Chapter I

*Leishmania donovani* targets tumor necrosis factor receptor-associated factor (TRAF) 3 for impairing TLR4-mediated host response
**Introduction**

*Leishmania* are intracellular protozoan parasites that are transmitted by the sand fly vector and gain entry into mammalian phagocytes for intracellular replication. Despite the varied clinical manifestations and the homing of the organisms to different organs, all forms of leishmaniasis are caused by species of the protozoan genus *Leishmania* and affected over 12 million people worldwide, with disease severity ranging from mild cutaneous lesions at the bite wound to death from visceral Leishmaniasis. *Leishmania* spp. are digenetic organisms shuttling between a flagellated promastigote in the gut of the sandfly and an intracellular amastigote in the mammalian host. Promastigotes attach to macrophages via a receptor-mediated mechanism and are taken up by phagocytosis into a phagosome, which fuses with lysosomes to form the phagolysosome. Once inside the macrophage, the promastigotes undergo significant biochemical and metabolic changes, which result in the obligatory intracellular form of the parasite – the amastigotes (Contreras et al, 2010). Macrophages being the pivotal constituents of the innate immune system play a vital role for recognition and elimination of microbial pathogens. Recognition of invading microorganisms by the innate immune system is a first and essential step in their successful elimination. At the molecular level this recognition is mediated by a specific interaction of a so-called pathogen-associated molecular pattern (PAMP) and a distinct member of the Toll-like receptor family (Medzhitov et al, 1998). All TLRs contain a leucine-rich extracellular domain that is supposed to mediate ligand binding, as well as a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain that upon activation recruits adaptor proteins, such as MyD88, via homomeric interaction of TIR domains(Karin & Gallagher, 2009). The N-terminal death domain of MyD88 subsequently binds to and activates the interleukin receptor-associated kinase 1/4 (IRAK1/4), TNF receptor-associated factor 6 (TRAF6), ubiquitin-conjugating enzyme 13 (Ubc13), and cellular inhibitor of apoptosis 1/2 (cIAP1/2) (Dong et al, 2006). Here lies a complex signaling event that is precisely timed and critically regulated by ubiquitination events. This is followed by subsequent release and translocation of the entire complex to the cytosol resulting in activation of TGF-β-associated kinase 1 (TAK1) and downstream mitogen activated protein kinases (MAPKs) ultimately resulting in IKK/NF-κB activation (Karin & Gallagher, 2009). In the present study, we identified tumor necrosis factor receptor associated factor (TRAF) 3, a negative regulator of TLR4 signaling, as a target utilized by *Leishmania* to deactivate TLR4 signaling mediated macrophage activation. We used LPS as a
cognate ligand for TLR4—and compared the signaling responses between LPS treated RAW 264.7 cells with only *L. donovani* infected and *L. donovani* + LPS co-stimulated macrophages. TRAF3, which was ubiquitinated at lys 48 position and subsequently degraded following LPS treatment, found to be persist in *L. donovani* and *L. donovani* + LPS co-stimulated cells due to defective lys 48 ubiquitination. Lys 63-linked ubiquitination of upstream proteins in the cascade (cIAP1/2 and TRAF6), mandatory for TRAF3 degradation, was also reduced in post-infection. This may be attributed to reduced association between ubiquitin-conjugating enzyme Ubc13 and TRAF6 during infection. Inhibition of TRAF3 before infection by shRNA in BALB/c mice showed enhanced IL-12 and TNF-α and decreased spleen parasite burden, thereby marking reduction in disease progression. Our findings identified TRAF3 as a novel molecular regulator exploited by *Leishmania* for successful infection.

**MATERIALS AND METHODS**

**Reagents, antibodies, and constructs**

Ubiquitin lys-48-specific mAb and ubiquitin lys-63-specific mAb were purchased from Cell Signaling (Danvers, MA, USA). The rest of the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytochalasin D, HRP-conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 was purchased from Sigma-Aldrich. The pNF-κB-luciferase plasmid containing 5 copies of NF-κB consensus sequences (pNF-κB-LUC) was obtained from Stratagene, (La Jolla, CA, USA). TLR2-neutralizing antibody was obtained from Invivogen (San Diego, CA, USA).

**Cells and parasites**

*L. donovani* (MHOM/IN/1983/AG83) parasites were cultured as promastigotes in medium M199 (Invitrogen, Carlsbad, CA, USA) with Hanks’ salt containing HEPES (12 mM), l-glutamine (20 mM), 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). BALB/c mice (6 to 8 wk old) were euthanized for isolation of BMDMs from the femurs and tibiae as described previously (Kar et. al., 2010). The adherent murine macrophage cell line RAW 264.7 was cultured at 37°C with 5% CO2 in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin, and 100
U/ml penicillin. For *in vitro* infection, cells were infected with *L. donovani* promastigotes at a parasite:cell ratio of 10:1 (Kar et al., 2010) and incubated for the specified periods.

