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Subversion of Innate Immune Responses by *Brucella* through the Targeted Degradation of the TLR Signaling Adapter, MAL

Dola Sengupta,*† Alicia Koblansky,*†‡ Jennifer Gaines,§ Tim Brown,§ A. Phillip West,*† Dekai Zhang,*†‡ Tak Nishikawa,*† Sung-Gyoo Park,*†‡ R. Martin Roop, II,§ and Sankar Ghosh*†‡

Gram-negative bacteria belonging to the *Brucella* species cause chronic infections that can result in undulant fever, arthritis, and osteomyelitis in humans. Remarkably, *Brucella* sp. genomes encode a protein, named TcpB, that bears significant homology with mammalian Toll/IL-1 receptor domains and whose expression causes degradation of the phosphorylated, signal competent form of the adapter MyD88-adapter–like (MAL). This effect of TcpB is mediated through its box 1 region and has no effect on other TLR adapter proteins such as MyD88 or TIR-domain containing adapter protein–inducing IFNβ. TcpB also does not affect a mutant, signal-incompetent form of MAL that cannot be phosphorylated. Interestingly, the presence of TcpB leads to enhanced polyubiquitination of MAL, which is likely responsible for its accelerated degradation. A *Brucella abortus* mutant lacking TcpB fails to reduce levels of MAL in infected macrophages. Therefore, TcpB represents a unique pathogen-derived molecule that suppresses host innate-immune responses by specifically targeting an individual adapter molecule in the TLR signaling pathway for degradation. *The Journal of Immunology*, 2010, 184: 000–000.

Detection of microbes by TLRs is a critical step in activation of the innate immune response and is essential for robust priming of the adaptive immune response (1, 2). TLRs localize to the plasma membrane or to endocytic membranes and recognize certain molecular elements of pathogens called pathogen associated molecular patterns (PAMPs). On ligand with PAMPs, TLRs undergo conformational changes that allow them to engage intracellular adapter molecules, thus initiating a cascade of signaling events that culminates in activation of key transcription factors, such as NF-κB and AP-1. These transcription factors in turn trigger the production of cytokines, chemokines, and antimicrobial peptides that eventually help contain and clear the infection (3). So far, 13 TLRs have been identified in mammals and they all share a leucine-rich extracellular region (LRR) and an intracellular signaling domain known as the Toll/IL-1 receptor (TIR) domain. Although the LRR domain is responsible for binding to PAMPs, it is the TIR domain that binds to TIR-domain containing adapter molecules to initiate signaling (2, 4). Five TIR-domain containing adapter molecules have been described: MyD88 (5), MyD88-adapter–like (MAL)(6) [also known as TIRAP (7)], TIR-domain containing adapter protein–inducing IFNβ, TRIF (also known as TICAM1) (8, 9), TRIF-related adaptor molecule (TRAM; also known as TICAM2) (10–12), and sterile armadillo-motif–containing protein (SARM) (13, 14). Although MyD88 and TRIF can bind to TLRs independent of other adapters, MAL and TRAM can bind only in conjunction with MyD88 and TRIF, respectively. Therefore, MAL and TRAM act as bridging adapters that connect MyD88 and TRIF to their respective TLRs (12, 15). SARM functions only by interaction through TRIF and acts a negative regulator (16). These adapters have unique signaling properties, and their differential use by TLRs results in specialized signaling outcomes that permit a tailored, robust response against the pathogen (2, 17).

The genus *Brucella* is comprised of Gram-negative bacteria that survive and replicate predominantly in macrophages in a broad range of mammalian hosts from cows to humans, causing brucellosis, a zoonotic disease in the latter host (18). The hallmarks of animal brucellosis, in both domesticated and wild animals, are abortion, infertility, and reproductive failures. Infection in humans causes chronic debilitating fever (also known as Malta fever), chills, malaise, arthritis, dementia, and in serious cases, endocarditis and neurologic disorders (19, 20). Human brucellosis primarily occurs either through contact with infected animals or through consumption of dairy products from infected animals, and is caused primarily by *B. melitensis*, *B. abortus*, and *B. suis* strains (21). Brucellosis is the leading zoonosis on a worldwide scale and constitutes a major public health threat in regions of the world where *Brucella* infections are uncontrolled in food animals (20).
**Brucella** sp. have a low infectious dose and are easily aerosolized, and human infections with these bacteria are debilitating and difficult to treat (22, 23). Consequently, these bacteria are presently listed as class B agents on the National Institute of Allergies and Infectious Diseases list of etiologic agents of concern with respect to their potential use in bioterrorism (24, 25).

