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

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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 poly-ubiquitination 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 ligatiion 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-kB 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 as 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 causes chronic debilitating fever (also known as Malta fever), chills, malaise, arthritis, dementia, and in serious cases, endocarditis and neurologic disorders (19, 20). **Brucella** 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 TLR 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 (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 pDNA3.1 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 (Invirotgen, 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 polyethyleneimine (PEI), lipofectamine, or Fugene 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 cells where lipofectamine or Fugene 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, 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 + 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 preclarified with PGS beads with rotation for 30 min at 4°C. Precleared lysates (50 μl) were saved for immunoblotting later. The remainder of the preclarified 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 (Na₈V₃O₈) 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], Irak-1, TRAF 6), NF-kB-luciferase reporter construct (100 ng/well) and Renilla luciferase reporter (50 ng/well) constructs were cotransfected using PEI or Fugene 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 lysate...
was assayed using the dual luciferase assay kit according to the suggested protocol. The level of Renilla luciferase activity was used to normalize NF-kB-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 × 10^6 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) or absence of TcpB as described previously. Half of the samples were treated 24 h later with MG132 (20 mM) and further characterization, grown overnight 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 X protein lysis buffer (0.3% [w/v] SDS, 200 mM EDTA, and 0.5% NP-40) and 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 × 10^4 Brucellae. 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 brucellae 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 log_{10} brucellae/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 in the Brucella 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 highly similar to the box I 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) to transcription/translation of this plasmid yielded a protein of MW DNA and cloned it into the pcDNA3 expression vector. In vitro MAL, and other TLRs (42, 43) (Fig. 1) predicted a conformation similar to TIR domains from MyD88, the box 2 region, computer modeling of the TIR region of TcpB A MAL (Fig. 1) served proline that is absolutely critical for signaling via TLR4 and conserved, and lacks some characteristic residues such as the conserved canonical TIR sequence (Fig. 1A). The box 2 region is also poorly conserved, and lacks some characteristic residues such as the conserved proline that is absolutely critical for signaling via TLR4 and MAL (Fig. 1A). Despite the lack of a box 3 region and divergence in the box 2 region, computer modeling of the TIR region of TcpB predicted a conformation similar to TIR domains from MyD88, MAL, and other TLRs (42, 43) (Fig. 1B).

To further investigate the potential functional role of TcpB, we amplified the TcpB coding region from B. melitensis 16M genomic DNA and cloned it into the pcDNA3 expression vector. In vitro transcription/translation of this plasmid yielded a protein of MW ~26 kDa (Fig. 1C), as predicted by the open-reading frame in the DNA sequence. The open-reading frame was then fused in frame with GST in a pGEX vector, and recombinant GST-TcpB was generated in E.coli BL21 cells. The purified GST-TcpB was used to generate rabbit polyclonal antisera, which recognized two bands of ~25 kDa when TcpB was expressed in transfected 293 cells (Fig. 1D). 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). Preincubation of the Ab with excess GST TcpB also abrogates detection of these bands (lane 5). E. coli 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. 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.

Expression of the Brucella TcpB can inhibit signaling through TLR4

It has been reported previously that B. abortus is recognized by TLR2 and TLR4 (44, 45). To test whether TcpB might have any effect on activation of NF-κB via the TLR4 signaling pathway, 293T cells were transfected with a constitutively active CD4/TLR4 chimera in the presence or absence of TcpB, and activation of NF-κB was determined by measuring activity of a NF-κB-driven luciferase reporter construct. Coexpression of TcpB inhibited the activation of NF-κB by CD4/TLR4 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 3, 1E) 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). 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.
on the plasma membrane. 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. 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). 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. 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
TcpB of *Brucella* Causes Degradation of MAL

**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 adapters. 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, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger cal microbial detection mechanism, while keeping the larger
downregulation of MAL is a potent means of inhibiting the expression of proinflammatory cytokines and chemokines down-stream 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 \textit{Brucella} also acts as a negative regulator of TLR4-mediated responses by degrading the signal competent form of MAL. By targeting MAL, it ensures that only

\begin{figure}[h]
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\caption{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-b tubulin (as loading control). The levels of HA-MAL were quantitated by densitometry and presented below each panel. C, Flas-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.
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{supplementary_figure5.png}
\caption{Endogenous MAL is degraded by TcpB transfected into 293 cells, as well as in J774 cells infected with \textit{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 \textit{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-b 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.
\end{figure}
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. 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

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References


