4 and -11 but not with caspase-3 or CARD-deficient caspase-4 (supplemental Fig. 1). Next, we performed an immunoprecipitation assay to confirm the interaction of TcpB with caspase-4. Total lysate of human embryonic kidney (HEK) 293T cells that overexpress FLAG-caspase-4 was incubated with purified MBP-TcpB fusion protein or MBP alone followed by immunoprecipitation of caspase-4 using anti-FLAG antibody. MBP-TcpB could be coimmunoprecipitated with FLAG-caspase-4, suggesting a potential interaction between caspase-4 and TcpB (Fig. 1B). MBP alone was not detected in immunoprecipitated samples, which indicated specific interaction between TcpB and caspase-4.

**TcpB induces degradation of inflammatory caspases**

TcpB negatively regulates TLR4–mediated innate immune signaling by targeting the degradation of the TLR4 adaptor protein TIRAP. Our experimental data revealed a positive interaction between TcpB and caspase-4. Therefore, we sought to determine whether TcpB induces degradation of caspase-4. HEK293T cells were cotransfected with an equal concentration of FLAG-caspase-4 and increasing concentrations of HA-TcpB(G158A) induced caspase-4. HEK293T cells were cotransfected with an equal concentration of FLAG-caspase-4 and increasing concentrations of HA-TcpB followed by cell lysis, extraction of total protein, and immunoblotting to analyze the levels of caspase-4. TcpB induced degradation of caspase-4 in a dose-dependent manner (Fig. 2A). Because caspase-11 is the murine orthologue of human caspase-4, we wished to examine whether TcpB also induces enhanced degradation of caspase-11. Cotransfection of HA-TcpB and FLAG-caspase-11 resulted in enhanced degradation of FLAG-caspase-11 with increasing concentrations of HA-TcpB (Fig. 2B). Next, we performed a pulse-chase analysis of the degradation of caspase-4 or -11 in the presence or absence of TcpB. HEK293T cells expressing FLAG-caspase-4 or -11 in the presence or absence of HA-TcpB were treated with the protein synthesis inhibitor cycloheximide. Subsequently, the degradation of caspase-4 or -11 was monitored at various time points. We observed gradual degradation of caspase-4 or -11 at increasing time points only in the presence of TcpB in cycloheximide-treated cells (Fig. 2, C and D, and supplemental Fig. 2, A and B). Next, we examined whether TcpB(G158A) is capable of inducing the degradation of caspase-4. Cotransfection of HEK293T cells with FLAG-caspase-4 and increasing concentrations of HA-TcpB(G158A) did not induce degradation of...
FLAG-caspase-4 (Fig. 2E), which indicates that an intact TIR domain is required for TcpB to induce the degradation of caspase-4. Intracellular LPS stimulates caspase-4/11, which leads to the activation of caspase-1 and release of proinflammatory cytokines such as IL-1β. Therefore, we wished to examine whether TcpB also promotes the degradation of caspase-1. Cotransfection of HEK293T cells with an equal concentration of FLAG-caspase-1 and increasing concentrations of HA-TcpB induced the degradation of caspase-1 (Fig. 2F). The degradation of caspase-1 was also observed in the presence of TcpB in cycloheximide-treated cells with increasing time points (supplemental Fig. 2C and D). As observed before, cotransfection with TcpB(G158A) did not induce degradation of caspase-1, indicating the requirement of a functionally intact TIR domain for promoting the degradation of caspase-1 (Fig. 2G).

Next, we analyzed the degradation of endogenous caspase-1 and -11 by TcpB in a mouse macrophage cell line. We previously reported that recombinant MBP-TcpB is cell-permeable and efficiently internalized by RAW264.7 macrophages (26) (supplemental Fig. 2E). To analyze whether MBP-TcpB enhances the degradation of endogenous caspase-1 and -11, we treated RAW264.7 macrophages with MBP-TcpB or MBP followed by infection with Salmonella enterica serovar Typhimurium, which is a potent inducer of the non-canonical inflammasome pathway. Subsequently, the cells were lysed and subjected to immunoblotting followed by detection of endogenous caspase-1 and -11 using the respective antibodies. Macrophages treated with MBP-TcpB showed diminished levels of caspase-1 and -11 compared with the cells incubated with MBP alone (Fig. 2H). To examine the specificity of TcpB for inducing the degradation of caspases, we cotransfected an equal concentration of FLAG-caspase-3 with increasing concentrations of MYC-TcpB followed by analysis of the degradation of FLAG-caspase-3 with increasing concentrations of MYC-TcpB followed by analysis of the degradation of FLAG-caspase-3 (supplemental Fig. 2F). Taken together, our experimental data clearly indicate that TcpB induces degradation of
TcpB subverts non-canonical inflammasome activation

A

B

C

D

E

F

G

H

J. Biol. Chem. (2017) 292(50) 20613–20627
TcpB subverts non-canonical inflammasome activation

Inflammatory caspases, which mediate non-canonical inflammasome signaling.

**TcpB promotes ubiquitination of inflammatory caspases**

Selective protein degradation in eukaryotic cells is achieved by ubiquitination of target proteins and their degradation by the 20S proteasome complex. TcpB is reported to promote the ubiquitination of the TLR4 adaptor protein TIRAP, which leads to its enhanced degradation. This process negatively regulates the LPS-induced signaling through TLR4 (24). Given that TcpB promotes degradation of caspases 1, 4, and 11, we analyzed whether TcpB induces their ubiquitination by *in vivo* ubiquitination assay. HEK293T cells were cotransfected with MYC-TcpB; FLAG-caspase-1, -4, or -11; and HA-ubiquitin followed by immunoprecipitation of FLAG-caspases and immunoblotting. Ubiquitin-conjugated FLAG-caspases were detected by probing the membrane with anti-HA antibody. We observed an enhanced ubiquitination of caspases 11, 4, and 1 in the presence of TcpB (Fig. 3, A, B, and C). The experimental data indicate that TcpB induces ubiquitination of inflammatory caspases that may promote their degradation through proteasomes.

**TcpB suppresses caspase-4/11–mediated pyroptosis and proinflammatory responses induced by LPS**

Caspase-4/11 recognizes the presence of intracellular LPS, which is derived from Gram-negative bacterial pathogens and activates the non-canonical inflammasome pathway. Caspase-4/11 binds to intracellular LPS, which leads to their oligomerization, which induces pyroptotic cell death and secretion of proinflammatory cytokines such as IL-1α and IL-1β (2, 7, 27). Given that TcpB induces degradation of inflammatory caspases, we wished to examine whether TcpB suppresses caspase-4/11–mediated pyroptosis and proinflammatory cytokine secretion. First, we analyzed whether TcpB suppresses intracellular LPS-induced pyroptotic cell death in a murine macrophage cell line (RAW264.7), primary bone marrow-derived macrophages (BMDMs), and a human monocytic cell line (THP1). Cells were primed with Pam3CSK4 followed by treatment with purified MBP-TcpB or MBP alone for 4 h. Next, the cells were transfected with *E. coli* LPS, which is a potent inducer of pyroptotic cell death via activation of the non-canonical inflammasome pathway mediated by caspase-4/11. Induction of pyroptotic cell death by LPS was detected by measuring the lactate dehydrogenase (LDH) released by the cells. Macrophages treated with MBP-TcpB released decreased levels of LDH compared with the cells treated with MBP alone (Fig. 4A).

