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

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ABSTRACT Intramacrophage pathogen Leishmania donovani escapes host immune response by subverting Toll-like receptor (TLR) signaling, which is critically regulated by protein ubiquitination. In the present study, we identified tumor necrosis factor receptor-associated factor (TRAF) 3, degradative ubiquitination of which is essential for TLR4 activation, as a target for Leishmania to deactivate LPS-mediated TLR4 signaling. We used LPS-treated RAW 264.7 cells and compared the TLR4-mediated immune response in these cells with L. donovani and L. donovani + LPS costimulated macrophages. TRAF3, which was ubiquitinated (2.1-fold over control) at lys 48 position and subsequently degraded following LPS treatment, persisted in L. donovani and L. donovani + LPS costimulated 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 postinfection. 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 (10.8- and 8.1-fold over infected control) and decreased spleen parasite burden (61.3% suppression, P<0.001), thereby marking reduction in disease progression. Our findings identified TRAF3 as a novel molecular regulator exploited by Leishmania for successful infection.—Gupta, P., Giri, J., Srivastav, S., Chande, A. G., Mukhopadhya, R., Das, P. K., Ukil, A. Leishmania donovani targets tumor necrosis factor receptor-associated factor (TRAF) 3 for impairing TLR4-mediated host response. FASEB J. 28, 1756–1768 (2014). www.fasebj.org

Key Words: macrophage • ubiquitination • signalosome

TOOL-LIKE RECEPTORS (TLRs), which recognize pathogen-associated molecular pattern (PAMP), are the means for first line of host defense leading to pathogen clearance via enhanced proinflammatory response. Cognate ligand binding to TLRs leads to recruitment of myeloid differentiation primary response gene 88 (MyD88), 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) (1). 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 (2).

TRAF3 is an E3 ubiquitin ligase that regulates TLR pathway through distinct protein ubiquitination at specific residues (3). In contrast to other TRAF members, TRAF3 is particularly known as a negative regulator of NF-κB signaling in response to TLR ligation in bone marrow-derived macrophages (BMDMs), plasmacytoid dendritic cells (pDCs), and murine embryonic fibroblasts (MEFs) (4, 5). TRAF3-null cells demonstrate prolonged activation of NF-κB and subsequent release of proinflammatory cytokines (4, 5). Moreover, TRAF3-null mice have been shown to illustrate postnatal

Abbreviations: BMDM, bone marrow-derived macrophage; cIAP1/2, cellular inhibitor of apoptosis 1/2; iNOS, inducible nitric oxide synthase; LDU, Leishman-Donovan unit; LPG, lipophosphoglycan; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NEM, N-ethyl maleimide; TAK1, TGF-β associated kinase 1; TLR, Toll-like receptor; TRAF3/6, tumor necrosis factor receptor associated factor 3/6; Ubc13, ubiquitin-conjugating enzyme 13

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lethality with hyperinflammatory phenotype before death on d 10 (6). However, this potent defense mechanism of the host cell puts strong selective pressure on *Leishmania* parasites, which have, in turn, evolved strategies to modulate the host TLR signaling cascade in their own favor. Several reports have documented the role of TLRs and the importance of the pathway in curbing the progression of disease (7). Mice lacking TLR4 (8) or MyD88 (9) show high susceptibility to *Leishmania* infection, which can be attributed to NF-κB inactivation and proinflammatory cytokine down-regulation. Similar reports support the role of TLR4 and endosomal TLR in activating host macrophages during *Leishmania panamensis* infection (10).

We reported earlier that *Leishmania donovani*, the causative agent of fatal visceral leishmaniasis, could suppress TLR2-mediated signaling by exploiting host deubiquitinating enzyme A20 despite possessing a potent TLR2 ligand, lipophosphoglycan (LPG; ref. 11). However, *Leishmania* could also suppress TLR4-mediated proinflammatory cytokine production in BMDMs, and the inhibitory effects might be extended to the immune response elicited by other TLRs as well (11). In the present study, we tried to elucidate the molecular mechanism behind infection-induced down-regulation of macrophage TLR4 activation. Our study showed that *Leishmania* infection alters the ubiquitination pattern of TRAF3 required for the effective cytosolic translocation of the TLR4-anchored multiprotein complex, ultimately leading to infection-induced down-regulation of host immune response.

**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). Cytokalasin 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). The pNF-κB reporter assay 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 (12). 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 (12) and incubated for the specified periods.

**Infection in mice**

*In vivo* infection was performed by injecting 10⁷ *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; ref. 13). Splenocytes were isolated and cultured as described earlier (12). 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 RNeasy 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, Tgfβ, inducible nitric oxide synthase (inos), Tbet*, 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 ΔΔCt 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, cIAP1/2, and A20-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 × 10⁵ 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 (14). 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 × 10⁸ infectious U/ml as described earlier (15). shRNA target sequence selection and nonrelevant control vector use were the same as reported previously (16). 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 10⁵ 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 (11). For ubiquitination analysis, 10 mM N-ethyl maleimide (NEM; Sigma) was added to the lysis buffer.

Densitometric analysis

Semi-quantification of ubiquitinated proteins was done using ImageJ 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 ± so. 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 (3). 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 proinflammatory cytokines Il-12 and Tnfα (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). All the experiments were further repeated in BMDMs, where a similar profile of cytokine production was observed (Supplemental Fig. S1). LPS-induced inos mRNA expression was also significantly decreased (57.4%, P<0.01) when LPS and L. donovani were coadministered (Fig. 1B). As iNOS and proinflammatory 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 coincubation 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-coadministered cells (59.4, 70.2, and 54.9%, respectively) at 30 min postinfection (Fig. 1D). Furthermore, LPS also caused significant induction of TAK1 phosphorylation (7.5-fold over control at 30 min postinfection; Fig. 1E), the MAP3 kinase that controls the activation of p38, JNK, and NF-κB (17) and this increase was significantly reversed in LPS- and L. donovani-coadministered cells (69.3% reduction; Fig. 1E). These results suggest that Leishmania parasites could effectively down-regulate TLR4-mediated inflammatory responses even if they were coadministered 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 (17), 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 (3), 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 signal 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 immunoprecipitated 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 immunoprecipitated 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 (3). 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

Figure 1. Effect of L. donovani and LPS coadministration on macrophage activation. A) RAW 264.7 cells were incubated for 24 h with either LPS (100 ng/ml) alone, and/or L. donovani promastigote (cell/parasite ratio, 1:10). Expression of Il-12 and Tgrfα was evaluated by TaqMan analysis at the mRNA level, normalized to β-actin and expressed as fold change compared with control. B) RAW cells were treated as in A for 24 h, and inos mRNA levels were measured by real-time PCR analysis. C) RAW cells were transfected with pNF-κB luciferase plasmid (1 μg) and 0.5 μg of pCMV-β-gal. After 24 h of transfection, cells were treated with L. donovani, LPS, or LPS along with L. donovani for 24 h, lysed, and processed for luciferase activity. D, E) Macrophages were treated separately with LPS and/or L. donovani for various time periods. Whole-cell lysates were subjected to SDS-PAGE followed by Western blotting to detect phosphorylation of p38, ERK1/2, and JNK (D) and TAK1 (E). 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.
as well as LPS and/or L. donovani-administered cells documented a steady level of TRAF3 expression as studied up to 60 min postinfection (Fig. 2C) whereas in LPS-treated cells, TRAF3 started disappearing after 10 min. Similar observation was obtained in BMDM (Supplemental Fig. S2). However, levels of cIAP1/2, TRAF6, and Ubc13 in the whole cell lysate did not show any significant change \((P>0.05; \text{Fig. 2D})\). 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.

**Effect of TRAF3 inhibition on NF-\(\kappa\)B activation and parasite survival during \(L.\) donovani infection**

Persistence of TRAF3, a negative regulator of the MAP kinase pathway (3), in the infected sample prompted us to determine its functional role by siRNA-mediated gene silencing. The efficacy of knockdown was deter-
mined 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 (18). 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, II-12, and *Tnfa* 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 samples).