**Infection in mice**

*In vivo* infection was performed by injecting $10^7$ *L. donovani* promastigotes, *via* the tail vein of female BALB/c mice. Parasite burdens were detected by Giemsa-stained impression smears of spleen and liver taken from infected mice. Organ parasite burden expressed as Leishman-Donovan units (LDU) was calculated as the number of amastigotes/1000 nucleated cells × organ weight (in grams). Splenocytes were isolated and cultured as described earlier (Kar et al., 2010). All the animal care and experimental procedures were carried out in strict accordance with the recommendations of the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The protocol has been approved by the Committee on the Ethics of Animal Experiments of Indian Institute of Chemical Biology (permit 147-1999).

**Analysis of gene expression by real-time PCR**

Total RNA was extracted using RNasey mini kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. cDNA was prepared from 1 µg of total RNA using the superscript first-strand synthesis system for the RT-PCR kit (Invitrogen). Quantitative real-time PCR (ABI 7500 Fast real time PCR system; Applied Biosystems, Foster City, CA, USA) was performed using TaqMan Fast Universal PCR master mix (Applied Biosystems) with TaqMan probes for *IL-12*, *TNF-α*, inducible nitric oxide synthase (inos), *T-bet*, and Gata-3 (Applied Biosystems). The following amplification conditions were maintained throughout the amplification process: 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantitation was performed by the comparative $\Delta \Delta Ct$ method, and data were normalized to β-actin mRNA level and expressed as a fold change compared with uninfected controls.

**Cytokine analysis by ELISA**

ELISA was performed using a sandwich ELISA kit (Quantikine M; R&D Systems, Minneapolis, MN, USA). The detection limit of these assays was >5.1, >2.5, >4, >4.6, >2, >1.5, and >2 pg/ml for TNF-α, IL-12p70, IL-10, TGF-β, IL-4, IL-13, and IFN-γ, respectively.
NF-κB reporter assay

RAW 264.7 macrophages were transfected with 1 µg NF-κB luciferase reporter vector along with 0.5 µg pCMV-β-gal in serum-free medium using Lipofectamine (Invitrogen) according to manufacturer’s instruction. Cells were harvested using reporter lysis buffer (Promega, Madison, WI, USA), and NF-κB luciferase activity was assessed by a luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

RNA-mediated interference by siRNA transfection

TRAF3-, TRAF6- and cIAP1/2-specific siRNAs were purchased from Santa Cruz Biotechnology. RAW 264.7 cells were seeded in 6-well tissue culture plates at a density of 2 × 10^6 cells/plate in antibiotic and serum-free normal growth medium, followed by transfection of siRNA at a concentration of 15 pmol/100 µl siRNA transfection medium (Santa Cruz Biotechnology) as per manufacturer’s instruction. Scrambled siRNA served as control.

shRNA construct

The TRAF3-specific shRNA cassette, driven by the promoter of the small nuclear RNA U6, was produced by PCR-mediated amplification of the TRAF3 gene, and the selection of shRNA target sequences was based on published guidelines (Pei et.al., 2006). The shRNA cassette was thereafter cloned into a self-inactivating lentiviral vector pCRI.LV. Virus produced was concentrated by ultracentrifugation, and virus titer was calculated at 2 × 10^6 infectious U/ml as described earlier (Santhosh et. al., 2008). shRNA target sequence selection and nonrelevant control vector use were the same as reported previously (Basu Ball et. al., 2011). For in vivo knockdown of TRAF3, spleen and liver tissues of anesthetized BALB/c mice were injected with 50 µl of the 1000× vector concentrate of TRAF3-specific shRNA construct, 3 d before infection.

Preparation of membrane and cytosolic fractions

Cells were suspended in a buffer containing 20 mM Tris (pH 7.4), 5 mM MgCl₂, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 µg/ml of aprotinin for 20 min at 4°C and disrupted in a Dounce homogenizer (15 strokes). After nuclei were removed by centrifugation at 1000 g for 10 min at 4°C, the supernatants were centrifuged at 10^5 g for 1 h at 4°C, and the cytosol fraction was collected. The pellets containing cellular membranes were
resuspended in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Nonidet P-40. Immunoblotting and immunoprecipitation Cells (RAW 264.7 cells and BMDMs) were lysed in lysis buffer (Cell Signaling), and the concentration of proteins in the supernatants was estimated using a Bradford reagent. For immunoprecipitation, 500 µg of cell lysates was precleared by incubation with protein A/G plus agarose (Santa Cruz Biotechnology) for 1 h followed by incubating overnight with specific primary antibody at 4°C. Then, 25 µl of protein A/G plus agarose beads was added to the mixture and incubated for 4 h at 4°C. Immune complexes were processed and immunoblotted as described previously. For ubiquitination analysis, 10 mM N-ethyl maleimide (NEM; Sigma) was added to the lysis buffer.

**Densitometric analysis**
Semiquantification of ubiquitinated proteins was done using Image J software (U.S. NIH, Bethesda, MD, USA; http://rsb.info.nih.gov). Densitometric analyses for all other experiments were carried out using Quantity One software (Bio-Rad, Hercules, CA, USA). Band intensities were quantified, and the values were normalized to endogenous control were expressed in arbitrary units as indicated in bar graphs adjacent to figures.

**Statistical analysis**
All experiments were performed ≥ 3 times. Macrophage cultures were set in triplicates, and results are expressed as means ± sd. A Student’s t test was employed to evaluate the statistical significances of differences among pair of data sets, and a value of P < 0.05 was considered to be significant.

