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The *Brucella* effector protein TcpB induces degradation of inflammatory caspases and thereby subverts noncanonical inflammasome activation in macrophages

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Running title: TcpB subverts noncanonical inflammasome activation

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ABSTRACT

The inflammasome contains intracellular receptors that recognize various pathogen-associated molecular patterns and play crucial roles in innate immune responses to invading pathogens. Noncanonical 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 nonprofessional 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 TIR domain-containing adaptor protein (TIRAP). TcpB is a cell-permeable protein with multiple functions, and its intracellular targets other than TIRAP 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 noncanonical inflammasome activation and suppressed pyroptosis and secretion of IL-1α and β induced by intracellular LPS delivery. The intact TIR domain was essential for TcpB to subvert the noncanonical 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 typhimurium*–induced pyroptosis and proinflammatory cytokine secretion in macrophages. Since 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 invaded microorganisms (1). Inflammasomes are comprised of Pathogen Recognition Receptors (PRR) belong to the family of Nucleotide-binding domain leucine-rich repeat containing (NLR) or absent in melanoma 2 like receptors (ALR) or pyrin, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) adaptor molecule and caspase-1(2,3). NLR senses various microbial or physiological stimuli that leads to the assembly of inflammasome complexes. This molecular assembly activates pro-caspase-1 into active caspase-1 enzyme, which in turn activates pro-inflammatory 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 inflammasome also leads to a lytic form of programmed cell death termed as pyroptosis (5).

Recent studies identified a non-canonical form of inflammasome activation, which represents a new paradigm in the understanding of innate immune mechanism (6). In the 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, 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-1 β 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 have played essential role in LPS-induced pyroptosis (11,12).

Activation of inflammasome by intracellular pathogens or physiological stress leads to secretion of pro-inflammatory cytokines and pyroptosis. This process accomplishes two types of host defense mechanisms viz. induction of inflammation that activates various anti-microbial 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 the inflammasome activation remains 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 hetero-dimerization 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 *E. 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, causing abortion, infertility and undulant fever (20). *Brucella* establish replication niche in macrophages and subvert host's innate and adaptive immune responses (21,22). The *Brucella* effector, TcpB is a TIR domain-containing protein that is encoded by all the known species of *Brucella*. TcpB harbors a phosphoinositide phosphate-binding domain at the N-terminus and a TIR domain at the C-terminus (23). TcpB suppresses NF-κB activation and pro-inflammatory cytokine secretion mediated by TLR2 and 4 receptors (16,23,24). TcpB induces targeted ubiquitination and degradation of TLR2/4 adaptor protein TIRAP to suppress the 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 caspase-1, 4 and 11 that attenuated LPS or *Salmonella*-induced non-canonical inflammasome signaling. Further, 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 employing mate and plate technique.

AH109 strain of yeast harboring 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-alpha gal. Subsequent sequencing of isolated plasmids indicated caspase-4 as one of the interacting partners of TcpB. Further, we 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. 1*A*). Our yeast-two hybrid analysis did not indicate interaction between TcpB and caspase-4 deficient for CARD domain or caspase-3 (Fig. 1*A*). 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 (Suppl Fig. 1). Next, we performed immunoprecipitation assay to confirm the interaction of TcpB with caspase-4. Total lysate of HEK293T cells that overexpresses FLAG-caspase-4 was incubated with purified maltose binding protein (MBP)-TcpB fusion protein or MBP alone followed by immunoprecipitation of caspase-4 using anti-FLAG antibody. MBP-TcpB could be co-immunoprecipitated with FLAG-caspase-4 suggesting a potential interaction between caspase-4 and TcpB (Fig. 1*B*). 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 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 co-transfected with 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. 2*A*). Since the caspase-11 is the murine orthologue of human caspase-4, we wished to examine whether TcpB induces enhanced degradation of caspase-11 also. Co-transfection of HA-TcpB and FLAG-caspase-11 resulted enhanced degradation of FLAG-caspase-11 with increasing concentrations of HA-TcpB (Fig. 2*B*). 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, D* and Suppl Fig. 2*A, B*). Next, we examined whether $TcpB^{G158A}$ is capable of inducing the degradation of caspase-4. Co-transfection of HEK293T cells with FLAG-caspase-4 and increasing concentrations of HA- $TcpB^{G158A}$ did not induce degradation of FLAG-caspase-4 (Fig. 2*E*), which indicates that an intact TIR domain is required for TcpB to induce the degradation of caspase-4. Intracellular LPS stimulates caspase-4/11 that leads to the activation of caspase-1 and release of pro-inflammatory cytokines such as IL-1β. Therefore, we wished to examine whether TcpB promotes the degradation of caspase-1 also. Co-transfection of HEK293T cells with equal concentration of FLAG-Caspase-1 and increasing concentrations of HA-TcpB induced the degradation of caspase-1 (Fig. 2*F*). The degradation of caspase-1 was also observed in presence of TcpB in cycloheximide treated cells with increasing time points (Suppl Fig. 2*C, D*). As observed before, co-transfection 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. 2*G*).

Next, we analyzed the degradation of endogenous caspase-1 and 11 by TcpB in mouse macrophage cell line. We previously reported that recombinant MBP-TcpB is cell permeable and it is efficiently internalized by RAW264 macrophages (26) (Suppl Fig. 2*E*). To analyze whether MBP-TcpB enhances the degradation of endogenous caspase-1 and 11, we treated RAW264 macrophages with MBP-TcpB or MBP followed by infection with *Salmonella typhimurium*, which is a potent inducer of non-canonical inflammasome pathway. Subsequently, the cells were lyzed 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 to the cells incubated with MBP alone (Fig. *2H*). To examine the specificity of TcpB for inducing the degradation of caspases, we co-transfected equal concentration of FLAG-caspase-3 with increasing concentrations of MYC-TcpB followed by analysis of the degradation of FLAG-caspase-3. TcpB did not induce the degradation of FLAG-caspase-3 (Suppl Fig. 2*F*). Taken together, our experimental data clearly indicate that TcpB induces degradation of inflammatory caspases, which mediates the 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 20S proteasome complex. TcpB is reported to be promoting the ubiquitination of TLR4 adaptor protein TIRAP which leads to its enhanced degradation. This process negatively regulates the LPS-induced signaling through TLR4 receptor (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 co-transfected 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 pro-inflammatory responses induced by LPS-Caspase-4/11 recognizes the presence of intracellular LPS, which is derived from Gram negative bacterial pathogens and activates non-canonical inflammasome pathway. Caspase-4/11 binds to intracellular LPS that leads to their oligomerization, which induces pyroptotic cell death and secretion of pro-inflammatory 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 caspases-4/11-mediated pyroptosis and pro-inflammatory cytokine secretion. First, we analyzed whether TcpB suppresses intracellular LPS-induced pyroptotic cell death in murine macrophage cell line (RAW264), primary bone marrow-derived macrophages (BMDMs) and the human monocytic cell line (THP1). Cells were primed with Pam3CSK4 followed by treatment with purified MBP-TcpB or MBP alone for 4 hours. Next, the cells were transfected with *E. coli* LPS, which is a potent inducer of pyroptotic cell death by activating 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 to the cells treated with MBP alone (Fig 4*A*).

To further verify this observation, primed RAW264 cells were treated with MBP-TcpB or MBP followed by LPS transfection and staining with Zombie Red dye which is non-permeable to live cells but permeant to the 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 and displayed labelling only on the cell periphery (Fig. 4*B*). RAW264 cells treated with MBP alone displayed increasing amounts of total protein labelling and appeared brighter, indicating pyroptotic cell death (Fig. 4*B*). These experimental data imply that TcpB suppresses intracellular LPS-induced pyroptotic cell death in macrophages. Next, we examined whether TcpB affects the cytotoxicity induced by paraformaldehyde (PFA), 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 (Suppl Fig. 3*A*).