**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 II-12 and *Tnfa* 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 ± s.n. *P*<0.05, **P*<0.01, ***P*<0.001; Student’s *t* test.
treated LPS- and L. donovani-administered cells, \( P<0.001 \); Fig. 5D) as well as reduced parasite survival (76.3% reduction as compared with control siRNA treatment, \( P<0.001 \); Fig. 3E). TRAF3 silencing was found to have no effect on L. donovani uptake, as revealed by using cytochalasin D (10 \( \mu \)g/ml for 30 min before treatment) as the negative control (\( P>0.05 \); Supplemental Fig. S3C). 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. To ascertain the role of A20, an ubiquitin editing enzyme (19), in TLR4-mediated signaling during infection, A20 was silenced by siRNA-mediated knockdown in RAW264.7 macrophages and cells were incubated with either LPS and/or L. donovani for 24 h. Silencing of A20 significantly increased NF-\( \kappa \)B, IL-12, and TNF-\( \alpha \) production (Supplemental Fig. S3A, B) in LPS- and L. donovani-treated cells at the mRNA level (4.5-, 4.4- and 6.9-fold respectively over control siRNA-treated LPS- and L. donovani-administered cells, \( P<0.001 \)). Administration of TLR2 neutralizing antibodies (1 \( \mu \)g/ml, 1 h before treatment with LPS and L. donovani) did not alter the level of these cytokines suggesting thereby that this proinflammatory response was mediated by TLR4 and not by TLR2. This is not surprising, as A20 is upregulated in L. donovani infection (11) and is known to inhibit TLR4 signaling by deubiquitinating TRAF6.

Ubiquitination pattern of signalosome complex proteins during L. donovani infection

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 2 ubiquitin 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 post-translational modification of proteins determines their fate in cell signaling (20). 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 (21). 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 maximum 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 an unaltered MyD88 independent TLR4 signaling (5) 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- and 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 in 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). We repeated these experiments in BMDMs, and similar ubiquitination profile of TRAF6, cIAP1/2, and TRAF3 was observed in case of LPS and L. donovani treatment (Supplemental Fig. S4A). However, for total ubiquitination, no significant change in ubiquitination pattern was observed for LPS- and L. donovani- and LPS-treated cells (Supplemental Fig. S4B). 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.

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 ubiqui-
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 (*P*/H11021 0.01; 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 (*P*/H11021 0.05; 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*/H11021 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. Hence, our next aim was to determine the underlying mechanism for this modulation.

Ub13 is a ubiquitin-conjugating enzyme that catalyzes the attachment of ubiquitin polymers onto target proteins, where the ubiquitin chain is linked by noncanonical K-63 instead of canonical K48 (19). Infection by *L. donovani* led to defective K63-linked ubiquitination of TRAF6 as well as reduced levels of Ub13 (Fig. 2B). We therefore investigated the kinetics of Ub13 association with TRAF6 during infection by coimmunoprecipitation. In contrast to LPS-treated cells, where Ub13 was strongly associated with TRAF6 till 30 min postinfection (Fig. 5M), it started dissociating from TRAF6 within 10 min after infection in LPS- and *L. donovani*-treated cells (Fig. 5N) suggesting thereby a possible explanation for reduced ubiquitination of TRAF6. All these results suggest that *Leishmania* inhibits the Ub13-mediated K63-linked ubiquitination of TRAF6, which led to decreased ubiquitination of cIAP1/2 and ultimately prevents cIAP1/2-induced TRAF3 degradation.

Figure 4. Effect of *L. donovani* infection on ubiquitination of cIAP1/2, TRAF3, and TRAF6. A, C) 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-cIAP1/2 (A) or anti-TRAF6 (C) 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-TRAF3 (B), anti-cIAP1/2 (D), or anti-TRAF6 (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 ± sn. 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 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 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 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 postinfection 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-α, and IFN-γ (1010±90, 780±87, and 680±70 pg/ml compared with 97±11, 96±8, and 94±9 pg/ml in control shRNA-treated infected mice, P<0.001) along with marked reduction of IL-10, TGF-β, and IL-13 (390±42, 379±33, and 283±27 pg/ml compared with 995±86, 908±98, and 648±65 pg/ml) in control shRNA-transfected infected mice, P<0.001; Fig. 6E, F) at 4 wk postinfection, 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±14 pg/ml compared with 392±41 pg/ml in control shRNA-treated infected mice, P<0.001; Fig. 6G). Finally, 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 postinfection in the spleen and liver of infected mice (Fig. 6H, I). These results suggest that inhibition of TRAF3 following L. donovani infection facilitates proinflammatory cytokine response leading to parasite suppression, which specifies TRAF3 as a negative regulator exploited by Leishmania for disease progression.

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 (23). TLR4-knockout studies showed increased parasite survival (8), and TLR4-mediated IL-12 signaling is found to be important for resistance against the parasite (24). Although most of the TLR4-associated proteins positively regulate macrophage defense (25), TRAF3, as an E3 ubiquitin ligase and an essential component of TLR signaling, negatively regulates TLR4-mediated host activation, possibly to avoid unregulated inflammatory response (3). 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 (26, 27), 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 (5), remained unaltered in the infected condition, supporting the fact that Leishmania can selectively modulate inflammatory responses (28, 29) to cater its own needs. The negative regulatory role of TRAF3 in macrophage activation was documented by gene-knockdown studies (26), 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 (26, 30). K63-linked ubiquitination of cIAP1/2 is in turn catalyzed by TRAF6, whose autoubiquitination-mediated activation catalyzes ubiquitination of cIAP1/2, accentuating their activity as TRAF3 K48-specific E3 ligases (26, 30). 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 (31), the E2-conjugating enzyme Ubc13 has also been implicated in TRAF6 self-ubiquitination and TRAF6-induced NF-kB activation (20, 21, 23, 32, 33). 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 (GPL), and cysteine proteases (CPs), are few (34, 35). Recent reports suggest that some exosomes released by *Leishmania*, which are supposed to contain molecules like GP63 and elongation factor-1α (EF-1α), act as master players in sabotaging host cell signaling (36). 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-α. 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 (6). 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-translational 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.

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Signal Transduction:
*Leishmania donovani* Prevents Oxidative Burst-mediated Apoptosis of Host Macrophages through Selective Induction of Suppressors of Cytokine Signaling (SOCS) Proteins

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Leishmania donovani Prevents Oxidative Burst-mediated Apoptosis of Host Macrophages through Selective Induction of Suppressors of Cytokine Signaling (SOCS) Proteins

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Background: Leishmania inhibits oxidative burst-mediated apoptosis of macrophages during phagocytosis.

Results: L. donovani induces (SOCS) 1 and 3, which suppress macrophage apoptosis through thioredoxin-mediated stabilization of protein-tyrosine phosphatases.

Conclusion: Leishmania exploits macrophage SOCS proteins for inhibition of apoptosis, thus protecting its niche for survival and replication.

Significance: This study demonstrates a novel anti-apoptotic mediator for parasite infection.

One of the mechanisms for establishment of infection employed by intra-macrophage pathogen-like Leishmania is inhibition of oxidative burst-mediated macrophage apoptosis to protect its niche for survival and replication. We tried to elucidate the underlying mechanism for this by using H2O2 for induction of apoptosis. Leishmania donovani-infected macrophages were much more resistant to H2O2-mediated apoptosis compared with control. Although infected cells were capable of comparable reactive oxygen species production, there was less activation of the downstream cascade consisting of caspase-3 and -7 and cleaved poly(ADP)-ribose polymerase. Suppressors of cytokine signaling (SOCS) 1 and 3 proteins and reactive oxygen species scavenging enzyme thioredoxin, known to be involved in stabilization of protein-tyrosine phosphatases, were found to be induced during infection. Induction of SOCS proteins may be mediated by Egr1, and silencing of Socs1 and -3 either alone or in combination resulted in reduced thioredoxin levels, enhanced activation of caspases, and increased apoptosis of infected macrophages. The induction of protein-tyrosine phosphatases, thioredoxin, SOCS, and Egr1 in L. donovani-infected macrophages was found to be unaffected by H2O2 treatment. SOCS knocked down cells also displayed decreased parasite survival thus marking reduction in disease progression. Taken together, these results suggest that L. donovani may exploit SOCS for subverting macrophage apoptotic machinery toward establishing its replicative niche inside the host.