**RESULTS**

**Modulation of TLR4 signaling pathway by *L. donovani***
One of the crucial strategies by which *Leishmania* parasites may neutralize the defensive machinery of host macrophages is to tamper with the proximal events taking place at the TLR signaling pathway. Stimulation of macrophages with LPS, the ligand for TLR4, leads to transient formation of membrane-associated signaling complexes (Tseng et al, 2010). However, the scenario may be modified after *L. donovani* infection; therefore, we thought it worthwhile to administer LPS and *Leishmania* together in order to understanding how the signaling was manipulated by *Leishmania* in RAW 264.7 cells. LPS treatment (100 ng/ml) induced the up-
regulation of pro-inflammatory cytokines IL-12 and TNF-α (6.7- and 12.6-fold increase respectively compared with control, \( P < 0.001 \); Fig. 1A). On the contrary, production of both the cytokines was significantly reduced (71.2 and 80.9% respectively, \( P < 0.001 \)) when *Leishmania* and LPS were administered together (Fig. 1A). LPS induced iNOS mRNA expression was also significantly decreased (57.4%, \( P < 0.01 \)) when LPS and *L. donovani* were co-administered (Fig. 1B). As iNOS and pro-inflammatory cytokine gene expressions are primarily regulated by the transcription factor NF-κB, we measured the activity of NF-κB by luciferase reporter assay. On LPS stimulation, luciferase activity was enhanced by 7.8-fold over control cells whereas co-incubation of cells with LPS and *L. donovani* led to 73.6% attenuation of luciferase activity compared with LPS-stimulated cells (\( P < 0.001 \); Fig. 1C). Since MAP kinases play a major role in the activation of NF-κB, we studied the status of various MAP kinases. LPS induced strong phosphorylation of all the 3 MAP kinases (5.1-, 7.2-, and 3.6-fold for p38, ERK, and JNK, respectively), which was substantially decreased in *L. donovani*- and LPS-co-administered cells (59.4, 70.2, and 54.9%, respectively) at 30 min post-infection (Fig. 1D). Furthermore, LPS also caused significant induction of TAK1 phosphorylation (7.5-fold over control at 30 min post-infection; Fig. 1E), the MAP3 kinase that controls the activation of p38, JNK, and NF-κB (Lee et al, 2000) and this increase was significantly reversed in LPS- and *L. donovani*-co-administered cells (69.3% reduction; Fig. 1E). These results suggest that *Leishmania* parasites could effectively down-regulate TLR4-mediated inflammatory responses even if they were co-administered with LPS.
**L. donovani** inhibits translocation of TLR4-associated signaling complex into the cytosol

To ascertain the mechanisms responsible for suppressing LPS-stimulated macrophage activation, we studied TLR4-associated signaling complexes. For this, both the membrane and cytosolic fractions were prepared from either LPS or LPS- and *L. donovani*-coadministered RAW 264.7 cells. Membrane fractions were immunoprecipitated with anti-TLR4 antibody. Since cytosolic translocation of TAK1 is necessary for its activation and subsequent downstream signaling (Lee et al, 2000), we immunoprecipitated the cytosolic fractions with anti-TAK1 antibody to identify which of the signalosome components translocated from membrane to cytosol along with TAK1.
Individual components in both fractions were detected by Western blotting using respective antibodies (Fig. 2A, B). Immediately after LPS stimulation (within 0–5 min), MyD88, cIAP1/2, Ubc13, TRAF3, TRAF6, and TAK1, proteins known to be associated with TLR, were found in the membrane fraction, which persisted up to 10 min (Fig. 2A, left panel). The scenario was quite opposite in the cytosolic fraction that revealed appearance of membrane to cytosolic translocation of the entire signaling complex containing MyD88, TRAF6, cIAP1/2, Ubc13, and TAK1 10 min after LPS stimulation (Fig. 2A, right panel). Interestingly, TRAF3 was not detected in the immune-precipitated cytosolic complex (Fig. 2A, right panel). On the other hand, all the proteins except Ubc13 could be detected in the membrane fraction of LPS- and L. donovani-administered cells as studied up to 30 min (Fig. 2B, left panel), without any apparent cytosolic translocation (Fig. 2B, right panel). Significant activation of TAK1 (3.1-fold increase in phospho-TAK1 level over control, $P<0.001$) was observed in LPS-treated immuno-precipitated cytosolic fraction (Fig. 2B, left panel) whereas no phospho-TAK1 could be detected in LPS- and L. donovani-treated samples (Fig. 2B, right panel). These results suggest that LPS-induced TLR4-associated signaling complex translocates to the cytosol without TRAF3 and activates TAK1-mediated downstream signaling. This is in agreement with the hypothesis that TRAF3 degradation is one of the essential criteria for successful translocation of the membrane bound complex to the cytosol and subsequent phosphorylation of TAK1 (Tseng et al, 2010). In LPS- and L. donovani treated cells, this cytosolic translocation is completely inhibited possibly because of the persistent presence of TRAF3 in the TLR4-anchored signaling complex (Fig. 2B, left panel). This explained our earlier observation as to why TAK1 was not activated in LPS- and L. donovani-administered samples (Fig. 1E). Another interesting observation was decreased Ubc13 level in the membrane fraction of infected cells (Fig. 2B, left panel) without any apparent translocation to the cytosol (Fig. 2B, right panel), which might be indicative of probable degradation or dissociation of Ubc13 from TLR4-associated signalosome complex during infection. Since inhibition of TRAF3 degradation in the membrane could be responsible for impaired translocation of the entire signalosome complex, we determined the expression of TRAF3 in the whole cell lysate. L. donovani as well as LPS L. donovani-administered cells documented a steady level of TRAF3 expression as studied up to 60 min post-infection (Fig. 2C) whereas in LPS treated cells, TRAF3 started disappearing after 10 min. However, levels of cIAP1/2, TRAF6, and Ubc13 in the whole cell lysate did not show any significant change
These results suggest that *L. donovani* may interfere with the translocation of the TRAF3-cIAP1/2-TRAF6 signalosome complex into the cytosol by inhibiting TRAF3 degradation.

