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IRAK-M regulates the inhibition of TLR-mediated macrophage immune response during late in vitro *Leishmania donovani* infection

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Intramacrophage protozoan parasite *Leishmania donovani*, causative agent of visceral leishmaniasis, escapes Toll-like receptor (TLR) dependent early host immune response by inducing the deubiquitinating enzyme A20, which is sustained up to 6 h postinfection only. Therefore, *Leishmania* must apply other means to deactivate late host responses. Here, we elucidated the role of IL-1 receptor-associated kinase M (IRAK-M), a negative regulator of TLR signaling, in downregulating macrophage proinflammatory response during late hours of in vitro infection. Our data reveal a sharp decline in IRAK1 and IRAK4 phosphorylation at 24 h postinfection along with markedly reduced association of IRAK1–TNF receptor associated factor 6, which is mandatory for TLR activation. In contrast, IRAK-M was induced after A20 levels decreased and reached a maximum at 24 h postinfection. IRAK-M induction coincided with increased stimulation of TGF-β, a hallmark cytokine of visceral infection. TGF-β-dependent signaling-mediated induction of SMAD family of proteins, 2, 3, and 4 plays important roles in transcriptional upregulation of IRAK-M. In infected macrophages, siRNA-mediated silencing of IRAK-M displayed enhanced IRAK1 and IRAK4 phosphorylation with a concomitant increase in downstream NF-κB activity and reduced parasite survival. Taken together, the results suggest that IRAK-M may be targeted by *L. donovani* to inhibit TLR-mediated proinflammatory response late during in vitro infection.

Keywords: Host defense · IRAK-M · *Leishmania* · Macrophage · Toll-like receptors

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Introduction

Visceral leishmaniasis caused by the protozoan parasite *Leishmania donovani* is associated with immunological dysfunctions of macrophages [1]. In spite of multiple intracellular signal transduction pathways stringently regulating macrophage effector functions, the parasite gets access into the host macrophages and develops several strategies to secure its own survival and replication [2]. The innate immune response against *L. donovani* infection begins with the recognition of molecular structures related to this pathogen by Toll-like receptors (TLRs) [3]. Upon activation, TLRs associate with the myeloid differentiation factor 88 (MyD88), which further recruits IL-1 receptor associated kinase (IRAK) proteins and TNF receptor associated factor 6 (TRAF6) [4]. This is followed by a series of events culminating in the degradation of IκB, thereby allowing NF-κB to be translocated to the nucleus and to activate the transcription of proinflammatory cytokines such as IL-12 and TNF-α [5]. Although inflammatory response is

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critical to control the growth of pathogenic organisms, uncontrolled stimulation of TLRs can lead to disproportionate inflammation. Therefore, TLR signaling is tightly regulated in order to avoid such detrimental inflammatory responses [6]. Several negative regulators of TLRs have been implicated in preventing excessive TLR signaling, including A20, SOCS, ST-2, SIGIRR, and IRAK-M.

In our earlier study, we demonstrated that despite the presence of potent TLR2 ligand lipophosphoglycan (LPG) on the surface of the parasites, *L. donovani* escapes TLR-mediated proinflammatory response, by induction of the deubiquitinating enzyme A20 [7]. However, it is of interest to note that *L. donovani* induced A20 levels in macrophages remain significant only up to 6 h postinfection. Hence, A20 levels may be implicated only during early hours of in vitro infection and since macrophages are capable of producing proinflammatory cytokines incessantly when challenged by a pathogen, *Leishmania* must have developed alternative strategies to suppress host's proinflammatory activity, after the depletion of A20 levels. We observed that although A20 was rapidly found to degrade after 6 h of infection, *L. donovani* still exercised a sustained inhibition of NF-κB-mediated proinflammatory response in host macrophages. In the present study, we aimed at deciphering the mechanism applied by the parasite to suppress proinflammatory response after the depletion of A20. We observed reduction in IRAK1 phosphorylation correlated with the upregulation of IRAK-M during delayed hours of *L. donovani* infection.

IRAK-M (or IRAK-3) is a kinase-deficient member of the TLR/IRAK family and negatively regulates TLR-mediated signaling through MyD88-dependent TLRs, including TLR2, TLR4, TLR7/8, and TLR9 [8, 9]. IRAK-M null mice produce more proinflammatory cytokines after bacterial infection and are more susceptible to the lethal effect of sepsis [10]. It has also been reported that impairment in innate lung antibacterial response to *Pseudomonas aeruginosa* is mediated by IRAK-M [11]. Recently, increased IRAK-M expression was reported in the lungs of mice with primary pneumococcal pneumonia [12]. The purpose of this study therefore was to characterize the role of IRAK-M in *L. donovani* infection and to determine whether IRAK-M influences disease progression in visceral leishmaniasis. We report that IRAK-M expression in macrophages is induced upon *L. donovani* infection and TGF-β is the agent which may trigger this induction. A better understanding of how *L. donovani* affects macrophage defense by regulating downstream immune events may provide additional insight into *Leishmania* pathogenesis and also enhance our understanding of the host response to intracellular pathogen in general.

Results

Leishmania maintains a sustained inhibition on proinflammatory response even after A20 depletion

In our previous study, we demonstrated the role of deubiquitinating enzyme A20 in suppressing the TLR-mediated immune response. However, since A20 is an early response protein [13], we monitored the expression kinetics of A20 in the course of *L. donovani* infection. A20 mRNA levels reached a maximum at 3 h postinfection (8.7-fold more than control macrophages, $p < 0.001$) followed by a gradual decline with barely detectable levels at 24 h postinfection (Fig. 1A). Similar trend was observed in A20 protein levels (Fig. 1B). Since the deubiquitination of TRAF6 by A20 is responsible for the inhibition of TLR-mediated immune response during *L. donovani* infection, we next sought to determine the time kinetics of deubiquitinase activity of A20. A20 deubiquitinase activity was markedly reduced at 6 h of infection and barely detectable at later time points (Fig. 1C). However, it was of interest to note that in spite of barely detectable levels of A20 expression as well as deubiquitinase activity after 6 h, a consistent reduction in NF-κB activation was observed till 48 h postinfection (79.1 and 58.9% reduction, $p < 0.001$ at 24 and 48 h, respectively, as compared with LPG treatment) (Fig. 1F). In order to ascertain whether *Leishmania* inhibit NF-κB proinflammatory response in macrophages or prevents the induction of NF-κB, we performed NF-κB-dependent luciferase assay during very early time points of infection. It was observed that *L. donovani* infection led to an increase in NF-κB activity (1.4-, 2.2-, and 2.0-fold over control) at 5, 10, and 15 min postinfection, respectively, (Fig. 1D) after which the activity was markedly decreased (Fig. 1E and F). This is probably because of induction of macrophage's very early innate immune response which at later times is inhibited by *Leishmania*. However, this very early induction of NF-κB was not reflected at the cytokine levels as measured up to 48 h postinfection (Fig. 1G–I). Probably a little prolonged NF-κB induction may be required to achieve a detectable secreted cytokine level. Next, we checked the effect of LPG on the activation of NF-κB in *L. donovani* infected macrophages by luciferase assay. Although LPG administration in control uninfected macrophages showed significant NF-κB induction (Fig. 1E and F), it failed to restore the activation of NF-κB in *L. donovani* infected macrophages (Fig. 1E and F). Preincubation of LPG with polymyxin B (10 U/mL for 2 h), an LPS inhibitor, did not alter NF-κB induction, suggesting thereby that induction of NF-κB by LPG in control uninfected macrophages is not due to the presence of LPS as contaminant (data not shown). We also observed a significant suppression in the levels of NF-κB-mediated proinflammatory cytokines IL-12 (88.1% reduction, $p < 0.001$, as compared with LPG-treated macrophages at protein level) and TNF-α (72.4% reduction, $p < 0.001$, as compared with LPG-treated macrophages at protein level) at 24 h postinfection (Fig. 1I). These results suggested that though *Leishmania*-induced A20 levels diminished after 6 h of infection, the parasite still exercised a sustained inhibition on the NF-κB-mediated proinflammatory response of macrophages, indicating an alternative mechanism.