In addition to inducing pyroptosis, activation of caspase-4/11 by LPS leads to secretion of pro-inflammatory cytokines *viz.*, IL-1α and IL-β (29). Therefore, we analyzed the effect of TcpB on

secretion of caspase-4/11-induced inflammatory cytokines in macrophages. Primed BMDMs or RAW264 macrophages were treated with MBP-TcpB or MBP alone followed by LPS transfection and quantification of secreted IL-1α and β by ELISA. Macrophages treated with MBP-TcpB secreted diminished levels of IL-1α and β compared to MBP treated cells (Fig. 4*C*, *D* and Suppl Fig. 3*B*). Taken together, these experimental data indicate that TcpB suppresses intracellular LPS-induced pyroptosis and secretion of pro-inflammatory cytokines in macrophages.

Next, we analyzed whether $TcpB^{G158A}$, which is deficient in inducing the degradation of caspase-4/11 can suppress the LPS-induced cell death and secretion of pro-inflammatory cytokines. Primed RAW264 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-β by macrophages (Fig. 4*E*).

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 cells were treated with Z-LEVD-FMK followed by treatment with MBP-TcpB or MBP protein. Subsequently, 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. 4*F*). The synergistic effect of TcpB was observed when the cells were treated with MBP-TcpB in the presence of Z-LEVD-FMK (Fig. 4*F*). These experimental data imply that TcpB targets caspase-4/11 to attenuate pyroptosis and secretion of pro-inflammatory cytokines mediated by non-canonical inflammasome pathway.

Our experimental data indicated that TcpB induces the degradation of caspase-1. Therefore, we examined the effect of TcpB on caspase-4/11-independent inflammatory pathway where the role of caspase-1 is crucial. Nigericin activates NLRP3 inflammasome that leads 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 BMDM cells. Primed BMDM cells were incubated with MBP-TcpB or MBP alone followed by treatment with nigericin and quantification of LDH and IL-1β. Diminished level of LDH and IL-1β was observed in BMDM cells treated with TcpB compared to MBP treated cells (Fig. 4*G*). The experimental data indicate that TcpB is capable of suppressing other inflammasome-signaling pathways, which is mediated by caspase-1.

TcpB attenuates IL-1β secretion in Brucella-infected macrophages-Brucella are stealthy intracellular pathogen that efficiently evade or suppress host innate immune responses. *Brucella* are weak inducers of TLR4 signaling owing 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 inflammasome, we performed macrophage infection studies followed by analysis of LDH and IL-1β secretion by infected macrophages. We employed *Brucella neotomae* for our studies, which was isolated from desert wood rat and non-pathogenic to human (33). Infection dynamics of *B. neotomae* and the human-pathogenic species, *B. melitensis* were reported to be similar in murine macrophages and both the species induced mortality in IRF-$1^{-/-}$ mice in 9-12 days (34,35). We analyzed the growth dynamics of *B. neotomae* in RAW264 at various time points, which indicated that *B. neotomae* could efficiently replicate in murine macrophages (Fig. 5*A*). Next, we analyzed the induction of pyroptosis by *B. neotomae* in RAW264. We used *Salmonella typhimurium* as the positive control as it induces pyroptotic cell death in murine macrophages (10,36). Primed RAW264 cells were infected with *B. neotomae* or *S. typhimurium* followed by measurement of LDH released into the media. Macrophages infected with *S. typhimurium* released elevated levels of LDH whereas *B. neotomae* infected cells released minimal LDH (Fig. 5*B*). Next, we stained the *B. neotomae* or *S. typhimurium* infected macrophages with Zombie Red dye to visualize the extent of pyroptosis induced by these pathogens. Infection with *S. typhimurium* resulted more Zombie Red positive cells compared to the infection with *B. neotomae* (Fig. 5*C*). These experimental data imply that *B. neotomae* is a weak inducer of pyroptotic cell death.

B. abortus was reported to induce IL-1β secretion in LPS primed BMDMs at 17 hours post infection (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 hours post infection (Suppl. 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 or MBP- TcpB^{G158A} or MBP alone followed by *B. neotomae* infection and analysis of IL-1β levels at 24 hours post infection. The BMDMs treated with MBP-TcpB secreted diminished levels of IL-1β compared to cells treated with MBP alone or MBP-TcpB^{G158A} (Fig. 5*D*). 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. 5*E*), which suggests that the induction of IL-1β is mediated by activation of non-canonical inflammasome signaling. Previous studies demonstrated enhanced level of IL1-β in IRF-1$^{-/-}$ mice infected with *B. melitensis* deficient in *TcpB*. Taken together, the data indicate that TcpB plays essential role in the suppression of pro-inflammatory responses mediated by non-canonical inflammasome activation in *Brucella*-infected macrophages.

TcpB attenuates Salmonella-induced pyroptosis and inflammatory responses- S. typhimurium induces pyroptotic cell death and secretion of pro-inflammatory cytokines in mouse macrophages through the activation of caspoase-11 (38). Therefore, we wished to examine whether TcpB attenuates caspase-11 activation in *Salmonella* infected macrophages. Primed RAW264 or BMDMs were treated with MBP-TcpB or TcpB^{G158A} or MBP alone followed by infection with *S. typhimurium* and measurement of LDH, IL-1α and β by ELISA. LDH and pro-inflammatory cytokine levels were significantly less in *S. typhimurium* infected macrophages treated with TcpB-MBP (Fig 6*A*). As observed previously, MBP-TcpB^{G158A} failed to suppress *S. typhimurium*-induced cell death or cytokine release (Fig. 6*B*). Next, we analyzed the synergistic effect of TcpB in the presence of Z-LEVD-FMK in the context of *S. typhimurium* infection. An enhanced suppression of LDH and pro-inflammatory cytokine release were observed in the samples treated with MBP-TcpB and Z-LEVD-FMK suggesting that TcpB targets caspase-11 to attenuate *S. typhimurium*-induced cell death and cytokine secretion (Fig. 6*C*). These experimental data imply that TcpB is capable of suppressing *S. 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 pro-inflammatory responses mediated by TLR2 and 4 receptors and inhibits CD8$^+$ T cell killing of *Brucella* epitope specific target cells (16,23,41). TcpB also plays essential role in *Brucella*-induced unfolded protein response, which contributes to the intracellular replication of *Brucella* (42). The first hint about the molecular mechanism of TcpB-mediated TLR4 suppression was emerged upon the demonstration of molecular mimicry of TcpB where it mimicked the phosphoinositide phosphate- and cytoskeleton-binding properties of the TLR4 adaptor protein TIRAP (23). Subsequently, TcpB-TIRAP interaction and enhanced ubiquitination and degradation of TIRAP by TcpB were demonstrated to describe the TcpB-mediated suppression of TLR4 signaling (24). However, the mechanism by which TcpB ubiquitinate target proteins remains obscure. It is possible that TcpB recruits host ubiquitin ligases for ubiquitination and subsequent degradation of target proteins.

Since 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 caspse-4/11 serves as essential components for intracellular LPS signaling (7). Recognition of intracellular LPS from Gram negative bacteria by caspases-4/11 drives a non-canonical form of

inflammatory response that leads to pyroptotic cell death and secretion of pro-inflammatory cytokines (6). Given that TcpB attenuates extracellular LPS signaling mediated by TLR4, we sought to analyze whether TcpB subvert 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, which indicated that TcpB promotes ubiquitination and degradation of casepase-4 and 11. Recently, targeting caspase-4/11 for subverting the intracellular LPS signaling was demonstrated for two bacterial pathogens. Effector proteins OspC3 of *Shigella* and NleF of Enteropathogenic *E.coli* targeted caspases-4/11 to attenuate the inflammatory responses. Both the proteins were reported to be binding to the catalytic site of caspases-4/11 that leads to its inhibition (18,19). However, TcpB subverts caspases-4/11-mediated signaling in a unique manner where it induces ubiquitination and degradation of caspase-4 and 11. TcpB targeted caspase-1 also for enhanced ubiquitination and degradation. This may be accounted 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 caspases-mediated cytotoxicity and pro-inflammatory responses. It appears that TcpB interacts with the CARD domain of inflammatory caspases.

