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TLR4-mediated activation of MyD88 signaling induces protective immune response and IL-10 down-regulation in *Leishmania donovani* infection

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In visceral leishmaniasis, a fragmentary IL-12 driven type 1 immune response along with the expansion of IL-10 producing T-cells correlates with parasite burden and pathogenesis. Successful immunotherapy involves both suppression of IL-10 production and enhancement of IL-12 and nitric oxide (NO) production. As custodians of the innate immunity, the toll-like receptors (TLRs) constitute the first line of defense against invading pathogens. The TLR-signaling cascade initiated following innate recognition of microbes shapes the adaptive immune response. Whereas numerous studies have correlated parasite control to the adaptive response in *Leishmania* infection, growing body of evidence suggests that the activation of the innate immune response also plays a pivotal role in disease pathogenicity. In this study, using a TLR4 agonist, a *Leishmania donovani (LD)* derived 29 kDa β 1,4 galactose terminal glycoprotein (GP29), we demonstrated that the TLR adaptor myeloid differentiation primary response protein-88 (MyD88) was essential for optimal immunity following *LD* infection. Treatment of *LD*-infected cells with GP29 stimulated the production of IL-12 and NO while suppressing IL-10 production. Treatment of *LD*-infected cells with GP29 also induced the degradation of IKB and the nuclear translocation of NF- B, as well as rapid phosphorylation of p^{38} MAPK and p54/56 JNK. Knockdown of TLR4 or MYD88 using siRNA showed reduced inflammatory response to GP29 in *LD*-infected cells. Biochemical inhibition of p^{38} MAPK, JNK or NF- B, but not p42/44 ERK, reduced GP29-induced IL-12 and NO production in *LD*-infected cells. These results suggested a potential role for the TLR4-MyD88–IL-12 pathway to induce adaptive immune responses to *LD* infection that culminated in an effective control of intracellular parasite replication.

Keywords: Interleukin-10, *Leishmania donovani*, Mitogen activated protein kinase, Myeloid differentiation primary response protein, Nuclear factor kappa beta, Th1 immune response, Toll like receptor, Visceral leishmaniasis

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania* affects 12 million people worldwide. The obligate intracellular parasite is transmitted by sandflies that infect primarily macrophages in the vertebrate host and cause cutaneous, muco-cutaneous or visceral form of the disease. Kala-azar or human visceral leishmaniasis (VL) is caused by the protozoan parasite *Leishmania donavani* (*LD*), or *L. infantum* (chagasi)[1]. Protective immunity is associated with a predominant IL-12 driven Th1 immune response and IFN- production, while T-cell derived IL-10 determines disease outcome. Both *Leishmania* promastigotes and amastigotes suppress macrophage IL-12, superoxide and nitric oxide production[2] and this inability to produce IL-12 is the primary cause of non-healing disease[3].

Pattern recognition receptors (PRRs), including Toll like receptors (TLR) recognition of highly conserved structural motifs referred to as pathogen-associated molecular patterns (PAMPs) triggers the innate immune system. PAMPs interact with TLRs to secrete cytokines, including IL-12, which promotes the differentiation of T-helper 1 (Th 1) cells that

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Abbreviations: sAC, splenic adherent cell; ERK, extra-cellular signal-regulated kinases; GP29, 29 kDa β-1,4-galactose terminal glycoprotein of *Leishmania donovani;* IRAK, IL-1 receptor-associated kinase; IRF, interferon regulatory transcription factor; JNK, c-Jun NH_2-terminal kinase; KD, knock down; LD, *Leishmania donovani;* LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MD2, myeloid differentiation factor 2; NF- B, nuclear factor kappa beta; PAMP, pathogen-associated molecular patterns; PRR, pattern recognition receptor; TIRAP, toll/interleukin-1 receptor domain-containing adapter protein; TLR, toll-like receptors; TRAF6, TNF receptor associated factor 6; VL, visceral leishmaniasis; WT, wild type.

produce IFN- and facilitate cell-mediated immune response[4]. In leishmaniasis, which affects 10 million people, TLR4 is required for proper parasite control, due to the induction of IL-12[5,6].