Leishmania donovani disrupts IRAK–TRAF6 complex during established infection through IRAK-M induction

We next tried to elucidate whether any discrepancy in the upstream TLR proteins might be responsible for the sustained

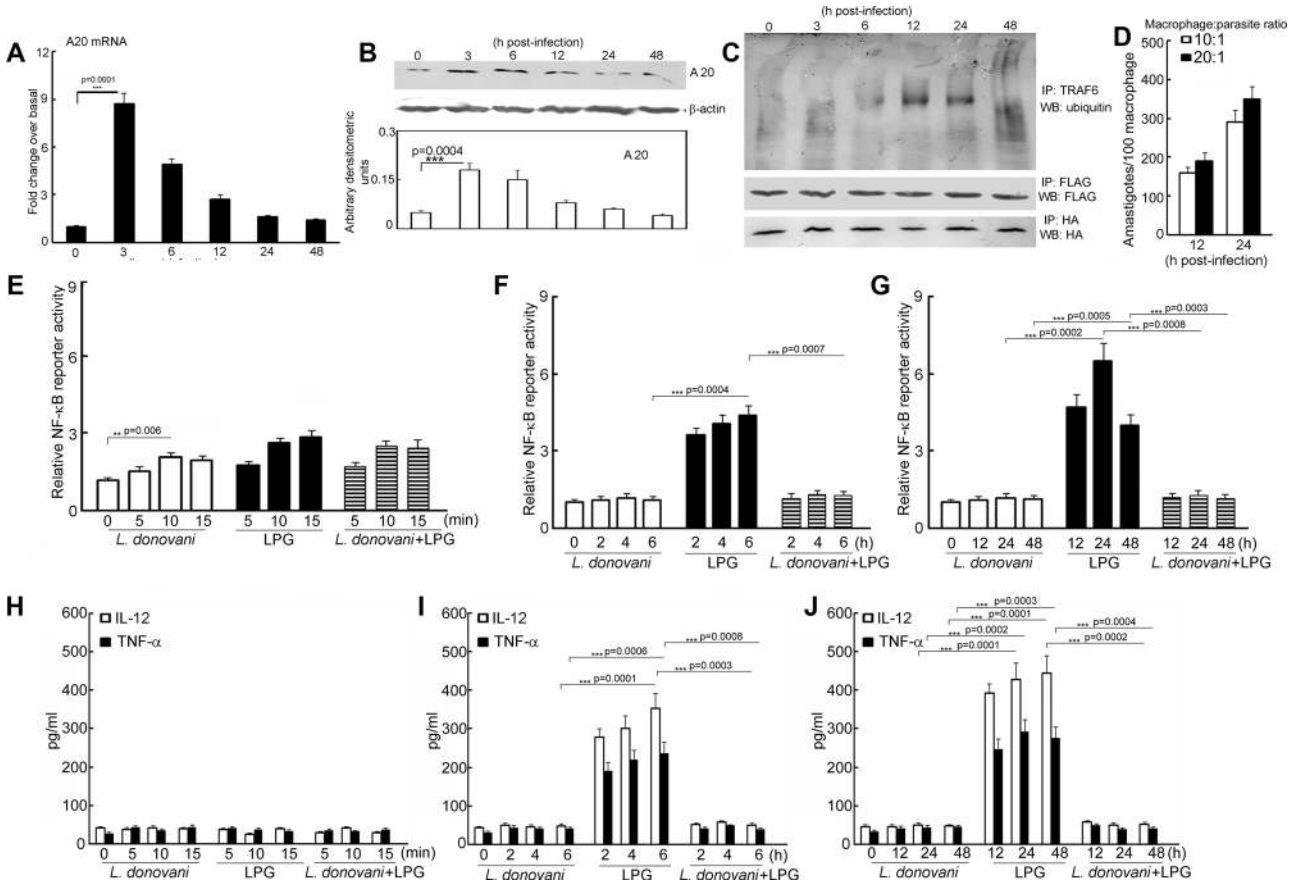

Figure 1. Effect of *L. donovani* infection on A20 expression and NF-κB-mediated cytokine expression. (A, B) RAW 264.7 cells were infected with *L. donovani* promastigotes (macrophage: parasite 1:20) for various time periods as indicated and the expression of A20 was evaluated at both (A) mRNA and (B) protein levels by real-time PCR and Western blotting, respectively. (A) mRNA levels were normalized to GAPDH and expressed as fold change compared with control. (C) HEK-293T cells were co-transfected with FLAG-TRAF6 and HA-ubiquitin. Cell lysates were immunoprecipitated with anti-FLAG Ab followed by incubation with A20 (immunoprecipitated from infected macrophages for various time points). The ubiquitination of TRAF6 was analyzed by immunoblotting with anti-HA Ab. (D) Cells were infected with promastigotes at macrophage: parasite ratio of 1:20 and 1:10 for indicated time periods and intracellular parasite number was determined by Giemsa staining. (E–G) RAW264.7 cells were transfected with pNF-κB luciferase plasmid (1 μg) and 0.5 μg of pCMV-β-gal. Twenty-four hours posttransfection, cells were subjected to either *L. donovani* infection or LPG administration (6 μg/mL) for (E) 5, 10, and 15 min; (F) 2, 4, and 6 h; and (G) 12, 24, and 48 h followed by lysis and processed for estimation of luciferase activity. (H–J) Macrophages were incubated either with purified LPG (6 μg/mL) or with *L. donovani* and expressions of IL-12 and TNF-α were analyzed by ELISA at the protein level after (H) 5, 10, and 15 min; (I) 2, 4, and 6 h; and (J) 12, 24, and 48 h. The cultures were set in triplicate and the experiments were done at least three times each. The results are representative of three individual independent experiments performed at different times. The error bars represent mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test.