Programmed cell death, or apoptosis, is a signal-dependent physiological suicide mechanism that preserves homeostasis by maintaining the delicate balance between cell proliferation and cell death (1). Apart from serving all these diverse spectra of functions, it serves as a defense mechanism against viruses and probably other infectious agents, such as intracellular bacteria and parasites (2). In plants, insects, and mammals, the rapid induction of apoptosis in response to pathogen entry represents an evolutionarily conserved protective response against infections. Conversely, as pathogens are under great selective pressure to defeat the host defense systems, they have evolved a variety of ways to specifically antagonize apoptotic death of the invaded host cell, allowing them more time to replicate (3–5). Moreover, it would be to the advantage of the invading organism to subvert the apoptotic machinery; hence not destroying its niche before egression. Therefore, even though apoptosis, induced in infected cells by cytotoxic immune effector cells, is a critical defense against intracellular pathogens, many viral, bacterial, and protozoan pathogens have developed mechanisms to invade and multiply within host cells without inducing apoptosis (6–10). Various parasites undermine the apoptotic progression that includes Chlamydia, Escherichia coli, Mycobacterium tuberculosis, Toxoplasma gondii, Plasmodium berghei, and Leishmania species (11–17). Leishmania donovani was the first parasite reported to enhance host cell viability by inhibiting growth factor deprivation-induced apoptosis. One potential mechanism behind this inhibition has been through the activation of NF-κB and PI3K/Akt pathways (17, 18).

Leishmania species cause a spectrum of diseases ranging from nonlethal cutaneous leishmaniasis (Leishmania major) to fatal visceral leishmaniasis (L. donovani). The Leishmania parasites are internalized by macrophages into phagolysosomes, where they display the remarkable ability to survive and replicate within this hostile environment. However, it is of interest to note that once internalized into macrophages, the parasite has to face severe oxidative stress inside the macrophages due to extensive production of reactive oxygen species (ROS) (19).

The abbreviations used are: ROS, reactive oxygen species; SOCS, suppressors of cytokine signaling; PTP, protein-tyrosine phosphatase; SHP, Src homology; DCFDA, 2',7'-dichlorofluorescein diacetate; PARP, poly(ADP)-ribose polymerase; Z, benzoylloxy carbonyl; fmk, fluoromethyl ketone; pNPP, p-nitrophenyl phosphate; pna, p-nitroanilide; Pi, propidium iodide.

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**This article contains supplemental Figs. 1–3.**

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Large quantities of ROS have been implicated as microbicidal agents in pathological situations and ultimately result in apoptosis of the macrophages harboring the pathogen, thereby resulting in parasite clearance (20). Although *Leishmania* promastigotes are susceptible to oxygen intermediates generated in vitro, they succeed in establishing infection either by avoiding or resisting the toxic effects of superoxide and other ROS generated during phagocytosis (21, 22). Recent studies have revealed that ROS leads to transient oxidation and inactivation of protein-tyrosine phosphatases (PTPs) that compose a large, structurally diverse family of receptor-like and nontransmembrane enzymes that are specific regulators of signal transduction, which, in conjunction with the protein-tyrosine kinases, exert exquisite control over various biological functions (23, 24). All PTPs contain catalytic cysteine residues on the ROS-sensitive site, and the ROS-mediated oxidation of cysteine residues results in their inactivation. These phenomena are reversible during the redox regulation such that the oxidized PTPs are readily reduced back by thioredoxin and/or glutathione, which act in the ROS scavenging system. In various studies, a role of thioredoxin in cell protection from the ROS-induced apoptosis has been reported in mammalian systems (25, 26). These PTP-stabilizing enzymes such as thioredoxin are reported to act in coordination with members of the suppressors of cytokine signaling (SOCS) family in the inhibition of ROS-mediated apoptotic signaling cascade (27). It has been reported that SOCS suppress cytokine signal transduction by binding to phosphorylated tyrosine residues on cytokine receptor chains, and the physiological importance of SOCS1 and SOCS3 is demonstrated by the lethal phenotypes observed in knock-out mice (28). Silencing of SOCS proteins has been reported to promote apoptosis in various malignancies. Moreover, recent studies have also shown the implication of SOCS-mediated anti-apoptotic signaling in several diseases (29–32). In addition to the modulation of PTPs, the mechanisms of ROS-induced apoptosis involve diverse downstream enzymes, including mitogen-activated protein kinases (MAPKs) and the associated signaling pathways. However, the precise role that different MAPK members play during ROS-induced apoptosis and the mechanistic link between ROS-mediated modulation of PTPs and MAPK activation are not known.

In this study, using hydrogen peroxide as an inducing agent for ROS-mediated apoptosis, we tried to elucidate the mechanism used by the parasite to counteract oxidative burst and the consequent suppression of oxidative burst-mediated host cell apoptosis. Hydrogen peroxide treatment failed to bring about apoptosis of macrophages infected with *L. donovani*. It was observed that although infected cells were capable of ROS production during early hours, there was complete abrogation of the downstream caspase cascade that was found to be mediated by SOCS proteins. Silencing of these proteins resulted in reduced thioredoxin levels and increased apoptosis in infected macrophages through de-activation of PTPs. SOCS knockdown cells also displayed decreased parasite survival, thus marking reduction in disease progression. Taken together, these results suggest that *L. donovani* employs differential induction of host SOCS proteins to subvert macrophage apoptotic machinery triggered by parasite internalization-mediated oxidative burst, thus establishing its replicative niche inside the host.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Parasites**—The pathogenic promastigotes of *L. donovani* strain (MHOM/IN/1983/AG83) were maintained in Medium 199 (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin. The murine macrophage cell line RAW 264.7 was maintained at 37 °C, 5% CO₂ in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). *In vitro* infection experiments were carried out with the RAW 264.7 cell line using stationary phase promastigotes at a 10:1 parasite/macrophage ratio.

**Reagents, Antibodies, and Constructs**—All antibodies were from Santa Cruz Biotechnology and Cell Signaling Technology. All other chemicals were from Sigma, unless indicated otherwise.

**Apoptosis Detection by Annexin V Staining**—RAW 266.7 cells (2 × 10⁴) were infected with *L. donovani* promastigotes for different time periods. One group of infected macrophages for each time point of infection was treated with H₂O₂. After an hour of treatment, the culture media were replaced, and cells were incubated overnight at 37 °C, 5% CO₂. The cells were washed twice with PBS. Apoptosis was then determined using annexin-V–FLUOS staining kit (Roche Applied Science) as per the manufacturer’s instructions. Cells were analyzed on FACS Canto II™ cell sorter using 488 nm excitation and 530 nm emissions for FITC and >600 nm for PI fluorescence using FACS Diva software.

**Immunoprecipitation and Immunoblotting**—Cells were lysed in lysis buffer (Cell Signaling Technology), and the protein concentrations in the cleared supernatants were estimated using a protein assay (Bio-Rad). Immunoprecipitation was performed as described previously (33). Briefly, pre-cleared cell lysates (500 μg) were incubated overnight with specific primary antibody at 4 °C. For co-immunoprecipitation studies, pull-down with unrelated antibodies served as control. 25 μl of protein A/G plus agarose beads (Santa Cruz Biotechnology) were added to the mixture and incubated for 4 h at 4 °C. Immune complexes were collected and washed three times with ice-cold lysis buffer and once with lysis buffer without Triton X-100. The immunoprecipitated samples and cell lysates were resolved by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Millipore). 30 μg of protein from the whole cell lysate of each sample were loaded as input. The membranes were blocked with 5% BSA in wash buffer (TBS, 0.1% Tween 20) for 1 h at room temperature and probed with primary antibody overnight at dilution recommended by the suppliers. Membranes were washed three times with wash buffer and then incubated with alkaline phosphatase-conjugated secondary antibody and detected by hydrolysis of 5-bromo-4-chloro-3’-indolyphosphate chromogenic substrate according to the manufacturer’s instructions.

**Estimation of ROS Production**—Intracellular ROS generation was measured using the oxidant sensitive green fluorescent dye 2’,7’-dihydrodichlorofluorescein diacetate.
SOCS Proteins in Macrophage Apoptosis by L. donovani

(H_2DCFDA) (Molecular Probes). Measurement of fluorescence in cells was made by counting at least 10,000 events/test using a FACScalibur flow cytometer (BD Biosciences), with a fluorescein isothiocyanate filter, and the cells were gated out based on their fluorescent property. Samples were examined by FACScalibur, and the results were analyzed using CellQuest software (BD Biosciences).