To further verify this observation, primed RAW264.7 cells were treated with MBP-TcpB or MBP followed by LPS transfection and staining with Zombie Red dye, which is non-permeant to live cells but permeant to cells with compromised cell plasma membranes (28). Zombie Red dye binds to primary amine group of proteins, and most of the cells treated with TcpB-MBP excluded the Zombie Red dye and displayed labeling only on the cell periphery (Fig. 4B). RAW264.7 cells treated with MBP alone displayed increasing amounts of total protein labeling and appeared brighter, indicating pyroptotic cell death (Fig. 4B). These experimental data imply that TcpB suppresses intracellular LPS-induced pyroptotic cell death in macrophages. Next, we examined whether TcpB affects paraformaldehyde (PFA)-induced cytotoxicity, which is independent of caspase-4/11. THP1 cells were incubated with MBP-TcpB or MBP alone followed by treatment of cells with PFA. TcpB did not affect the cell death induced by PFA, which suggests that TcpB attenuates cytotoxicity induced by inflammatory caspases (supplemental Fig. 3A).

In addition to inducing pyroptosis, activation of caspase-4/11 by LPS leads to secretion of proinflammatory cytokines, viz IL-1α and IL-1β (29). Therefore, we analyzed the effect of TcpB on secretion of caspase-4/11–induced inflammatory cytokines in macrophages. Primed BMDMs or RAW264.7 macrophages were treated with MBP-TcpB or MBP alone followed by LPS transfection and quantification of secreted IL-1α and IL-1β by ELISA. Macrophages treated with MBP-TcpB secreted diminished levels of IL-1α and IL-1β compared with MBP-treated cells (Fig. 4, C and D, and supplemental Fig. 3B). Taken together, these experimental data indicate that TcpB suppresses intracellular LPS–induced pyroptosis and secretion of proinflammatory cytokines in macrophages.

Next, we analyzed whether TcpB(G158A), which is deficient in inducing degradation of caspase-4/11, can suppress the LPS-induced cell death and secretion of proinflammatory cytokines. Primed RAW264.7 macrophages were treated with wild-type MBP-TcpB or MBP-TcpB(G158A) protein followed by intracellular delivery of LPS by transfection and measurement of LDH release and secreted cytokines. MBP-TcpB(G158A) failed to suppress the pyroptotic cell death and secretion of IL-1α and IL-1β by macrophages (Fig. 4E).

**Figure 2.** TcpB induces degradation of human caspase-4 (A) and its mouse orthologue caspase-11 (B). HEK293T cells were cotransfected with an equal concentration of FLAG-caspase-4 or -11 and increasing concentrations of HA-TcpB. Cells were harvested 24 h post-transfection followed by lysis and immunoblotting (IB). FLAG-caspase-4/11 was detected using anti-FLAG antibody. Actin served as the loading control. TcpB induced degradation of caspase-4/11 in a dose-dependent manner. C and D, pulse-chase analysis of TcpB-induced caspase-4 degradation. HEK293T cells were transfected with FLAG-caspase-4 alone (C) or with HA-TcpB (D). Twenty-four hours post-transfection, cells were treated with cycloheximide for 1 h followed by harvesting the cells at the indicated time points and immunoblotting. The gradual degradation of FLAG-caspase-4 was detected in the presence of HA-TcpB with increasing time points in cycloheximide-treated cells (D). E, TcpB(G158A) did not induce the degradation of caspase-4. Cotransfection of HEK293 cells with FLAG-caspase-4 and increasing concentrations of HA-TcpB(G158A) did not change the levels of caspase-4, indicating the inability of TcpB(G158A) to induce the degradation of caspase-4. F, TcpB promotes degradation of caspase-1. HEK293T cells were cotransfected with an equal concentration of FLAG-caspase-1 and increasing concentrations of HA-TcpB. FLAG-caspase-1 underwent degradation with increasing concentrations of HA-TcpB. G, TcpB(G158A) failed to induce degradation of caspase-1. HEK293T cells were cotransfected with FLAG-caspase-1 and HA-TcpB(G158A). FLAG-caspase-1 did not undergo degradation in the presence of TcpB(G158A). H, TcpB enhances degradation of endogenous caspase-1 and -11 in mouse macrophages. RAW264.7 cells were treated with MBP-TcpB or MBP followed by infection with S. enterica Typhimurium for 5 h. Subsequently, the cells were harvested and subjected to immunoblotting to detect endogenous caspase-1 and -11. An enhanced degradation of endogenous caspase-1 and -11 was observed in MBP-TcpB-treated cells compared with cells treated with MBP alone. Immunoblots in A–G are representative of three independent experiments. The immunoblot in H is representative of two independent experiments. The right panels of the immunoblots show the densitometry analysis of caspase bands normalized to actin.
Next, we examined the effect of TcpB on LPS-induced non-canonical inflammatory responses in the presence of Z-LEVD-fmk, which inhibits caspase-4/11 activity. RAW264.7 cells were treated with Z-LEVD-fmk followed by treatment with MBP-TcpB or MBP protein. Subsequently, the non-canonical inflammasome pathway was activated by LPS transfection. Treatment of macrophages with Z-LEVD-fmk suppressed the pyroptotic cell death and inflammatory cytokine secretion (Fig. 4F). A synergistic effect of TcpB was observed when the cells were treated with MBP-TcpB in the presence of Z-LEVD-fmk (Fig. 4F). These experimental data imply that TcpB targets caspase-4/11 to attenuate pyroptosis and secretion of proinflammatory cytokines mediated by the non-canonical inflammasome pathway.

Our experimental data indicated that TcpB induces the degradation of caspase-1. Therefore, we examined the effect of TcpB on the caspase-4/11–independent inflammatory pathway where the role of caspase-1 is crucial. Nigericin activates the NLRP3 inflammasome, leading to caspase-1–dependent activation of IL-1β secretion (30). We analyzed the effect of TcpB on nigericin-induced cell death and IL-1β secretion in BMDMs. Primed BMDMs were incubated with MBP-TcpB or MBP alone followed by treatment with nigericin and quantification of LDH and IL-1β secretion (30). We analyzed the effect of TcpB on nigericin-induced cell death and IL-1β secretion in BMDMs. These experimental data identify that TcpB is capable of suppressing other inflammasome-signaling pathways, and this is mediated by caspase-1.