inhibition of NF-κB-mediated proinflammatory response even in the absence of A20. No significant change was observed in the expressions of adaptor protein MyD88 and the two kinases IRAK1 and IRAK4 throughout the course of infection (Fig. 2A). Since ligand binding to TLRs results in phosphorylation of IRAK4 which in turn phosphorylates IRAK1, we next checked the phosphorylation status of both these proteins. Both p-IRAK4 and p-IRAK1 levels peaked at 6 h postinfection (3.1- and 2.6-fold, respectively, $p < 0.001$) followed by a sharp decrease (93.3 and 94.8% reduction, $p < 0.001$ for p-IRAK4 and p-IRAK1, respectively, at 24 h postinfection as compared with the maxima at 6 h postinfection) (Fig. 2B). IRAK1 kinase activity also revealed a similar pattern (Fig. 2C). IRAK1/4 phosphorylation was also analyzed following LPG administration with time points corresponding to that used in the case of *L. donovani* infection. However, in contrast to *L. donovani* infected macrophages, a significant induction in both IRAK1 and IRAK4 phosphorylation was observed following LPG administration at 3 h posttreatment, slightly decreased at 6 h, significantly decreased at 12 h, and then slow induction started appearing 24 h onward (Fig. 2B, right panel). Since IRAK1 phosphorylation is a necessary prerequisite for its release from MyD88 and subsequent attachment to TRAF6 [14], we tried to check the association of IRAK1 with MyD88 and TRAF6 by co-immunoprecipitation studies. IRAK1 was found to be strongly associated with TRAF6 up to 6 h of *L. donovani* infection after which there was a significant reduction in TRAF6-bound IRAK1 with a concomitant increase in MyD88-bound IRAK1 at corresponding time periods (Fig. 2D). These data suggested that reduction in IRAK1 phosphorylation might result in failure of IRAK1 to dissociate from MyD88 and subsequently attach with TRAF6.

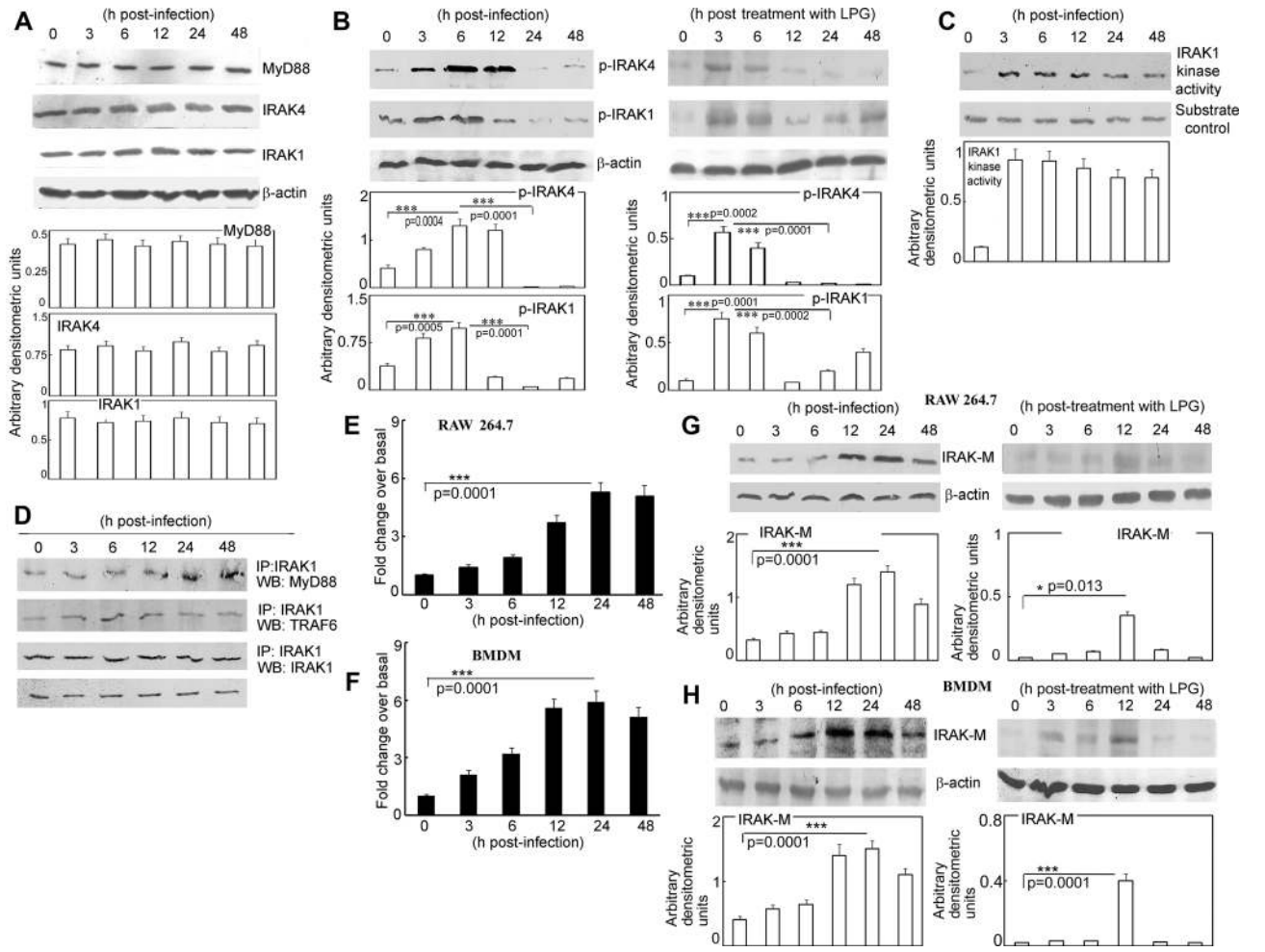

Figure 2. Effect of *L. donovani* infection on IRAK1–TRAF6 association and IRAK-M expression. (A, B) RAW 264.7 macrophages were infected with *L. donovani* (cell/parasite ratio, 1:20) for indicated times, and whole-cell lysates were subjected to SDS-PAGE followed by Western blotting to analyze the expression of (A) MyD88, IRAK4, and IRAK1 and (B) phosphorylated IRAK4 and IRAK1. (B, right panel) Phosphorylated IRAK4 and IRAK1 expressions were also analyzed in macrophages treated only with LPG for indicated time periods. (C, D) RAW cells were infected with *L. donovani* for indicated times, and whole-cell lysates were subjected to immunoprecipitation with anti-IRAK1 antibody. Immunoprecipitated IRAK1 was subjected to evaluation of kinase activity by incubation in kinase assay buffer containing 10 μCi of γ-32P (C) and expression of MyD88, TRAF6, and IRAK1 (D) by Western blotting with respective antibodies. (E–H) Both RAW and BMDM were infected with *L. donovani* for various time periods as indicated and the expression of IRAK-M was analyzed at mRNA and protein levels by real-time PCR analysis and Western blotting, respectively. IRAK-M mRNA levels in RAW (E) and BMDM (F), whereas IRAK-M protein levels in RAW (G) and BMDM (H). (G, H, right panel) Protein level expressions of IRAK-M were also determined after the treatment with LPG for indicated time periods in RAW and BMDM. IP: immunoprecipitation using the indicated Ab; WB: Western blot analysis using the indicated Ab. Results are representative of three separate individual experiments and the error bars represent mean ± SD ($n = 3$). ***$p < 0.001$; Student's *t*-test.