Phosphatase Assay—Macrophages were lysed in PTP lysis buffer (50 mM Hepes, pH 7.4, containing 0.5% Triton X-100, 10% glycerol, 1 mM benzamidin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A) and kept on ice for 45 min. Lysates were cleared by centrifugation, and protein content was determined by protein assay (Bio-Rad). 10 μg of protein extract were incubated in phosphatase reaction buffer (50 mM Hepes, pH 7.5, 0.1% 2-mercaptoethanol, 10 mM pNPP) for 30 min. Absorbance was read at 405 nm. In a separate set of experiments, PTP activity was further determined by the capacity of protein lysates to dephosphorylate a monophosphorylated phosphotyrosine peptide substrate (TRDIPYETDYIRK) for 10 min at 37°C. Free inorganic phosphate was detected with malachite green (Sigma), and absorbance was taken at 620 nm. To evaluate specific activities of SHP-1, SHTB1, SHTB-2, and CD45, the proteins were immunoprecipitated using respective antibodies, and specific PTP activity was then evaluated by pNPP hydrolysis as described above. Nonspecific hydrolysis of pNPP by lysates was assessed in nonimmune IgG immunoprecipitates and subtracted from the values obtained for enzyme immunoprecipitates.

Real Time PCR—Total RNA from RAW 264.7 cells was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. 1 μg of DNA was used as template for cDNA synthesis using the SuperScript first strand synthesis system for the RT-PCR kit (Invitrogen). Quantitative real-time PCRs (ABI 7500 Fast Real Time PCR system, Applied Biosystems) were performed using TaqMan Fast Universal PCR master mix (Applied Biosystems). TaqMan probes for Socs1, Socs2, Socs3, and C/EBPα were purchased from Applied Biosystems. The ABI 7500 Fast Sequence detector was programmed with the following PCR amplification conditions: 40 cycles of 95°C for 15 s and 60°C for 1 min. β-Actin was chosen as an internal control for variability in amplification because of differences in initial mRNA concentrations. Relative quantitation was performed using the comparative ΔΔCt method, and data were normalized to β-actin mRNA levels and expressed as a fold change compared with uninfected controls.

siRNA Transfection—RAW 264.7 cells (2 × 10⁶) were transfected with 1 μg of either Egr1 or SHP1 or PTB1B or thioredoxin or SOCS1 and/or SOCS3 siRNA according to the manufacturer’s instructions (Santa Cruz Biotechnology). Scrambled siRNA was used as control. Following silencing, cells were infected with L. donovani promastigotes as described earlier.

Caspase-3 Activity Assay—Cells were washed twice with ice-cold PBS, resuspended in 50 μl of ice-cold lysis buffer (1 mM N-dithiothreitol, 0.03% Nonidet P-40 (v/v), in 50 mM Tris, pH 7.5), kept on ice for 30 min, and finally centrifuged at 14,000 × g for 15 min at 4°C. 10 μg of total protein was incubated with the caspase-3 substrate (Ac-DEVD-pNA) for 1 h at 37°C. The absorption was measured by spectrometry at 405 nm.

Electrophoretic Mobility Shift Assays (EMSA)—10 μg of nuclear extracts from control as well as treated cells were pre-incubated with 1 μg of poly(dl-dc) in a binding buffer (25 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl) for 10 min at room temperature. 0.5 ng of [α-32P]dCTP-labeled Egr1 oligonucleotide probe was then added to the reaction mixture followed by incubation for 30 min. Oligonucleotide probe with a mutated Egr1-binding site (Santa Cruz Biotechnology) was used for competition experiments. The DNA-protein complex was then electrophoresed on 6% nondenaturing polyacrylamide gels in 0.5× TBE buffer (50 mM Tris, 50 mM borate, and 1 mM EDTA) and analyzed by autoradiography.

Fluorescence Microscopy—Macrophages (5 × 10⁶) were plated onto 18-mm² coverslips kept in 30-mm Petri plates and cultured overnight. The cells were then infected with L. donovani promastigotes, washed twice in PBS, and fixed with methanol for 15 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 and incubated with Egr1 antibody for 1 h at 4°C. After washing, coverslips were incubated with Texas Red-conjugated secondary antibody (1 h, 4°C). The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/ml) in PBS plus 10 μg/ml RNase A to label the nucleus, mounted on slides, and visualized under Olympus BX61 microscope at a magnification of 1000, and the images thus captured were processed using ImagePro Plus (Media Cybernetics).

Chromatin Immunoprecipitation (ChIP) Assay—Cells were cross-linked with 1% formaldehyde and harvested into lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1X protease inhibitor mixture) and sonicated, followed by immunoprecipitating with rabbit anti-Egr1 antibody. Immunoprecipitation with a normal rabbit IgG served as a negative control. Immunoprecipitated cell lysates were incubated with protein A/G plus agarose, washed, and then heated at 65°C for 1.5 h to reverse the cross-linking. DNA fragments were purified, and PCR amplification was performed using 5 μl of DNA (recovered from ChIP) with 35 cycles of denaturation at 95°C (30 s), annealing at 60°C (50 s), and extension at 72°C (50 s) with a final extension at 72°C for 10 min. Amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The following primer pairs were used to amplify putative Egr1-binding sites in the Socs1 and Socs3 promoter regions, respectively, SOCS1 sense 5'-CGGGGCCCTCAGTTTCTCC-3' and antisense 5'-ATCAGGCTTAAACCAAGGGA-3', and SOCS3 sense 5'-TGAATTAGGGAGCCCCACAC-3' and antisense 5'-TACCTAGTCCCCAGCGGAAT-3'.

Densitometric Analysis—Densitometric analyses for all experiments were carried out using QUANTITY ONE software (Bio-Rad). Band intensities were quantitated densitometrically, and the values were normalized to endogenous control and expressed in arbitrary units. The ratios of optical density of particular bands/endogenous control are indicated as bar graphs adjacent to figures.

Statistical Analysis—Data shown are representative of at least three independent experiments unless otherwise stated as n values given in the legend. Macrophage cultures were set in triplicate, and the results are expressed as the mean ± S.D.
Student’s t test was employed to assess the statistical significance of differences among a pair of data sets with a p value of <0.05 considered to be significant.

RESULTS

*L. donovani* Inhibits H$_2$O$_2$-induced Apoptosis of Host Macrophages during Phagocytosis—Internalization of a pathogen into macrophages generally causes a huge oxidative burst that results in apoptosis of host cells toward clearance of pathogen burden (20). To determine whether the intra-macrophage parasite *L. donovani* can evade this strategy of host defense, thus protecting its niche for survival and replication, we measured the ROS production in macrophages infected with *L. donovani* during early hours of infection. To this end, RAW 264.7 cells were infected with *L. donovani* promastigotes for the indicated time periods, washed with PBS, and incubated for 30 min with the green fluorescent dye H$_2$DCFDA, and fluorescence levels of 50,000 cells were counted. A gate was established that delineated approximately the upper 5% of fluorescent cells. *L. donovani* infection was found to cause 68.8 ± 8.4, 71.6 ± 5.8, 61.6 ± 7.4, and 40.2 ± 3.2% ROS production in RAW 264.7 macrophages at 5, 10, 15, and 30 min post-infection, respectively (Fig. 1A). To determine whether this initial oxidative outburst could lead to macrophage apoptosis, cells were infected with *L. donovani* for the indicated time points, washed to remove un-internalized parasites, and incubated overnight at 37°C, and the percentage of apoptotic cells was measured by annexin V-PI flow cytometric analysis. Host cell apoptosis in infected macrophages was found to be considerably higher at 5 min of infection (55.4 ± 7.8% annexin V-positive cells, p < 0.0001), which was significantly reduced during later time points (38.8 ± 5.9%, p < 0.0001, and 6.9 ± 0.8%, p < 0.0002, at 10 and 15 min, respectively) (Fig. 1B). To determine whether this amount of ROS produced during early hours of *L. donovani* infection was capable of causing macrophage apoptosis, cells were administered with the indicated concentrations of H$_2$O$_2$ to mimic the initial oxidative burst condition in the case of *L. donovani* during 5–30 min of infection. Interestingly, 62.1 ± 8.1% of ROS produced by 200 μM H$_2$O$_2$ (Fig. 1C), which was very close to that produced by *L. donovani* at 15 min post-infection (61.6%), could cause a significant induction in apoptotic macrophage populations (68.9 ± 5.2% annexin V-positive cells, p < 0.0001) (Fig. 1D) as compared with 6.9% in case of *L. donovani* infection. Because *L. donovani* could inhibit host cell apoptosis despite significant ROS production during phagocytosis, we administered H$_2$O$_2$ (400 μM for 1 h) and compared the induction of apoptosis in normal and *L. donovani*-infected macrophages at various time points of infection. H$_2$O$_2$ exposure resulted in 79.6 ± 9.7% annexin V-positive cells in the case of normal macrophages (p < 0.0002) (Fig. 1E). In contrast, *L. donovani*-infected cells showed a much lower extent of apoptosis on exposure to H$_2$O$_2$ (11.6 ± 0.8, 8.2 ± 1.3, 7.7 ± 0.4, 8.8 ± 1.3, and 7.7 ± 0.9% annexin V-positive cells at 2, 4, 6, 12 and 24 h after infection) (Fig. 1E). To investigate whether the inhibition in host cell apoptosis was dependent upon pathogen internalization, cells were administered with cytochalasin D (which prevents the uptake but not the attachment of the parasite) prior to infection. Cytochalasin D treatment (2 μM) caused 54.7 ± 7.7, 70.7 ± 4.9, and 82.3 ± 5.8% reduction in internalized parasites as compared with untreated infected cells at 2, 4, and 6 h post-infection, respectively (p < 0.01) (Fig. 1F). Parasite-mediated suppression of apoptosis was reversed on treating the cells with cytochalasin D. Cytochalasin D treatment in macrophages showed a much higher extent of apoptosis than infected macrophages (33.1 ± 6.2, 35.1 ± 5.1, and 50.7 ± 5.9% more apoptotic cells as compared with *L. donovani*-infected macrophages at 2, 4, and 6 h post-infection, p < 0.01) (Fig. 1G). This suggests that internalization of the parasite is a necessary prerequisite for suppression of host cell apoptosis. To determine whether this inhibition of apoptosis was mediated through the inhibition of ROS production by the parasite, we measured ROS levels at similar time points in H$_2$O$_2$-administered infected cells. *L. donovani*-infected cells were found to produce significant levels of ROS up to 6 h, with a maximum of 55.0 ± 3.7% at 2 h post-infection (Fig. 1H). ROS production by macrophages incubated for various time periods (2–24 h) was found to be comparable with that in control cells (0 h of incubation) after treatment with H$_2$O$_2$ for 1 h (supplemental Fig. 1) thereby suggesting that merely incubating cells before H$_2$O$_2$ treatment does not make them refractory to ROS production. Inhibition of ROS generation may therefore be attributed to parasite infection. Taken together, these results suggest that *L. donovani* can successfully counteract oxidative burst-mediated apoptosis in macrophages, which is not mediated by inhibition of ROS production.