We previously reported that TcpB is a cell permeable protein that translocates into the macrophages (26). TcpB harbors a putative protein transduction domain at the N-terminus, which may facilitate its entry into the cells. Recombinant TcpB fused with MBP translocated into mouse macrophages and suppressed the 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 plasmamembrane and enters into the macrophages to attenuate pyroptosis and inflammation by promoting the degradation of capase-4 and 11. The property of TcpB to cross the plasmamembrane 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 the *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 to *S. typhimurium*. However, *B. neotomae* induced IL-1β secretion in BMDMs, which was 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 to induce IL-1β secretion in *B. neotomae*-infected BMDMs, which may occur upstream of NLRP3. Consistently, a recent study reported activation of the NLRP3 by caspase-4/11 to induce IL-1β processing and secretion (10). *S. typhimurium* is a potent inducer of non-canonical inflammasome that leads to pyroptosis and secretion of pro-inflammatory cytokines by the infected macrophages. Treatment of macrophages with recombinant TcpB protein suppressed the pyroptosis and cytokine secretion by *S. typhimurium*-infected macrophages. This confirms that TcpB targets caspase-11 for subverting the cell death and inflammatory responses. A TIR domain-containing protein (tlpA) was reported in S*almonella enterica* serovar Enteritidis, which suppresses TLR4 mediated NF-κB activation and secretion of IL-1β by macrophages (14). It will be interesting to examine whether tlpA also targets non-canonical inflammasome pathway to attenuate IL-1 β secretion by macrophages.

TcpB efficiently suppresses pro-inflammatory cytokine signaling mediated by TLR4 receptor. Our experimental data suggest dual functions for TcpB where it attenuates pro-inflammatory cytokines induced by both the

extracellular and intracellular activation of pathogen recognition receptors. 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 will help the bacteria to establish a replicative niche for sustained infection. However, the detection of intracellular *Brucella* by the PPRs including inflammasome results in secretion of pro-inflammatory cytokines, which activates various anti-microbial responses in the host. Therefore, suppression of pro-inflammatory responses induced by the infected macrophages is essential for *Brucella* to survive in the host. It appears that TcpB plays essential role in suppression of these host innate immune responses for the chronic persistence of *Brucella* in the host. In agreement with this, *B. melitensis* deficient in TcpB was reported to induce elevated levels of pro-inflammatory cytokines in mice compared to the wild-type *B. melitensis* (23).

In summary, our studies demonstrate that TcpB attenuates caspases-4/11-mediated non-canonical inflammasome signalling in mouse and human macrophages. TcpB promotes ubiquitination and degradation of caspase-1, 4 and 11 that attenuates intracellular LPS-induced or bacteria-induced pyroptosis and secretion of inflammatory cytokines. Studies imply that TcpB play major role in the innate immune suppression of *Brucella* by attenuating both the TLR-4- and caspase-4/11-mediated defence mechanisms of the host. The detection of intracellular LPS by caspase-4/11 plays major role in LPS-induced septicaemia in addition to recognition of extracellular LPS by TLR4 (43). Since 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), 1X Penicillin-Streptomycin solution (Gibco) and 100 µg/ml Normocin (Invivogen) was used for culturing Human Embryonic Kidney (HEK) 293T (American Type Culture Collection) and THP1 cells (American Type Culture Collection). RPMI (Sigma) supplemented with 10% fetal bovine serum and 1X Penicillin-Streptomycin solution (Gibco) was used to culture RAW264 (American Type Culture Collection). Cells were grown in a 37° C humidified atmosphere of 5% CO_2. To isolate Bone marrow derived macrophages (BMDM) from mouse, bones were collected from the hind leg of 4-6 week old C57BL6 mice followed by isolation of bone marrow cells using the standard procedure (44). For differentiation, cells were cultured in RPMI supplemented with 10% fetal bovine serum, 1X Penicillin-Streptomycin solution and 25 ng/ml of Mouse-Colony Stimulating Factor (R&D Systems). All the 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, pGBKT7 (Clontech) in fusion with the GAL4 DNA binding domain to generate pBDTcpB (bait). Yeast strain AH109 (Clontech) was transformed with pBDTcpB construct using lithium acetate/single-stranded carrier DNA/polyethylene glycol method. 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 the Y187 harboring human cDNA library was mated according to the manufactures instructions. The diploid yeast colonies were selected on SD agar with quadruple amino acid drop-out (-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 drop out 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 L of LB medium with glucose (2 %) and ampicillin (100 µg/mL) was inoculated with overnight grown *E. coli* BL21 cells (0.1 %) harbouring the pMALTcpB or pMALTcpB^{G158A} mutant plasmid. The culture was grown at 37^{o} C until 0.6 OD at OD600 followed by

induction with IPTG (0.5 mM). After the induction, cells were grown at 25° C for 5 h. Amylose affinity chromatography was employed for purification. Cells were collected by centrifugation and sonicated followed by clarification by centrifugation at 16,000g for 20 min. The supernatant was collected and passed through a column harboring 5 ml of amylose resin (NEB). The column was then washed with the 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% glycerol. The concentration of MBP-TcpB or MBP-TcpB^{G158A} was estimated using Bradford reagent (Sigma). The purified proteins were aliquoted and stored at -80 freezer after snap freezing in liquid nitrogen.

Co-Immunoprecipitation-HEK293T cells ($3x10^6$) 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 X100, 1 mM EDTA and 1X protease inhibitor cocktail (Pierce) followed by clarification of the lysate by centrifugation at 12,000 rpm for 20 minutes. To determine the interaction between TcpB and Caspase-4, purified MBP-TcpB or MBP alone was mixed with the HEK293T lysate containing HA-caspase-4. The lysates were pre-cleared with Protein G plus agarose beads and mixed with 5 µg of anti-HA antibody (Santa Cruz Biotechnology #SC-7392) followed by incubation overnight at 4° C on a rotator. Next, Protein G plus agarose was added into the samples and incubated further for 3 hours at 4° C on a rotator. Subsequently, agarose beads were washed three times with TNT buffer (20 mM Tris [pH8.0], 150 mM NaCl, 1% Triton X100) and re-suspended in 30 µl of SDS sample buffer (BioRad) and boiled for 10 minutes followed by SDS PAGE and immunoblotting. The membrane was probed with horseradish peroxidase (HRP)-conjugated anti-MBP antibody (1:5,000, New England Biolabs #E8038S) in 5% milk in TBST overnight at 4° C. Subsequently, the membrane was washed 3 times with TBST for 5 minutes each and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 minutes, followed by acquiring the luminescent signal using a Chemi-documentation system (Syngene).

Co-transfections to examine the degradation of inflammatory caspases- HEK293T cells (0.5×10^6) co-transfected with 300 ng of pCMV-FLAG-Caspase-4 or pCMV-FLAG-Caspase-11 or pCMV-FLAG-Caspase-1 (pCMV-FLAG-Caspase11 and 1 were gift from Junying Yuan (Addgene plasmid # 21145 and #21142) and increasing concentrations (300 ng, 600 ng, 900 ng and 1.2 µg) of HA-TcpB in 12 well plates. 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 loaded on 12% Tris-Glycine SDS PAGE gel followed by immunoblotting. The membrane was probed with HRP conjugated anti-FLAG antibody (1:5,000, Sigma #A8592) to detect FLAG tagged Caspases and HRP conjugated anti-HA antibody (1:5,000, Sigma #H6533) for detecting HA-TcpB.