IRAK-M, a negative regulator of the TLR pathway, prevents the dissociation of IRAK1 from MyD88 and its subsequent association with TRAF6 [15]. We, therefore, sought to determine the levels of IRAK-M after *L. donovani* infection. Our data revealed an induction in IRAK-M levels reaching maxima (5.3- and 4.4-fold over control, $p < 0.001$ at mRNA and protein level respectively) at 24 h postinfection (Fig. 2E and G, left panel). We further validated these findings in Bone-marrow derived macrophages (BMDM) which also depicted an increase in IRAK-M expression, both at mRNA and protein level (Fig. 2F and H, left panel). However, IRAK-M expression was found to increase a little after 12 h of LPG treatment at both mRNA and protein levels (Fig. 2G and H, right panels). These results suggest that the formation of IRAK1–TRAF6 complex might be disrupted during late hours of *L. donovani* infection through induction of IRAK-M.

Role of TGF-β signaling cascade in the induction of IRAK-M by *L. donovani*

TGF-β is known to have a broad suppressive influence on the immune system, partly through the cross-talk between its signaling intermediates and the components of the TLR cascade [16]. Few studies also documented the direct involvement of TGF-β in the induction of IRAK-M [16, 17]. Interestingly, *Leishmania* infection

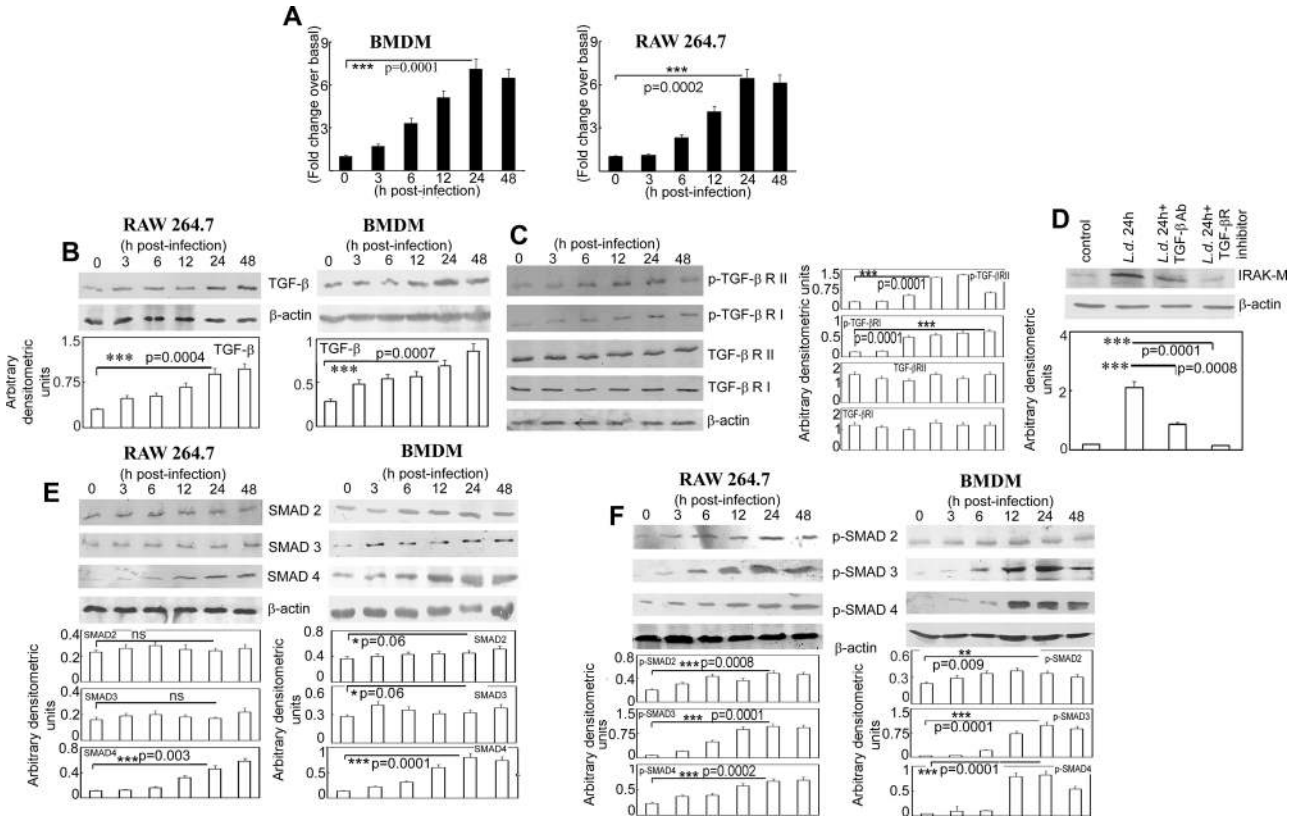

Figure 3. Role of TGF-β signaling cascade in the induction of IRAK-M by *L. donovani*. (A, B) Both RAW 264.7 and BMDM were incubated with *L. donovani* for various time periods as indicated and the expression of TGF-β was analyzed both at (A) mRNA and (B) protein levels by real-time PCR analysis and Western blotting, respectively. (C) RAW 264.7 cells were infected with *L. donovani* for various time periods. Whole-cell lysates were subjected to SDS-PAGE followed by evaluation of the phosphorylation of TGF-β RI and RII by Western blotting using respective antibodies. (D) RAW 264.7 macrophages were infected with *L. donovani* for 24 h along with either anti-TGF-β Ab or TGF-βR inhibitor. Whole-cell lysates were subjected to analysis of IRAK-M expression by Western blotting. (E, F) Macrophages (both RAW and BMDM) were infected with *L. donovani* for indicated times, and whole-cell lysates were subjected to Western blotting for the expression of (E) SMAD2, SMAD3, and SMAD4 and (F) phosphorylated SMAD2, SMAD3, and SMAD4 using respective antibodies. Results are representative of three individual independent experiments and the error bars represent mean ± SD ($n = 3$). ns: not significant; $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test.