*L. donovani* Infection Inhibits the Caspase Cascade through De-phosphorylation of MAPKs—To ascertain whether H$_2$O$_2$-induced apoptotic response was mediated by MAPKs, we sought to determine the phosphorylation-mediated activation of MAPKs in H$_2$O$_2$-treated infected cells. There was significant decrease in phosphorylated forms of p38 (41.4, 84.1, and 73.4% reduction at 2, 4, and 6 h post-infection, respectively, as compared with H$_2$O$_2$-administered control macrophages, p < 0.05) (Fig. 2A, left panel). The levels of phospho-ERK1/2 in *L. donovani*-infected cells were found to be reduced significantly after 4 h and continued to decrease until 24 h (68.7 and 73.7% reduction in p-ERK1 and p-ERK2 at 6 h post-infection, p < 0.001) upon H$_2$O$_2$ treatment as compared with H$_2$O$_2$-treated uninfected cells (Fig. 2A, left panel). However, reduction in p-JNK was observed only at 2 h post-infection (54.6% reduction, p < 0.001) (Fig. 2A, left panel). High basal levels of p-p38, p-ERK, and p-JNK obtained in H$_2$O$_2$-treated normal macrophages (Fig. 2A, left panel) may be attributed to peroxide treatment as H$_2$O$_2$-untreated normal macrophages did not show any phosphorylation of p38, ERK, and JNK (supplemental Fig. 2). The phosphorylation of all three MAPKs was markedly abrogated in *L. donovani*-infected cells in the absence of H$_2$O$_2$ treatment (Fig. 2A, right panel), suggesting that *Leishmania* strongly inhibits MAPK activation. We further checked the activation of caspases following *L. donovani* infection and found that whereas control macrophages following H$_2$O$_2$ treatment showed high levels of active initiator caspase-9 and -7, there was a marked reduction (56.4 and 31.1% reduction, p < 0.05) of these caspases at 6 h post-infection with a gradual increase in the level of pro-caspases (Fig. 2B, left panel). However, *L. donovani* infection in the absence of H$_2$O$_2$ treatment depicted no...
cleavage of pro-caspase-9 and -7 (Fig. 2B, right panel). We further checked the expression and activity of caspase-3, which is the main effector caspase involved in the apoptotic signaling cascade. There was a significant reduction in cleaved caspase-3 expression (21.7, 59.8, and 80.4% reduction at 2, 4, and 6 h post-infection, respectively) (Fig. 2C, left panel) with a concomitant decrease in its activity (44.6, 52.1, and 68.3% reduction at 2, 4, and 6 h post-infection, respectively, p < 0.01) (Fig. 2D) as compared with control cells after H$_2$O$_2$ treatment. Further confirming the inhibition of ROS-mediated apoptosis by *Leishmania*. Administration of caspase-9 inhibitor Z-LEHD-fmk to infected macrophages before H$_2$O$_2$ treatment did not result in further increases in the levels of downstream pro-caspase-7 and -3 (Fig. 2E), thereby suggesting that the gradual increase in the **FIGURE 1.** Effect of *L. donovani* infection on macrophage ROS generation and apoptosis. A, C, and H, macrophages were either infected with *L. donovani* (L.d.) promastigotes with a parasite/macrophage ratio of 1:1 for the indicated time periods (A) or treated with various concentrations of H$_2$O$_2$ for 1 h (C) or infected with promastigotes followed by treatment with H$_2$O$_2$ (400 μM) for 1 h (H). Cells were washed, and ROS generation was measured by H$_2$DCFDA staining followed by flow cytometric analysis. The H$_2$DCFDA-positive cells are indicated as the percentage of gated cells. B, D, and E, macrophages were either infected with *L. donovani* promastigotes (B) or treated with various concentrations of H$_2$O$_2$ for 1 h (D) or infected with promastigotes followed by treatment with H$_2$O$_2$ (400 μM) for 1 h (E). Cells were washed and incubated overnight at 37 °C, and the extent of apoptosis was analyzed by annexin V-tagged FITC-PI flow cytometry. Dual parameter dot plot of FITC fluorescence (x axis) versus PI fluorescence (y axis) is represented as logarithmic fluorescence intensity. Quadrants are as follows: upper left, necrotic cells; lower left, live cells; lower right, apoptotic cells; upper right, necrotic or late phase of apoptotic cells. F and G, control or cytochalasin D (2 μM)-pretreated RAW 264.7 cells were infected with *L. donovani* promastigotes for different time periods as indicated. The number of parasites per 100 macrophages was evaluated by Giemsa staining (F), and apoptosis was quantified by flow cytometry (G). Results are representative of three individual experiments, and the error bars represent mean ± S.D. (n = 3). **, p < 0.01; ***, p < 0.001 by Student’s t test.
level of pro-caspases observed post-infection (Fig. 2, B and C) was not due to new synthesis. However, the cleavage of these two caspases was markedly abrogated in the presence of Z-LEHD-fmk suggesting that the stabilization of pro-caspases during *L. donovani* infection may be due to the blockage of their proteolytic cleavage. This was also supported by the fact that an increase in the level of pro-caspases coincided with the gradual decrease in active forms of respective caspases (Fig. 2, B and C). Moreover, *L. donovani* infection also led to reduction in cleaved PARP levels (73.3% reduction at 6 h post-infection, \( p < 0.0001 \)) (Fig. 2F) as compared with control cells after \( \text{H}_2\text{O}_2 \) treatment. *Leishmania* infection at similar time points, in the absence of peroxide, resulted in almost complete inhibition of the cleavage of both caspase-3 and PARP (Fig. 2, C, right panel, and F, lower panel) further suggesting that *L. donovani* suppresses the MAPK-mediated apoptotic signaling cascade independent of \( \text{H}_2\text{O}_2 \) administration. These results suggested that *L. donovani* infection could modulate ROS-dependent caspase cascade thus protecting host cells from oxidative burst-mediated apoptosis.

