Published in final edited form as:
*J Immunol*. 2010 January 15; 184(2): 956–964. doi:10.4049/jimmunol.0902008.

# Subversion of innate immune responses by *Brucella* through the targeted degradation of the TLR signaling adapter, MAL

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## Abstract

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 TIR (Toll/IL-1 receptor) domains and whose expression causes degradation of the phosphorylated, signal competent form of the adapter 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 TRIF. TcpB also does not affect a mutant, signal-incompetent form of MAL that cannot be phosphorylated. Interestingly the presence of TcpB leads to enhanced polyubiqitination 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.

Detection of microbes by toll-like receptors (TLR) is a critical step in activation of the innate immune response and is essential for robust priming of the adaptive immune response (2, 3). TLRs localize to the plasma membrane or to endocytic membranes and recognize certain molecular elements of pathogens called Pathogen Associated Molecular Patterns (PAMPs). Upon ligation 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, AP1 etc. These transcription factors in turn trigger the production of cytokines, chemokines and antimicrobial peptides that eventually help contain and clear the infection (4). 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-1Receptor (TIR) domain. While 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 (3, 5) Five TIR-domain containing adapter molecules have been described: MyD88 (6), MyD88-adapter-like, MAL(7) (also known as TIRAP (8)), TIR-domain containing adapter protein inducing IFNβ, TRIF (also known as TICAM1) (9, 10), TRIF-related adaptor molecule, TRAM (also known

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as TICAM2)(11–13) and sterile armadillo-motif-containing protein (SARM)(14, 15). While 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 (13, 16). SARM functions only by interaction through TRIF and acts a negative regulator (17). These adapters have unique signaling properties, and their differential utilization by TLRs results in specialized signaling outcomes that permit a tailored, robust response against the pathogen (3, 18)

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 (19). 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 neurological disorders(20). (21)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.(22). 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 (21). *Brucella* spp. have a low infectious dose, are easily aerosolized and human infections with these bacteria are debilitating and difficult to treat (23, 24). Consequently, these bacteria are presently listed as Class B agents on the NIAID list of etiologic agents of concern with respect to their potential use in bioterrorism (25, 26).

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 lipopolysaccharide (LPS) of these bacteria, for instance, elicits a reduced and delayed inflammatory response in infected hosts compared to the endotoxin of some other Gram negative bacteria and this property has been proposed to allow the brucellae to employ a mechanism for a “stealthy” mode of entry into host macrophages (27). The *Brucella* LPS O-side chain also forms complexes with MHCII and interferes with the capacity of *Brucella* infected macrophages to serve as antigen presenting cells (1). Once the brucellae have been engulfed by host macrophages, they employ 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 Toll/IL-1 receptor (TIR) domains and interfere with TLR2 and TLR4 signalling 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[53]. *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 IRF-$1^{-/-}$ mouse model of infection and unlike wild-type 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. Here 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

### Antibodies

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 antibody, anti-M2 monoclonal antibody, and anti β-tubulin monoclonal antibody was from Sigma. Rabbit monoclonal anti MAL antibody for detecting endogenous MAL was from Epigenetics. Rabbit Polyclonal anti VSV antibody was from Zymed. Anti MyD88 antibody for detecting the endogenous protein was from Santa Cruz Biotechnology. Protein –G Sepharose, GST sepharose beads were from Pharmacia. Horseradish-peroxidase labeled secondary antibodies were purchased from Jackson laboratories. Dual Luciferase assay kit and TnT *In Vitro* Transcription and Translation kits were from Promega. Restriction enzymes and other DNA modifying enzymes were from New England Biolabs.

### Plasmids

TcpB was amplified by PCR from genomic DNA of B. *melitensis* 16M and cloned into pcDNA3.1 V5B as described below. TcpBm was created by deleting 9 bp from Box 1 of wt TcpB using the QuikChange II Site-Directed Mutagenesis Kit from Stratagene and wt TcpB as template.

CD4/TLR4 construct has been described before. HA tagged MAL and MAL-DN clones were kindly provided by Dr. Luke O' Neill.

### Alignment of sequences and computation 3D modeling

Multiple alignment of TIR domain was domain was done by Align X software (Invitrogen). Tertiary structure of TIR domain of TcpB, MAL and TLR4 were predicted bu 3D-JIGSAW (version 2.0) server with TLR 2-TIR domain structure (PDB ID: 1FYX) as 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 PEI, Lipofectamine or Fugene as indicated in the figure legends. For PEI mediated transfection, 24 hrs after seeding HEK 293 cells, DNA was mixed with PEI (2 ug PEI per ug DNA used) and diluted with Optimem. After incubation for 20 mins at room temperature, the DNA-PEI complexes were added to the cells. Where Lipofectamine or Fugene were used, transfections were done according to manufacturer's protocol. Cells were harvested 24–48 hrs later in Lysis buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1% Tirton-X100 (TNT) + protease inhibitors. Equal amounts of each sample was fractioned by 12% SDS_PAGE and transferred to Immobilon. After blocking in TTBS with 5% nonfat milk (NFM) for 30 min at room temperature, the blots were incubated with primary antibody (in TTBS+ 5%NFM) overnight at 4° C (unless noted otherwise), washed 3 times with TTBS and incubated with appropriate HRP conjugated secondary antibody (1: 10, 000 dilution) for 30 minutes at room temperature. After three washes with TTBS, bands were detected by ECL according to manufacturer's recommendation.

### Immunoprecipitation

HEK293 cells were seeded ($0.8\times10^6$ cells/well) into 6 well plates 24hrs prior to transfection. DNA were transfected using either Lipofectamine or PEI as described above. Cells were harvested after ~30 hrs in 250 ul of TNT buffer + protease inhibitors. After 15 minutes incubation on ice, samples were spun at 14, 000 rpm for 15 minutes at 4° C to remove cellular debris. 300 ug of the supernatants were then pre-cleared with Protein G sepahrose beads with rotation for 30 minutess at 4 °C. 50 ul of the pre-cleared lysates were saved for immunoblotting later. Remainder of the pre-cleared lysates were then incubated overnight with appropriate antibody and protein G sepharose at 4° C. Immune complexes were collected by spinning at 5000x g for 2 minutes at 4° C. Washed beads 3× with TNT, added 30 ul of 6× SDS-loading dye, boiled for 1 minute and fractionated on SDS-Polyacrylamide gel. Samples were immunoblotted with the indicated antibodies as described above. For analysis with the same antibody as used for Immunoprecipitation, loaded only 5ul of each sample. Used the reminder for analysis with other antibodies. Pre-immune lysates were analyzed in identical manner.