There is a considerable amount of evidence that indicates that the capacity of *Brucella* to avoid or interfere with components of the host innate, and acquired immune responses plays a critical role in their virulence. The lipid A moiety of the LPS of these bacteria, for instance, elicits a reduced and delayed inflammatory response in infected hosts compared with the endotoxin of some other Gram-negative bacteria and this property has been proposed to allow the brucellae to use a mechanism for a “stealthy” mode of entry into host macrophages (26). The *Brucella* LPS O-side chain also forms complexes with MHC class II and interferes with the capacity of *Brucella*-infected macrophages to serve as APCs (27). Once the brucellae have been engulfed by host macrophages, they use a type IV secretion system to redirect the membrane-bound compartment within which they reside from the endolysosomal pathway into a pathway where this compartment maintains extensive interaction with the endoplasmic reticulum (28–30). Two effectors that appear to be secreted into the host cell cytoplasm by the *Brucella* T4SS have recently been described but the biological function of these effectors is presently unknown (31, 32). The brucellae also produce a periplasmic cyclic β-1,2-glucan that assists these bacteria in modifying their phagosome in such a fashion that it avoids fusion with lysosomes in host cells (33).

The interactions of *Brucella* strains with TLR4 and TLR2 on host cells has been shown to influence the induction of innate immune responses during infection (34). Several recent reports have described the activity of a *Brucella* protein designated TcpB (also known as Btp1) (35, 36) that shares significant amino acid with mammalian TIR domains and interferes with TLR2 and TLR4 signaling pathways when expressed in mammalian cells. TcpB has also been shown to resemble the TIR domain adapter protein MAL, by being able to bind phosphoinositides (37). *Brucella tcpB* mutants do not exhibit attenuation in cultured murine macrophages or immunocompetent mouse models of chronic infection. These mutants do, however, display delayed virulence in the IFN regulatory factor (IRF-1−/−) mouse model of infection, and unlike wild-type (wt) strains they do not inhibit the maturation of murine dendritic cells suggesting that TcpB plays a role in the early interactions of *Brucella* strains with their host cells. In this study, we present evidence that the *Brucella* TcpB targets the TLR adapter protein MAL for degradation, thereby suppressing TLR4 signaling and providing a molecular mechanism for subversion of innate immune responses by these bacteria.

**Materials and Methods**

**Abs**

Rabbit polyclonal anti-TcpB antisera (raised by injecting rabbits with GST-TcpB protein) were affinity purified against GST-TcpB. Anti-HA mouse ascites fluid, anti-HA rabbit polyclonal Ab, anti-M2 mAb, and anti-β-tubulin mAb was from Sigma-Aldrich (St. Louis, MO). Rabbit monoclonal anti-MAL Ab for detecting endogenous MAL was from Epitomics (Burlingame, CA). Rabbit polyclonal anti-VSV Ab was from Zymed (San Francisco, CA). Anti-MyD88 Ab for detecting the endogenous protein was from Santa Cruz Biotechnology (Santa Cruz, CA). Protein-G Sepharose (PGS) and GST Sepharose beads were from Pharmacia (Peapack, NJ). HRP-labeled secondary Abs were purchased from The Jackson Laboratory (Bar Harbor, ME). Dual luciferase assay kit and TNT in vitro transcription and translation kits were from Promega (Madison, WI). Restriction enzymes and other DNA-modifying enzymes were from New England Biolabs (Ipswich, MA).

**Plasmids**

TcpB was amplified by PCR from genomic DNA of *B. melitensis* 16M and cloned into pcDNA3.1 V5B as described later in this paper. TcpBm was created by deleting 9 bp from box 1 of wt TcpB using the QuickChange II Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) and wt TcpB as template.

CD4/TLR4 construct has been described previously. HA-tagged MAL and MAL-DN clones were kindly provided by L. O’Neill (Trinity College, Dublin, Ireland).

**Alignment of sequences and computation three-dimensional modeling**

Multiple alignment of TIR domain was domain was performed by Align X software (Invitrogen, San Diego, CA). Tertiary structure of TIR domain of TcpB, MAL, and TLR4 were predicted by 3D-JIGSAW (version 2.0) server with TLR 2-TIR domain structure (PDB ID: 1FYX) as a model. The predicted structures were further refined for energy minimization by using deepview/Swiss-pdbviewer version 3.7.