TcpB attenuates IL-1β secretion in Brucella-infected macrophages

*Brucella* are stealthy intracellular pathogens that efficiently evade or suppress host innate immune responses. *Brucella* are weak inducers of TLR4 signaling due to their unconventional LPS, which is several hundred times less toxic than that of enterobacterial species (31, 32). To examine whether *Brucella* activate non-canonical inflammasomes, we performed macrophage infection studies followed by analysis of LDH and IL-1β secretion by infected macrophages. We used *Brucella neotomae*, which was isolated from desert wood rat and is non-pathogenic to human (33), for our studies. Infection dynamics
of *B. neotomae* and the human-pathogenic species *Brucella melitensis* were reported to be similar in murine macrophages, and both species induced mortality in IRF-1−/− mice in 9–12 days (34, 35). We analyzed the growth dynamics of *B. neotomae* in RAW264.7 cells at various time points and found that *B. neotomae* could efficiently replicate in murine macrophages (Fig. 5A). Next, we analyzed the induction of pyroptosis by *B. neotomae* in RAW264.7 cells. We used *S. enterica* Typhimurium as the positive control as it induces pyroptotic cell death in murine macrophages (10, 36). Primed RAW264.7 cells were infected with *B. neotomae* or *S. enterica* Typhimurium followed by measurement of LDH released into the medium. Macrophages infected with *S. enterica* Typhimurium released elevated levels of LDH, whereas *B. neotomae*–infected cells released minimal LDH (Fig. 5B). Next, we stained the *B. neotomae*– or *S. enterica* Typhimurium–infected macrophages with Zombie Red dye to visualize the extent of pyroptosis induced by these pathogens. Infection with *S. enterica* Typhimurium resulted in more Zombie Red–positive cells compared with infection with *B. neotomae* (Fig. 5C). These experimental data imply that *B. neotomae* is a weak inducer of pyroptotic cell death. *Brucella abortus* has been reported to induce IL-1β secretion in LPS-primed BMDMs at 17 h postinfection (37). We analyzed induction of IL-1β secretion by *B. neotomae* in BMDMs and the effect of TcpB on IL-1β secretion by *B. neotomae*–infected macrophages. BMDMs were primed with LPS followed by infection with *B. neotomae* and quantification of IL-1β levels. In our infection studies, *B. neotomae* induced maximum levels of IL-1β in BMDMs at 24 h postinfection (supplemental Fig. 4).

Next, we analyzed whether TcpB could attenuate *B. neotomae*–induced IL-1β secretion in macrophages. LPS-primed BMDMs were treated with MBP-TcpB, MBP-TcpB(G158A), or MBP alone followed by *B. neotomae* infection and analysis of IL-1β levels 24 h postinfection. The BMDMs treated with MBP-TcpB secreted diminished levels of IL-1β compared with cells treated with MBP alone or MBP-TcpB(G158A) (Fig. 5D). Next, we examined whether *B. neotomae*–induced IL-1β secretion is mediated by caspase-11. BMDMs were treated with Z-LEVD-fmk followed by infection with *B. neotomae* and quantification of IL-1β levels. Z-LEVD-fmk suppressed IL-1β secretion by *B. neotomae*–infected BMDMs (Fig. 5E), which suggests that the induction of IL-1β is mediated by activation of the non-canonical inflammasome signaling. Previous studies demonstrated an enhanced level of IL-1β in IRF-1−/− mice infected with TcpB-deficient *B. melitensis*. Taken together, the data indicate that TcpB plays an essential role in the suppression of proinflammatory responses mediated by non-canonical inflammasome activation in *Brucella*-infected macrophages.

**TcpB subverts non-canonical inflammasome activation**

**TcpB attenuates Salmonella-induced pyroptosis and inflammatory responses**

*S. enterica* Typhimurium induces pyroptotic cell death and secretion of proinflammatory cytokines in mouse macrophages through the activation of caspase-11 (38). Therefore, we wished to examine whether TcpB attenuates caspase-11 activation in *Salmonella*-infected macrophages. Primed RAW264.7 cells or BMDMs were treated with MBP-TcpB, TcpB(G158A), or MBP alone followed by infection with *S. enterica* Typhimurium and measurement of LDH, IL-1α, and IL-1β by ELISA. LDH and proinflammatory cytokine levels were significantly lower in *S. enterica* Typhimurium–infected macrophages treated with TcpB-MBP (Fig. 6A). As observed previously, MBP-TcpB(G158A) failed to suppress *S. enterica* Typhimurium–induced cell death or cytokine release (Fig. 6B). Next, we analyzed the synergistic effect of TcpB in the presence of Z-LEVD-fmk in the context of *S. enterica* Typhimurium infection. An enhanced suppression of LDH and proinflammatory cytokine release was observed in the samples treated with MBP-TcpB and Z-LEVD-fmk, suggesting that TcpB targets caspase-11 to attenuate *S. enterica* Typhimurium–induced cell death and cytokine secretion (Fig. 6C). These experimental data imply that TcpB is capable of suppressing *S. enterica* Typhimurium–induced non-canonical inflammasome signaling in mouse macrophages.

**Discussion**

*Brucella* evade and/or suppress host innate and adaptive immune responses to successfully invade and persist in the host (21, 39, 40). *Brucella* effector protein TcpB efficiently suppresses proinflammatory responses mediated by TLR2 and −4 and inhibits CD8+ T cell killing of *Brucella* epitope–specific target cells (16, 23, 41). TcpB also plays an essential role in *Brucella*-induced unfolded protein response, which contrib-