### Phosphatase Treatment

Indicated pre-Immune lysates were treated with 50 units of Calf Intestinal Phosphatase (CIP) in presence or absence of 4 mM sodium orthovanadate ($Na_2V_2O_5$) as inhibitor of CIP and incubated for 3 hr at 37° C. Samples were analyzed thereafter as described above.

### Luciferase Assay

HEK 293 cells ($0.8\times 10^5$/well) were seeded onto 24 well plates 24 h before transfection. Increasing amounts of TcpB (50, 100, 200ng/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. 24 hours later, cells were washed once with PBS and then harvested in 100 ul of Passive lysis buffer from the Dual Luciferase Assay kit. After incubation on ice for 15 min, 5 ul of the lysate was assayed using the Dual Luciferase assay kit according to the suggested protocol. 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 triplicates (CD4/TLR4) or duplicates (all others).

### Ubiquitination assay

HEK293 cells ($0.8 \times 10^6$ cells/well in 6 well plates) were transfected with HA MAL (300ng/well) and VSV-Ubiqitine gene (1.0 ug/well) in presence (1.2 ug/well) or absence of TcpB as described above. 24 hrs later, half of the samples were treated with MG132 (20 mM) for 4 h. Cells were washed and harvested in PBS and spun at 2500 rpm for 3 mins. Pelleted cells were resuspended in 300 ul SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl, pH 8.0 and boiled for 10 mins. They were then sonicated for 5 secs pulses, 1 sec pauses for a total of 30 secs, followed by centrifugation at 16,000xg for 15 min. Supernatant was transferred to a new tube. To 300 mg of total protein (volume made up to 500 ul with $ddH_2O$) added 500 ml 2× IP buffer (2% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, 8.0, 1 mM EDTA,).5% NP-40). Preclearing and immunoprecipitation of the lysates thereafter were carried out as described above.

### Construction of TcpB

TcpB open reading frame was amplified from *B. melitensis* 16M genomic DNA using the following primers:

Fwd: 5' CGGGGTACCATGTCTAAAGAGAAACAAAGCC-3'

Rev: 5' GAAAGAACTGCATTCCCTTATCAAGAATTCCG-3'

The ~750 nt, amplified product was gel purified and ligated to pCDNA 3.1-V5-His B vector and transformed into *E.coli* DH5α cells. Putative colonies containing the correct plasmids were identified by analysis of the minipreps of the resulting bacterial colonies.

### Construction of the *Brucella abortus tcpB* mutant strain

The *tcpB* open reading frame was PCR amplified from *Brucella abortus* 2308 chromosome, using the following primers.

FWD 5'-CTTGAGAGGCACCGCTCAAT-3'

REV 5'-CAGATTTGCGCAGATTGCAT-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* DH5a with blue/white selection, putative colonies containing the desired plasmid were grown up and stocked in 25% glycerol/*Brucella* broth. Following confirmation of the transformation product, the plasmid pTB*tcpB* was cut with the blunt cutters EcoRV and NruI, which removed an internal fragment of 577 bp from the *tcpB* coding region. The kanamycin resistance cassette (*aph3a*) from pKS-Kn was inserted into the resulting blunt site and this plasmid was given the designation pTB*tcpB*D. This plasmid was dialyzed and electroporated into *B. abortus* 2308 using previously described methods (37). The resulting transformants were cultured on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) and 45 μg/ml kanamycin and subsequently patched onto SBA with containing kanamycin and 25 μg/ml ampicillin. Colonies that were resistant to kanamycin, but sensitive to ampicillin were selected for further characterization, grown overnight in *Brucella* 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 (*aph3a*), 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 aph3a- and complete *tcpB*-specific primers, but no products 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 (38) 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 wt *B.abortus* 2308 and *B.abortus tcpB* mutant strains

Infection of macrophages with *Brucella* was done in biosafety level 3 laboratory according to the protocol described by Gee, et al. (Infect Immun. 2005 May;73(5):2873–80). Briefly, J774 cells grown in T75 flasks were cultivated in DMEM supplemented with 5% fetal calf serum (FCS) at 37°C in 5% $CO_2$. Following 24h of growth, the confluent mononlayers were infected with 2308 or the *tcpB* mutant. *Infectious strains were opsonized for 30 min with a subagglutinating dilution (1:1,500) of hyperimmune C57BL6J mouse serum at a multiplicity of infection (MOI) of 50 *Brucella*e per macrophage (MOI of 50:1) and incubated at 37°C with 5% $CO_2$ for 1h to allow for phagocytosis of the *Brucella*e. Cell culture medium was removed and replaced with fresh medium supplemented with 5% FCS and 50 μmg/ml of

gentamicin to kill any remaining extracellular bacteria and incubated at 37°C with 5% $CO_2$ for 2h. 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 of gentamicin. Cell culture medium was replaced with fresh medium every 24h. At 48h 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 5m incubation at room temperature, macrophage lysates were centrifuged for 30m at 10K rpm, supernatant collected, filtered, and bacterial and cellular debris discarded. J774 lysates were then boiled for 15 minutes. J774 lysate protein concentrations were then determined by 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' 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 analysed by western blot analysis with anti MAL antibody (1:1000 dilution) as described in Methods and Material.

### Western blot anlaysis of cutures of wt *B. abortus* and *B.abortus tcpB* mutants

*Brucella abortus* cultures were inoculated at $1\times10^3$ CFU/ml in *Brucella* broth and incubated at 37°C under 5% $CO_2$ with shaking. Bacteria were pelleted at 48, 72, and 96h and resuspended in 1 mL 1X protein lysis buffer (0.3% (w/v) SDS, 200 mM DTT, 22 mM Tris-base, 28 mM Tris-HCl, pH 8.0), boiled for 30 minutes and lysed by bead beating 6 times for 40 seconds. Samples were then centrifuged for 20 minutes at 14K rpm and supernatant collected. Total cell protein concentrations were determined by the Bradford method and equal amounts of protein were separated onto an SDS-PAGE gel. After transfer to nitrocellulose membrane, TcpB protein was detected using anti TcpB antibody (1:1000) and goat-anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (1:10,000). Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate.