**L. donovani** Induces Macrophage PTP Activity through SOCS-mediated Induction of Thioredoxin—Considering the kinase-phosphatase balance involved in maintaining cellular homeostasis and decreased phosphorylation of ERK and p38 in *L. donovani*-infected cells after \( \text{H}_2\text{O}_2 \) treatment, we sought to determine the total PTP activity in macrophages following infection. Macrophages were analyzed for PTP activity by the capacity of total cell lysates to dephosphorylate pNPP as well as a synthetic tyrosine monophosphorylated peptide substrate. There was significant increase in PTP activity (4.8-, 6.4-, and 5.9-fold at 2, 4, and 6 h post-infection, \( p < 0.001 \)) upon \( \text{H}_2\text{O}_2 \) treatment as compared with \( \text{H}_2\text{O}_2 \)-treated uninfected cells (Fig. 3A). Infection of macrophages in the absence of \( \text{H}_2\text{O}_2 \) also showed induction in PTP activity (5.2-, 6.7-, and 6.1-fold at 2, 4, and 6 h post-infection, \( p < 0.001 \)), which was comparable with that obtained after \( \text{H}_2\text{O}_2 \) treatment (Fig. 3A). Similar trends were noted in case of specific PTP activity as observed by the dephosphorylation of synthetic tyrosine monophosphorylated peptide (Fig. 3B). We then checked the individual activity and protein level expression of PTPs known to be involved in the ROS-mediated signaling cascade like SHP-1, SHP-2, CD45, and PTP1B (Fig. 3C). Of all the PTPs tested, the activities of SHP-1 and PTP1B were found to be significantly elevated reaching a maximum of 5.6-fold at 6 h post-infection for SHP-1 and 6.3-fold at 4 h post-infection for PTP-1B (\( p < 0.0001 \)) (Fig. 3C). Protein expression levels of these two proteins were also increased in \( \text{H}_2\text{O}_2 \)-treated infected cells (Fig. 3D, left panel).
donovani infection in the absence of H₂O₂ also depicted a similar pattern of induction for SHP1 and PTP1B, with a maxima of 5.1-fold at 6 h post-infection for SHP1 and 5.4-fold at 4 h post-infection for PTP1B (Fig. 3E). The protein level expressions of both SHP1 and PTP1B were also elevated during infection in the absence of peroxide treatment, thereby suggesting that PTP induction by Leishmania might be independent of H₂O₂ treatment (Fig. 3D, right panel). Because thioredoxin is known to have a role in stabilizing the PTPs, we sought to determine whether L. donovani infection had any effect on macrophage

FIGURE 3. Effect of L. donovani infection on PTP activity, thioredoxin, and SOCS expression. Macrophages were infected with L. donovani for the indicated time periods. One group of infected macrophages from each time point was subjected to H₂O₂ treatment for 1 h. A and B, total and specific PTP activities were evaluated by the capacity of cell lysates to hydrolyze pNPP (A) or a synthetic tyrosine phosphopeptide (B). Absorbance values were taken at 405 and 620 nm, respectively. C and E, activity of the indicated PTPs were determined by the capacity of immunoprecipitated samples to hydrolyze pNPP in the presence (C) and absence (E) of H₂O₂. Results are expressed as the relative increase (n-fold) over PTP activity in control cells. D and F, cells were processed as above and then subjected to Western blotting with respective antibodies for various PTPs (D) and thioredoxin (F). G, cells processed as above were immunoprecipitated with anti-thioredoxin antibody followed by immunoblotting with the indicated antibodies. 30 μg of each sample was loaded as a whole cell lysate input control. H–J, expression of various SOCS proteins was determined at mRNA levels in the presence (H) and absence (I) of H₂O₂ and protein level in the presence (H, left panel) and absence (H, right panel) of H₂O₂. IP, immunoprecipitation using the indicated antibody; IB, immunoblot analysis using the indicated antibody; WCL, whole cell lysate. Results are representative of three individual experiments, and the error bars represent mean ± S.D. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 by Student’s t test.
thioredoxin levels. Following H$_2$O$_2$ treatment, *L. donovani*-infected macrophages showed significantly enhanced expression of thioredoxin (2.1-, 8.6-, and 9.1-fold as compared with control macrophages at 2, 4, and 6 h post-infection; *p* < 0.05) (Fig. 3F, *left panel*). Although infected macrophages (without H$_2$O$_2$ treatment) demonstrated similar thioredoxin levels at 2, 4, and 6 h post-infection (Fig. 3F, *right panel*), thioredoxin induction was found to persist up to 24 h in the absence of peroxide as compared with infected H$_2$O$_2$-treated cells where this was considerably reduced after 6 h of infection. We then checked for the role of thioredoxin in the regulation of PTP activity by studying the molecular interaction between them. Co-immunoprecipitation studies revealed strong association of thioredoxin with both SHP-1 and PTP1B at 4 and 6 h post-infection as compared with control cells both in the presence and absence of H$_2$O$_2$ (Fig. 3G, *left and right panel*). Because thioredoxin is known to be regulated by members of SOCS family of proteins, which play a substantial role in the regulation of ROS-mediated apoptotic signaling cascade, we studied whether *Leishmania* could modulate the expression levels of SOCS proteins under H$_2$O$_2$ treatment. Real-time PCR analysis of various members of the SOCS family proteins revealed marked elevation in mRNA levels of *Socs1* and *Socs3* with maximum levels (6.1-fold at 6 h for *Socs1* and 5.9-fold at 4 h for *Socs3*, respectively; *p* < 0.001) (Fig. 3H) without any apparent alteration in the expression levels of *Socs2* and CIS. Similar induction of *Socs1* and *Socs3* expression was also observed at protein levels (Fig. 3I, *left panel*) as studied by immunoblot analysis with maximum expression at 6 h post-infection (*p* < 0.01). However, although the induction of *Socs1* was found to be stable up to 12 h, *Socs3* expression was short lived as seen by the sharp reduction in the level of this protein after 6 h of infection. Moreover, both *Socs1* and *Socs3* were induced in *L. donovani*-infected macrophages even in the absence of peroxide treatment with a maxima of 5.9- and 5.7-fold at mRNA levels at 6 and 4 h post-infection, respectively (Fig. 3I). Similar induction was revealed at protein levels (Fig. 3I, *right panel*), thereby suggesting that H$_2$O$_2$ treatment may not be required for the induction of SOCS proteins by *Leishmania*. These results suggest that *L. donovani* may exploit host SOCS1 and SOCS3 to induce thioredoxin thereby enhancing the activity of PTP.