**Figure 4.** A, TcpB suppresses pyroptotic cell death induced by intracellular delivery of LPS. RAW264.7 cells, BMDMs, or THP1 cells were primed with Pam3CSK4 followed by treatment with MBP-TcpB or MBP alone. Cells were then transfected with LPS followed by quantification of LDH released in the media. TcpB-treated macrophages released diminished levels of LDH compared with cells treated with MBP alone, indicating the suppression of pyroptotic cell death induced by non-canonical inflammasome signaling. “Lipo+LPS” refers to intracellular LPS and therefore caspase-4/11 detection; whereas “LPS” refers to extracellular LPS and hence TLR4-mediated detection. B, TcpB-treated cells resist pyroptotic cell death induced by LPS. Primed RAW264.7 macrophages were treated with TcpB-MBP or TcpB alone followed by LPS transfection. Cells were then stained with Zombie Red dye to visualize pyroptotic cell death. Cells treated with TcpB excluded the dye and displayed peripheral labeling compared with MBP-treated cells, which had taken up the dye and appeared bright. Images are representative of three independent experiments. Scale bar, 30 μm. C and D, TcpB attenuates secretion of proinflammatory cytokines induced by intracellular delivery of LPS. Primed RAW264.7 cells (C) or BMDMs (D) were treated with MBP-TcpB or MBP alone followed by LPS transfection and quantification of IL-1α and IL-1β levels by ELISA. Macrophages treated with TcpB secreted diminished levels of IL-1α and IL-1β compared with MBP-treated cells. E, TcpB(G158A) could not suppress cell death and secretion of proinflammatory cytokines induced by LPS. Primed RAW264.7 cells were treated with MBP-TcpB, MBP-TcpB(G158A), or MBP alone followed by LPS transfection and analysis of cytotoxicity and IL-1α and IL-1β secretion. MBP-TcpB(G158A) could not suppress cell death and secretion of proinflammatory cytokines compared with wild-type TcpB. F, synergistic effect of TcpB in the presence of caspase-4 inhibitor. Primed RAW264.7 cells were treated with MBP-TcpB or MBP in the presence or absence of Z-LEVD-fmk (10 μM) followed by LPS transfection and analysis of LDH, IL-1α, and IL-1β. TcpB exhibited an enhanced suppression of LDH, IL-1α, and IL-1β release in the presence of Z-LEVD-fmk, indicating its synergistic effect. G, TcpB suppresses nigericin-induced cell death and IL-1β secretion by macrophages. Primed BMDMs were incubated with MBP-TcpB or MBP alone followed by treatment with nigericin and quantification of released LDH and IL-1β. Diminished levels of LDH and IL-1β were observed in BMDMs treated with TcpB compared with MBP-treated cells. The data were analyzed using Sigma Plot software, and statistical significance was determined using a t test. The data are presented as mean ± S.D. from at least three independent experiments (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
utes to the intracellular replication of *Brucella* (42). The first hint regarding the molecular mechanism of TcpB-mediated TLR4 suppression emerged upon demonstration of molecular mimicry by TcpB: it mimicked the phosphoinositide phosphate- and cytoskeleton-binding properties of the TLR4 adapter protein TIRAP (23). Subsequently, TcpB-TIRAP interaction and enhanced ubiquitination and degradation of TIRAP by TcpB were demonstrated to underlie the TcpB-mediated suppression of TLR4 signaling (24). However, the mechanism by which TcpB ubiquitinates target proteins remains obscure. It is
possible that TcpB recruits host ubiquitin ligases for ubiquitination and subsequent degradation of target proteins. Because TcpB efficiently suppressed the extracellular LPS–induced signaling, it was envisaged that TcpB may affect the intracellular LPS signaling as well. Recent studies demonstrated that caspase-4/11 serves as an essential component for intracellular LPS signaling (7). Recognition of intracellular LPS from Gram-negative bacteria by caspase-4/11 drives a non-canonical form of inflammatory response that leads to pyroptotic cell death and secretion of proinflammatory cytokines (6). Given

Figure 5. A, multiplication of B. neotomae in RAW264.7 cells. Cells were infected with the indicated m.o.i. followed by enumeration of CFUs for various time points. B. neotomae efficiently multiplied in RAW264.7 cells. B, B. neotomae induced minimal cell death in macrophages. RAW264.7 macrophages were infected with B. neotomae or S. enterica Typhimurium followed by analysis of LDH secreted by macrophages. B. neotomae induced a minimal level of LDH in infected macrophages. C, staining of B. neotomae– or S. enterica Typhimurium–infected macrophages with Zombie Red dye. B. neotomae–infected macrophages exhibited minimal staining compared with S. enterica Typhimurium–infected cells, indicating minimal induction of pyroptosis by B. neotomae. The image is representative of three independent experiments. Scale bar, 30 μm. D, TcpB suppresses IL-1β secretion in B. neotomae (Bru)–infected BMDMs. BMDMs were primed with LPS followed by treatment with MBP-TcpB, MBP-TcpB(G158A), or MBP alone and infection with B. neotomae. Secretion of IL-1β by infected BMDMs was analyzed 24 h postinfection. BMDMs treated with MBP-TcpB secreted less IL-1β compared with cells treated with MBP-TcpB(G158A) or MBP alone. E, B. neotomae–induced secretion of IL-1β in BMDMs is caspase-11–dependent. Primed BMDMs were treated with Z-LEVD-fmk followed by infection with B. neotomae. IL-1β secretion by infected cells was analyzed 24 h postinfection. Treatment with Z-LEVD-fmk suppressed the secretion of IL-1β by B. neotomae–infected BMDMs. The data were analyzed using Sigma Plot software, and statistical significance was determined using a t test. The data are presented as mean ± S.D. from at least three independent experiments (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Figure 6. TcpB suppresses Salmonella–induced activation of non-canonical inflammasome. RAW264.7 cells (A) were primed with Pam3CSK4 followed by MBP-TcpB or MBP protein treatment and infection with S. enterica Typhimurium. TcpB attenuated LDH, IL-1α, and IL-1β secretion by RAW264.7 cells compared with cells treated with MBP alone. B, TcpB(G158A) mutant could not attenuate Salmonella–induced inflammasome activation. Primed BMDMs were treated with wild-type MBP-TcpB, MBP-TcpB(G158A) mutant protein, or MBP alone followed by S. enterica Typhimurium infection. MBP-TcpB(G158A) mutant did not exhibit suppression of Salmonella–induced release of LDH or secretion of IL-1α and IL-1β by macrophages compared with wild-type MBP-TcpB. C, synergistic effect of TcpB in the presence of caspase-4 inhibitor in Salmonella (Sal)–induced inflammasome activation. Primed RAW264.7 cells were treated with MBP-TcpB in the presence or absence of Z-LEVD-fmk followed by S. enterica Typhimurium infection. An enhanced suppression of LDH, IL-1α, and IL-1β was observed by TcpB in the presence of Z-LEVD-fmk. The data were analyzed using Sigma Plot software, and statistical significance was determined using a t test. The data are presented as mean ± S.D. from at least three independent experiments (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
that TcpB attenuates extracellular LPS signaling mediated by TLR4, we sought to analyze whether TcpB subverts LPS-induced intracellular signaling mediated by caspase-4/11. This assumption was further strengthened by identification of a positive interaction between TcpB and human caspase-4 in a high-throughput yeast two-hybrid screening. To suppress the TLR4 signaling, TcpB interacted with TIRAP and induced its degradation. Therefore, we examined the fate of caspase-4/11 in the presence of TcpB and found that TcpB promotes ubiquitination and degradation of caspase-4/11. Recently, targeting caspase-4/11 for subverting intracellular LPS signaling was demonstrated for two bacterial pathogens. Effector proteins OspC3 of *Shigella* and NleF of enteropathogenic *E. coli* targeted caspase-4/11 to attenuate the inflammatory responses. Both proteins were reported to bind to the catalytic site of caspase-4/11, leading to its inhibition (18, 19). However, TcpB subverts caspase-4/11-mediated signaling in a unique manner where it induces ubiquitination and degradation of caspase-4/11. TcpB also targeted caspase-1 for enhanced ubiquitination and degradation. This may account for the attenuation of nigericin-induced NLRP3 inflammasome signaling by TcpB. Our experimental data suggest that the intact TIR domain is essential for degradation and subsequent suppression of caspase-mediated cytotoxicity and proinflammatory responses. It appears that TcpB interacts with the CARD of inflammatory caspases.