### Spleen colonization data

Spleen colonization data were obtained as described before (Jason M. Gee, 2005). Briefly, 6- to 8-week old female BL6 mice were infected via the intraperitoneal route with approximately $5\times10^4$ brucellae. At 1, 2,3 and 4 weeks postinfection, 5 mice per experimental group were sacrificed by isoflurane overdose. Immediately following euthanasia, spleens were harvested aseptically and homogenized in sterile PBS. Spleen homogenates were then serially diluted 10-fold in sterile 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 log10 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 *Brucella 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, 39). For clarity, we will use the designation TcpB. As shown in Figure 1a, 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 canonical TIR sequence. 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. 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 (40, 41) (Figure 1b).

To further investigate the potential functional role of TcpB, we amplified the TcpB coding region from *Brucella melitensis* 16 M genomic DNA and cloned it into the pcDNA3 expression vector. *In vitro* transcription/translation of this plasmid yielded a protein of MW ~26 Kd (Fig 1c), as predicted by the open reading frame in the DNA sequence. The open reading fame was then fused in frame with glutathione-S-transferase in a pGEX vector, and recombinant GST-TcpB was generated in *Escherichia coli* BL21 cells. The purified GST-TcpB was used to generate rabbit polyclonal antisera, which recognized two bands of ~25 kd when TcpB was expressed in transfected 293 cells (Figure 1d, lane 1). Both of these bands are specific for TcpB as they are not detected by the pre-immune sera (Fig. 1d, lane 4), nor are they detected in lysates transfected with an empty pcDNA3 plasmid (Fig 1d, lane 2). Moreover, pre-incubation of the antibody with the GST-TcpB protein abolishes the detection of TcpB expressed in 293 cells (Fig 1d lane 5). These two TcpB bands could result from differential post-translational modifications, or might represent a product of premature termination of translation, but are unlikely to be due to differential phosphorylation (Supplementary Figure 4).

## Expression of the Brucella TcpB can inhibit signaling through TLR4

It has been reported previously that *Brucella abortus* is recognized by TLR2 and TLR4 (42, 43). 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. Co-expression 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 amino acids (phenylalanine-isoleucine-serine) in Box 1 region of TcpB-TIR (Fig 1d, lane 3 and Fig 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 over-expression of MAL, MyD88, TRIF, IRAK-1, or TRAF6 (Fig 2 c–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 on the plasma membrane. Remarkably TcpB was also shown to resemble MAL by being able to bind phosphoinositides[53]. 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 (44). We therefore wanted to test if 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 co-immunoprecipitated MAL or MyD88 using immunoblotting with antibodies 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 MyD88 and MAL binding, (Fig 3d) which suggests TcpB doesn't 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 upon expression in cells, where the slower migrating band corresponds to a phosphorylated form (45). As expected, in the absence of TcpB, expression of HA MAL generated two bands (Figure 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; Fig 4b. lanes 1–4, top two panels). At the highest concentration of TcpB tested, the lower band is also reduced (Fig 4b, lanes 1and 4). The extent of reduction was more pronounced after 45 hours than after 24 hours (Fig 4b). Treatment with calf Intestinal phosphatase (CIP) abolishes the slower migrating band confirming that the upper band corresponds to a phosphorylated version of MAL (Fig 4e and Supplementary Figure 4). The effect of TcpB on MAL is dependent on the intact Box1 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 since TcpB had no detectable effect on co-expressed 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) (7, 8, 46). We also wanted to test whether TcpB had a similar effect on endogenous MAL. Using a rabbit monoclonal antibody 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 above 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 (47). To test if TcpB might also lead to enhanced, ubiquitin-dependent degradation of MAL, we expressed HA-MAL, TcpB and VSV-ubiquitin in presence or absence of the proteasome inhibitor, MG132. Although MAL expressed by itself is ubiquitinatinated to a low level, the extent of ubiquitination is dramatically increased in the presence of TcpB (Fig 6, lane 5). This suggests that the phosphorylated form of MAL is most likely degraded by TcpB through a process that involves polyubiquitination. However, unlike SOCS1, which is an E3 ubiquitin ligase (48, 49), TcpB has no obvious similarity to known ubiquitin ligases. Hence, it is unclear if TcpB itself is responsible for enhanced ubiquitination of MAL, or whether it acts by recruiting some other ubiquitin ligase. If TcpB itself induces degradation of MAL via polybiquitination, then the functional domain responsible remains to be identified.

## Reduction of MAL levels in J774 cells infected with virulent Brucella abortus 2308 is linked to the presence of TcpB

To determine if the native TcpB has an effect on MAL levels in mammalian cells infected with *Brucella* strains, we evaluated the levels of this protein in the murine macrophage-like cell line J774 infected with virulent *B. abortus* strain 2308 and an isogenic *tcpB* deletion mutant designated KB2 (Supplementary Figure 1). This cell line have been used extensively

as a model for the interactions of bacterial pathogens including *Brucella* strains with host macrophages (30, 50). Immunoblotting of *Brucella* lysates with the TcpB specific antibody revealed that TcpB could only be detected in wild-type *B. abortus* 2308 but not the *tcpB* mutant (Supplementary Figure 2). To test the effect of TcpB encoded by the bacteria on endogenous MAL, we infected J774 macrophages with wild-type and *tcpB* mutant of *B.abortus* 2308. As shown in Figure 5B, the MAL specific antibody could detect only one band in the J774 cell lysates. However, when J774 cells were infected with wild type *B. abortus* 2308, MAL was significantly diminished suggesting that the visible band in these cells was most likely the phosphorylated form (Fig 5b, lane 1 vs. lane 3). In contrast, infection with the *B. abortus tcpB* mutant showed significantly less reduction of MAL levels (Fig 5B, lane 2 vs. lane 3). These results further support the model that TcpB causes the degradation of MAL. The level of MyD88 expression in the J774 cells remained unchanged in absence or presence of *B. abortus* 2308 or *tcpB* infection (Figure 5C). These results further bolster our contention that TcpB does not exert its effect through interaction with MyD88.