**Transfection and immunoblotting**

DNA was transfected into HEK 293 cells using polyethylénimine (PEI), lipofectamine, or Fugiene as indicated in the figure legends. For PEI-mediated transfection, 24 h after seeding HEK 293 cells, DNA was mixed with PEI (2 μg PEI per μg DNA used) and diluted with OptiMEM. After incubation for 20 min at room temperature, the DNA–PEI complexes were added to the wells Where lipofectamine or Fugiene were used, transfections were performed according to manufacturer’s protocol. Cells were harvested 24–48 h later in lys buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton-X100 (TNT) plus protease inhibitors. Equal amounts of each sample was fractioned by 12% SDS–PAGE and transferred to Immobilon. After blocking in Tween 20 and Tris-buffered saline (TTBS) with 5% nonfat milk for 30 min at room temperature, the blots were incubated with primary Ab (in TTBS + 5% nonfat milk) overnight at 4°C (unless noted otherwise), washed three times with TTBS, and incubated with appropriate HRP-conjugated secondary Ab (1:10,000 dilution) for 30 min at room temperature. After three washes with TTBS, bands were detected by ECL according to manufacturer’s recommendation.

**Immunoprecipitation**

HEK 293 cells were seeded (0.8 × 10^6 cells/well) into 6-well plates 24 h prior to transfection. DNA was transfected using either lipofectamine or PEI as described previously. Cells were harvested after ∼30 h in 250 μl TNT buffer plus protease inhibitors. After 15 min incubation on ice, samples were spun at 14,000 rpm for 15 min at 4°C to remove cellular debris. Supernatants (300 μg) were then preincubated with PGS beads with rotation for 30 min at 4°C. Precleared lysates (50 μl) were saved for immunoblotting later. The remainder of the precleared lysates were then incubated overnight with appropriate Ab and PGS at 4°C. Immune complexes were collected by spinning at 5000 × g for 2 min at 4°C. Washed beads three times with TNT, added 30 μl 6× SDS–loading dye, boiled for 1 min, and fractionated on SDS-PAGE. Samples were immunoblotted with the indicated Abs as described previously. For analysis with the same Ab as used for immunoprecipitation, loaded only 5 μl of each sample. Used the reminder for analysis with other Abs. Preimmune lysates were analyzed in identical manner.

**Phosphatase treatment**

Indicated preimmune lysates were treated with 50 U calf intestinal phosphatase (CIP) in presence or absence of 4 mM sodium orthovanadate (Na2V2O5) as inhibitor of CIP and incubated for 3 h at 37°C. Samples were analyzed thereafter as described previously.

**Luciferase assay**

HEK 293 cells (0.8 × 10^6/well) were seeded onto 24-well plates 24 h before transfection. Increasing amounts of TcpB (50, 100, and 200 ng/ well) or TcpB mutant (TcpBm), one stimulant plasmid DNA (CD4/TLR4 [250 ng/well], MAL [25 ng/well], MyD88 [5 ng/well], TRIF [5 ng/well], IκB-1, TRAF 6), NF-κB-luciferase reporter construct (100 ng/well) and Renilla luciferase reporter (50 ng/well) constructs were cotransfected using PEI or Fugiene according to the manufacturer’s recommendation. Total amount of DNA transfected was maintained constant (800 ng) by addition of various amounts of the empty vector. Cells were washed once with PBS 24 h later and then harvested in 100 μl of passive lysis buffer from the dual luciferase assay kit. After incubation on ice for 15 min, 5 μl of the lystate
was assayed using the dual luciferase assay kit according to the suggested protocol. The level of Renilla luciferase activity was used to normalize NF-κB-luciferase activity to serve as control for transfection. Results are expressed as mean fold stimulation over unstimulated controls. Each assay was repeated at least three times, each time in triplicate (CD4/TLR4) or duplicate (all others).

**Ubiquitination assay**

HEK293 cells (0.8 × 10⁶ cells/well in 6-well plates) were transfected with plasmids encoding HA MAL (300 ng/well) and VSV-Ubiquitin (1.0 µg/well) in the presence (1.2 µg/well) of absence of TcpB as described previously. Half of the samples were treated 24 h later with MG132 (20 mM) for 4 h. Cells were washed and harvested in PBS and spun at 2500 rpm for 3 min. Pelleted cells were resuspended in 300 µl SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl, pH 8.0, and boiled for 10 min. They were then sonicated for 5-s pulses and 1-s pauses for a total of 30 s, followed by centrifugation at 16,000 × g for 15 min. Supernatant was transferred to a new tube. Five hundred microliters 2 × IP buffer (2% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, 8.0, 1 mM EDTA, and 0.5% NP-40) was added to 300 µg total protein (volume made up to 500 µl with ddH₂O). Preclearing and immunoprecipitation of the lysates thereafter were carried out as described previously.