We previously reported that TcpB is a cell-permeable protein that translocates into macrophages (26). TcpB harbors a putative protein transduction domain at the N terminus that may facilitate its entry into the cells. Recombinant TcpB fused with MBP translocated into mouse macrophages and suppressed NF-κB activation (26). Similarly, the macrophages treated with MBP-TcpB secreted diminished levels of LDH and inflammatory cytokines upon activation of caspase-4/11 by intracellular delivery of LPS or infection with intracellular bacterial pathogens. This indicates that TcpB crosses the plasma membrane and enters into the macrophages to attenuate pyroptosis and inflammation by promoting the degradation of caspses 4 and 11. The property of TcpB to cross the plasma membrane may allow the protein to translocate into neighboring cells from the *Brucella*-infected cells to exert its immune suppression properties in the host. This may contribute to the minimal activation of host immune responses observed during *Brucella* infection.

*B. abortus* did not induce pyroptotic cell death in mouse macrophages as a minimal level of LDH could be detected in BMDMs infected with *B. abortus* (37). In agreement with this observation, minimal pyroptotic cell death and LDH release were observed in BMDMs infected with *B. neotomae* compared with *S. enterica* Typhimurium. However, *B. neotomae* induced IL-1β secretion in BMDMs, which appeared to be caspase-11–dependent as treatment of cells with Z-LEVD-fmk or MBP-TcpB suppressed the *B. neotomae*–induced IL-1β secretion. The secretion of IL-1β by BMDMs infected with *B. abortus* was partially dependent on NLRP3 and AIM2 inflammasomes (37). However, our studies suggest a role for caspase-11 in the induction of IL-1β secretion in *B. neotomae*–infected BMDMs that may occur upstream of NLRP3. Consistent with our results, a recent study reported activation of NLRP3 by caspase-4/11 that induced IL-1β processing and secretion (10). *S. enterica* Typhimurium is a potent inducer of non-canonical inflammasomes that leads to pyroptosis and secretion of proinflammatory cytokines by the infected macrophages. Treatment of macrophages with recombinant TcpB protein suppressed the pyroptosis and cytokine secretion by *S. enterica* Typhimurium–infected macrophages. This confirms that TcpB targets caspase-11 for subverting cell death and inflammatory responses. A TIR domain-containing protein (tlpA) was reported in *S. enterica* serovar Enteritidis that suppresses TLR4-mediated NF-κB activation and secretion of IL-1β by macrophages (14). It will be interesting to examine whether tlpA also targets the non-canonical inflammasome pathway to attenuate IL-1β secretion by macrophages.

TcpB efficiently suppresses proinflammatory cytokine signaling mediated by TLR4. Our experimental data suggest dual functions for TcpB where it attenuates proinflammatory cytokines induced by both extracellular and intracellular activation of PRRs. Macrophages are the primary target cells of *Brucella* for replication to facilitate their chronic persistence in the host. Inhibition of cell death in *Brucella*-infected cells helps the bacteria to establish a replicative niche for sustained infection. However, the detection of intracellular *Brucella* by the PRRs, including inflammasomes, results in secretion of proinflammatory cytokines, which activate various antimicrobial responses in the host. Therefore, suppression of proinflammatory responses induced by the infected macrophages is essential for *Brucella* to survive in the host. It appears that TcpB plays an essential role in suppression of these host innate immune responses for the chronic persistence of *Brucella* in the host. In agreement with this, TcpB-deficient *B. melitensis* has been reported to induce elevated levels of proinflammatory cytokines in mice compared with the wild-type *B. melitensis* (23).

In summary, our studies demonstrate that TcpB attenuates caspase-4/11–mediated non-canonical inflammasome signaling in mouse and human macrophages. TcpB promotes ubiquitination and degradation of caspases 1, 4, and 11, which attenuates intracellular LPS-induced or bacterium-induced pyroptosis and secretion of inflammatory cytokines. Studies suggest that TcpB play a major role in the innate immune suppression of *Brucella* by attenuating both the TLR4- and caspase-4/11–mediated defense mechanisms of the host. The detection of intracellular LPS by caspase-4/11 plays a major role in LPS-induced septicemia in addition to recognition of extracellular LPS by TLR4 (43). Because TcpB suppresses both TLR4- and caspase-4/11–mediated inflammatory responses, it may serve as an ideal drug candidate for treatment of sepsis.

**Experimental procedures**

**Cell culture and transfections**

Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Sigma), 1× penicillin-streptomycin solution (Gibco), and 100 μg/ml Normocin (Invi-vogen) was used for culturing HEK293T (American Type Culture Collection) and THP1 cells (American Type Culture Collection). RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin solu-
activation was used to culture RAW264.7 cells (American Type Culture Collection). Cells were grown in a 37 °C humidified atmosphere of 5% CO₂. To isolate BMDMs from mice, bones were collected from the hind leg of 4–6-week-old C57BL/6 mice followed by isolation of bone marrow cells using the standard procedure (44). For differentiation, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin solution, and 25 ng/ml mouse colony-stimulating factor (R&D Systems). All DNA transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

**Screening using yeast Mate and Plate technique**

TcpB gene from *B. melitensis* was cloned into the yeast two-hybrid vector pGBKKT7 (Clontech) in fusion with the GAL4 DNA-binding domain to generate pBDTcpB (bait). Yeast strain AH109 (Clontech) was transformed with pBDTcpB construct using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method. The normalized Mate and Plate human cDNA library cloned into a GAL4 AD vector (prey) and transformed into yeast strain Y187 was purchased from Clontech. Next, AH109 harboring pBDtcpB and Y187 harboring human cDNA library were mated according to the manufacturer’s instructions. The diploid yeast colonies were selected on SD agar with quadruple amino acid dropout (− Ade/− His/− Leu/− Trp) medium (Clontech) containing X-α-Gal (Clontech). Prey plasmids were rescued from the blue colonies of diploid yeast that grew on dropout medium with X-α-Gal, and the insert was identified by sequencing. Nucleotide and predicted amino acid sequences were analyzed using various bioinformatics tools.