**Figure 2.** Modulation of signalosome translocation during *L. donovani* infection. A, B) RAW 264.7 cells were stimulated with either LPS (100 ng/ml; A) or LPS and *L. donovani* (B). Membrane and cytosolic fractions were prepared from whole-cell lysate and immunoprecipitated (IP) with anti-TLR4 and anti-TAK1 antibodies, respectively. Kinetics of signalosome translocation was ascertained by immunoblotting with antibodies against individual signalosome components. C) Cells were infected with parasites at 1:10 ratio and/or stimulated with LPS (100 ng/ml) for indicated times, and whole-cell lysates were subjected to Western blot analysis with anti-TRAF3 antibody; 0 min sample served as control. D) Cells were infected with parasites and/or stimulated with LPS for indicated times, and whole-cell lysates were subjected to Western blot analysis with indicated antibodies. One set of representative data is shown of the 3 independent experiments, expressed as means ± sd. **P < 0.01, ***P < 0.001; Student's t test.
Effect of TRAF3 inhibition on NF-κB activation and parasite survival during *L. donovani* infection

Persistence of TRAF3, a negative regulator of the MAP kinase pathway (Tseng et al. 2010), in the infected sample prompted us to determine its functional role by siRNA-mediated gene silencing. The efficacy of knockdown was determined by Western blotting, which showed 74.2% down-regulation in *L. donovani*-infected macrophages (Fig. 3A). In TRAF3-silenced infected cells, there were significant increase in the levels of phosphorylated TAK1 and p38 (3.8- and 4.3-fold compared with control siRNA-treated samples; Fig. 3B), the 2 kinases primarily involved in the host response against infection (Takaesu et al, 2001). NF-κB reporter activity was also markedly increased in TRAF3-silenced LPS- and *L. donovani*-administered cells (4.2-fold over control siRNA-treated samples, *P*<0.001; Fig. 3C). The MAP kinase and NF-κB reporter activity in TRAF3-silenced LPS-treated cells were almost comparable to those of LPS-treated cells (Fig. 3B, C). The levels of NF-κB, *Il-12*, and *Tnf*-α expression observed in TRAF3-silenced *L. donovani*-infected cells were higher than control siRNA-treated infected cells (3.2-, 2.2-, and 3.5-fold respectively, *P*<0.001; Fig. 3C, D). Moreover, silencing of TRAF3 in LPS- and *L. donovani*-administered cells led to enhanced levels of IL-12 and TNF-α (4.1- and 6.5-fold, respectively, over control siRNA treated LPS- and *L. donovani*-administered cells, *P*<0.001; Fig. 3D) as well as reduced parasite survival (76.3% reduction as compared with control siRNA treatment, *P*<0.001; Fig. 3E). These results suggest that prolonged presence of TRAF3 in case of *L. donovani* infection might play a major role in the modulation of TLR4-mediated MAP kinase activation.
Figure 3. Role of TRAF3 in infection. Cells were transfected (24 h) with either control or TRAF3 siRNA followed by *L. donovani* infection and/or stimulation with LPS (100 ng/ml) for 30 min. A) TRAF3 expression was determined by Western blotting. B) Levels of total and phosphorylated TAK1 and p38 were measured by immunoblotting in transfected cells. C) Cells were transfected with control or TRAF3 siRNA along with pNF-κB-luciferase plasmid (1 µg) and 0.5 µg of pCMV-β-gal. After 24 h of transfection, cells were stimulated with LPS (100 ng/ml) and/or infected with *L. donovani* for 24 h and assayed for luciferase activity. D) Cells were transfected with control or TRAF3 siRNA for 24 h and then treated with LPS (100 ng/ml) and/or infected with *L. donovani* for 24 h, followed by real-time PCR analysis of *Il-12* and *Tnf-α* levels as fold change. E, F) Cells were transfected with control or TRAF3 siRNA, infected with either *L. donovani* alone or *L. donovani* and LPS for 24 h, and intracellular parasite numbers (E) and percentage of infection (F) were determined by Giemsa staining. One set of representative data is shown of 3 independent experiments, expressed as means± sd. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s t test.
Role of UBC 13 and individual ubiquitin ligases in the modulation of the signalosome complex