**Construction of TcpB**

TcpB open-reading frame was amplified from *B. melitensis* 16M genomic DNA using the following primers: Fwd: 5′-CGGTTGACAGGCTCAGACCGCACAGAACCCGCAATCTGCGGAT-3′ and Rev: 5′-CGATTGTCGCTGATCCGCATGTTCCCG-3′. The ~750 nt, amplified product was gel purified and ligated to pCNDNA 3.1-V5-His B vector and transformed into *Escherichia coli* DH5α cells. Putative colonies containing the correct plasmids were identified by analysis of the minipreps of the resulting bacterial colonies.

**Construction of the B. abortus tcpB mutant strain**

The tcpB open-reading frame was PCR amplified from *B. abortus* 2308 chromosome, using the following primers: Fwd.: 5′-CTTGTTAGGACACCGCTCAAT-3′ and Rev: 5′-CAGATTGTCGCTGATCCGCATGTTCCCG-3′. This resulted in a product of 1961-bp that was ligated into pGEM-T Easy (Promega) according to manufacturer protocols. The ligation mixture was transformed into *E. coli* DH5α with blue/white selection, putative colonies containing the desired plasmid were grown and stocked in 25% glycerol/Brucella broth. After confirmation of the transformation product, the plasmid pTBtcpB was cut with the blunt cutters EcoRV and NraI, which removed an internal fragment of 577 bp from the tcpB coding region. The kanamycin resistance cassette (aph3β) from pKKS-Kn was inserted into the resulting blunt site and this plasmid was given the designation pTBtcpBD. This plasmid was dialyzed and electroporated into *B. abortus* 2308 using previously described methods (38). The resulting transformants were cultivated on 5% L agar supplemented with 0.5% ampicillin, 10 µg/ml tetracycline, and 20 µg/ml kanamycin. Plasminogen was added to 5% with 100 µl/ml of 2308 broth and stored at −80°C in Brucella broth supplemented with 25% glycerol. Chromosomal DNA was isolated from all of these putative mutants and PCR analysis of the mutated tcpB region was performed using primers specific for the kanamycin resistance gene (aph3β), the ampicillin resistance gene (bla) from the pGEM plasmid, the entire tcpB gene, and a 400-bp region representing the sequences deleted from the tcpB coding regions. This analysis yielded the expected products with the aph3β- specific primers and tcpB-specific primers with the bla- or 400-bp deleted region-specific primers. One mutant was given the designation KB2 and selected for further analysis. The crystal violet exclusion assay (39) was performed on KB2 to ensure that this strain had retained is smooth lipopolysaccharide phenotype. Brucella strains that spontaneously lose their LPS O-chain (this changes them from the “smooth” to the “rough” LPS phenotype) are highly attenuated in animal models.

**Infection of J774 cells with *B. abortus* tcpB mutants**

Infection of macrophages with *Brucella* was performed in a biosafety level 3 laboratory according to the protocol described by Gee et al. (40). Briefly, J774 cells grown in T75 flasks were cultivated in DMEM supplemented with 5% FCS at 37°C in 5% CO₂. After 24 h of growth, the confluent monolayers were infected with 2308 or the tcpB mutant. Infectious strains were opsonized for 30 min with a subagglutinating dilution (1:100) of hyperimmune C57BL/6 mouse serum at a multiplicity of infection (MOI) of 50 *Brucella* per macrophage (MOI of 50:1) and incubated at 37°C with 5% CO₂ for 1 h to allow for phagocytosis of the *Brucella*. Cell culture medium was removed replaced with fresh medium supplemented with 5% FCS and 50 µg/ml gentamicin to kill any remaining extracellular bacteria and incubated at 37°C with 5% CO₂ for 2 h. The macrophages were then washed with PBS supplemented with 0.5% FCS and maintained thereafter in DMEM with 5% FCS and 12.5 µg/ml gentamicin. Cell culture medium was replaced with fresh medium every 24 h. At 48 h post-infection, macrophages were washed with PBS supplemented with 0.5% FCS and lysed with protein sample buffer supplemented with 0.1% deoxycholic acid. After 5 min incubation at room temperature, macrophage lysates were centrifuged for 30 min at 10,000 rpm, supernatant collected, frozen and lyophilized and cellular debris discarded. J774 lysates were then boiled for 15 min. J774 lysate protein concentrations were then determined by the Bradford method. Where necessary, the lysates were further concentrated by acetone precipitation as follows. Four volumes of ice cold acetone added to one volume of lysate, and stored overnight at −20°C. Proteins were recovered by centrifugation at 14,000 rpm for 15 s and samples were resuspended in protein lysis buffer (0.3% [w/v] SDS, 200 mM DTT, 22 mM Tris-base, 28 mM Tris-HCl, pH 8.0). Samples were then analyzed by Western blot analysis with anti-MAL Ab (1:1000 dilution) as described in Materials and Methods.