in macrophages is correlated with a massive induction of TGF-β production [18]. We, therefore, wanted to investigate whether this infection-triggered TGF-β could play a role in the induced expression of IRAK-M in *L. donovani* infected macrophages. As seen in Figure 3A, the level of TGF-β mRNA was induced during infection reaching a maximum (6.4-fold over control, $p < 0.001$) at 24 h postinfection. The kinetics of TGF-β mRNA induction strongly corresponded to that of IRAK-M induction in *L. donovani* infected cells (Fig. 2E). Similar trend was observed in the case of TGF-β protein expression (Fig. 3B). This was further validated in BMDM, which also showed a significant induction (7.1- and 2.5-fold in TGF-β mRNA and protein, respectively, $p < 0.001$) (Fig. 3A (left panel) and B (right panel)). We next studied the effect of *L. donovani* infection on the phosphorylation status of TGF-β receptors I and II. *Leishmania donovani* infection strongly induced the phosphorylation of both these receptors, without causing any significant change in their expression. The phosphorylation of TGF-βRII was found to be maximum (4.6-fold over control, $p < 0.0001$) at 24 h, whereas that of TGF-βRI reached a maximum (5.2-fold over control, $p < 0.0001$) at 48 h postinfection (Fig. 3C). To further ascertain the role of TGF-β in IRAK-M induction, we studied the effect of inhibiting the TGF-β cascade on the expression of IRAK-M. Administration of TGF-β neutralizing antibodies as well as inhibitors of TGF-β receptor kinase activity resulted in a significant decrease in IRAK-M expression (83.6% and 87.1%, respectively, $p < 0.001$) (Fig. 3D). Since the anti-inflammatory effect of TGF-β is mediated through TGF-β receptors and SMAD family of proteins, we next studied the status of various SMAD proteins during *L. donovani* infection in RAW 264.7 cells. *Leishmania donovani* infection was found to induce the levels of SMAD4 with a maximum (5.5-fold over control, $p < 0.001$) at 24 h postinfection (Fig. 3E, left panel), whereas the expressions of SMAD2 and SMAD3 during infection did not show any significant change (Fig. 3E, left panel). However, a significant induction in the phosphorylation of all the three SMAD proteins was observed (2.3-, 3.4-, and 3.1-fold, $p < 0.001$, at 24 h postinfection for SMAD2, SMAD3, and SMAD4, respectively) (Fig. 3F, left panel). These experiments were repeated in BMDM, which also yielded similar results (Fig. 3E and F, right panels). All these results suggest that the TGF-β-SMAD cascade might be activated to induce IRAK-M during *L. donovani* infection.

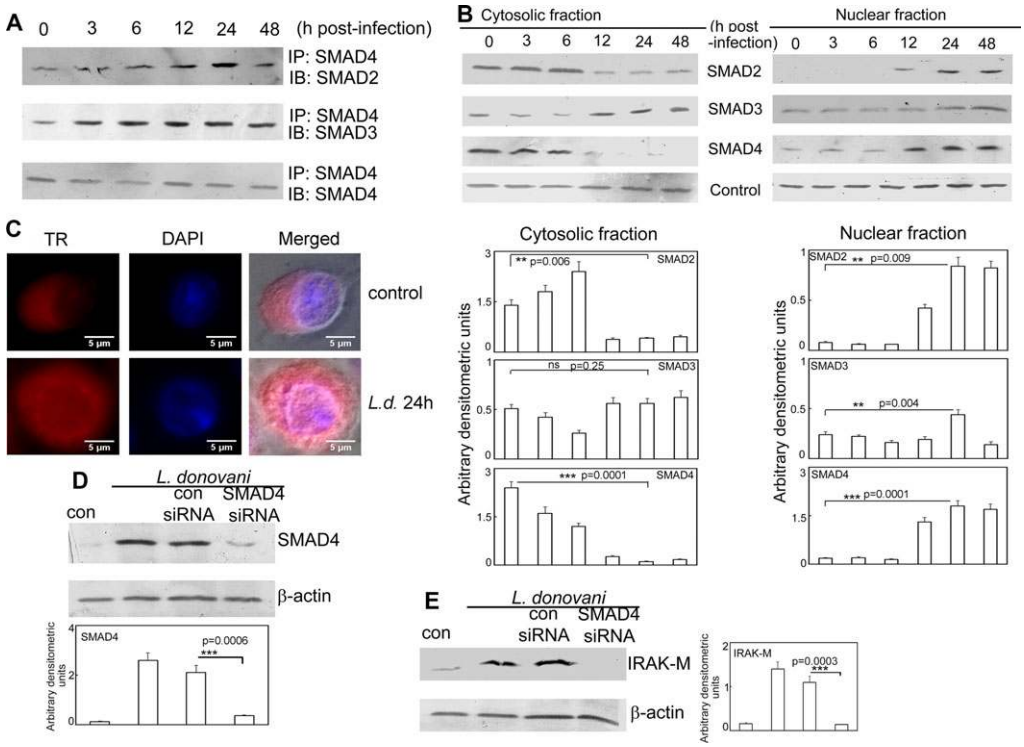

Figure 4. Transcriptional regulation of IRAK-M in *L. donovani* infected macrophages. (A) Macrophages were infected with *L. donovani* for indicated time periods and whole-cell lysates were subjected to immunoprecipitation with anti-SMAD4 antibody. Immunoprecipitates were subjected to Western blotting for the expression with SMAD2, SMAD3, and SMAD4 using respective antibodies. (B) Cells were treated as above, nuclear and cytosolic extracts were prepared, and expression of SMAD4 was analyzed by Western blotting. (C) Macrophages were subjected to *L. donovani* infection as above, and stained with anti-SMAD4 monoclonal antibody followed by secondary APC-conjugated antibody. Nuclei were stained with DAPI, and cells were analyzed under fluorescence microscope. Magnification 1000×. (D, E) Macrophages were transfected (24 h) with either control or SMAD4 siRNA followed by infection with *L. donovani* promastigotes for 24 h. Expression of (D) SMAD4 and (E) IRAK-M were evaluated by Western blot analysis. Results are representative of three individual independent experiments and the error bars represent mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test.