IP study (fig 2B,) could not detect any significant level of UBC 13 in the membrane fraction of LPS- and L. donovani-administered cells as studied up to 30 min (Fig. 2B, left panel), without any apparent cytosolic translocation (TAK1 attached complex). Since, Ubc13 is an ubiquitin-conjugating enzyme of TRAF6, catalyzes the attachment of ubiquitin polymers on to target proteins, we therefore investigated the kinetics of Ubc13 association with TRAF6 during infection by coimmunoprecipitation. In contrast to LPS treated cells, where Ubc13 was strongly associated with TRAF6 till 30 min post-infection (Fig. 5M), it started dissociating from TRAF6 within 10 min after infection in LPS- and L. donovani-treated cells (Fig. 5N). Reduced k63-linked ubiquitination of TRAF6 in infection (fig. 4B) validated this observation. Since the TRAF6-cIAP1/2-TRAF3 signaling complex did not translocate to the cytosol following L. donovani infection, which may be attributed to the persistence of TRAF3, we checked for any discrepancies in the assembly of this complex in the whole-cell lysate. To this end, we studied the interaction of TRAF3 with the other 2ubiquitin ligases by coimmunoprecipitation studies. Both cIAP1/2 and TRAF6 strongly coimmunoprecipitated with TRAF3 after 10 min of LPS stimulation, followed by degradation of TRAF3 at 30 min (Fig. 4A). In contrast, steady level expression of TRAF3 was observed even at 30 min in LPS- L. donovani-treated cells and despite the consistent presence of TRAF3, its association with cIAP1/2 and TRAF6 was significantly decreased (P<0.05) compared with LPS-treated cells (Fig. 4A). In addition to TRAF3 persistence, decreased association of TRAF3 with cIAP1/2 and TRAF6 may also contribute to the defective signalosome translocation.

We therefore determined the ubiquitination pattern of these proteins since ubiquitin-dependent posttranslational modification of proteins determines their fate in cell signaling (Lorick et al, 1999). While K48-linked polyubiquitination of a protein is associated with its proteasomal degradation, K63-linked polyubiquitination is primarily associated with activating the protein to participate in protein-protein interaction, thus triggering downstream signaling pathways (Komander, 2009). TRAF3 was immunoprecipitated from both LPS- and LPS- and L. donovani-coadministered RAW264.7 cells followed by Western blotting with total, K48-linked, and K63-linked anti-ubiquitin antibodies. Unlike LPS-treated cells, where a time-dependent increase in K48-linked TRAF3 ubiquitination was found with a maxima at 10 min, LPS- and L. donovani-
treated cells showed a decrease in K48-linked ubiquitination of TRAF3 ($P<0.01$; Fig. 3B). The similar K63-linked TRAF3 ubiquitination as observed in both LPS and LPS- and *L. donovani*-treated cells (Fig. 4B) may indicate a unaltered MyD88 independent TLR4 signaling during infection. Since *Leishmania* infection impaired the association of TRAF3 with either cIAP1/2 or TRAF6, at the same time inhibiting K48-mediated degradation of TRAF3, we checked whether there was any discrepancy in the association of TRAF6-cIAP1/2 complex. Coimmunoprecipitation studies revealed markedly reduced association ($P<0.01$) between TRAF6 and cIAP1/2 in LPS- *L. donovani*-treated cells as compared with LPS treated cells (Fig. 4C). We then studied the ubiquitination pattern of cIAP1/2 and TRAF6 and found a time-dependent increase of total and K63-linked ubiquitination of both these proteins in LPS-treated cells, which was significantly decreased in LPS- and *L. donovani*-coadministered cells (Fig. 4D, E). A simultaneous decrease in the K63-linked ubiquitination pattern of TRAF6 and cIAP1/2 and K48-linked ubiquitination of TRAF3 in infected cells indicates a potential ubiquitination-dependent modulation of the host proteins by the parasite, thus making them incompetent to trigger the downstream signaling cascade.
Figure 4. Effect of *L. donovani* infection on Ubc 13 mediated TRAF6 ubiquitination and modulation of cIAP1/2, TRAF3 ubiquitination. A, C, D) RAW cells were infected with *L. donovani* and/or stimulated with LPS for indicated times, and whole-cell lysates were subjected to immunoprecipitation with anti-Ubc13 (A) or anti-TRAF3 (C) or anti-TRAF6 anti-antibody. Immunoprecipitates were subjected to Western blotting with indicated antibodies. B, D, E) For ubiquitination experiments, cells were infected with *L. donovani* promastigotes and/or stimulated with LPS for indicated times. Whole-cell lysates were prepared with NEM (10 mM), and 500 µg protein was boiled in 1% SDS for 5 min for disruption of noncovalently linked proteins. This was subjected to immunoprecipitation with anti-TRAF6 (B), anti-TRAF3 (D) anti-cIAP1/2 (E) antibodies, and immunoprecipitates were subjected to immunoblotting with anti-K63-linked ubiquitin and anti-K48-linked ubiquitin antibodies. Levels of cIAP1/2, TRAF3, and TRAF6 in the immunoprecipitates were detected by immunoblotting with respective antibodies. One set of representative data is shown of 3 independent experiments, expressed as means ± sd. IP, immunoprecipitation with indicated antibodies; IB, immunoblot with indicated antibodies. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s *t* test.
Role of individual ubiquitin ligases in the modulation of the signalosome complex