Following receptor ligand association, TLR signalling occurs through the sequential recruitment and activation of various adaptor molecules and kinases. Myeloid differentiation protein-88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) are the two key downstream adapter molecules recruited by TLRs to trigger downstream signaling events involved in innate immunity. TLR4 signals through both MyD88 and TRIF pathways[7], TLR3 signals through TRIF and rest of TLRs signal through only the MyD88 pathway[8-10]. Use of specific TLR ligands can programme cells to elicit protective immunity against infectious diseases.

In a previous study, we have identified a *Leishmania*-derived TLR4 agonist GP29[5] that induces TLR4-mediated TNF- and IL-12 production to suppress TLR2-mediated IL-10 production. This study provides a molecular basis of GP29-mediated IL-10 suppression. We show that GP29-mediated TLR4 stimulation triggers the MyD88-mediated inflammatory response that contributes to effective control of intracellular parasite replication.

Materials and Methods

Animals, parasites and animal infection

Six weeks old BALB/c mice (female, originally bought from Jackson Laboratory, Bar Harbour, Maine), reared in the Indian Institute of Chemical Biology facility were used, with prior approval of the Animal Ethics Committee of the Institute (Accreditation No. 147/1999/CPCSEA). C57BL/6-background IL-10 KO mice were a kind gift of Prof. A Surolia (National Institute of Immunology, New Delhi). Pentavalent antimony-responsive AG83 (MHOM/IN/83/AG83) was used for experimental infection[11]. Parasites were maintained in golden hamsters and promastigotes obtained after transforming amastigotes from infected spleen, were maintained in M199[11]. Animals were infected through the tail vein with 2×10^7 second passage *LD* promastigotes.

Purification of GP29

GP29 was purified essentially as described earlier[5]. In brief, complete soluble antigen (CSA) was prepared from attenuated *LD* clonal promastigotes in the presence of 0.04% Non-idet P-40[5,12]. Galactose terminal protein was purified by affinity chromatography on a *Erythrina crystagalli*-Sepharose column[5]. All reagents, including GP29 were tested for endotoxin contamination by the Limulus amoebocyte lysate (LAL) endpoint assay (QCL-1000; Bio-Whittaker, MD, USA), following the manufacturer's manual and were less than 0.1 EU/mL.

Infection of macrophage culture

Infections with 2nd passage promastigotes were carried out *in vitro* using the murine macrophage cell line RAW 264.7 or with splenic adherent cells from mice. Parasites were added to the macrophages at a 20:1 parasite/macrophage ratio as described previously[5]. MTT assay was used to assess cell viability using an MTT-based colorimetric assay kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

Treatment of M s with inhibitor

Infected cells were pretreated with inhibitors for 1 h prior to GP29 treatment. Cells were treated with inhibitors to p[38] (SB202190, 20 μM), JNK (SP600125, 20 μM), ERK1/2 (U0126, 20 μM), IRAK1/4 inhibitor I (20 μM), IKK inhibitor wedelolactone (20 μM) and BAY11-7082 (5 μM) for 1 h and with TRIF inhibitory peptide (Pepinh-TRIF, 100 μM) for 30 min, prior to treatment with GP29. Subsequent, identical steps were taken with the *LD* infected control groups.

Transfection of siRNA to RAW264.7 cells

Cells were transfected with 1 μg of appropriate siRNA or control siRNA according to the manufacturer's instructions (Santa Cruz Biotechnology).