**Protein expression and purification**

Expression and purification of TcpB fused with maltose-binding protein (MBP) was performed as described earlier (26). Briefly, 1 liter of LB medium with glucose (2%) and ampicillin (100 µg/ml) was inoculated with overnight-grown *E. coli* BL21 cells (0.1%) harboring pMALTcpB or pMALTcpB(G158A) mutant plasmid. The culture was grown at 37 °C until A₆₀₀ of 0.6 was reached followed by induction with isopropyl-1-thio-β-D-galactopyranoside (0.5 mM). After the induction, cells were grown at 25 °C for 5 h. Amylose affinity chromatography was used for purification. Cells were collected by centrifugation and sonicated followed by clarification by centrifugation at 16,000 × g for 20 min. The supernatant was collected and passed through a column harboring 5 ml of amylose resin (New England Biolabs). The column was then washed with sonication buffer followed by the same buffer containing decreasing concentrations of NaCl (750, 500, 250, and 100 mM). Protein elution was performed with elution buffer containing 50 mM Tris-HCl (pH 8.0) and 30 mM maltose. The eluted protein was concentrated using Centricon protein concentrator (Millipore) and dialyzed in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 10% glycercol. The concentration of MBP-TcpB or MBP-TcpB(G158A) was estimated using Bradford reagent (Sigma). The purified proteins were aliquoted and stored in a −80 °C freezer after snap freezing in liquid nitrogen.

**Coimmunoprecipitation**

HEK293T cells (3 × 10⁶) were transfected with pCMV-FLAG-caspase-4 using Lipofectamine 3000 reagent (Invitrogen) in 60-mm dishes. Forty-eight hours after transfection, cells were lysed at 4 °C in lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1× protease inhibitor mixture (Pierce) followed by clarification of the lysate by centrifugation at 12,000 rpm for 20 min. To determine the interaction between TcpB and caspase-4, purified MBP-TcpB or MBP alone was mixed with the HEK293T lysate containing FLAG-caspase-4. The lysates were precleared with Protein G Plus-agarose beads and mixed with 5 µg of anti-FLAG antibody (Sigma, catalog number F1804) followed by incubation overnight at 4 °C on a rotator. Next, Protein G Plus-agarose was added to the samples and incubated further for 3 h at 4 °C on a rotator. Subsequently, agarose beads were washed three times with TNT buffer (20 mM Tris (pH 8.0), 150 mM NaCl, and 1% Triton X-100), resuspended in 30 µl of SDS sample buffer (BioRad), and boiled for 10 min followed by SDS-PAGE and immunoblotting. The membrane was probed with horseradish peroxidase (HRP)-conjugated anti-MBP antibody (1:5,000; New England Biolabs, catalog number E8038S) in 5% milk in TBS-Tween 20 overnight at 4 °C. Subsequently, the membrane was washed three times with TBS-Tween 20 for 5 min each and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 min followed by acquiring the luminescence signal using a Chemi documentation system (Syngene).

**Cotransfections to examine the degradation of inflammatory caspases**

HEK293T cells (0.1 × 10⁶) cotransfected with 300 ng of pCMV-FLAG-caspase-4, pCMV-FLAG-caspase-11, or pCMV-FLAG-caspase-1 (pCMV-FLAG-caspase-11 and -1 were gifts from Junying Yuan (Addgene plasmids 21145 and 21142) and increasing concentrations (300 ng, 600 ng, 900 ng, and 1.2 µg) of HA-TcpB in a 12-well plate. Twenty-four hours after transfection, the cells were lysed in RIPA buffer, and the protein concentration was estimated using Bradford reagent (Sigma). Equal amounts of protein samples were subjected to 12% Tris-glycine SDS-PAGE followed by immunoblotting. The membrane was probed with HRP-conjugated anti-FLAG antibody (1:5000; Sigma, catalog number A8592) to detect FLAG-tagged caspases and HRP-conjugated anti-HA antibody (1:5000; Sigma, catalog number H6533) for detecting HA-TcpB. To analyze the degradation of endogenous caspase-1/11, RAW264.7 cells (0.1 × 10⁶) were seeded into 12-well plates followed by incubation of cells with purified MBP-TcpB or MBP alone for 4 h. Next, cells were infected with *S. enterica* Typhimurium for 5 h followed by lysis of cells in RIPA buffer and immunoblotting. Endogenous caspase-11 was detected using anti-caspase-11 antibody (1:1000; Cell Signaling Technology, catalog number 14340) followed by HRP-conjugated anti-rabbit secondary antibody (1:5000; Cell Signaling Technology, catalog number 7077S). Endogenous caspase-1 was detected using anti-caspase-1 antibody (1:1000; Cell Signaling Technology, catalog number 2225S) followed by HRP-conjugated anti-rabbit secondary antibody (1:5000; Cell Signaling Technology, cat-
TcpB subverts non-canonical inflammasome activation

Protein internalization

RAW264.7 (0.1 × 10⁶) mouse macrophages were seeded in a 12-well plate and incubated with various concentrations of purified MBP-TcpB or MBP alone for 5 h. Next, the cells were washed with PBS two times followed by treatment with trypsin-EDTA for 1 min. Cells were washed with PBS three more times and lysed in RIPA buffer followed by SDS-PAGE and immunoblotting.

Cell death and cytokine release assays

RAW264.7 cells, THP1 cells, or BMDMs (0.1 × 10⁶) were seeded in a 96-well plate and primed with Pam3CSK4 for 3 h. Next, the primed cells were incubated with purified MBP-TcpB or MBP protein (100 μg/ml) for 5 h in triplicates. Subsequently, the cells were transfected with LPS (2 μg/ml) using FuGENE HD (0.25% (v/v); Promega). Cells treated with FuGENE HD alone or LPS alone served as controls. Next, the plates were centrifuged at 800 × g for 5 min and incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 16–20 h followed by collection of culture supernatant. Cytotoxicity was analyzed by measuring the LDH levels (Takara) in the culture supernatants. The levels of IL-1α and IL-1β in the supernatants were analyzed by cytokine ELISA (R&D Systems). For analyzing the endogenous level of the processed form of IL-1β, RAW264.7 cells were seeded in a 12-well plate and primed with Pam3CSK4 for 2 h followed by treatment with MBP-TcpB or MBP alone for 5 h. Cells were transfected with LPS as described above. Four hours post-transfections, the cells were lysed and subjected to immunoblotting followed by immunoprobing using anti-mouse IL-1β antibody (1:1000; R&D Systems, catalog number AF-401-SP) to detect the precursor and mature forms of IL-1β. The blot was reprobed with anti-MBP-HRP antibody (1:5000; New England Biolabs, catalog number E8038S) to detect internalized MBP-TcpB and monoclonal anti-β-actin-peroxidase conjugate antibody to detect actin. To analyze the effect of TcpB in the presence of caspase-4/11 inhibitor, cells were treated with Z-LEVD-fmk (10 μM; Biovision) for 1 h followed by incubation with MBP-TcpB or MBP and LPS transfection. For analysis of nigericin-induced cell death and IL-1β secretion, BMDMs (0.05 × 10⁶) were seeded in a 24-well plate and primed with LPS (100 ng/ml) for 4 h followed by treatment with MBP or MBP-TcpB protein for 5 h. Next, the cells were stimulated with nigericin (10 μM) for 90 min followed by collection of supernatant and quantification of LDH and IL-1β levels. To analyze PFA-induced cytotoxicity, THP1 cells (0.1 × 10⁶) were seeded in a 12-well plate and pretreated with MBP-TcpB or MBP for 5 h. Next, the cells were treated with 2% PFA in the presence of MBP-TcpB or MBP for 24 h followed by Annexin V-FITC and propidium iodide staining using an ApoAlert Annexin V apoptosis detection kit (Takara) according to the manufacturer’s instructions. Stained cells were analyzed using a flow cytometer (BD LSRFortessa).