The defective ubiquitination pattern of all the 3 ubiquitin ligases TRAF6, cIAP1/2, and TRAF3 under infected condition prompted us to determine the interdependency of these 3 proteins by siRNA-mediated silencing in LPS-treated cells. The efficacy of knockdown was determined by Western blotting, which showed 81.6, 77.9 and 83.5% reduction in the protein level expressions of TRAF3, cIAP1/2, and TRAF6 respectively (Fig. 5A–C). Knockdown of TRAF3 in LPS-treated cells did not cause any change in K63-linked ubiquitination of both TRAF6 and cIAP1/2 (Fig. 5D, E), suggesting that K63-linked ubiquitination of these 2 proteins does not depend on TRAF3. However, knockdown of cIAP1/2 in LPS treated cells resulted in significant decrease in K48-linked ubiquitination of TRAF3 (P<0.01), without having any effect on the ubiquitination of TRAF6, suggesting the role of cIAP1/2 in only TRAF3 ubiquitination (Fig. 5F, G). Interestingly, silencing of TRAF6 in LPS-treated cells resulted in substantial decrease in K63-linked ubiquitination of cIAP1/2, and the level was comparable to that in LPS and L. donovani-coadministered cells (P0.01; Fig. 5H). This indicates a potential role of TRAF6 in cIAP1/2 ubiquitination. Moreover, silencing of TRAF6 also resulted in significantly reduced K48-linked ubiquitination of TRAF3 (P0.05; Fig. 5I) with a concomitant increase in the level of TRAF3 comparable to that found in LPS- and L. donovani-treated cells (Fig. 5J). Collectively, these data suggest that TRAF3 may act downstream of both cIAP1/2 and TRAF6, and cIAP1/2 lies between the 2 TRAF family proteins. Silencing of TRAF6 significantly increased the level of TRAF3 in LPS-treated macrophages (P<0.001; Fig. 5J), but the TRAF6 protein level was found to be comparable in LPS- and LPS- and L. donovani-treated cells (Fig. 5K). Therefore, instead of the unmodified protein, altered K63-linked ubiquitination of TRAF6 (Fig. 5L) may be responsible for TRAF3 persistence during infection. All these results suggest that Leishmania inhibits the K63-linked ubiquitination of TRAF6, which led to decreased ubiquitination of cIAP1/2 and ultimately prevents cIAP1/2-induced TRAF3 degradation.
Role of TRAF3 in infection in BALB/c mouse model

From our *in vitro* observations, it was revealed that persistence of TRAF3 in the signalosome complex during infection led to the deactivation of MAPK pathway, thereby facilitating parasite survival within host macrophages. We therefore evaluated the role of TRAF3 in the disease progression of visceral Leishmaniasis during *in vivo* situation. Splenocytes from control and infected mice were isolated after 2, 4 and 6 wk, respectively, and 1 set of splenocytes isolated from both control and infected mice was further stimulated by LPS (100 ng/ml), and TRAF3 expression was evaluated in all the 4 groups. Although *L. donovani* infection showed a steady level of TRAF3 at 2, 4, and 6 wk of infection, similar to control mice, LPS stimulation of
splenocytes isolated from both control and 2-wk-infected mice depicted a marked reduction in TRAF3 levels (68.8 and 61.6%, respectively, \( P<0.05 \)) as compared with untreated controls (Fig. 6A). However, when splenocytes from 4-wk-infected mice were subjected to LPS administration, enhanced TRAF3 expression was found, suggesting infection-induced stabilization of TRAF3 \emph{in vivo} (Fig. 6A). We then checked whether inhibition of TRAF3 could reverse the parasite triggered anti-inflammatory response, and for this, silencing of TRAF3 was achieved through administration of lentiviral vector-mediated shRNA. To this end, mice were injected with either control or TRAF3-specific shRNA, followed by \emph{L. donovani} infection for up to 6 wk. TRAF3 shRNA-treated mice showed apparently no sign of illness and remained healthy throughout the course of the experiment. The specificity and efficacy of shRNA on TRAF3 expression in spleen and liver were evaluated by immunoblot analysis that showed substantial reduction (80.6% and 66.3%, respectively, \( P<0.001 \)) of TRAF3 expression at 4 wk post-infection in comparison to control shRNA treatment (Fig. 6B). Since the disease progression in the case of visceral Leishmaniasis is dependent on differentiation of Th1/Th2 subtypes, with a role of Th1-dependent cytokines in parasite clearance, we next examined the effect of TRAF3 knockdown on the differentiation of Th1 and Th2 subpopulations. Gata-3, which is a marker for Th2 cell lineage differentiation, showed 41.2% decrease in expression after TRAF3 shRNA treatment as compared with control shRNA (Fig. 6C). Moreover, it was observed that T-bet, a transcription factor known to direct Th1 lineage commitment was induced in infected mice following TRAF3 knockdown (2.9-fold more than control shRNA treatment; Fig. 6D). TRAF3 inhibition in infected mice also showed significantly increased levels of IL-12, TNF-\( \alpha \), and IFN-\( \gamma \) (1010 \( \pm \) 90, 780 \( \pm \) 87, and 680 \( \pm \) 70 pg/ml compared with 97 \( \pm \) 11, 96 \( \pm \) 8, and 94 \( \pm \) 9 pg/ml in control shRNA-treated infected mice, \( P<0.001 \)) along with marked reduction of IL-10, TGF-\( \beta \), and IL-13 (390 \( \pm \) 42, 379 \( \pm \) 33, and 283 \( \pm \) 27 pg/ml compared with 995 \( \pm \) 86, 908 \( \pm \) 98, and 648 \( \pm \) 65 pg/ml) in control shRNA-transfected infected mice, \( P<0.001 \); Fig. 6E, F) at 4 wk post-infection, thereby suggesting that knockdown of TRAF3 in infected mice shifted the Th1/Th2 balance in favor of the host. Since IL-4 induces differentiation of naive helper T cells (Th0 cells) to Th2 cells (22), the level of IL-4 was also measured and found to be significantly reduced in TRAF3 shRNA-treated infected mice (160 \( \pm \) 14 pg/ml compared with 392 \( \pm \) 41 pg/ml in control shRNA treated infected mice, \( P<0.001 \); Fig. 6G). Finally, \emph{in vivo} silencing of TRAF3 resulted in markedly reduced parasite burden (61.3 and 54.1% reduction of spleen and liver parasite burden, respectively, compared with control shRNA-treated infected animal, \( P<0.001 \)) at 6 wk post-infection in the spleen and liver of infected mice (Fig. 6H, I). These results suggest that
inhibition of TRAF3 following *L. donovani* infection facilitates pro-inflammatory cytokine response leading to parasite suppression, which specifies TRAF3 as a negative regulator exploited by *Leishmania* for disease progression.