RT-PCR

Total RNA was isolated from RAW 264.7 cells using the RNeasy minikit (QIAGEN) and was individually analyzed (three cover slips/group) by RT-PCR[11]. RNA (1 μg) from different experimental groups was reverse transcribed into cDNA by random hexamers (Invitrogen) using Superscript II (Invitrogen). The synthesized cDNA was analyzed for the expression of IL-10, IL-12 and iNOS with gene-specific primers in a thermocycler (Perkin Elmer model 9700) with a hot start at 94°C for 7 min in a final volume of 50 μl. Each gene of interest was normalized to the ß-Actin gene and the fold change was compared relative to infected control.

ELISA

Cytokine levels in the RAW 264.7 cells were measured using a sandwich ELISA kit (Quantikine M; R&D Systems, Minneapolis, MN, USA) as described previously[5]. Spleen cells were stimulated with GP29 (5 µg/mL) for 72 h. The detection limit of these assays was <2.5 and <4 pg/mL for IL-12p70 and IL-10, respectively. IRF-3 activity was examined by ELISA using an IRF-3 activity kit (Active Motif. Inc.)

Measurement of NO

RAW 264.7 cells (10^6/mL) were suspended in phenol-red free RPMI medium and incubated with or without GP29 (5 µg/mL) for 72 h in 5% CO_2 incubator at 37 C. The culture supernatant was analyzed for its nitrite (NO_2^-) content by using Griess reagent[11]. Each experiment was performed in triplicate and the data represented as mean ± standard deviation.

Preparation of nuclear and cytoplasmic extracts

The nuclear and cytoplasmic extracts were prepared from normal and infected macrophages in the presence or the absence of GP29 by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit, from Thermo Scientific as per manufacturer's protocol.

Fluorescence microscopy

RAW 264.7 cells (5×10^5) were plated on to 18 mm^2 coverslips kept in 30-mm Petri plates and cultured overnight. The cells were then infected with *L. donovani* promastigotes, washed twice in PBS, treated with GP29 (5 µg/mL) for the indicated time and fixed with methanol for 15 min at room temperature. The cells were then permeabilized with 0.1% Triton X and incubated with NF- B p65 Ab for 1 h at 4 C. After washing, coverslips were incubated with FITC-conjugated secondary Ab (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 slide and visualized under an Olympus BX61 microscope at a magnification of X1000 and the images thus captured were processed using ImagePro Plus (Media Cybernetics).

Immunoblotting

Cells were lysed in lysis buffer (Cell Signaling Technology) and the protein concentrations in the cleared supernatants were estimated using a Bio-Rad protein assay (Bio-Rad). The cell lysates (80 µg protein/well) were resolved by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Millipore). The membranes were blocked with 5% w/v milk in TBS-Tween (0.05% Tween 20 in 10 mM Tris/100 mM NaCl, pH 7.5) for 1 h at room temperature and probed with primary Ab overnight at a dilution recommended by the suppliers. Membranes were washed three-times with wash buffer and then incubated with HRP-conjugated secondary Ab and detected by ECL detection system (Thermo Scientific) according to the manufacturer's instructions.

Statistical analysis

Data shown were representative of at least three independent experiments unless otherwise stated as n values given in the legends of figures. RAW cultures were set in triplicate and the results were expressed as the mean ± SD. Student t test was used to assess the statistical significances of differences among pairs of data sets with a p value < 0.05 considered to be significant.

Results

Activation of MyD88 mediated pro-inflammatory signaling

TLR4 signals through both MyD88 and TRIF[7]. To investigate the importance of MyD88 and TRIF in GP29-mediated anti-leishmanial effect, siRNA-mediated knock-down (KD) system and TRIF inhibitory peptide Pepinh-TRIF were used, respectively. Twenty-four h after siRNA treatment, MyD88 protein levels in RAW 264.7 cells were decreased by more than 85% (Fig. 1a, *upper panel*). In a previous study, we have observed the maximum microbicidal and anti-leishmanial activity of GP29 at 5 µg/mL[5]. Hence, in this study the dose of 5 µg/mL was used. MyD88 KD, but not TRIF inhibition failed to contain intracellular parasite replication in GP29-treated infected cells (Fig. 1a).