The recombinant *Brucella* TcpB exhibits a clear impact on the activity of MAL when this protein is expressed in mammalian cells. The differential effects of infection of J774 cells with virulent *B. abortus* 2308 and the isogenic *tcpB* mutant also support the proposition that TcpB is secreted into the cytoplasm of infected host phagocytes where it has an effect on cellular levels of MAL. Based on these results, it was initially somewhat 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 (Supplementary Figure 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[53]. 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 over expressed 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 (21). 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 destroy host immune function. By utilizing TcpB to specifically inhibit MAL signaling, *Brucella* sp. can effectively dampen a critical microbial detection 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, down-regulation of MAL is a potent means of inhibiting the expression of pro-inflammatory 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 signal competent form of MAL. By targeting MAL, it ensures that only the TLR2 and TLR4 pathways (that respond to gram negative bacteria) are affected but not the other pathways that respond to different pathogens via different PAMPs.

Recently two other groups also have reported on TcpB (35, 39). A more recent study reported that TcpB was most similar to MAL, in particular in its ability to bind phosphoinositides[53]. In agreement with our work, these studies also reported that TcpB inhibits TLR4 and TLR2 mediated signaling to NF-κB although the mechanism by which TcpB functioned was not determined. Our work is the first demonstration of a mechanism by which TcpB inhibits signaling by TLR4 and activation of NF-κB, namely through targeted degradation of MAL. Moreover, in contrast to findings by the other groups, we see no effect of TcpB on activation of NF-κB activation by MyD88. Our immunoprecipitation data also shows that over expressed MyD88 and TcpB do not interact, nor does TcpB cause degradation of MyD88.

It has been demonstrated that upon LPS stimulation, MAL gets phosphorylated by Bruton's Kinase at Tyr- 86, Tyr-106 and Tyr 187 and such phosphorylation is critical for signaling and activation of NF-κB. Substitution of tyrosine with phenylalanine or alanine at positions 86 and 187 not only abolishes phosphorylation of MAL but also renders MAL signaling-deficient (45, 46, 51). LPS stimulation of cells also leads to SOCS1 mediated degradation of MAL and this degradation is absolutely dependent on phosphorylation of MAL (47). Thus, phosphorylation of MAL followed by its degradation affords the cells an efficient means of down-regulating robust MAL-MyD88 mediated TLR signaling. The recent finding that LPS-tolerized cells do not exhibit MAL phosphorylation and are signaling-deficient upon secondary stimulation with LPS also supports such a model (45, 46, 51). Our data showing that TcpB degrades phospho-MAL and inhibits TLR4 signaling is also consistent with this notion. Despite its effect on signaling by MAL, Piao et al have shown that the phosphorylation deficient MAL mutants Y86A, Y106A and Y187A, have no effect on post receptor stimulation of NF-κB by MyD88, IRAK-2 or TRAF-6 (46). This result also strongly supports our finding that TcpB has no effect on post-receptor activation of NF-κB by MyD88 and other adapters.

MAL is required for recruitment of MyD88 from the cytosol and delivering it to the plasma membrane via its PIP 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. (44, 52). The phosphoinositide binding property of TcpB[53] 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 (46, 52), 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 due to unregulated overactive signaling event (as seen in case of *Salmonella* poisoning) or due to fatal or severe sepsis, as seen in case of LPS tolerization.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We would like to thank Dr Ian Strickland and Matthew Hayden for their help with experiments as well as stimulating discussions.

This paper was supported by grants from NIH to SG (R21-AI63373, RO1- AI059440 and R37-AI33443).

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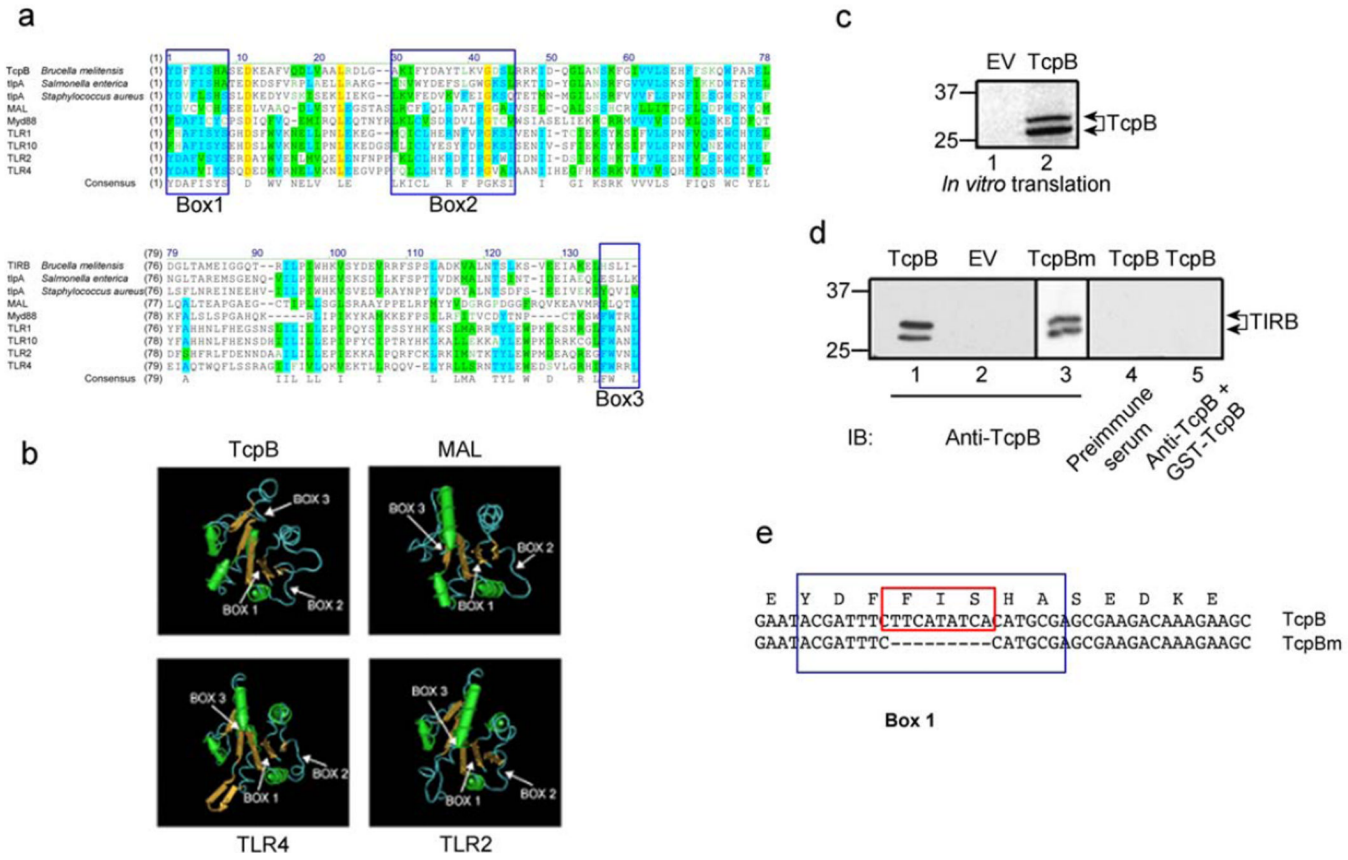