**Western blot analysis of cultures of *B. abortus* and *B. abortus tcpB mutants**

*Brucella abortus* cultures were inoculated at 1 × 10⁶ CFU/ml in Brucella broth and incubated at 37°C under 5% CO₂ with shaking. Bacteria were pelleted at 48, 72, and 96 h, resuspended in 1 ml 1× protein lysis buffer (0.3% [w/v] SDS, 200 mM DTT, 2 mM Tris-base, 28 mM Tris-HCl, pH 8.0), boiled for 30 min, and lysed by bead beating six times for 40 s. Samples were then centrifuged for 20 min at 14,000 rpm and supernatant collected. Total cell protein concentrations were determined by the Bradford method and equal amounts of protein were separated onto a SDS-PAGE gel. After transfer to nitrocellulose membrane, TcpB protein was detected using anti-TcpB Ab (1:1000) and goat anti-rabbit Ig G conjugated to HRP (1:10,000). Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, IL).

**Spleen colonization data**

Spleen colonization data were obtained as described previously (40). Briefly, 6–8-wk-old female BL6 mice were infected via the i.p. route with approximately 5 × 10⁴ *Brucella* at 1, 2, 3, and 4 wk postinfection, five mice per experimental group were sacrificed by isoflurane overdose. Immediately after euthanasia, spleens were harvested aseptically and homogenized in sterile PBS. Spleen homogenates were then serially diluted 10-fold in PBS and plated onto SBA to determine the number of *Brucella* present at each time point. Spleen homogenates were also plated in parallel on SBA supplemented with the appropriate antibiotics to confirm the identity of the isolates and monitor the stability of the plasmid in MEK2Cm. Mean averages of counts from each test group were determined, and the data were expressed as log₁₀ *Brucella*/spleen.

**Results**

**TIR domain containing Brucella protein inhibits signaling by the TLR4 pathway**

Using the sequence of the TIR domain of hTLR4 to search for related coding sequences in the *B. melitensis* 16M genome sequence, we identified a coding sequence homologous to mammalian TIR-domain sequences and originally gave it the designation TIRB (TIR-domain-like protein of *Brucella*). This protein, encoded by BMEI1674 in the *B. melitensis* 16M genome and BAB1_0279 in the *B. abortus* 2308 sequence, has subsequently been given the designations TcpB and BtpA in the literature (35, 36, 41). For clarity, we will use the designation TcpB. As shown in Fig. 1A, box I, alignment of the TIR domain of TcpB with other TIR domains from various TLRs and adapters such as MyD88 and MAL indicates that TcpB is most similar to the box 1 region. However, unlike the TIR domains of these other proteins, TcpB lacks a well-defined box 3 region, missing even the most highly conserved amino acid residues of the
amplified the TcpB coding region from ∼26 kDa (Fig. 1). In vitro transcription/translation of this plasmid yielded a protein of MW ∼25 kDa (lane 1), as predicted from the sequence and not seen with empty vector (lane 1). Rabbit polyclonal Ab raised and affinity purified against GST-TcpB detects two bands ∼25 kDa when TcpB is expressed in 293 cells (lane 1). These bands are not detected in 293 cells expressing empty vector (lane 2) or when preimmune serum was used for immunoblotting (lane 4). Precipitation of the Ab with excess GST TcpB also abrogates detection of these bands (lane 5). DNA corresponding to 3 aa residues FIS (red box) within the conserved box 1 region of TcpB-TIR (Fig. 1D) did not have a similar inhibitory effect (Fig. 1A, lane 3, E). E. coli TcpB is recognized by rabbit polyclonal Ab raised and affinity purified against GST-TcpB in a dose dependent manner, as shown in Fig. 2A. Expression of a mutated form of TcpB, TcpBm, that contains a replacement of 3 aa (phenylalanine-isoleucine-serine) in box 1 region of TcpB-TIR (Fig. 1D, lane 1, E) did not have a similar inhibitory effect (Fig. 2A), suggesting that the box 1 region of TcpB is necessary for its function. Interestingly, however, TcpB could not inhibit the activation of NF-κB by overexpression of MAL, MyD88, TRIF, IRAK-1, or TRAF6 (Fig. 2C–F, respectively). These data indicate that TcpB likely inhibits TLR4 signaling by interfering with an event preceding the binding of TIR-containing adapters MAL, TRAM, MyD88, or TRIF.