Furthermore, MyD88 KD resulted in a significant decrease in IL-12 (66.17 ± 1.23%) and NO (89.12 ± 1.98%) production in GP29-treated cells. In contrast, TRIF-blockade did not affect GP29-mediated IL-12 and NO production post-GP29 treatment (Fig. 1b). In keeping with the higher intracellular parasite number, IL-10 production was high in both treated (684.71 ± 32.66 pg/mL) and infected (765.15 ± 36.01 pg/mL) MyD88 KD cells, compared to the wild type (WT) (165 ± 48.02 pg/mL) or control siRNA transfected (146.14 ± 39.68 pg/mL) GP29-treated counterparts (Fig. 1c).

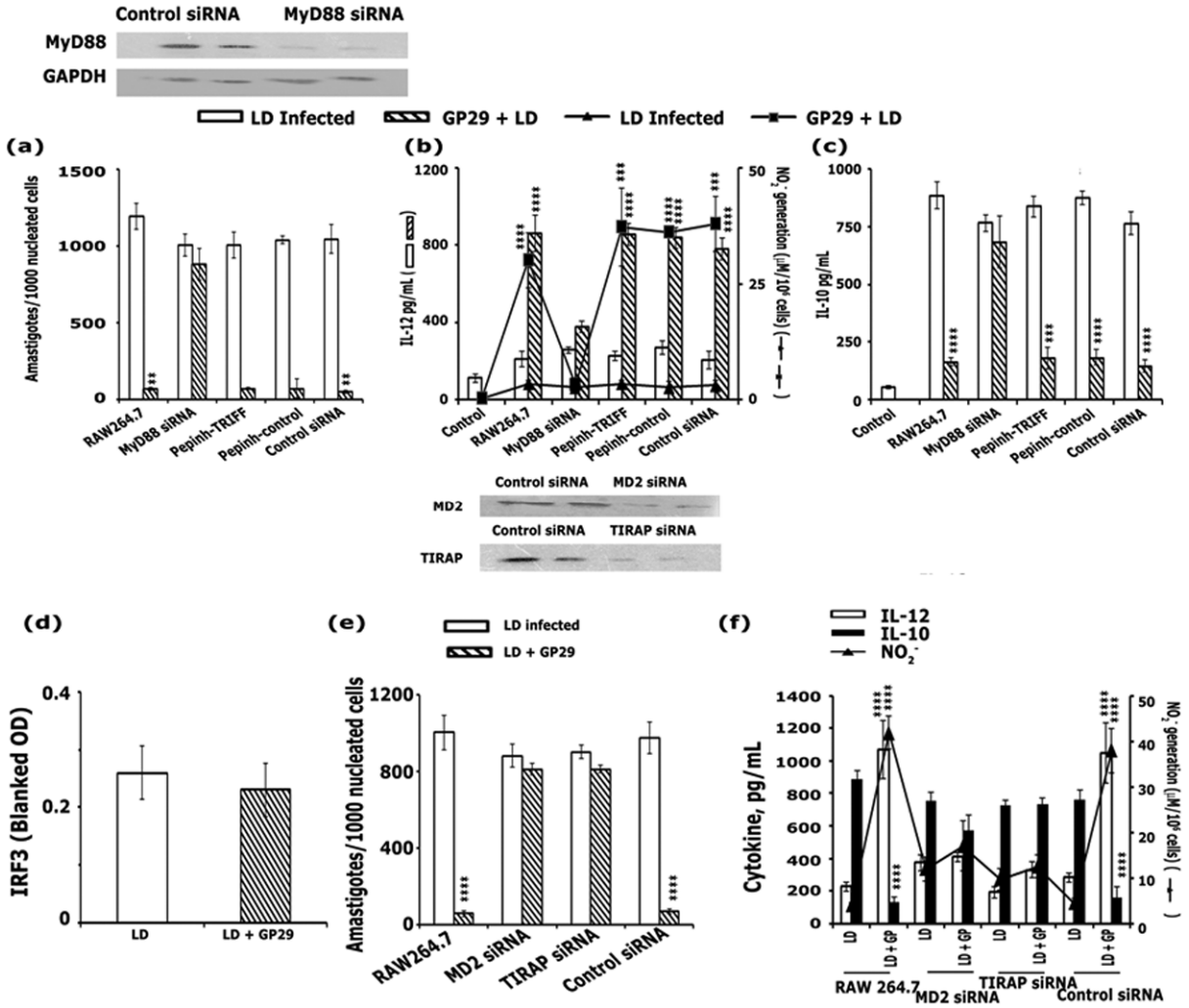

Fig. 1—GP29-mediated anti-leishmanial effect is MyD88-dependent but TRIF independent [RAW 264.7 cells were transfected with siRNAs specific to MyD88. A control group was transfected with control siRNA. After 24 h of transfection, cells were recovered and their MyD88 level assessed in Western blots. GAPDH in total proteins was used as loading controls. Blots are representative of three separate experiments (*upper panel)*. WT RAW 264.7 cells or siRNA MyD88 cells were infected with *LD* promastigotes for 24 h at a parasite/Mφ ratio of 20:1. 24 h parasitized cells were exposed to GP29 for 24 h. In a parallel set, RAW 264.7 cells were incubated with Pepnih-TRIF (100 μM) for 6 h prior to *LD* infection. (a) Intracellular parasite number was determined by Giemsa staining and expressed as amastigotes/1000 nucleated cells; (b, c) IL-12 and IL-10 cytokine levels were assessed by ELISA and NO_2^- production was determined by Griess reagent; (d) IRF-3 activity was examined by ELISA using an IRF-3 activity kit; (e, f) RAW cells were transfected with TIRAP and MD2 siRNA and analyzed by Western blot as