To analyze the degradation of endogenous Caspase-11, J774 cells ($0.5x10^6$) were seeded into 12-well plate followed by incubation of cells with purified MBP-TcpB for 5 hours. Next, cells were lyzed in RIPA buffer and subjected to immunoblotting. Endogenous caspase-11 was detected using anti-caspase-11antibody (1:1000, Cell Signaling Technology #14340) followed by HRP-conjugated anti-rat secondary antibody (1:5,000, Cell signaling technology #7077S). Endogenous caspase-1 was detected using anti-caspase-1 antibody (1:1,000, Cell signaling technology #2225S) followed by HRP-conjugated anti-rabbit secondary antibody (1:5,000, Cell signaling technology #7074S). Actin was detected using Monoclonal Anti-β-Actin−Peroxidase Conjugate antibody (1:25,000, Sigma #A3854).

In vivo ubiquitination assays- HEK293T cells ($1x10^6$) were co-transected with 1.5 µg of pCMV-MYC-TcpB, 1.5 µg of pCMV-FLAG-Caspase-4 or pCMV-FLAG-Caspase-11 and 1.5 µg of pCMV-HA-ubiquitin (gifted by Dr. Shigeki Miyamoto) with various combinations. Total DNA

concentration was maintained at 4 µg using the empty vector. Twenty hours after the transfection, the cells were treated with proteosome inhibitor MG132 (Sigma) at a concentration of 20 µm for 4 hours. Next, cells were washed with PBS and lysed in 300 µl of lysis buffer containing 20 mM Tris-HCI [pH 7.4] and the 1% SDS (24). Cell lysates were transferred into Eppendorf tubes and boiled for 10 minutes, followed by clarification of the lysates by centrifugation at 13,000 rpm for 15 minutes. Cell lysates were diluted with buffer containing 20mM Tris-HCl [pH 7.5]; 150 mM NaCl, 2% Triton X100 and 0.5% NP40 (24). Five micrograms of anti-FLAG antibody was added into the lysates and incubated overnight at 4° C on a rotator. Immunoprecipitation and western analysis were performed with samples as described before. The membrane was probed with HRP-conjugated anti-HA and anti-FLAG antibodies to detect HA-Ubiquitin and FLAG-Caspases, respectively. TcpB was detected in whole cell lysate using HRP-conjugated anti-MYC antibody (1:5,000, Sigma #7077S).

Protein internalization-RAW 264.7 ($0.5x10^6$) mouse macrophages were seeded in 12-well plate and incubated with various concentrations of purified MBP-TcpB or MBP alone for 5 hours. Next, the cells were washed with PBS for two times followed by treatment with trypsin EDTA for 1 minute. Cells were washed with PBS for three more times and lysed in the RIPA buffer followed by SDS PAGE and immunoblotting.

Cell death and cytokine release assays-RAW 264.7 cells or THP1 or BMDMs ($0.5x10^5$) were seeded in 96-well plate and primed with Pam3CSK4 for 3 hours. Next, the primed cells were incubated with purified MBP-TcpB or MBP protein (100 µg/ml) for 5 hours in triplicates. Subsequently, the cells were transfected with LPS (2µg/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 800g for 5min and incubated at 37° C humidified atmosphere of 5% CO_2 for 16-20 hours followed by collection of culture supernatant. Cytotoxicity was analyzed by measuring the LDH levels (Takara) in the culture supernatants. The levels of IL-α 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, RAW 264.7 cells were seeded into 12-well plate and primed with Pam3CSK4 for 2 hours, followed by treatment with MBP-TcpB or MBP alone for 5 hours. Cells were transfected with LPS as described before. Four hours post-transfections, the cells were lyzed and subjected to immunoblotting followed by immunoprobing using anti-mouse-IL-1β antibody (1:1000, R&D Systems, # AF-401-SP) to detect pre-and mature form of IL-1 beta. The blot was re-probed with anti-MBP-HRP antibody (1:5000, New England BioLabs, #E8038S) to detect internalized MBP-TcpB and the Monoclonal Anti-β-Actin−Peroxidase Conjugate antibody to detect actin. To analyze effect of TcpB in the presence of caspase-4/11 inhibitor, cells were treated with Z-LEVD-FMK (10 µM, Biovision, USA) for 1 hour followed by incubation with MBP-TcpB or MBP and LPS transfection. For analyzing nigericin induced cell death and IL-1β secretion, BMDM cells ($0.05x10^6$) were seeded in 24-well plate and primed with LPS (100 ng/ml) for 4 hours followed by treatment with MBP or MBP-TcpB protein for 5 hours. 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 the PFA induced cytotoxicity, THP1 cells ($0.1x10^6$) were seeded into 12-well plate and pre-treated with MBP-TcpB or MBP for 5 hours. Next, the cells were treated with 2% PFA in the presence the MBP-TcpB or MBP for 24 hours and followed by Annexin V FITC and PI staining using ApoAlert Annexin V Apoptosis detection kit (Takara) as per manufacturer's instructions. Stained cells were analyzed using a flow cytometer (BD LSRFortessa).

Bacterial infections studies-Salmonella typhimurium (ATCC 14028) was cultured in LB broth (HiMedia) overnight at 37°C. Three hours before the infection, the *S. typhimurium* culture was diluted (1:50) with fresh LB broth containing 300 mM NaCI and grown without shaking for 3 hours at 37° C to induce SPI-1 gene expression. *Brucella neotomae* (ATCC 23459) was cultured in Brucella broth (BD) and the cells were harvested in stationary phase by centrifugation at 6000g for 5 minutes.

For bacterial infections, RAW 264.7 cells or BMDMs (1 x 10^4) were seeded in 96-well plate followed by priming and protein treatment as described before. Harvested bacterial cells were washed two times with PBS and finally resuspended PBS. *S. typhimurium* (MOI of 100) or *B. neotomae* (MOI of 100 or 1000) was added into the wells followed by centrifugation of plates at 800g for 10 min to spin down the bacteria onto the cells. Cells were infected for 90 minutes, followed by treatment with gentamicin at 100µg/ml and 10µg/ml for *S. typhimurium* or *B. neotomae*, respectively to kill the extracellular bacteria. The cells were maintained at 50 µg/ml and 5µg/ml gentamycin for *S. typhimurium* or *B. neotomae*, respectively. Next, the culture supernatant was collected at various time points and analyzed the levels of LDH and IL-α and β. For analyzing TcpB-mediated suppression of IL-1α and β in *Brucella*-infected cells, cells were treated with MBP-TcpB or MBP alone for three hours followed by infection with *B. neotomae* (MOI of 1000) as mentioned before. Infected cells were maintained in MBP-TcpB or MBP for 24 hours. Culture supernatant was collected 12 hours post infection followed by addition of fresh media with proteins.

Zombie Red dye staining of macrophages- RAW 264.7 cells (1x10^5) were seeded in glass-bottom petri plates (Eppendorf) and allowed to adhere overnight. Priming of the cells and protein treatment were performed as described earlier. Next, the cells were transfected with LPS for 12 hours, followed by staining with the Zombie Red dye (Biolegend) as per manufacturer's protocol. Briefly, cells were washed thrice with PBS and stained with Zombie Red dye at 1:200 dilution for 30 minutes at room temperature. Cells were washed two times with PBS and fixed with 4% para formaldehyde solution for 15 min. Next, cells were washed three times with PBS and mounted in ProLong Gold Antifade Reagent with DAPI (Thermofisher). Zombie red dye is excited by yellow/green laser (561nm) and has fluorescence emission at 624nm. Cells were analyzed using the confocal microscope (Leica TCS SP8) in 63x 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 were determined using *t*-test.