**Figure 6.** Effect of TRAF3 inhibition on parasite survival and Th1/Th2 cytokine balance. A) Splenocytes were isolated from control or 2-, 4-, and 6-wk infected BALB/c mice (10^7 parasites/animal). One set of control and infected splenocytes from each system was further stimulated with LPS (100 ng/ml) for 30 min. Splenocyte lysates were assayed for the expression of TRAF3 at various time points by immunoblotting. B) To achieve TRAF3 knockdown *in vivo*, 50 µl of 1000× lentiviral vector concentrate of TRAF3-specific shRNA or control shRNA (GFP-encoding shRNA) was transfected into spleen and liver tissue in anesthetized BALB/C mice. Expression of TRAF3 in control or TRAF3 shRNA-treated infected mice was assayed by immunoblotting. C, D) Mice were administered either control or TRAF3 shRNA, followed by infection with *L. donovani*. mRNA was isolated from each group, and expression of Gata-3 (C) and T-bet (D) was estimated by real-time PCR. E–G) Levels of IL-12, TNF-α, and IFN-γ (E); IL-10, TGF-β, and IL-13 (F); and IL-4 (G) were similarly measured in splenocytes of different groups (control or infected or infected plus control shRNA and infected plus TRAF3 shRNA) of mice at indicated time points by ELISA. H, I) Spleen (H) and liver (I) parasite burden were evaluated weekly in different groups as described in Materials and Methods and represented as mean ± SD LDU for 5 animals. One set of representative data is shown of 3 independent experiments, expressed as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s t test.
DISCUSSION

It has always been intriguing how a unicellular pathogen like *Leishmania* manages to evade host cell defense and successfully survive and replicate. Accumulating evidence indicates a significant contribution of TLR4 in curbing *Leishmania* growth in both phases of immune response (Faria et al., 2012). TLR4-knockout studies showed increased parasite survival (Kropf et al., 2004), and TLR4-mediated IL-12 signaling is found to be important for resistance against the parasite (Kropf et al., 2004). Although most of the TLR4-associated proteins positively regulate macrophage defense (Shibolet and Podolsky, 2007), TRAF3, an E3 ubiquitin ligase and an essential component of TLR signaling, negatively regulates TLR4-mediated host activation, possibly to avoid unregulated inflammatory response (Tseng et al., 2010). Our study brings forth the important role of TRAF3 in enabling the parasite to make macrophage defenses submissive, through defective translocation of TLR4-anchored multiprotein complex (Fig. 7). Our data revealed that this translocation stagnancy was because of reduced TRAF3 degradation during infection. The persistent TRAF3 level during infection was inversely related to its K48-linked ubiquitination, which is a prerequisite for CD40-, MAPK-, and other TNFR-mediated macrophage activation (Vallabhapurapu et al., 2008; Medzhitov et al., 1998), and the absence of K48 ubiquitination in leishmaniasis is in line with this notion. On the other hand, K63-linked TRAF3 ubiquitination, responsible for IL-10 activation through the MyD88-independent TRIF pathway (Oganesyan et al., 2006), remained unaltered in the infected condition, supporting the fact that *Leishmania* can selectively modulate inflammatory responses (Ruse and Knaus, 2006; Ruhland et al. 2007) to cater its own needs. The negative regulatory role of TRAF3 in macrophage activation was documented by gene-knockdown studies (Vallabhapurapu et al., 2008), and this is in accordance with our observation that the absence of TRAF3 reversed infection-induced anti-inflammatory effects in terms of proinflammatory mediators and intramacrophage parasite survival.