Bacterial infections studies

S. enterica Typhimurium (ATCC 14028) was cultured in LB broth (HiMedia) overnight at 37 °C. Three hours before the infection, the S. enterica Typhimurium culture was diluted (1:50) with fresh LB broth containing 300 mM NaCl and grown without shaking for 3 h at 37 °C to induce SPI-1 gene expression. B. neomycinae (ATCC 23459) was cultured in Brucella broth (BD Biosciences), and the cells were harvested in stationary phase by centrifugation at 6000 × g for 5 min.

For bacterial infections, RAW264.7 cells or BMDMs (1 × 10⁶) were seeded in a 96-well plate followed by priming and protein treatment as described above. Harvested bacterial cells were washed two times with PBS and finally resuspended in PBS. S. enterica Typhimurium (m.o.i. of 100) or B. neomycinae (m.o.i. of 100 or 1000) were added into the wells followed by centrifugation of plates at 800 × g for 10 min to spin down the bacteria onto the cells. Cells were infected for 90 min followed by treatment with gentamicin at 100 and 10 μg/ml for S. enterica Typhimurium and B. neomycinae, respectively, to kill the extracellular bacteria. The cells were maintained at 50 and 5 μg/ml gentamycin for S. enterica Typhimurium and B. neomycinae, respectively. Next, the culture supernatant was collected 12 h post-infection, and the levels of LDH, IL-1α, and IL-1β were analyzed. For analyzing TcpB-mediated suppression of IL-1α and -β in Brucella-infected cells, cells were treated with MBP-TcpB or MBP alone for 3 h followed by infection with B. neomycinae (m.o.i. of 1000) as described above. Infected cells were maintained in MBP-TcpB or MBP for 24 h. Culture supernatant was collected 12 h postinfection followed by addition of fresh medium with proteins.

Zombie Red dye staining of macrophages

RAW264.7 cells (1 × 10⁶) were seeded in glass-bottom Petri plates (Eppendorf) and allowed to adhere overnight. Priming of the cells and protein treatment were performed as described
TcpB subverts non-canonical inflammasome activation

above. Next, the cells were transfected with LPS or infected with *B. neotomae* or *S. enterica* Typhimurium for 12 h followed by staining with Zombie Red dye (Biolegend) according to the manufacturer’s protocol. Briefly, cells were washed thrice with PBS and stained with Zombie Red dye at 1:200 dilution for 30 min at room temperature. Cells were washed two times with PBS and fixed with 4% paraformaldehyde solution for 15 min. Next, cells were washed three times with PBS and mounted in ProLong Gold antifade reagent with DAPI (Thermo Fisher). Zombie Red dye is excited by yellow/green laser (561 nm) and has fluorescence emission at 624 nm. Cells were analyzed using a confocal microscope (Leica TCS SP8) with a 63× oil immersion objective, and the images were analyzed using Leica Application Suite X and Thermo Scientific HCS Studio 2.0 cell analysis software.

Statistical analysis

Data were analyzed using Sigma Plot software, and statistical significance was determined using a t test.

Ethics statement

For collection of BMDMs, C57BL6 mice were housed at the hired animal house facility of the National Institute of Animal Biotechnology at Teena Biolabs Private Ltd., Hyderabad, India. Teena Biolabs Private Ltd. has been registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (registration number 177/PO/cb/99/CPCSEA). All experimental protocols were approved by the Institutional Animal Ethics Committee of Teena Biolabs Private Ltd. (approval number TBPL-NIAB/05/2016).

**Author contributions—**G. R. conceived and designed the study and wrote the paper. P. J., S. N., and S. M. performed experiments. N. R. performed MBP-TcpB and MBP expression and purification. P. J., S. N., and G. R. analyzed the experimental data. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Avery August (Cornell University) for critical reading of the manuscript and insightful comments. We thank Shashikant Gawai and Rama Devi for help with confocal microscopy and mouse experiments, respectively.