Ethical statement- For collection of Bone Marrow Derived Macrophages (BMDMs, C57BL-6 mice were housed at the hired animal house facility of National Institute of Animal Biotechnology at the Teena Biolabs Private Limited, Hyderabad, India. Teena Biolabs Private Limited 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 the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Teena Biolabs Private Limited (Approval number: TBPL-NIAB/05/2016).

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CONFLICT OF INTEREST:-

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS:-

GR conceived and designed the study and wrote the paper. PJ, SN and SM performed experiments. NR performed MBP-TcpB or MBP expression and purification. PJ, SN and GR analyzed the experimental data. All authors reviewed the results and approved the final version of the manuscript.

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FOOT NOTES

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The abbreviations used are: TLR, Toll-like receptor; TcpB, TIR domain-containing protein from Brucella; LPS, lipopolysaccharide; PFA, Paraformaldehyde.

FIGURE LEGENDS

Figure 1 *(A)*. Yeast-two hybrid screening to examine the interaction between TcpB and caspases. AH109 yeast strain was transformed with bait plasmid harbouring TcpB fused with DNA binding domain and the prey plasmid harbouring caspases-4/11/3/caspase-4ΔCARD fused with the DNA activation domain. Both the bait and prey plasmids were selected on SD/-Leu/-Trp dropout medium followed by analyzing their interaction by streaking the yeast on SD/-Ade/-His/-Leu/-Trp medium containing X-α-Gal. The growth of yeast with blue colour on quadruple amino acid drop-out medium indicates the interaction between TcpB and caspases. TcpB interacted with caspase-4 or 11 but not with caspase-3 or caspase-4 deficient for CARD

domain. Yeast harbouring Lamin fused with BD and T-antigen fused with AD served as the negative control, whereas the yeast carrying p53 fused with BD and T-antigen fused with AD served as the positive control; (*B*) Confirmation of TcpB-caspase-4 interaction by co-immunoprecipitation. Lysate of HEK293 cells overexpressing FLAG-caspase-4 was mixed with purified MBP-TcpB or MBP alone followed by immunoprecipitation of FLAG-caspase-4 using anti-FLAG antibody and immunoblotting. MBP-TcpB co-immunoprecipitated with FLAG-capase-4, which suggests the interaction between MBP-TcpB and caspase-4. MBP alone could not be co-immunoprecipitated with FLAG-caspase-4 indicating the specificity of TcpB-caspase-4 interaction. Immunoblot is representative of three independent experiments.

FIGURE 2. TcpB induces degradation of human caspase-4 (*A*) and its mouse orthologue caspase-11 (*B*). HEK293T cells were co-transfected with equal concentration of FLAG-caspase-4 or 11 and increasing concentrations of HA-TcpB. Cells were harvested 24 hours post-transfection followed by lysis and immunoblotting. FLAG-Caspase-4 or 11 was detected using anti-FLAG antibody. Actin served as the loading control. TcpB induced degradation of caspase-4 or 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 hour, followed by harvesting the cells at 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 capase-4. Co-transfection of HEK293 cells with FLAG-caspase-4 and increasing concentrations of HA-$TcpB^{G158A}$ did not change the levels of casase-4 indicating the inability of $TcpB^{G158A}$ to induce the degradation of caspase-4; (*F*) TcpB promotes degradation of caspase-1. HEK293T cells were co-transfected with 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 co-transfected with FLAG-Caspase-1 and HA-$TcpB^{G158A.}$ FLAG-Caspase-1 did not undergo degradation in presence of $TcpB^{G158A.}$; (*H*) TcpB enhances degradation of endogenous caspase-1 and 11 in mouse macrophages. RAW264 cells were treated with MBP-TcpB or MBP, followed by infection with *Salmonella typhimurium* overnight. 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 to the cells treated with MBP alone. Immunoblots (*A* to *G*) are representative of three independent experiments. Immunoblot (*H*) is representative of two independent experiments. Right panels of the immunoblots show the densitometry analysis of caspase bands normalized to actin.

FIGURE 3. TcpB promotes ubiquitination of caspase-11 (*A*), 4 (*B*) and 1 (*C*). HEK293T cells were co-transfected with various combinations of MYC-TcpB, FLAG-caspases and HA-Ubiquitin as indicated. 24 hours post-transfection, cells were treated with MG132 (except for lanes 8 to 9 in Fig. 3*A*) followed by cell lysis and immunoprecipitation of FLAG-caspases. Next, the samples were analyzed by immunoblotting. FLAG-caspases conjugated with HA-Ubiquitin was detected using anti-HA antibody. An enhanced ubiquitination of FLAG-caspase- 11, 1 and 4 were detected in the presence MYC-TcpB. MG132 treated cells showed slightly enhanced levels of FLAG-caspase-11 in the presence of TcpB compared to untreated cells (*A*). We used similar concentrations of FLAG-caspases and MYC-TcpB that resulted in accumulation of ubiquitinated FLAG-caspases in the immunoprecipitated samples. Immunoblots are representative of two independent experiments.

FIGURE 4. (*A*) TcpB suppresses pyroptotic cell death induced by intracellular delivery of LPS. RAW264 or 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 to the 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; whilst “LPS" refers to

extracellular LPS and hence TLR4-mediated detection; *(B)* TcpB treated cells resist pyroptotic cell death induced by LPS. Primed RAW264 macrophages were treated with MBP-TcpB or MBP alone followed by LPS transfections. 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 to MBP treated cells, which had taken up the dye and appeared bright. Images are representative of three independent experiments. Scale bar 30 μm; (*C & D*) TcpB attenuates secretion of pro-inflammatory cytokines induced by intracellular delivery of LPS. Primed RAW264 (*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 to MBP treated cells; (*E*) $TcpB^{G158A}$ could not suppress cell death and secretion of pro-inflammatory cytokines induced by LPS. Primed RAW264 cells were treated with MBP-TcpB or 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 pro-inflammatory cytokines compared to the wild-type TcpB; (*F*) Synergetic effect of TcpB in presence of caspase-4 inhibitor. Primed RAW264 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. TcpB suppresses nigericin induced cell death and IL-1β secretion in macrophages; (G) TcpB suppresses nigericin-induced cell death and IL-1β secretion by macrophages. Primed BMDM cells were incubated with MBP-TcpB or MBP alone followed by treatment with nigericin and quantification of released LDH and IL-1β. Diminished level of LDH and IL-1β was observed in BMDM cells treated with TcpB compared to MBP treated cells. The data were analyzed using Sigma Plot software and statistical significance were determined using *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 5. (*A*) Multiplication of *B. neotomae* in RAW264 cells. Cells were infected with indicated MOI followed by enumeration of CFUs for various time points. *B. neotomae* efficiently multiplied in RAW264 cells; (*B*) *B. neotomae* induced minimal cell death in macrophages. RAW264 macrophages were infected with *B. neotomae* or *S. typhimurium* followed by analysis of secreted LDH by macrophages. *B. neotomae* induced minimal level of LDH in infected macrophages; (*C*) Staining of *B. neotomae* or *S. typhimurium* infected macrophages with Zombie Red dye. *B. neotomae* infected macrophages exhibited minimal staining compared to *S. typhimurium* infected cells, indicating minimal induction of pyroptosis by *B. neotomae*. Image is representative of three independent experiments. Scale bar 30 μm; (*D*) TcpB suppresses IL-1β secretion in *B. neotomae* infected BMDMs. BMDMs were primed with LPS followed by treatment with MBP-TcpB or MBP-$TcpB^{G158A}$ or MBP alone and infection with *B. neotomae*. Secretion of IL-1β by infected BMDMs were analyzed at 24 hours post infection. BMDMs treated with MBP-TcpB secreted less IL-1β compared to 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 were analyzed at 24 hours post infection. 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 were determined using *t*-test. The data are presented as mean ±s.d. from at least three independent experiments (**$P < 0.01$, ***$P < 0.001$).