Transcriptional regulation of IRAK-M in *L. donovani* infected macrophages

Upon stimulation by TGF-β, SMAD2 and SMAD3 are phosphorylated and assembled into stable heteromeric complexes with SMAD4 followed by their translocation into the nucleus and subsequent DNA binding [19]. To this end, the association of SMAD2 and SMAD3 with SMAD4 was studied by co-immunoprecipitation. A strong association of SMAD4 with both SMAD2 and SMAD3 was observed in macrophages after 3 h of *L. donovani* infection as compared with uninfected controls and these associations were found to be stable as studied up to 48 h (Fig. 4A). To study the nuclear translocation of SMADs, we analyzed the expression of SMAD2, 3, and 4 at the protein level in both nuclear and cytosolic fractions of infected macrophages. An induction in the protein level expressions of all the three SMADs was observed (7.2-, 2.6-, and 6.9-fold more protein expression of SMAD2, 3, and 4, respectively, as compared with control cells, $p < 0.01$) in nuclear fractions at 24 h postinfection (Fig. 4B). The nuclear translocation of SMAD4 following infection was further ascertained by fluorescence microscopy using anti-SMAD4 antibody. In control macrophages, the signal for SMAD4 was distributed throughout the cell but did not co-localize with 4′,6-diamidino-2-phenylindole (DAPI) stained nuclei, indicating its cytosolic localization (Fig. 4C). On the contrary, *L. donovani* infection for 24 h resulted in an increase in the nuclear localization of SMAD4 as evident by markedly enhanced co-localization of SMAD4 signal (red) with DAPI-stained nuclei (blue) (Fig. 4C). To further validate the role of SMAD4 in the induction of IRAK-M in infected macrophages, we used an in vitro siRNA knockdown system for SMAD4. As shown in Figure 4D, SMAD4 was effectively downregulated by siRNA (82.9% reduction in expression as compared with control siRNA-treated cells, $p < 0.001$). SMAD4 knockeddown cells showed markedly decreased expression of IRAK-M (87.2% reduction as compared with control siRNA-treated cells, $p < 0.001$) (Fig. 4E). These results suggest that induction of IRAK-M may be mediated by SMAD4.

Effect of IRAK-M knockdown on proximal TLR signaling, cytokine production, and parasite survival

To ascertain further the effect of IRAK-M in the suppression of TLR signaling by *L. donovani* during established infection, we studied

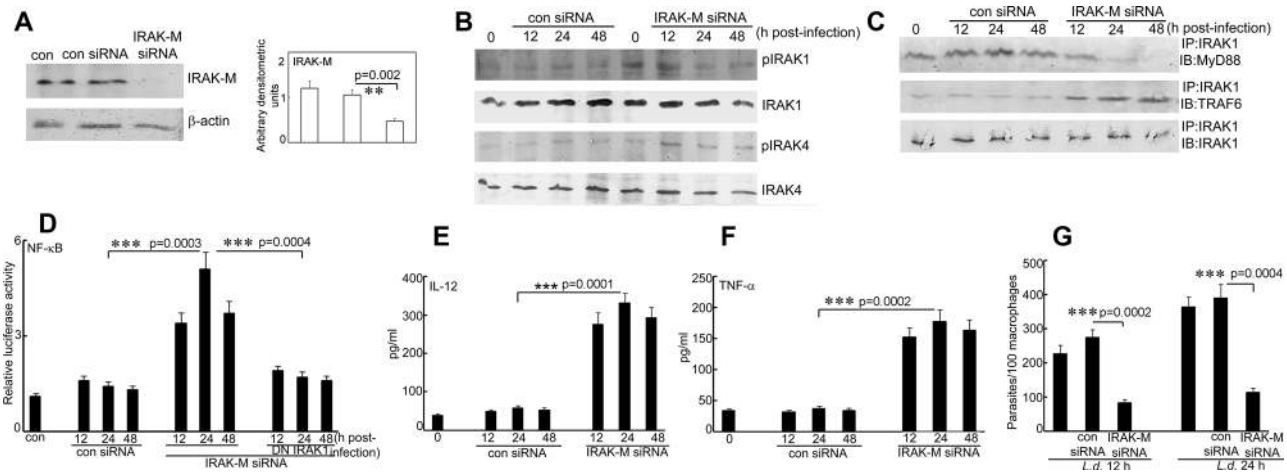

Figure 5. Effect of IRAK-M knockdown on proximal TLR signaling, cytokine production, and parasite survival. (A) RAW 264.7 macrophages were transfected (24 h) with either control or IRAK-M siRNA followed by infection with *L. donovani* promastigotes for 24 h. IRAK-M expression was evaluated by immunoblot analysis. (B, C) Control or IRAK-M siRNA transfected macrophages were infected with *L. donovani* promastigotes for various time periods as indicated. (B) p-IRAK1 and p-IRAK4 levels were analyzed by Western blotting. (C) The cell lysates were immunoprecipitated with anti-IRAK1 antibody and the immunoprecipitate was analyzed for MyD88 and TRAF6 expression by Western blotting. (D–G) RAW 264.7 cells were transfected with control or IRAK-M siRNA along with pNF-κB-luciferase plasmid (1 μg) and 0.5 μg of pCMV-β-gal (24 h). Cells were then infected with *L. donovani* promastigotes for various time periods as indicated. Infected macrophages were then transfected with DN IRAK1 construct. (D) Relative luciferase activity is shown. (E) IL-12 and (F) TNF-α concentration was measured by ELISA. (G) Intracellular parasite number was determined by Giemsa staining. (A–G) Results are representative of three individual independent experiments done at different times and the error bars represent mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test. DN: dominant negative.

the outcome of silencing of IRAK-M on the progression of the TLR cascade in infected macrophages. RAW 264.7 macrophages were transfected with IRAK-M-specific siRNA prior to infection. As shown in Figure 5A, transfection with IRAK-M siRNA resulted in 73.9% reduction ($p < 0.01$) of IRAK-M expression at the protein level. Since IRAK-M might have a role in dephosphorylation of IRAK1 following infection with *L. donovani*, we tried to monitor the phosphorylation status of IRAK1 in case of IRAK-M-silenced macrophages after *L. donovani* infection. Knockdown of IRAK-M increased the IRAK1 phosphorylation (4.7-fold over control siRNA treatment, $p < 0.001$) (Fig. 5B). We also checked for IRAK-4 phosphorylation in similar conditions. It was observed that silencing of IRAK-M led to an induction of IRAK4 phosphorylation but the level of phospho-IRAK4 was much less compared with that of phospho-IRAK1 (Fig. 5B). Co-immunoprecipitation studies revealed strong association of IRAK1 with TRAF6 with a concomitant decrease in IRAK1-MyD88 association in IRAK-M knockeddown infected cells, thereby suggesting that in the case of IRAK-M-silenced macrophages there is reversal of IRAK1–TRAF6 association (Fig. 5C). In consistence with the above data, NF-κB-dependent luciferase activity was found to be enhanced in the case of IRAK-M knockeddown *L. donovani* infected macrophages (3.6-fold over control siRNA administered infected macrophages, $p < 0.001$) at 24 h postinfection (Fig. 5D). The treatment of IRAK-M knockeddown cells with dominant negative construct for IRAK1 24 h prior to infection resulted in 77.4 and 49.6% reduction ($p < 0.001$) in NF-κB-dependent luciferase activity at 24 and 48 h postinfection, respectively, suggesting that the NF-κB activation may be mediated by IRAK1 (Fig. 5D). Since the induction of IRAK-M in *L. donovani* infected macrophages is associated with inhibition of TLR-mediated proinflammatory response, we studied the effect of IRAK-M silencing on the modulation of proinflammatory cytokine expression, which determines the fate of disease progression in visceral leishmaniasis. It was of interest to note that inhibition of IRAK-M led to marked induction in the level of these cytokines (5.8- and 4.8-fold for IL-12 and TNF-α, respectively, compared with control siRNA-treated cells, $p < 0.001$) at 24 h postinfection (Fig. 5E and F). We then examined the effect of IRAK-M silencing on the intracellular multiplication of the amastigotes. As shown in Figure 5G, silencing of IRAK-M significantly reduced the multiplication of amastigotes (63.5% and 74.9% reduction, $p < 0.001$) in parasite suppression compared with control siRNA-treated cells at 24 and 48 h postinfection, respectively) further accounting for the significance of IRAK-M in disease progression in visceral leishmaniasis. Taken together, these results suggest that *L. donovani* infection might exploit macrophage IRAK-M expression during delayed hours of in vitro infection leading to a pathogen friendly cytokine response resulting in enhanced parasite survival.