**Transcriptional Regulation of SOCS Proteins by *L. donovani***—The Egr group of transcription factors is known to regulate the transcription of SOCS family members, and therefore we checked for the expression of Egr1 at both protein and mRNA levels. It was interesting to note that Egr1 mRNA expression was induced in infected macrophages (1.7-, 3.9-, and 5.7-fold over control at 2, 4, and 6 h post-infection, *p* < 0.05), which coincided with SOCS induction (Fig. 4A). A similar trend was observed for infected cells in the absence of H$_2$O$_2$ (Fig. 4A). Induction of Egr1 protein expression was found to be comparable in *L. donovani*-infected macrophages in the presence and absence of H$_2$O$_2$ (Fig. 4B, *left and right panels*). This activation was further ascertained by the nuclear translocation of Egr1 as observed by fluorescence microscopy using anti-Egr1 antibody. In control macrophages, with or without H$_2$O$_2$ treatment, the signal for Egr1 was distributed throughout the cell but did not co-localize with DAPI-stained nuclei indicating its cytosolic localization (Fig. 4C). On the contrary, *L. donovani* infection for 6 h resulted in an increase in the nuclear localization of Egr1 (irrespective of H$_2$O$_2$ treatment) as evident by markedly enhanced co-localization of Egr1 signal (red) with DAPI-stained nuclei (blue) (Fig. 4C). Because the binding of a transcription factor to DNA is necessary for regulating the transcription of a gene, we checked for the Egr1-DNA binding. Analysis of DNA-protein interaction through EMSA depicted strong Egr1-DNA binding (Fig. 4D) at 6 h post-infection, indicating that Egr1 may have a role in elevated expression of SOCS proteins following *Leishmania* infection. To further ascertain the nuclear translocation of Egr1, we analyzed the expression of Egr1 at a protein level in both nuclear and cytosolic fractions. The results showed 3.8- and 3.7-fold more protein expression in nuclear fractions at 6 h post-infection in the case of *L. donovani* infection and *L. donovani* + H$_2$O$_2$ treatment, respectively, as compared with control (Fig. 4E, *right and left panel*). However, although Egr1 levels persisted in the nuclear fraction of *L. donovani*-infected macrophages until 24 h post-infection (Fig. 4F, *right panel*), the level decreased considerably after 6 h of infection in the case of H$_2$O$_2$ treatment (Fig. 4F, *left panel*). This might be the reason why DNA-protein binding was not observed at 12 and 24 h post-infection in Fig. 4D. However, we obtained DNA-protein binding up to 24 h post-infection in absence of H$_2$O$_2$ (Fig. 4G), thereby suggesting that H$_2$O$_2$ may exercise a feedback control over Egr1-DNA binding during *L. donovani* infection. Competition experiments using the Egr1 probe with a mutated binding site resulted in complete abrogation of DNA-protein interaction demonstrating the specificity of Egr1-DNA binding (Fig. 4, *E and H*). Next, we examined the effect of *L. donovani* infection on the binding of Egr1 to *Socs1* and *Socs3* promoter regions through ChIP. We found a detectable increase in Egr1 binding to the *Socs1* promoter (Fig. 4I). Egr1 binding was increased in a time-dependent manner upon *L. donovani* infection. However, the binding of Egr1 to *Socs3* promoter was much less as compared with *Socs1* (Fig. 4J). Replacement of Egr1 antibody with control IgG in the ChIP assay failed to yield any amplicon suggesting the specificity of the experiment (Fig. 4, *I and J, lower panels*). To validate the role of Egr1 in the induction of SOCS in infected macrophages, we used an *in vitro* siRNA knockdown system for Egr1. As seen in Fig. 4K, Egr1 was effectively down-regulated by siRNA (88.1% reduction in expression as compared with control siRNA-treated cells, *p* < 0.001). Egr1 knockdown cells showed markedly decreased expression of SOCS1 (66.7% reduction as compared with control siRNA-treated cells, *p* < 0.01) (Fig. 4L). However, inhibition of Egr1 resulted in merely 30.4% reduction (*p* < 0.05) in SOCS3 expression (Fig. 4L). These results suggest that induction of SOCS1 and SOCS3 may be mediated by Egr1.

**Effect of SOCS Inhibition on Thioredoxin-mediated Apoptotic Signal**—To investigate whether induction of SOCS1 and SOCS3 was associated with an increase in thioredoxin-mediated PTP activity, an siRNA-mediated knockdown system was used. Macrophages were administered with either SOCS1 or SOCS3 siRNA alone or in combination, and the efficacy of siRNA treatment was determined by assessment of protein expressions by Western blotting. As seen in Fig. 5, *A* and *B*, expressions of both SOCS1 and SOCS3 were consider-
ably reduced by treatment with respective siRNAs (69.2 and 81.2% reduction for SOCS1 and SOCS3, respectively, \(p < 0.01\)) as compared with control siRNA treatment. To see the collective role of both SOCS1 and SOCS3 on the modulation of the apoptotic cascade by \(L.\) donovani, we used a combined knockdown system for SOCS1 and SOCS3 for all of our experiments.

**FIGURE 4.** Transcriptional regulation of SOCS proteins. Macrophages were infected with \(L.\) donovani for the indicated time periods. One group of infected macrophages from each time point was subjected to \(H_2O_2\) treatment for 1 h. A and B, Egr1 expression was determined at the mRNA level (A) and protein level (B) by real time PCR and Western blotting respectively. C, cells were stained with anti-Egr1 monoclonal antibody followed by secondary Texas Red-conjugated antibody. Nuclei were stained with DAPI, and cells were analyzed under fluorescence microscope. D, E, G, and H, labeled Egr1 probe (D and G) or Egr1 probe with a mutated binding site (E and H) was incubated with nuclear extracts prepared from cells treated as above. DNA binding was analyzed by EMSA. F, cells were treated as above; nuclear and cytosolic extracts were prepared, and expression of Egr1 was analyzed by immunoblotting. I and J, cells were infected with \(L.\) donovani for the indicated time periods and analyzed for Egr1 ChIP assay. PCR amplification of anti-Egr1 immunoprecipitates (IP) and total input chromatin are shown in the upper and lower panels, respectively. K and L, macrophages were transfected (24 h) with either control or Egr1 siRNA followed by infection with \(L.\) donovani promastigotes for 6 h. Expression of Egr1 (K) and SOCS1 and SOCS3 (L) was evaluated by immunoblot analysis. Results are representative of three individual experiments, and the error bars represent mean \(\pm S.D.\) (\(n = 3\)). *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\) by Student’s \(t\) test.
Combined siRNA treatment resulted in 46.2 and 75.1% reduction in SOCS1 and SOCS3, respectively (p < 0.05) (Fig. 5, A and B). SOCS knockdown cells showed markedly decreased thioredoxin levels (79.4% reduction in combined SOCS1/3 siRNA-treated cells as compared with control siRNA treatment) thereby suggesting that the expression of both SOCS1 and SOCS3 is a necessary prerequisite for thioredoxin induction (Fig. 5C). Consistent with these data, SOCS1/3 knockdown also resulted in marked reduction in PTP activity (63.3 and 76.5% reduction in total and specific PTP activity, respectively, p < 0.01) (Fig. 5, D and E) as well as reduction in the activity of both SHP-1 and PTP1B (55.1 and 38.6% reduction, respectively, as compared with control siRNA treatment, p < 0.05) (Fig. 5F). We also checked the expression of both SHP-1 and PTP1B at protein level and found 93.5 and 81.9% reduction in their levels, respectively (p < 0.01) (Fig. 5G). The decrease in thioredoxin-mediated PTP activity was further validated by checking the protein-protein interaction of thioredoxin with SHP-1 and PTP1B. Co-immunoprecipitation studies revealed strong association of thioredoxin with SHP-1 and PTP1B following infection, which was markedly reduced in the presence of SOCS1/3 siRNA (Fig. 5H). These results indicate that both SOCS1 and SOCS3 play a vital role in thioredoxin-mediated enhancement of PTP activity in H2O2-treated infected macrophages.

**Effect of SOCS Knockdown on MAPK-mediated Caspase Activation, Macrophage Apoptosis, and Parasite Survival—**

To ascertain the functional significance of SOCS in H2O2-treated...
L. donovani-infected cells, we examined the effect of SOCS knockdown on the MAPK-triggered caspase cascade and subsequent apoptotic parameters. SOCS1 and -3 silencing in L. donovani-infected cells led to enhanced expression of both p-p38 and p-ERK (3.1-, 3.4-, and 3.3-fold for p-p38, p-ERK1, and p-ERK2, respectively, over control siRNA-treated samples) (Fig. 6A). Because the de-phosphorylation of MAPKs, observed in case of Leishmania infection, may be mediated by thioredoxin, SHP1, and PTP1B, we studied the effect of silencing these proteins through the siRNA-mediated knockdown system on the activation of p38 and ERK in infected macrophages. Efficacy of siRNA was determined by Western blotting, which showed 59.2, 70.1, and 67.2% inhibition in the case of SHP1, PTP1B, and thioredoxin, respectively (Fig. 6B, C, and D). Knockdown of SHP1, PTP1B, and thioredoxin led to an increase of 7.2-, 7.4-, and 7.7-fold of p-p38, 2.1-, 2.3-, and 3.4-fold of p-ERK1 and 2.3-, 2.7-, and 4.2-fold of pERK2, respectively, over control siRNA-treated samples (Fig. 6E) thereby suggesting that the increase in phosphatase activity in L. donovani-infected cells might lead to the reduction in phosphorylation of MAPKs.
lated forms of p38 and ERK1/2. SOCS1/3 silencing either alone or in combination in L. donovani-infected cells depicted considerable enhancement in caspase-3 activity (3.2-, 2.7-, and 4.8-fold more than control siRNA-treated cells, in the case of SOCS1, SOCS3, and SOCS1 and -3 knockdown, respectively, \( p < 0.01 \)) (Fig. 6F). It was interesting to note that this increase in caspase-3 activity could be markedly reversed by administration of SB203580 and FR180204, inhibitors of p38 and ERK, respectively, thereby suggesting the active involvement of these two MAPKs in SOCS-mediated signaling (Fig. 6G). A comparison of apoptotic populations between control and SOCS siRNA-treated infected macrophages showed enhanced apoptosis in the latter upon \( \text{H}_2\text{O}_2 \) treatment (65.1, 59.2, and 57.4% apoptotic cells, in case of SOCS1, SOCS3, and SOCS1 and -SOCS3 knockdown, respectively, compared with 23.3% in control siRNA-treated cells) (Fig. 6H). In agreement with the previous data, administration of SB203580 and FR180204 resulted in marked reduction in apoptosis induced by SOCS1 and/or SOCS3 knockdown, whereas treatment with SP600125, the inhibitor of JNK, did not have any effect on the same (Fig. 6I). We then tried to evaluate whether this increase in apoptosis by SOCS knockdown could actually play a role in decreasing the persistence of infection. It was observed that silencing of SOCS1 and -3 either alone or simultaneously resulted in decreased intra-macrophage survival of parasites (62.1, 48.1, and 67.3% reduction in case of SOCS1, SOCS3, and SOCS1 and -3 knockdown, respectively, as compared with control siRNA treatment) (Fig. 6J). Moreover, administration of inhibitors for p38 and ERK along with SOCS siRNA resulted in reversal of apoptotic inhibition (Fig. 6J), thereby revealing an active participation of both p38 and ERK in the SOCS-mediated anti-apoptotic signaling. Collectively, all these results suggest that L. donovani may counteract oxidative burst-mediated apoptosis through up-regulation of SOCS1 and -3, thus allowing successful replication and survival of the parasites.