FIGURE 6. TcpB suppresses *Salmonella*-induced activation of non-canonical inflammasome. RAW264 (*A*) were primed with Pam3SCK4 followed by MBP-TcpB or MBP protein treatment and infection with *S. typhimurium*. TcpB attenuated LDH, IL-1α and IL-1β secretion by RAW264 compared to 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 or MBP-$TcpB^{G158A}$ mutant protein or MBP alone followed by *S. 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 to wild-type MBP-TcpB; (*C*) Synergistic effect of TcpB in the presence of caspase-4 inhibitor in *Salmonella* -induced inflammasome activation. Primed RAW264 cells were treated with MBP-TcpB in the presence or absence

of Z-LEVD-FMK followed by *S. 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 were determined using *t*-test. The data are presented as mean ±s.d. from at least three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

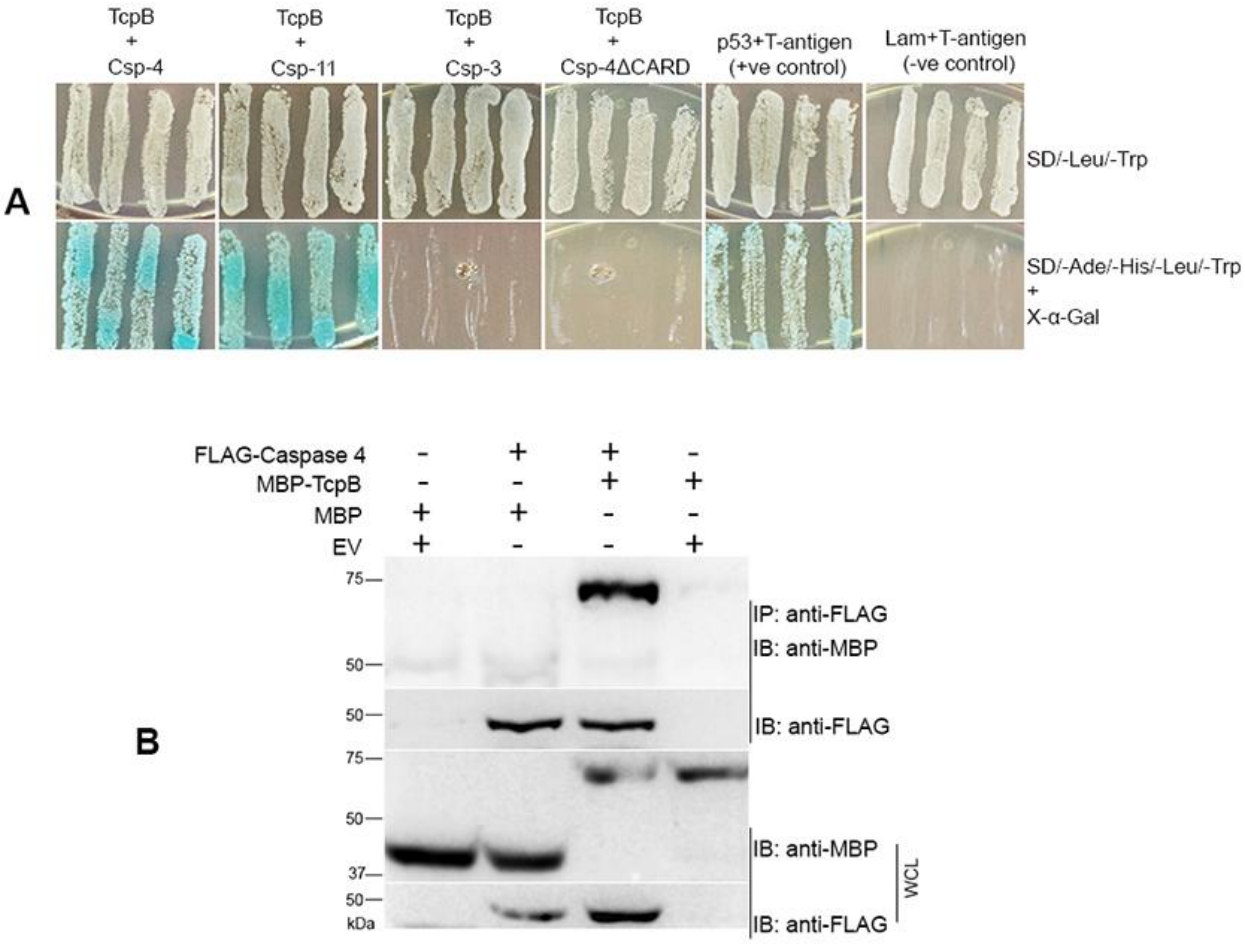
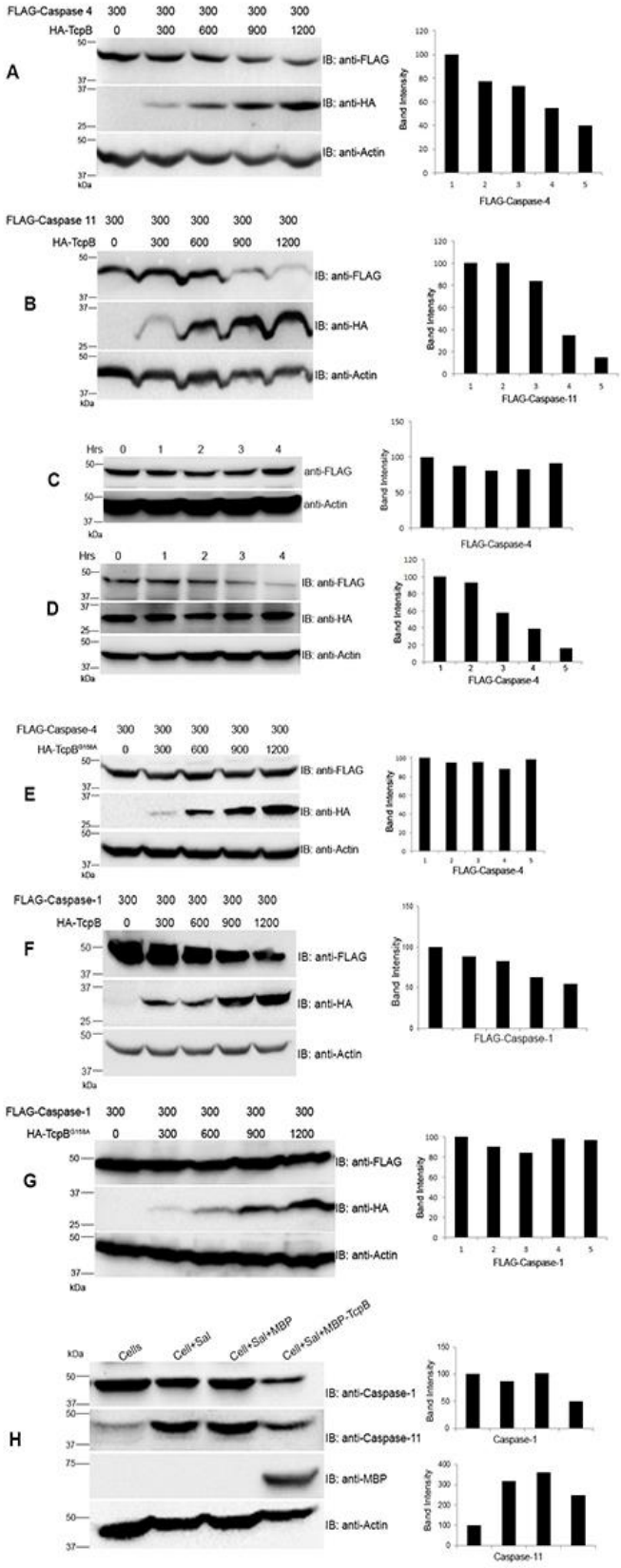