**Figure 1. Characterization of TcpB and TcpB mutant from *B. melitensis***

a. TcpB from *B.melitensis* contains a TIR domain that shares extensive homology with other TIR domain containing proteins, particularly in the Box 1 region. Conserved residues are indicated in blue on a turquoise background, while identical residues are indicated in red on a yellow background. Green background indicates blocks of similar residues while residues of weak similarity are indicated in green. B. 3D structural computation using TLR2 crystal structure as model reveals that like TLR2, MyD88 and MAL, TcpB also consists of β-sheets and α-helices that can be folded into very similar conformations as the others. C. *In vitro* transcription-translation of TcpB generates a band of ~25 kD (lane 2), as predicted from the sequence and not seen with empty vector (EV, lane 1). D. Rabbit polyclonal antibody raised and affinity purified against GST-TcpB detects two bands ~25 kD when TcpB is expressed in 293 cells (lane 1). These bands are not detected in 293 cells expressing empty vector (lane 2) or when pre-immune serum was used for immunoblotting (lane 4). Pre-incubation of the antibody with excess GST TcpB also abrogates detection of these bands (lane 5). E.DNA corresponding to three aa residues FIS (red box) within the conserved Box1 region of TcpB (marked by blue box) was deleted from wt TcpB and cloned into pCDNA 3.1 to give TcpBm that can be detected by anti-TcpB antibody (lane 3 in D).

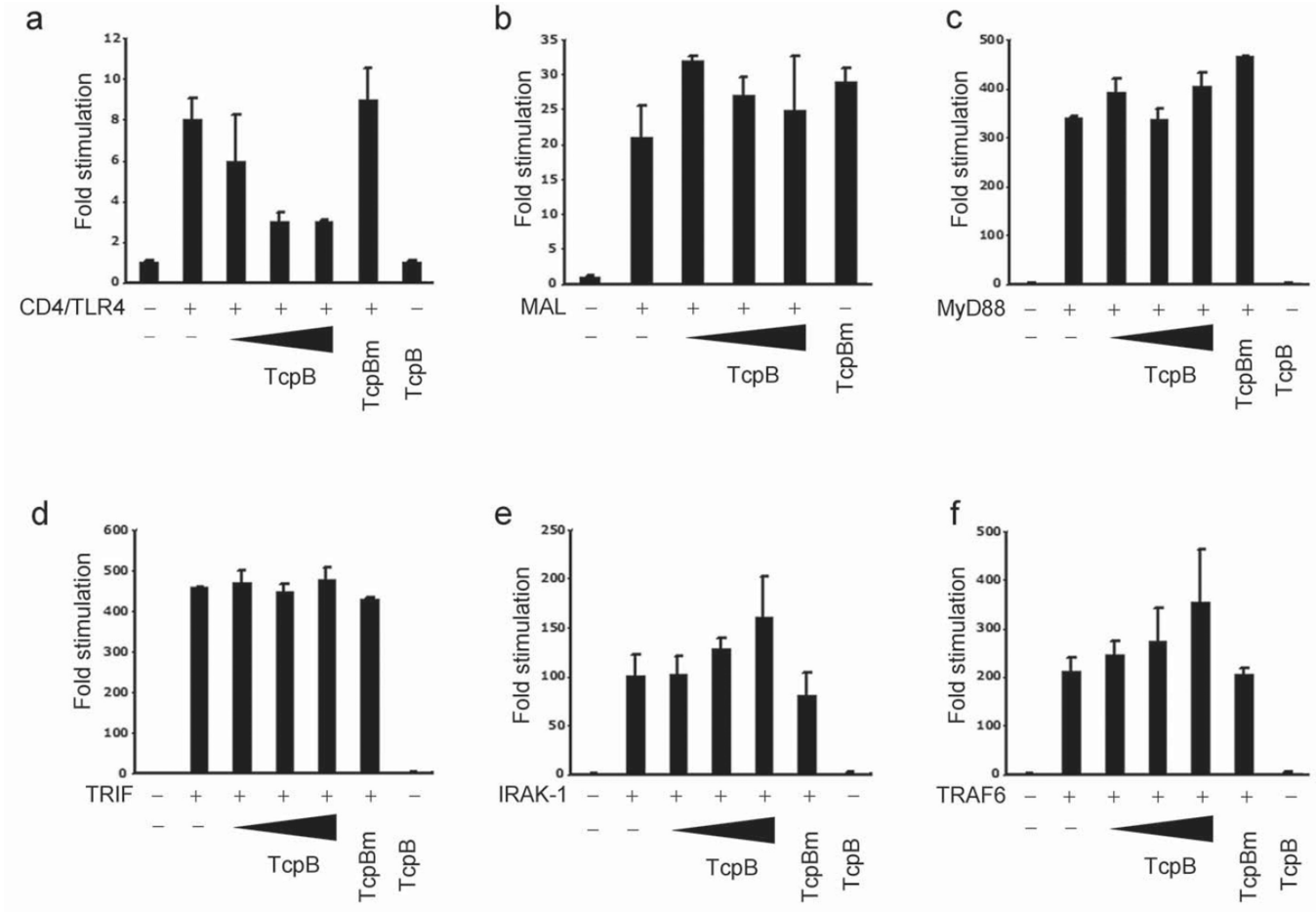


**Figure 2. TcpB inhibits NF-κB activation in 293 cells by CD4/TLR4, but not by MAL, MyD88, TRIF, IRAK1 or TRAF6**

293 cells were co-transfected with TcpB (50,100 or 200 ng/well) or TcpB mutant (200ng/well) with CD4/TLR4 (A), MAL (B), MyD88 (C), TRIF (D), Irak-1 (E), TRAF 6 (F) using Fugene and Luciferase activities were measured as described in Methods. TcpB alone lanes represent control transfection in absence of any of the stimulants. The data represent the summary of at least three experiments.