Decreased ubiquitination of TRAF3 was found to be associated with a decrease in total and K63- and K48-linked ubiquitination of cIAP1/2, the E3 ligase known to be responsible for TRAF3 ubiquitination (Vallabhapurapu et al., 2008; Matsuzawa et al., 2008). K63-linked ubiquitination of cIAP1/2 is in turn catalysed by TRAF6, whose autoubiquitination-mediated activation catalyzes ubiquitination of cIAP1/2, accentuating their activity as TRAF3 K48-specific E3 ligases (Vallabhapurapu et al., 2008; Matsuzawa et al., 2008). A decrease in the K63-linked
ubiquitination of TRAF6 during infection with no apparent change in total and K48-linked ubiquitination is indicative of signaling-mediated protein modification. This notion was further strengthened by selectively inhibiting each of the E3 ligases one at a time, which showed that the first ubiquitinating enzyme is TRAF6, and the ultimate modified one is TRAF3, and cIAP1/2 lies between these 2 TRAF family enzymes. Decreased ubiquitination could be a consequence of decreased association between these proteins, and markedly decreased TRAF6- cIAP1/2 association in the presence of increased TRAF3 validates this hypothesis. This may sound contradictory to our earlier observation in which all the signalosome-associated proteins remained attached under infected conditions. The only possible explanation could be that although all the components of the signalosome complex were associated, the strong and specific “1:1” interaction necessary for its translocation might be diminished. Failure in TRAF6 ubiquitination probably disrupts the proximity of TRAF6-cIAP1/2, thereby suppressing the TRAF3 K48-specific E3 ligase activity of cIAP1/2. Apart from A20, the ubiquitin editing enzyme responsible for TRAF6 deubiquitination (Sun et.al., 2011), the E2-conjugating enzyme Ubc13 has also been implicated in TRAF6 self-ubiquitylation and TRAF6-induced NF-B activation (Lorick et.al., 1999; Komander, 2009, Faria et.al., 2012; Shembade et.al, 2010; Kawai et.al, 2011). Decreased association of TRAF6-Ubc13 during infection suggested that L. donovani may inhibit ubiquitination of TRAF6 through Ubc13 dissociation/degradation. The molecule present in L. donovani responsible for this inhibitory effect is not known and can only be suggested at this juncture. Surface antigens that have been shown to play immunomodulatory roles, like hydrophilic acylated surface protein HASP), LPG, leishmanolysin (GP63), glycosylphospholipid (GIPL), and cysteine proteases (CPs), are few (Olivier et.al., 2005; Depledge et.al., 2010). Recent reports suggest that some exosomes released by Leishmania, which are supposed to contain molecules like GP63 and elongation factor-1(EF-1), act as master players in sabotaging host cell signaling (Silverman et.al., 2011). More detailed and in-depth studies of Leishmania and secreted exosomes are required to answer this. The critical role played by TRAF3 in infection was addressed in the BALB/c mouse model of visceral leishmaniasis. In infected mice, shRNA-mediated TRAF3 depletion showed significantly decreased parasite burden with increasing production of the proinflammatory cytokines IL-12 and TNF-. Even though TRAF3-null mice show postnatal lethality by d 10, histological evidence shows no gross defect in structure of organs like spleen, liver, thymus, and kidney (Xu et.al., 1996). shRNA mediated spleen-specific disruption of TRAF3 was persistent up to 6 wk after administration,
with no untoward inflammatory response as earlier reported for TRAF3-null mice. The present study has highlighted the role of TRAF3, which, if not completely indispensable, plays a critical role in the pathogenicity of *L. donovani*. This parasite targets the post-translation modulation of TRAF6, which, through a series of ubiquitination-dependent events, leads to the persistence of TRAF3 in the membrane and prevents macrophage proinflammatory activation, thus promoting parasite growth and survival (Fig. 7). Our study thus provides a mechanistic insight into modulation of host-defensive signaling machinery by *Leishmania* and presents TRAF3 as a potential therapeutic target in visceral leishmaniasis.

**Figure 7.** Modulation of macrophage membrane associated signaling events by *L. donovani*. LPS binding to TLR4 triggers the recruitment of MyD88, followed by formation of signalosome complex consisting of TRAF6, cIAP1/2, Ubc13, and TRAF3. Ubiquitination at K63 of TRAF6 enables it to ubiquitinate cIAP1/2, which in turn ubiquitinates TRAF3 at K48, leading to its proteasomal degradation. This is followed by subsequent release and translocation of the entire complex to the cytosol, resulting in activation of MAPK and NF-κB. *Leishmania* infection leads to Ubc13 dissociation from the complex, causing defective K63-linked ubiquitination of TRAF6. This leads to inadequate K63-linked ubiquitination of cIAP1/2, thereby resulting in insufficient K48-linked ubiquitination and subsequent reduction in TRAF3 degradation. As a result, TRAF3, as well as the signalosome complex, persists in the membrane, resulting in inappropriate TAK1 activation, which ultimately dampens the proinflammatory responses.