Figure 1



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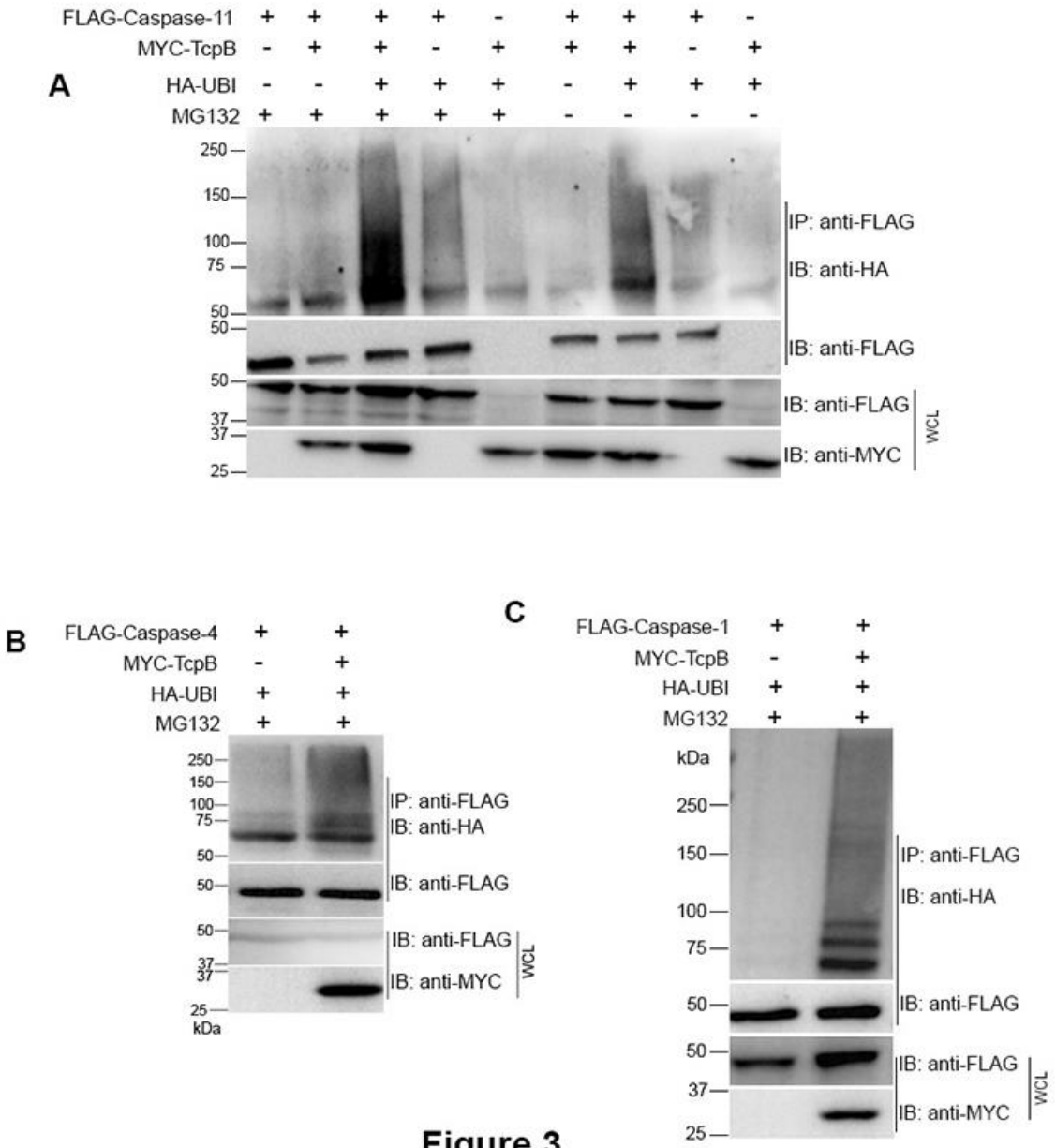

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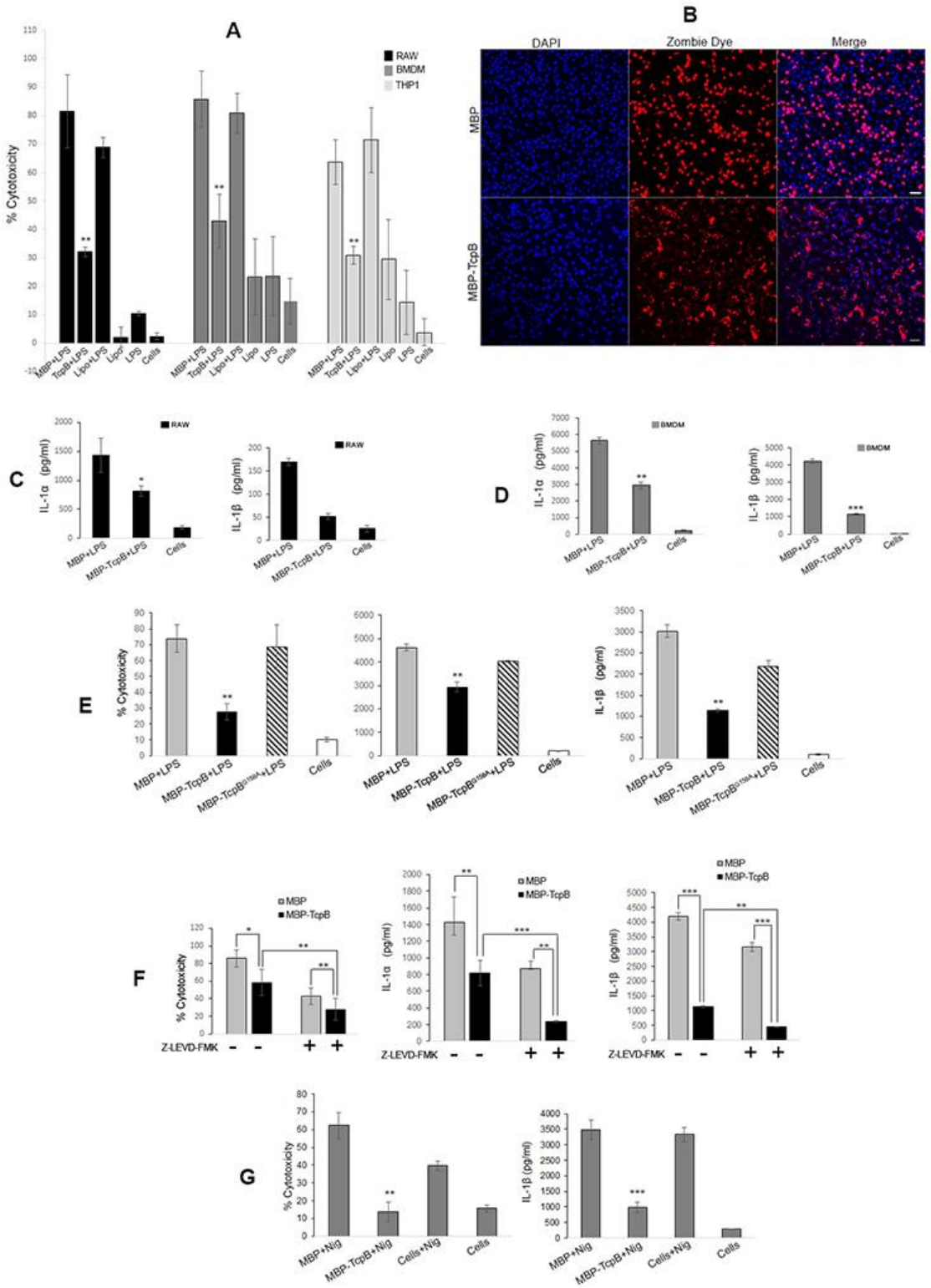