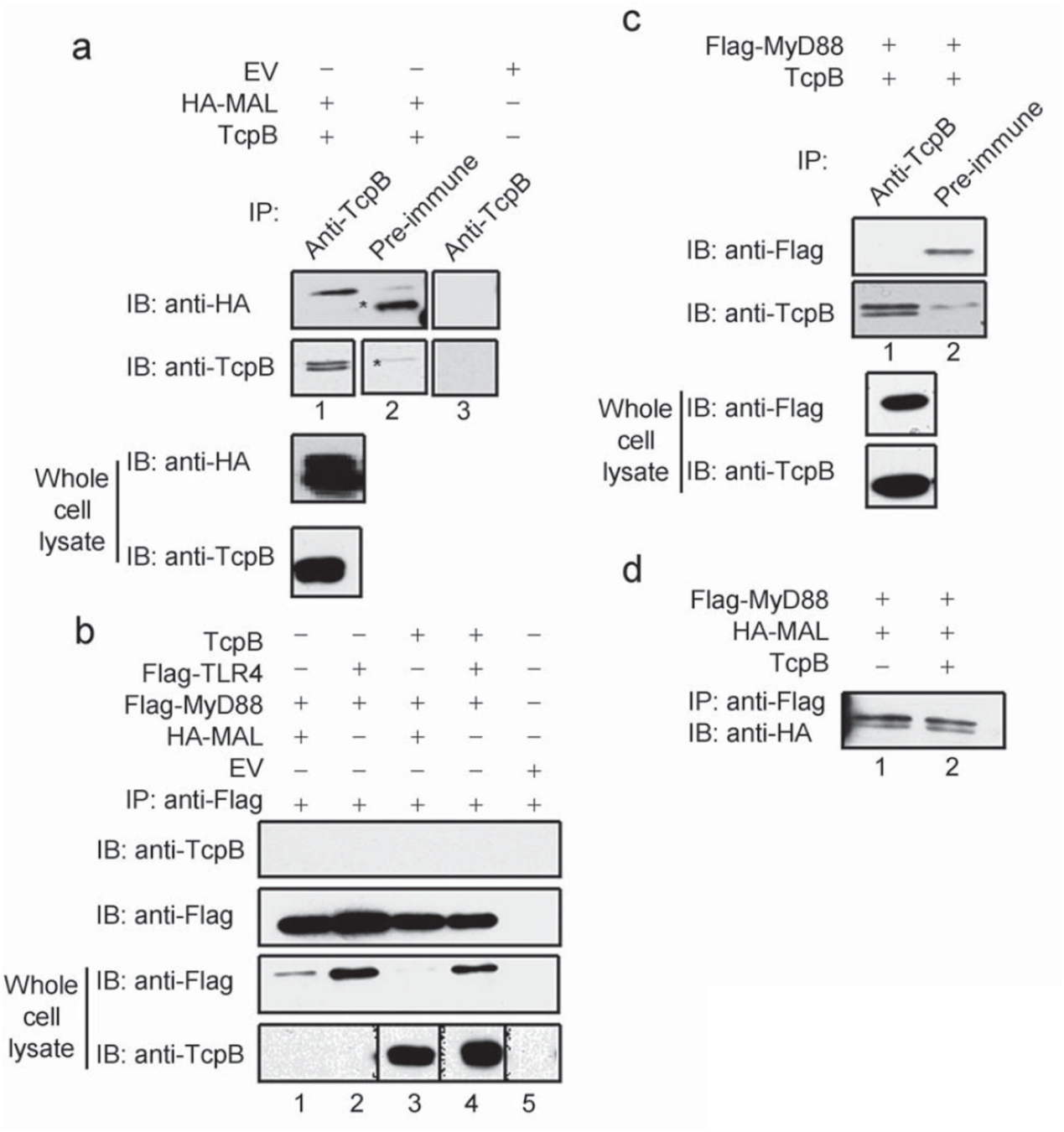


**Figure 3. TcpB co-immunoprecipitates with MAL but not with MyD88**

A–B. TcpB was co-transfected with HA-MAL (A) and Flag-MyD88 (B) into 293 cells using Lipofectamine. Cells were harvested after 24 hrs and immunoprecipitated with anti-TcpB antibody or pre immune serum as control. Immunoblot analysis with either anti HA antibody or anti-flag antibody showed that TcpB interacted only with MAL but not with MyD88. C, D. Flag MyD88 was co-transfected with HA-MAL (lanes 1 and 3) or Flag-hTLR4 (lanes 2 and 4) in absence (lanes 1 and 3) or presence (lanes 2 and 4) of TcpB in 293 cells using Lipofectamine. After immunoprecipitation with anti Flag agarose beads, samples were immunoblotted with anti-TcpB antibody, anti-Flag antibody to detect MyD88 and TLR4 (C)

or anti-HA antibody to detect MAL (D). Non-specific bands seen with pre-immune serum are indicated with asterik.