References

1. 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
2. Sharma, D., and Kanneganti, T. D. (2016) The cell biology of inflammasomes: mechanisms of inflammasome activation and regulation. *J. Cell Biol.* **213**, 617–629
3. Vanaja, S. K., Rathinam, V. A., and Fitzgerald, K. A. (2015) Mechanisms of inflammasome activation: recent advances and novel insights. *Trends Cell Biol.* **25**, 308–315
4. Man, S. M., and Kanneganti, T. D. (2016) Converging roles of caspases in inflammasome activation, cell death and innate immunity. *Nat. Rev. Immunol.* **16**, 7–21
5. Shi, J., Gao, W., and Shao, F. (2017) Pyroptosis: gasdermin-mediated programmed necrotic cell death. *Trends Biochem. Sci.* **42**, 245–254
6. Yang, J., Zhao, Y., and Shao, F. (2015) Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Curr. Opin. Immunol.* **32**, 78–83
7. Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., and Shao, F. (2014) Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **514**, 187–192
8. Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W. P., Muszytski, A., Forsberg, L. S., Carlson, R. W., and Dixit, V. M. (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246–1249
9. 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
10. Baker, P. J., Boucher, D., Bierschenk, D., Tebartz, C., Whitney, P. G., D’Silva, D. B., Tanzer, M. C., Monteleone, M., Robertson, A. C., Cooper, M. A., Alvarez-Diaz, S., Herold, M. J., Bedouï, S., Schroder, K., and Masters, S. L. (2015) NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-8. *Eur. J. Immunol.* **45**, 2918–2926
11. Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., and Shao, F. (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665
12. Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., and Lieberman, J. (2016) Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158
13. Akhter, A., Caution, K., Abu Khweek, A., Tazi, M., Abdurahman, B. A., Abdelaziz, D. H., Voss, O. H., Doseff, A. I., Hassan, H., Azad, A. K., Schlesinger, L. S., Wewers, M. D., Gavrilin, M. A., and Amer, A. O. (2012) Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization. *Immunity* **37**, 35–47
14. Newman, R. M., Salunkhe, P., Godzik, A., and Reed, J. C. (2006) Identification and characterization of a novel bacterial virulence factor that shares homology with mammalian Toll/interleukin-1 receptor family proteins. *Infect. Immun.* **74**, 594–601
15. Salcedo, S. P., Marchesini, M. I., Degos, C., Terwagne, M., Von Bargen, K., Lepidi, H., Herrmann, C. K., Santos Lacerda, T. L., Imbert, P. R., Pierre, P., Alexopoulou, L., Letesson, J. J., Comerci, D. J., and Gorvel, J. P. (2013) BtpB, a novel *Brucella* TIR-containing effector protein with immune modulatory functions. *Front. Cell. Infect. Microbiol.* **3**, 28
16. Ciril, C., Wieser, A., Yadav, M., Duerr, S., Schubert, S., Fischer, H., Stappert, D., Wantia, N., Rodriguez, N., Wagner, H., Svanborg, C., and Miethke, T. (2008) Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. *Nat. Med.* **14**, 399–406
17. Rosadini, C. V., and Kagan, J. C. (2015) Microbial strategies for antagonizing Toll-like-receptor signal transduction. *Curr. Opin. Immunol.* **32**, 61–70
18. Kobayashi, T., Ogawa, M., Sanada, T., Mimuro, H., Kim, M., Ashida, H., Akakura, Y., Yoshida, M., Kawalec, M., Reichhart, J. M., Mizushima, T., and Sasakawa, C. (2013) The *Shigella* OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection. *Cell Host Microbe* **13**, 570–583
19. Pallett, M. A., Crepin, V. F., Serafini, N., Habibzay, M., Kotik, O., Sanchez-Garrido, J., Di Santo, J. P., Shenoy, A. R., Berger, C. N., and Frankel, G. (2017) Bacterial virulence factor inhibits caspase-4/11 activation in intestinal epithelial cells. *Mucosal Immunol.* **10**, 602–612
20. Corbel, M. J. (1997) Brucellosis: an overview. *Emerg. Infect. Dis.* **3**, 213–221
21. Martirosyan, A., Moreno, E., and Gorvel, J. P. (2011) An evolutionary strategy for a stealthy intracellular *Brucella* pathogen. *Immunol. Rev.* **240**, 211–234
22. Oliveira, S. C., de Almeida, L. A., Carvalho, N. B., Oliveira, F. S., and Lacerda, T. L. (2012) Update on the role of innate immune receptors during *Brucella abortus* infection. *Vet. Immunol. Immunopathol.* **148**, 129–135

20626 J. Biol. Chem. (2017) 292(50) 20613–20627
TcpB subverts non-canonical inflammasome activation

23. Radhakrishnan, G. K., Yu, Q., Harms, J. S., and Splitter, G. A. (2009) \textit{Brucella} TIR domain-containing protein mimics properties of the Toll-like receptor adaptor protein TIRAP. \textit{J. Biol. Chem.} \textbf{284}, 9892–9898

24. Sengupta, D., Koblansky, A., Gaines, J., Brown, T., West, A. P., Zhang, D., Nishikawa, T., Park, S. G., Roop, R. M., and Gosh, S. (2010) Subversion of innate immune responses by \textit{Brucella} through the targeted degradation of the TLR signaling adapter, MAL. \textit{J. Immunol.} \textbf{184}, 956–964

25. Radhakrishnan, G. K., Harms, J. S., and Splitter, G. A. (2011) Modulation of microtubule dynamics by a TIR domain protein from the intracellular pathogen \textit{Brucella melitensis}. \textit{Biochem. J.} \textbf{439}, 79–83

26. Viganò, E., Diamond, C. E., Spreafico, R., Balachander, A., Sobota, R. M., and Mortellaro, A. (2015) Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. \textit{Nat. Commun.} \textbf{6}, 8761

27. Casson, C. N., Broggi, A., D'Agostino, J. A., Donadio, C. A., Shao, F., Wu, H., Springstead, J. R., and Kagan, J. C. (2016) An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. \textit{Science} \textbf{352}, 1232–1236

28. Stoenner, H. G. (1963) The behavior of \textit{Brucella suis} in reciprocal superinfection experiments in mice and guinea pigs. \textit{Am. J. Vet. Res.} \textbf{18}, 947–951

29. Covert, J., Mathison, A. J., Eskra, L., Banai, M., and Splitter, G. (2009) \textit{Brucella melitensis}, B. neotomae and B. ovis elicit common and distinctive macrophage defense transcriptional responses. \textit{Exp. Biol. Med.} \textbf{234}, 1450–1467

30. Stoenner, H. G. (1963) The behavior of \textit{Brucella neotomae} and \textit{Brucella suis} in reciprocal superinfection experiments in mice and guinea pigs. \textit{Am. J. Vet. Res.} \textbf{24}, 376–380

31. Goldstein, J., Hoffman, T., Frasch, C., Lizzio, E. F., Beining, P. R., Hochstein, D., Lee, Y. L., Angus, R. D., and Golding, B. (1992) Lipopolysaccharide (LPS) from \textit{Brucella abortus} is less toxic than that from \textit{Escherichia coli}, suggesting the possible use of \textit{B. abortus} or LPS from \textit{B. abortus} as a carrier in vaccines. \textit{Infect. Immun.} \textbf{60}, 1385–1389

32. Smith, J. A., Khan, M., Magnani, D. D., Harms, J. S., Durward, M., Radhakrishnan, G. K., Liu, Y. P., and Splitter, G. A. (2013) Active evasion of CTL mediated killing and low quality responding CD8 T cells contribute to persistence of brucellosis. \textit{PLoS One} \textbf{7}, e34925

33. Smith, J. A., Khan, M., Magnani, D. D., Harms, J. S., Durward, M., Radhakrishnan, G. K., Liu, Y. P., and Splitter, G. A. (2013) \textit{Brucella} induces an unfolded protein response via TcpB that supports intracellular replication in macrophages. \textit{PLoS Pathog.} \textbf{9}, e1003785

34. Aziz, M., Jacob, A., and Wang, P. (2014) Revisiting caspases in sepsis. \textit{Cell Death Dis.} \textbf{5}, e1526

35. Weischenfeldt, J., and Porse, B. (2008) Bone marrow-derived macrophages (BMM): isolation and applications. \textit{CSH Protoc.} \textbf{2008}, pdb.prot5080