Interaction with TcpB reduces the level of phosphorylated MAL

It has recently been demonstrated that MAL contains an N-terminal phosphoinositide-binding domain that allows it to recruit MyD88 from the cytosol and target it to domains close to TLR2 and TLR4.
Remarkably, TcpB was also shown to resemble MAL by being able to bind phosphoinositides (37). The TIR domains of TLR2/4, MyD88 and MAL then create a platform that enables MyD88 to bind IRAK4 via its death domain to initiate signaling (46). We therefore wanted to test whether TcpB might inhibit signaling by interfering with the interactions between the TIR domain of TLR4 with MAL or MyD88. We transfected cells with TcpB, HA-MAL, or Flag-MyD88, immunoprecipitated TcpB, and analyzed the presence of coimmunoprecipitated MAL or MyD88 by immunoblotting with Abs against the HA or Flag epitopes. The results show that TcpB interacts with MAL (Fig. 3A), but not with MyD88 (Fig. 3B). In addition, neither TLR4 nor MyD88 immunoprecipitates TcpB (Fig. 3C) suggesting that TcpB functions through its interaction with MAL or MyD88 by immunoblotting with Abs against the HA or Flag epitopes. The results show that TcpB interacts with MAL (Fig. 3A), but not with MyD88 (Fig. 3B). In addition, neither TLR4 nor MyD88 immunoprecipitates TcpB (Fig. 3C) suggesting that TcpB functions through its interaction with MAL. Moreover, TcpB expression had no effect on interactions of MyD88 and MAL (Fig. 3D), which suggests that TcpB does not block TLR4 signaling by disrupting this interaction. Finally, TcpB was unable to interact directly with IRAK-1, IRAK-2, or TRAF-6, other key players in the TLR4 signaling pathway (data not shown).

Previous studies have shown that two forms of MAL are detected on expression in cells, where the slower migrating band corresponds to a phosphorylated form (47). As expected, in the absence of TcpB, expression of HA MAL generated two bands (Fig. 4B, lane 1), but in presence of increasing amounts of TcpB, the level of the upper band was dramatically reduced (Fig. 4A, lanes 2 and 3, top panel; 4B, lanes 1–4, top two panels). At the highest concentration of TcpB tested, the lower band is also reduced (Fig. 4B, lanes 1 and 4). The extent of reduction was more pronounced after 45 h than after 24 h (Fig. 4B). Treatment with CIP abolishes the slower migrating band confirming that the upper band corresponds to a phosphorylated version of MAL (Fig. 4E and Supplemental Fig. 4). The effect of TcpB on MAL is dependent on the intact box 1 region of TcpB, as a mutation in this region abrogated the effect of TcpB on MAL (Fig. 4D). This effect of TcpB was specific for MAL because TcpB had no detectable effect on coexpressed MyD88 (Fig. 4C). Remarkably, TcpB has no effect on a MAL DN mutant in which proline 125 has been changed to histidine (P125H), thus rendering this mutant defective in both phosphorylation and signaling (Fig. 4F) (6, 7, 48). We also wanted to test whether TcpB had a similar effect on endogenous MAL. Using a rabbit mAb that recognizes endogenous MAL in 293 cells, we observed that the slower migrating form was abolished in presence of TcpB (Fig. 5A). Therefore, these results suggest that TcpB specifically abolishes the signal-competent, phosphorylated form of MAL, thereby affecting signaling through TLR4.

Interaction of MAL with TcpB leads to enhanced polyubiquitination of MAL

The results presented previously indicate that TcpB predominantly affects the phosphorylated version of MAL, although it is unclear if the observed reduction in the slower migrating form of MAL reflects reduced phosphorylation, enhanced dephosphorylation, or enhanced degradation. It has been shown previously that suppressor of cytokine stimulation-1 (SOCS-1) can negatively regulate signaling by TLR2 and TLR4 through degradation of MAL by an ubiquitination-dependent process (49). To test whether TcpB might also lead to enhanced, ubiquitin-dependent degradation of MAL, we expressed HA-MAL, TcpB, and VSV-ubiquitin in the...
The extent of ubiquitination is dramatically increased in the presence of TcpB, though MAL expressed by itself is ubiquitinated to a low level. Hence, it is unclear whether TcpB itself is responsible for enhanced ubiquitination of MAL, or whether it acts by recruiting some other ubiquitin ligase. Given this effect of TcpB on MAL it might seem surprising to find that B. abortus 2308 and the isogenic tcpB mutant exhibit equivalent patterns of intracellular survival and replication in cultured murine peritoneal macrophages and spleen colonization profiles in experimentally infected BALB/c and C57BL6 mice (Supplemental Fig. 3). These findings, however, are consistent with other published reports that other Brucella mutants do not display attenuation in cultured murine macrophages, HeLa cells or in mice with intact immune systems.