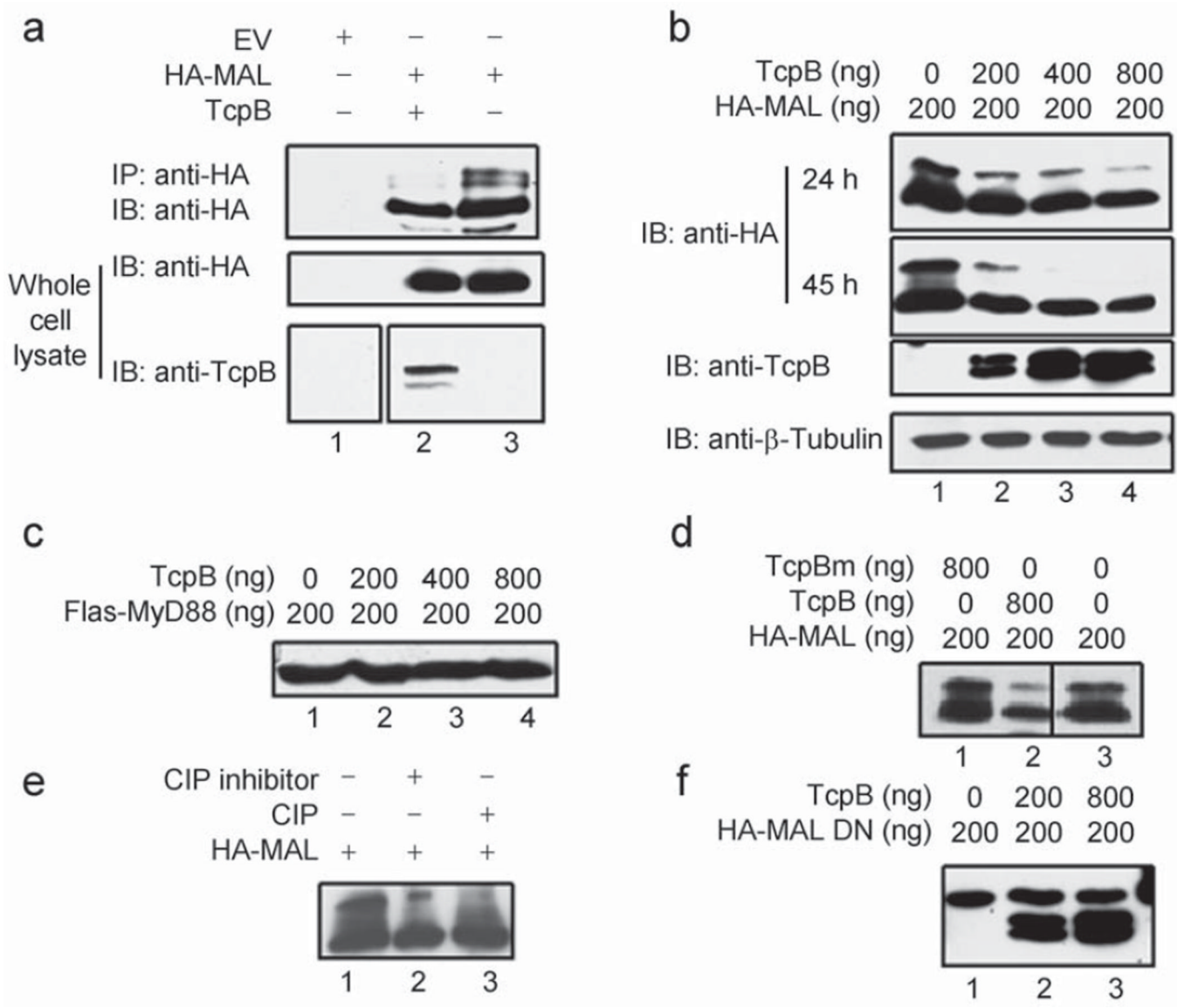


**Figure 4. Overexpression of TcpB in 293 cells lead to degradation of phosphorylated and signaling competent form of MAL**

A. HA-MAL was transfected into 293 cells in presence or absence of TcpB and immunoprecipitated with anti-HA antibody, and cell lysates were immunoblotted with anti-HA antibody. B. 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 hrs or 48hrs as indicated and immunoblotted with anti-HA antibody, or anti-TcpB antibody or anti-β tubulin (as loading control). The levels of the different proteins were quantitated by densitometry and presented below each panel. C, 200 ng of Flag-MyD88 was transfected into 293 cells together with 0, 200, 400 or 800 ng of TcpB. Cells were harvested after 24 hrs and immunoblotted with anti flag antibody. d. 293 cells were transfected with 200 ng of MAL in absence or presence of either 800 ng TcpB or TcpBm. Cells harvested after 24 hrs were immunoblotted with anti HA antibody. D. 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 48hrs and immunoblotted with anti-HA antibody. E. 293 cells were transfected with HA MAL. After 40 hrs, one third of the cell lysates were treated with

CIP alone and another one third with CIP in presence of CIP Inhibitor, $Na_2V_2O_5$. Samples were then immunoblotted with anti-HA. F. 200 ng of DN-MAL (signaling incompetent) were transfected into 293 cells in absence or presence of increasing amounts of TcpB. Cells were harvested after 24 hrs and cell lysates were immunoblotted with anti-HA antibody as well as anti-TcpB antibody. All transfections were done using PEI.