described above (e, *upper panel*). *LD*-infected MD2 siRNA or TIRAP siRNA RAW cells were treated with GP29 as described. Intracellular parasite number was determined by Giemsa staining (e) and expressed as amastigotes/1000 nucleated cells; (f) IL-12 and IL-10 cytokine levels were assessed by ELISA and NO_2^- production was determined by Griess. The results were representative of three independent experiments and data shown were means ± SD. ****p<0.0001; ***p<0.001 *versus* corresponding infected control; paired two-tailed Student's t-test]

IRF-3, a member of the interferon regulatory transcription factor (IRF) family is an important transcription factor mediated through the TLR-mediated TRIF-dependent pathway. IRF3 activity was not significantly increased on GP29 treatment, compared to infected controls (Fig. 1d). Taken together, these results suggested that GP29-mediated anti-leishmanial effect was MyD88-dependent, but TRIF-independent.

MyD88-dependent TLR4 signaling is triggered upon the formation of a homo-dimer mediated by the accessory protein myeloid differentiation factor 2 (MD2)[13,14]. To assess the involvement of MD2 in GP29-induced TLR4 activation, MD2 siRNA

transfected RAW264.7 cells ($MD2^{-/-}$ RAW) were infected with AG83 parasites prior to treatment with GP29. MD2 siRNA treatment for 24 h caused decrease in MD2 levels by more than 75% (Fig. 1 e, *upper panel*). $MD2^{-/-}$ gene silencing reduced the *in vitro* anti-leishmanial effect of GP29 by 69.73 ± 5.34% (Fig. 1e). Amount of nitrite and IL-12 production in $MD2^{-/-}$ RAW cells were also reduced (55.3% and 61.03%, respectively) (Fig. 1f), compared to GP29-treated infected control-siRNA transfected RAW cells.