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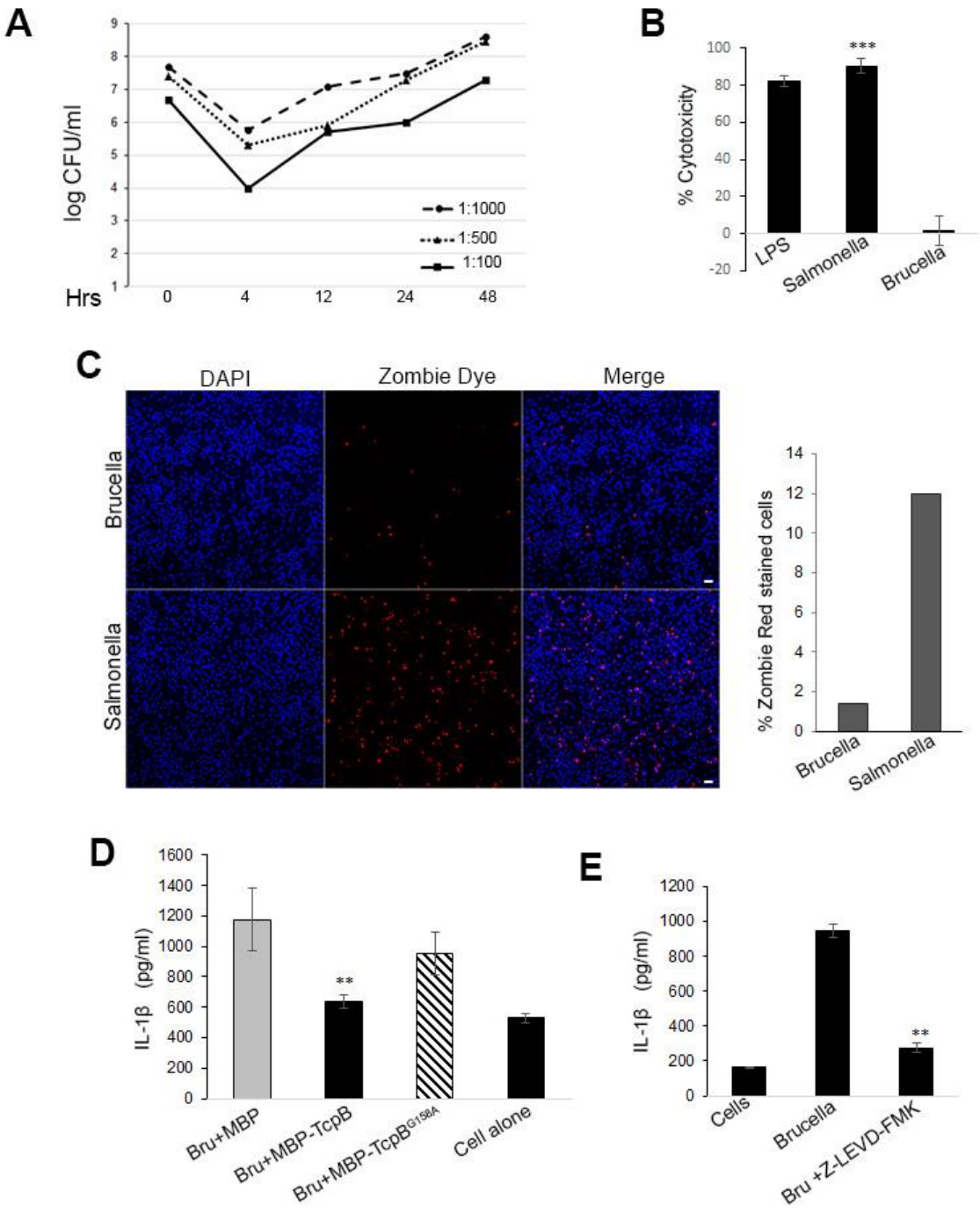

Figure 5

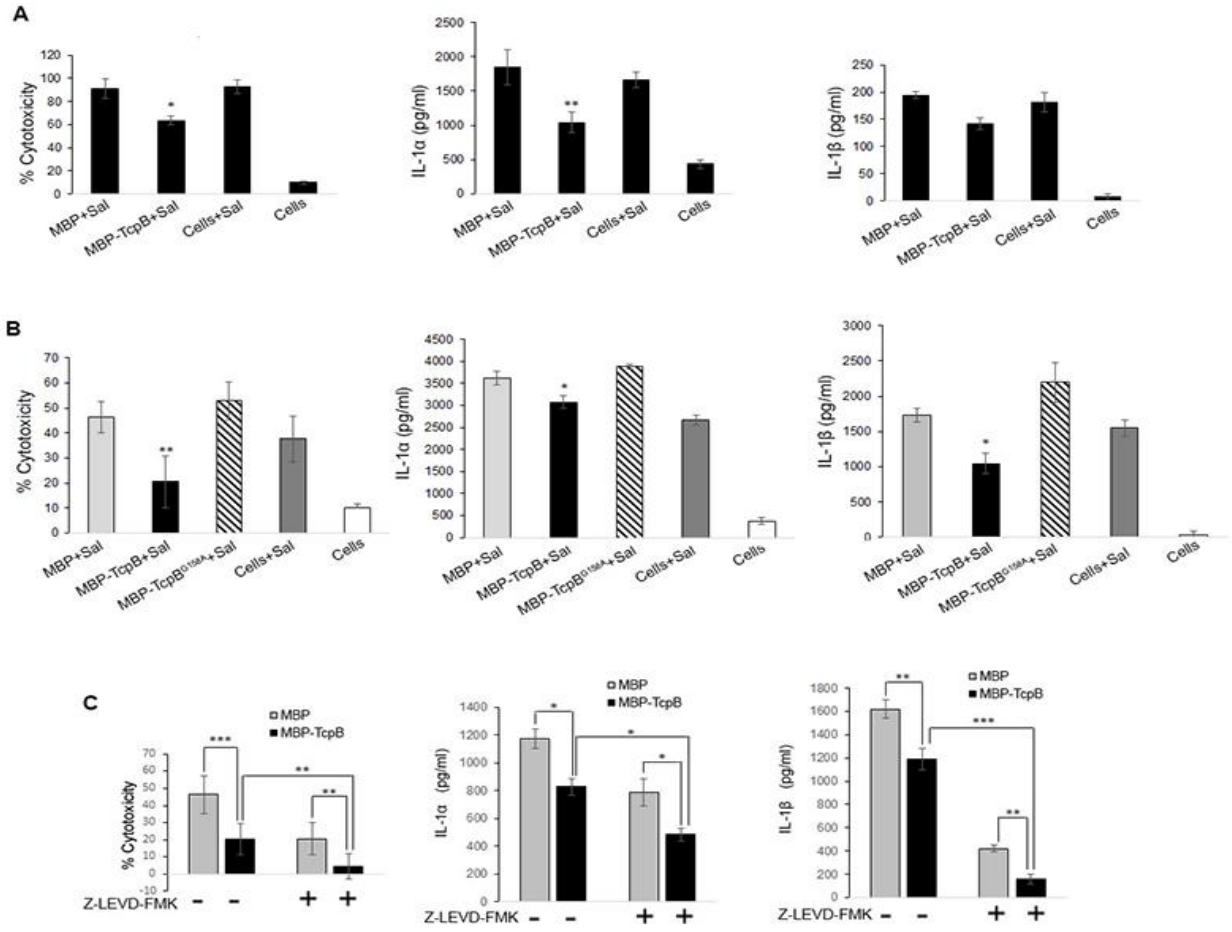

Figure 6

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

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