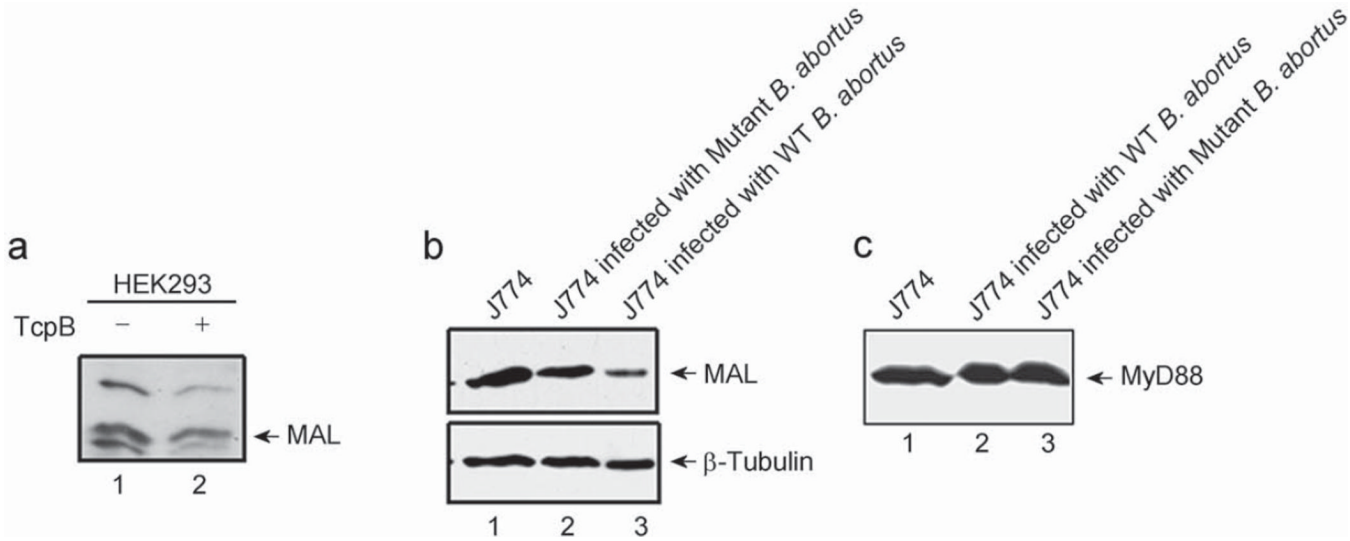


**Figure 5. Endogenous MAL is degraded by TcpB transfected into 293 cells, as well as in J774 cells infected with *B. abortus* 2308**

A. 293 cells were transfected with or without TcpB and cells were harvested after 40 hours. After fractionationation on 12% gels and transfer to membranes, they were immunoblotted with rabbit monoclonal anti-MAL antibody (1:1000). B. J774 cells were infected with either wild type *B. abortus* 2308 or a mutant form lacking boxes 1 and 2. 48 hrs after infection, did western blot analysis of the cell lysates with anti MAL antibody as described in A. Uninfected J774 cells were assayed in parallel. C. Same as in B, except that western blot analysis was done with anti MyD88 antibody that recognaized endogenous MyD88

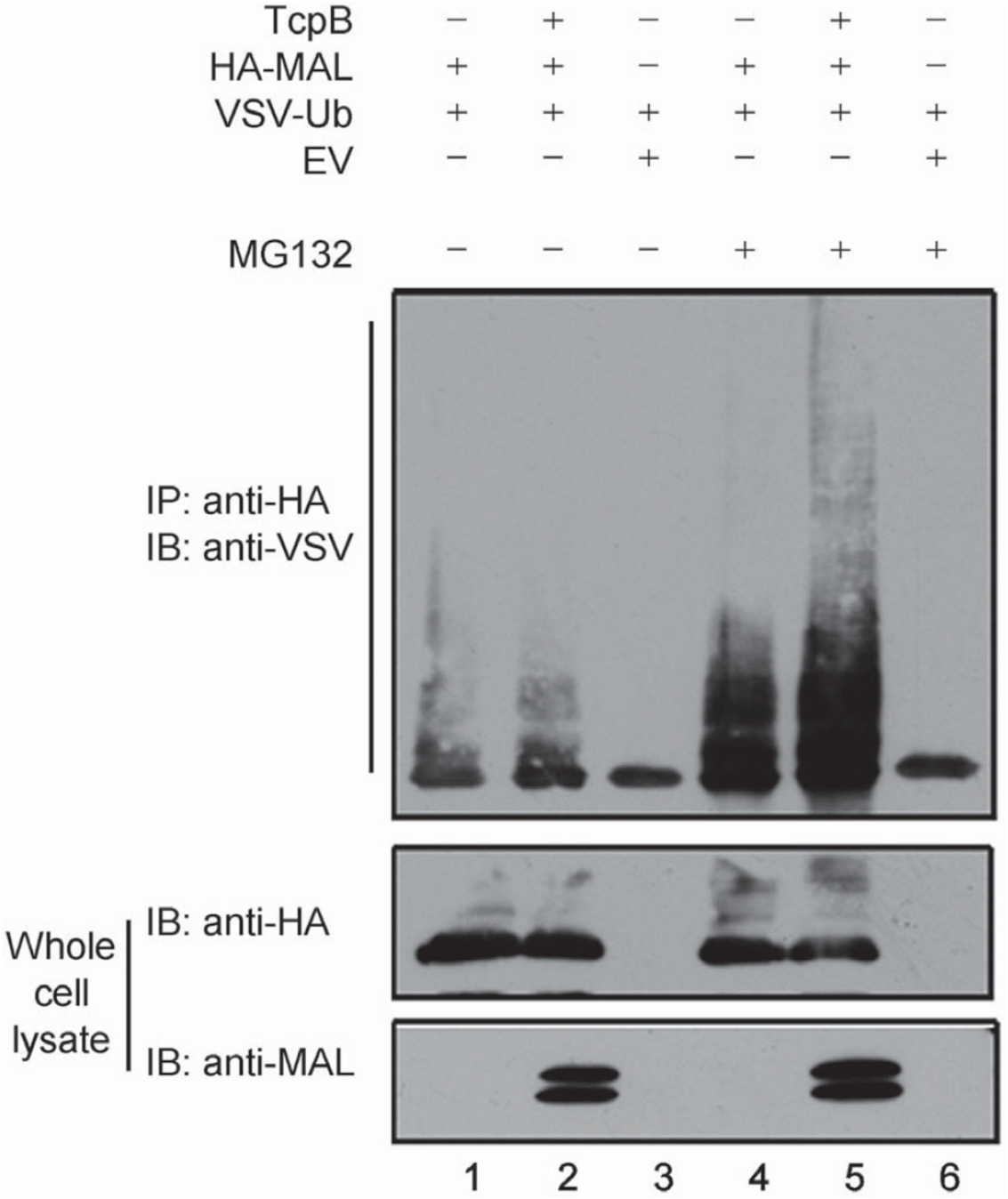


**Figure 6. TcpB promotes enhanced polyubiquitination of MAL**

293 cells were transfected with HA-MAL, VSV-Ubiquitin and TcpB using PEI. After 24 hrs, cells were treated with 20 mM MG132 where indicated for additional 4 hrs. Cell lysates were then immunoprecipitated with a-HA as described in methods and materials and immunoblotted with anti-VSV for detection of polyubiquitinated MAL.