Discussion

The Gram-negative bacteria *Brucella* encodes a protein named TcpB, that has significant homology to TIR domains, particularly in the box 1 region. Remarkably TcpB acts as a negative regulator of TLR4-mediated signaling by inducing targeted degradation of MAL, one of the components of the TLR signaling cascade that shares phosphoinositide binding property of TcpB (37). Other components of this pathway, such as MyD88, TLR4, IRAK 1 and 4, and TRAF6 are not affected by TcpB, as shown by luciferase reporter assays as well as immunoprecipitation studies with overexpressed proteins. Immunoblotting analysis of endogenous MAL also showed targeted degradation of MAL but not of other reporters. Given this effect of TcpB on MAL it might seem surprising that when coexpressed in HEK 293 cells, TcpB does not inhibit the activation of NF-κB. We believe that in transfected cells there is sufficient level of other signaling competent TIR-domain adapters, thereby making it difficult to detect significant inhibition of NF-κB activation by MAL in presence of TcpB.

Unlike infection by *Salmonella*, infection by *Brucella* is not usually fatal, but causes chronic debilitating disease (20). This bacteria, just like many other intracellular microbes, tries to avoid elicitation of a strong immunologic response that might destroy its replicative niche. However, the overall survival of the host could be compromised if the bacteria completely cripple the host immune system. Therefore, many microbes have evolved finely tuned mechanisms that interfere with host immunity to create a replication permissive environment, but not act to completely shut off host immune function. By using TcpB to specifically inhibit MAL signaling, *Brucella* sp. can effectively dampen a critical microbial infection mechanism, while keeping the larger innate immune response intact. Degradation of the phosphorylated MAL molecules would afford an excellent means of exerting regulated control on the TLR4-MAL-MyD88 signaling pathway. As MAL is a unique adapter in TLR2 and TLR4 signaling,
downregulation of MAL is a potent means of inhibiting the expression of proinflammatory cytokines and chemokines downstream of these receptors, while not inhibiting signaling via other TLRs. In fact, SOCS-1–mediated degradation of MAL after stimulation by LPS serves a similar purpose by helping to reset the cell to its basal state. TcpB in Brucella also acts as a negative regulator of TLR4-mediated responses by degrading the signaling competent form of MAL. By targeting MAL, it ensures that only

FIGURE 4. Overexpression of TcpB in 293 cells lead to degradation of phosphorylated and signaling competent form of MAL. A, HA-MAL was transfected into HEK 293 cells in presence or absence of TcpB and immunoprecipitated with anti-HA Ab, and cell lysates were immunoblotted with anti-HA Ab. B, HEK 293 cells were transfected with 200 ng of HA-MAL in absence or presence of increasing amounts (0, 200, 400, 800 ng) of TcpB. Cells were harvested after 24 h or 48 h as indicated and immunoblotted with anti-HA Ab, or anti-TcpB Ab or anti-β tubulin (as loading control). The levels of HA-MAL were quantitated by densitometry and presented below each panel. C, Flag-MyD88 (200 ng) was transfected into 293 cells together with 0, 200, 400, or 800 ng of TcpB. Cells were harvested after 24 h and immunoblotted with anti-flag Ab. D, HEK 293 cells were transfected with 200 ng of MAL in absence or presence of either 800 ng TcpB or TcpBm. Cells harvested after 24 h were immunoblotted with anti-HA Ab. D, HEK 293 cells were transfected with 200 ng of HA-MAL in absence (lane 3) or presence of 800 ng of TcpB (lane 2) or TcpBm (lane 1). Cells were harvested after 48 h and immunoblotted with anti-HA Ab. E, HEK 293 cells were transfected with HA MAL. After 40 h, one third of the cell lysates were treated with CIP alone and another one third with CIP in presence of CIP inhibitor, Na2V2O5. Samples were then immunoblotted with anti-HA. F, DN-MAL (signaling incompetent) (200 ng) were transfected into 293 cells in absence or presence of increasing amounts of TcpB. Cells were harvested after 24 h and cell lysates were immunoblotted with anti-HA Ab as well as anti-TcpB Ab. All transfections were performed using PEI.