**DISCUSSION**

For the successful survival of intracellular pathogens, protection of their niche, *i.e.* the host cell, is a necessary prerequisite. Apoptosis of infected cells is one of the classical defense mechanisms that result in elimination of the host cell along with the pathogen (34). Hence, many pathogens, including *Leishmania*, escape immune surveillance by developing mechanisms to suppress host cell apoptosis (35). However, phagocytosis of *Leishmania* promastigotes into macrophages results in a huge oxidative burst that normally should kill the host cell resulting in parasite clearance. Although inhibition of apoptosis by *L. donovani* has been reported, the fact that the parasite is able to deal with the oxidative stress after being phagocytosed and can protect the host macrophages from cell death is still an unexplored area. In this study using \( \text{H}_2\text{O}_2 \) as an inducer of ROS-mediated apoptosis, we tried to elucidate the intracellular signaling mechanisms used by *L. donovani* to overcome host-cell apoptosis. We observed that *Leishmania* could prevent ROS-mediated apoptosis of macrophages through the differential induction of SOCS proteins (SOCS1 and SOCS3) through thioredoxin in the inhibition of the apoptotic cascade. Because thioredoxin has a role in PTP stabilization, it might be possible that *L. donovani* may induce thioredoxin to protect the PTPs from being oxidized by ROS, thereby inhibiting the MAPK-driven caspase cascade.

The involvement of ROS in inducing cell death has been demonstrated in a number of studies (36, 37). In this study, we demonstrated that \( \text{H}_2\text{O}_2 \), which induces apoptosis in normal cells, could not do so in *L. donovani*-infected macrophages despite increased levels of ROS. *Leishmania* may achieve apoptotic inhibition via neutralization of ROS-mediated apoptotic signaling cascade rather than decreasing ROS production itself. Caspases that perform critically important roles in the induction of apoptosis are primarily triggered via two distinct but interconnected pathways, namely the mitochondrion-mediated and death receptor pathways (38). Both of these pathways eventually merge and lead to the activation of the downstream effector caspases-3 and -7, which ultimately execute apoptosis of the cell (38). In this study, we found that *Leishmania* markedly reduced the expression of both active initiator caspases-9 and -7 followed by suppression of the effector caspase-3 in host cells. MAPKs are known to have a precise role in the initiation of the caspase cascade, and there is evidence regarding their involvement in cleaving of inactive caspasess, thus rendering them active. Our study also revealed a significant reduction in the phosphorylation of ERK and p38 in *L. donovani* infection. MAPKs in turn are known to be regulated by PTPs; therefore, we studied the role of PTP in the MAPK-mediated apoptotic signaling cascade. ROS is reported to cause PTP inactivation by attacking thiol groups in the catalytic site of the PTP (39). The essential role of the active site cysteine residue in PTP-mediated catalysis provides a mechanism for redox-based regulation of PTP activity. The reversible oxidation and inactivation of PTP in response to \( \text{H}_2\text{O}_2 \) provide a well established mechanism for control of tyrosine phosphorylation-dependent signaling. \( \text{H}_2\text{O}_2 \) exposure to control macrophages resulted in inhibition of CD45, SHP-1, SHP-2, and PTP1B with a concomitant activation of ERK and p38. We found an enhancement of the specific activities of SHP-1 and PTP-1B following infection, which might contribute to de-phosphorylation of MAPK and consequent inhibition of the caspase cascade. However, the precise role that individual MAPK members play during ROS-induced apoptosis requires further investigation.

PTPs are known to be stabilized by a number of enzymes of the ROS-scavenging system. Thioredoxin might be involved in the stabilization of PTPs in *Leishmania* infection as its levels are increased in infected macrophages. The enhanced association of thioredoxin with SHP1 and PTP-1B may lead to the protection of thiol groups from ROS attack. The results of some recent studies demonstrate the role of SOCS family members in regulating thioredoxin expression during oxidative stress conditions. Moreover, SOCS1-transduced cells display elevated thioredoxin levels and a decrease in ROS generation induced by oxidative stress (27). Although specific interactions of SOCS1 with the transcription machinery of thioredoxin genes are not known, the role of SOCS proteins as transcriptional factors has been suggested in recent studies. This study indicated that infection by *L. donovani* resulted in induced expression of both SOCS1 and SOCS3 in macrophages, and transcription of these proteins may be regulated by the transcription factor Egr1.
SOCS Proteins in Macrophage Apoptosis by L. donovani

TGF-β has been reported to induce rapid induction of Egr1 in human skin fibroblasts (40), aortic smooth muscle cells (41), and mouse embryonic fibroblasts (42). Incidentally, L. donovani infection leads to the production of TGF-β, which is one of the major players in generating a Th2-biased immune response for establishment of infection (43, 44). It might be possible that during infection, high levels of TGF-β may be exploited by Leishmania to induce Egr1 levels. Our findings suggest that both SOCS1 and SOCS3 have a role in the inhibition of host cell apoptosis by L. donovani, which may be mediated through the induction of thioredoxin, which protects PTPs. These findings seem to be in good agreement with the fact that SOCS induction is correlated with cyto-protection (45). A number of emerging studies have revealed the implication of SOCS proteins in the regulation of cellular proliferation and apoptosis. For example, abolition of SOCS gene expression has been reported to induce apoptosis in liver and lymphoid organs (46, 47). To understand the mechanism responsible for increased apoptosis after SOCS down-regulation, we looked for changes in the expression levels of thioredoxin and phosphorylation of MAPKs and found that SOCS knockdown was correlated with increased phosphorylation of ERK and p38. Increased apoptosis was associated with activation of pro-apoptotic caspase-3, caspase-7, and caspase-9, and increased levels of cleaved poly(ADP-ribose) polymerase. Functional knockdown of SOCS resulted in reduced expression of thioredoxin suggesting a direct correlation between SOCS proteins and thioredoxin. In this context, we also observed a decrease in endogenous PTP activation in SOCS-siRNA-treated macrophages along with enhanced apoptosis of infected macrophages. However, either SOCS1 or SOCS3 did not co-immunoprecipitate with thioredoxin, SHP1, and PTP1B (supplemental Fig. 3) suggesting that SOCS do not directly associate with these proteins. SOCS1 has been reported to be associated with thioredoxin transcription as observed by elevated thioredoxin mRNA levels in SOCS1-overexpressing cells (27). It might therefore be possible that in L. donovani-infected macrophages, induced expression of SOCS led to enhanced thioredoxin transcription, thereby stabilizing the PTPs. The role of SOCS proteins during infection by various intracellular pathogens has been reported in a number of studies. For example, T. gondii induces endogenous SOCS1 and CIS, and this contributes to the parasite’s inhibition of IFN-γ (48). Also, infection with L. monocytogenes modulated IFN-γ signaling via induction of SOCS-3 (49). Our findings coincided with these reports as SOCS down-regulation resulted in decreased parasite survival thereby suppressing disease progression. Taken together, this study demonstrated that SOCS proteins play an important role in stabilizing the survival machinery of infected cells in the course of phagocytosis, and their down-regulation leads to increased cell death and diminished persistence of infection. This may provide a basis for a more rational design of therapies against visceral leishmaniasis.

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