Discussion

TLRs are critical players of innate and adaptive immune systems to combat invading microorganisms and activate multiple pathways leading to the induction of proinflammatory response and subsequent pathogen clearance [14]. However, the ubiquitous nature of pathogens is such that if left unchecked the host will be overwhelmed by immune activation. Hence, following TLR activation against invading pathogens, the intensity and duration

of its responses is tightly controlled at various points by different modulators, generally referred to as negative regulators [20]. Several pathogens exploit these negative regulators to suppress the TLR-mediated immune response, thereby leading to the successful establishment of infection [21–23]. Earlier, we demonstrated that macrophage deubiquitinating enzyme A20 is exploited by *L. donovani* to downregulate host immune response during early stage of infection [7]. However, A20 expression levels were decreased after reaching a maximum at 2 h postinfection, and *Leishmania* parasites remained under the threat of being exposed to TLR-induced host defense. It is, therefore, reasonable to speculate that after short-lived A20, *L. donovani* might employ a different negative regulator for sustained inhibition of TLR response during late hours of infection.

Present studies on macrophage TLR signaling cascade during *Leishmania* infection suggest that defective IRAK1 phosphorylation might prevent its association with TRAF6, thereby downregulating TLR activation. Interestingly, IRAK-M, a member of the IRAK family that lacks an active serine/threonine kinase domain, has been reported to inhibit the formation of IRAK1–TRAF6 complex by preventing the dissociation of IRAK1 and IRAK4 from the TLR complex, either by inhibiting phosphorylation of IRAK1 and IRAK4, or by stabilizing the TLR–MyD88–IRAK4 complex [24, 25]. This is in agreement with our findings of decreased IRAK1–TRAF6 association with a concomitant increase in IRAK1–MyD88 association, indicating a role of IRAK-M in the inability of IRAK1 to dissociate from TRAF6. In line with these findings, we observed a rapid induction in IRAK-M at both mRNA and protein levels and the kinetics of this induction coincided with IRAK1 dephosphorylation and reduced IRAK–TRAF6 association. This suggests that *Leishmania* maintains TRAF6 inactivation even after the absence of A20 probably through IRAK-M-mediated dephosphorylation of IRAK1. This is in agreement with the fact that IRAK-M is a proximal inhibitor of signaling receptors that rely on MyD88/IRAK-1/IRAK-4 for generating intracellular effects [26]. IRAK-M has recently been implicated in several diseases, where its induction is reported to facilitate either pathogen replication directly or indirectly by attenuating host immune response. For example, IRAK-M impaired host defense during pneumonia caused by *Klebsiella pneumonia* [12]. *Helicobacter pylori* infection led to the upregulation of IRAK-M, which was found to limit dendritic cell activation and proinflammatory cytokine production [27]. Moreover, IRAK-M overexpression promoted lung epithelial human rhino virus 16 (HRV-16) replication and autophagy [28].

The expression of IRAK-M is believed to be restricted to monocytes/macrophages [9], but recently reported to be expressed in dendritic and epithelial cells [27–29]. Our observations that *Leishmania*-induced inhibition of NF-κB and increased parasite survival was reversed by siRNA-mediated gene silencing of IRAK-M may be considered as another addition in this growing list of literature and are in agreement with a previous observation where IRAK-M$^{-/-}$ dendritic cells infected with *H. pylori* displayed more proinflammatory Th1 phenotype and less immunoregulatory IL-10 associated with increased MHC II expression [27]. *Leishmania* are known to evade host immune response by multiple mechanisms, one of which being upregulation of TGF-β, which may play an important role in IRAK-M regulation. In the present study, *Leishmania* infection upregulated the phosphorylation of both the TGF-β RI and II subunits and administration of TGF-β-specific antibody abrogated infection induced IRAK-M expression. Moreover, kinetics of TGF-β upregulation coincided with IRAK-M induction. Signaling by TGF-β family members occurs through phosphorylation of receptor-regulated SMAD proteins. Activated SMAD2 and SMAD3 then form heteromeric complexes with SMAD4 [30]. *Leishmania donovani* infection resulted in a strong association of SMAD2 and SMAD3 with SMAD4 with concomitant nuclear translocation of SMAD4. An increase in the expressions of SMAD2 and SMAD4 was observed in the nuclear fraction with corresponding reduction in the cytosolic fraction, thereby signifying enhanced nuclear translocation. However, comparatively low expression of SMAD3 was observed at 3 and 6 h post infection in the cytosolic fraction without any corresponding increase in the nuclear fraction. This aberrant expression of SMAD3 may be attributed to the fact that SMAD-mediated signals induced by TGF-β are tightly regulated by negative feedback mechanisms through inhibitory SMAD proteins. Accumulating data indicate that inhibitory SMAD proteins regulate the signals induced by the TGF-β superfamily through degradation of activated type I receptor and receptor-activated SMAD proteins [31, 32]. Hence, it might be possible that decrease in SMAD3 expression is a consequence of inhibitory SMAD activity. Although *L. donovani* was found to exploit natural negative regulators of TLR pathway for establishment of infection, all *Leishmania* species do not completely shut down the entire immune response. For example, *L. major* activates IL-1 alpha expression in macrophages through a MyD88-dependent pathway [33], *L. infantum* triggers a rapid NK-cell response in mice through TLR9-positive myeloid DC and IL-12 [34] and *L. mexicana* LPG activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4 [35]. Similarly, although LPG is known to be a potent TLR2 ligand, few reports are there which mention inhibitory effects of LPG on macrophage effector functions. For example, *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP Kinase [36]. Certain studies also demonstrate that *L. donovani* promastigotes require cell surface expression of LPG repeating units to evade the induction of ERK1/2 activation [37]. LPG from *L. donovani* promastigotes was also shown to be a potent inhibitor of purified protein kinase C (PKC) activity in vitro [38].

In conclusion, we uncovered another evasion mechanism by which *L. donovani* exercises a persistent inhibition on host's immune response leading to infection establishment. During late hours of in vitro infection, increased production of TGF-β led to TGF-β-mediated signaling of activated SMAD proteins resulting in upregulation of IRAK-M expression and downregulation of macrophage TLR signaling. Elucidation of these mechanisms in detail might be helpful in developing therapeutic agents with immunomodulatory capacity.