FIGURE 5. Endogenous MAL is degraded by TcpB transfected into 293 cells, as well as in J774 cells infected with B. abortus 2308. A, HEK 293 cells were transfected with or without TcpB and cells were harvested after 40 h. After fractionation on 12% gels and transfer to membranes, they were immunoblotted with rabbit monoclonal anti-MAL Ab (1:1000). B, J774 cells were infected with either wild-type B. abortus 2308 or a mutant form lacking boxes 1 and 2. Forty-eight hours postinfection, a Western blot analysis of the cell lysates was performed with anti-MAL Ab as described in A (upper panel) or anti-β tubulin Ab (lower panel). Uninfected J774 cells were assayed in parallel. C, Same as in B, except that Western blot analysis was performed with anti-MyD88 Ab that recognized endogenous MyD88.
MAL is required for recruitment of MyD88 from the cytosol and delivering it to the plasma membrane via its phosphoinositide-containing domain. There, a tertiary complex between TLR4, MAL, and MyD88 is presumably formed through interaction of their TIR domains. Such complexes can provide the scaffold for formation of further signaling complexes containing IRAK4, IRAK1, etc. (46, 54). The phosphoinositide binding property of TcpB (37) probably allows it to colocalize to the same region in the membrane and facilitate its ability to interact with MAL and cause its degradation. Conformational changes in TLR (induced by ligation with PAMP or other means) probably result in release of these complexes from TLR4, as well as activation of the signaling cascade. Although nothing is known about the fate of the released MAL molecule at this stage, it is possible that they are recycled back to the plasma membrane to be repositioned and be ready to initiate another cycle of signaling. It has been shown that in 293 cells unphosphorylated MAL shows increased association with TLR4 and MyD88 (48, 54), suggesting that perhaps phosphorylation of MAL is necessary for the release of MAL from the TLR complex. By degrading MAL, TcpB reduces the amount of available phosphorylated MAL, thereby, slowing down the signaling process, without completely shutting down the system. In mouse macrophage cell lines, where we do not detect phosphorylated forms of MAL, TcpB could be exerting similar effect simply by reducing the amount of available MAL molecules by degrading them. Such slowdown would allow the bacteria to survive inside the cell without the possibility of causing cell death because of unregulated overactive signaling event (as seen in case of Salmonella poisoning) or because of fatal or severe sepsis, as seen in case of LPS tolerization.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure Legends

Supplementary Figure 1
Genetic organization of the 1961 nt PCR fragment amplified from the chromosome of *B. abortus* 2308 and cloned into pGEMT-EZ (numbers are the nucleotide designations in the chromosome I sequence in GenBank), as described in Supplemental methods.

Supplementary Figure 2
TcpB is not expressed in *B. abortus tcpB* mutant. WT and *tcpBm* mutant cultures were grown in *Brucella* broth and harvested after indicated amount of time. West blot analysis was done with cell lysates using anti-TcpB antibody as described in methods and material.

Supplementary Figure 3
No differences in establishment of infection by *B. abortus* wt and *tcpBm* strains were observed up to 4 weeks post infection, as determined by the spleen colonization data, described in the supplementary method.

Supplementary Figure 4
TcpB does not appear to be phosphorylated. HEK-293 cells were transfected with 200ng of HA-MAL, 800ng of TcpB, and 1000ng of empty vector (EV) with PEI. After 24 hours, cells were harvested and either treated with the lamda phosphatase or left untreated; all samples were incubated for 2 hours. Cell lysates were analyzed by immunoblotting with anti-HA, anti-TcpB and anti-GAPDH antibodies. Data are representative of three independent experiments.
Supplementary Figure 1: Genetic organization of mutant *B. abortus* 2308 *tcpBm* lacking boxes 1 and 2

Genetic organization of the 1961 nt PCR fragment amplified from the chromosome of *B. abortus* 2308 and cloned into pGEMT-EZ (numbers are the nucleotide designations in the chromosome sequence in GenBank)
Supplementary Figure 2. Western Blot with Cell lysate from *B. abortus* 2308 and KB2
Supplementary Figure 3. Spleen colonization data for *B. abortus* 2308 containing WT and mutant TcpB
Supplementary Figure 4. Protein phosphatase treatment experiment

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MAL (phosphorylated) | MAL (unphosphorylated) | TcpB | GAPDH

Lane 1, 2, 3, 4, 5, 6, 7