Materials and methods

Cell culture and parasites

The maintenance of pathogenic promastigotes of *L. donovani* strain (MHOM/IN/1983/AG83) was done in Medium 199 (Invitrogen Life Technologies) supplemented with 10% FCS (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin. The murine macrophage cell line RAW 264.7 was maintained at 37°C/5% CO_2 in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The experiments involving in vitro infection were performed with RAW 264.7 and BMDM using stationary phase promastigotes at a 20:1 parasite/macrophage ratio for various time periods as described earlier [7]. BMDM was prepared from the femurs and tibias of 6- to 8-week-old BALB/c mice as described previously [7]. LPS was quantified by a sensitive colorimetric LPS assay (QCL 1000; Lonza). The parasite cultures and the final culture supernatants of infected BMDM and RAW 264.7 cells contained less than 95 pg/mL.

Reagents, antibodies, and constructs

All antibodies were purchased either from Santa Cruz Biotechnology or Cell Signaling Technology. The TGF-β antibody used in the study was goat polyclonal IgG anti-TGF beta 1 antibody and TGFβR-inhibitor used was TGFβRI Kinase inhibitor VII from Santa Cruz. The HA-ubiquitin construct was purchased from Sigma-Aldrich. FLAG-TRAF6-wt (Addgene plasmid 21624) was purchased from Addgene [39]. DN IRAK1 (1–217) was kindly provided by Dr. M. Muzio (Department of Immunology and Cell Biology, Mario Negri Institute, Milan, Italy) [40].

Real-time PCR

Total RNA from macrophages was isolated using the RNeasy mini kit (Qiagen) according to manufacturer's instructions, and cDNA was synthesized from total RNA using the SuperScript first strand synthesis system (Invitrogen). qPCR was performed as described previously [41].

In vitro deubiquitination assay

The deubiquitinase assay was performed as described earlier [7]. Briefly, expression plasmids FLAG-TRAF6 and HA-ubiquitin were co-transfected into HEK293 cells for 24 h; cells were harvested using RIPA buffer containing protease inhibitor cocktail and NEM (*N*-ethylmaleimide). Cell lysates were incubated with FLAG M2 beads overnight, followed by washing with Tris buffered saline (TBS) buffer. After elution, 15 μL of eluted Flag-TRAF6 was incubated for 4 h at 37°C with A20 immunoprecipitated from infected macrophages in deubiquitination assay buffer. The analysis of ubiquitination pattern was performed by Western blotting using an anti-HA, anti-FLAG, or anti-A20 Ab.

Transient transfection and NF-κB reporter assay

One microgram of NF-κB luciferase reporter vector (Stratagene) was co-transfected along with 0.5 μg pCMV-β-gal (Promega) in RAW 264.7 macrophages (2×10^6) in serum-free medium using Lipofectamine (Invitrogen) according to manufacturer's instruction, and NF-κB luciferase activity was assessed as previously described [14]. For siRNA transfection, RAW 264.7 cells (2×10^6) were transfected with 1 μg SMAD4/IRAK-M siRNA or control siRNA according to manufacturer's instruction (Santa Cruz Biotechnology). Following silencing, cells were infected with *L. donovani* promastigotes as described earlier [42].

Cytokine analysis by ELISA

The level of various cytokines in the culture supernatants was measured using a sandwich ELISA kit (Quantikine M; R&D Systems) as per the detailed instructions of the manufacturer.

Immunoblotting

Immunoblotting was performed with respective antibodies as described previously [7, 42].

LPG purification

LPG was isolated from stationary-phase promastigotes as described earlier [7]. Briefly, *L. donovani* promastigotes (10^8 cells) were extracted with chloroform/methanol/water (1:2:0.5, v/v). The insoluble fraction was used for LPG extraction with 9% 1-butanol in water, and the pooled supernatants were vacuum dried followed by octyl sepharose chromatography in HPLC using a 1-propanol gradient (5–60%) in 1% ammonium acetate. The isolated LPG was dissolved in PBS, and the concentration was determined by estimating the amount of hexose in the extract by a modified phenol–sulfuric acid assay. LPG was sonicated in RPMI 1640 before addition to cells; 2×10^6 macrophages were infected with 4×10^7 *L. donovani* parasites (parasite/macrophage ratio of 20:1) from which 6 μg of LPG is obtained. This amount of purified LPG (i.e., 6 μg) was therefore used for the treatment of macrophages. LPS content was found to be less than 25 pg/mL. The number of internalized parasites per 100 macrophages for parasite/macrophage ratio of 20:1 was 320 ± 40 as compared to 280 ± 32 in the case of 10:1, which is generally used for in vitro infection (Fig. 1D).

Fluorescence microscopy

Macrophages (5×10^5) were adhered onto 18-mm^2 coverslips kept in 30-mm Petri plates and kept overnight at 37°C. The cells were then infected with *L. donovani* promastigotes, washed twice in PBS, and fixed with methanol for 15 min at room temperature. Cells were permeabilized using 0.1% Triton X-100 and incubated with SMAD4 antibody for 1 h at 4°C. After washing, coverslips were incubated with TR-conjugated secondary antibody (1 h, 4°C). The cells were then stained with DAPI (1 μg/mL) in PBS plus 10 μg/mL RNase A to label the nucleus, mounted on slides, and visualized under an Olympus BX61 microscope at a magnification of 1000, and the images thus captured were processed using ImagePro Plus (Media Cybernetics).

IRAK1 kinase assay

Macrophages were lysed and the lysates were precleared with protein A/G agarose beads (Santa Cruz). IRAK1 antibody and protein A/G agarose beads were added to the supernatant and samples were incubated overnight at 4°C. Beads were collected and washed with the lysis buffer followed by washing with the kinase assay buffer (20 mM HEPES pH 7.5, 20 mM $MgCl_2$, 3 mM $MnCl_2$, and 10 mM β-glycerophosphate). The samples were incubated with kinase assay buffer containing 10 μCi of γ-^{32}P for 30 min at 30°C. The reaction was stopped by the addition of sample loading buffer (12.5% Tris-HCl pH 6.8, 10% glycerol, 10% SDS, 5% β-mercaptoethanol, and 0.05% bromophenol blue). Samples were boiled and ran on SDS-PAGE.

Densitometric analysis

Densitometric analyses for all experiments were carried out using QUANTITY ONE software (Bio-Rad).

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 triplicates and the results are expressed as the mean ± SD. Student's t-test was employed to assess the statistical significances of differences among pair of data sets. Statistical significance was considered to be a value of $p < 0.05$.

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Abbreviations: **IRAK**: IL-1 receptor-associated kinase · **LPG**: lipophosphoglycan · **MyD88**: myeloid differentiation factor 88 · **TRAF6**: TNF receptor associated factor 6

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