Activation of the MyD88-dependent pathway also requires the co-operation of a second adaptor molecule TIRAP (Toll/interleukin-1 receptor domain-containing adapter protein) for a successful TLR4-mediated inflammatory response[7]. To determine the role of TIRAP in GP29-mediated effecter responses, TIRAP gene was silenced using a sequence specific siRNA. 24 h TIRAP siRNA treatment reduced TIRAP levels by more than 80% (Fig. 1e, *upper panel*). TIRAP silencing diminished the anti-leishmanial effector responses of GP29 (Fig. 1e, f). There was a 67.1% and 81.75% reduction in IL-12 and NO production, respectively in GP29-treated TIRAP KD infected cells (Fig. 1f).

TLR4 mediated NF- B activation

In murine cutaneous leishmaniasis, MyD88 dependent pathways are required for an IL-12-mediated protective immune response[15]. The functional activation of MyD88 signaling leads to the production of pro-inflammatory cytokines like IL-12 and quick activation of nuclear factor-κB (NF-κB), MAPKs (mitogen activated protein kinase) and signaling elements like IL-1 receptor-associated kinase (IRAK)-1, IRAK4 and TNF receptor associated factor 6 (TRAF6)[16]. Activation of the transcription factor NF-κB involves cytoplasmic dissociation of the inhibitor protein IκB and translocation of the active NF-κB complex into the nucleus[7,16]. As a first insight into the probable importance of the NF-κB pathway in GP29-mediated anti-leishmanial response, we studied the cytosolic and nuclear distribution of NF-κB by immunoblotting in murine primary splenic adherent cells (sAC).

As evidenced from Fig. 2a, there was a time-dependent decrease in NF-κB p65 cytosolic expression with a concomitant increase in the nuclear expression of NF-κB p65 in GP29-treated *LD* infected cells. Time kinetics of the nuclear translocation of p65 was studied in GP29-treated *LD*-RAW 264.7 cells by fluorescence microscopy. *LD*-infected cells were treated with 5 µg/mL GP29 and after the appropriate incubation time, cells were fixed, permeabilized and stained with Ab against p65 (green). Nucleus was stained with DAPI (blue). Untreated cells showed (Fig. 2b i-iv) typical cytoplasmic distribution of NF-κB. In cells exposed to GP29, a time-dependent change in the cytoplasmic and nuclear staining intensity was evident. NF- B began to translocate after 15 min of activation (Fig. 2b v-viii) and by 45 min, NF-κB staining was co-localized with the nuclear stain indicating translocation had occurred (Fig. 2b ix-xii).

Fluorescence microscopic results were confirmed by the electrophoretic mobility shift assay (EMSA) at 15, 45 and 60 min of GP29 treatment. DNA binding activity of NF-κB was found to be markedly enhanced in *LD*-infected RAW cells, following GP29 treatment (8.7-, 9.27- and 6.1- fold increase following 15, 45 and 60 min of treatment, respectively) (Fig. 2c i). The specificity of binding was confirmed by incubating the nuclear extract with a 200-fold excess of unlabeled oligonucleotide, which resulted in complete displacement of the NF-κB-specific band (Fig. 2c i, *lane 6*). Since, maximum NF-κB-DNA binding activity was observed at 45 min of GP29 treatment, effect of GP29 on NF-κB translocation was also monitored by EMSA in $TLR^{-/-}$ RAW cells. TLR4 gene was silenced using a sequence specific siRNA. Twenty-four hours after siRNA treatment TLR4 levels decreased by more than 85% (Fig. 2c ii, *upper panel*). GP29 was unable to activate NF- B in $TLR^{-/-}$ RAW cells (Fig. 2c ii, *lane 1*). This indicated that GP29 induced a TLR4-mediated NF- B activation pathway.

NF- B activity in IL-$10^{-/-}$ M

The direct impact of GP29-mediated IL-10 suppression in controlling intracellular parasite growth was further substantiated by using $IL\text{-}10^{-/-}$ sACs. *LD*-infected sACs from IL-10 sufficient and deficient (IL-10 KO, B6.129P2-Il10tm1Cgn/J Nii) mice on a BL6 background were treated with 5 µg/mL GP29 for 45 min. GP29-treated (Fig. 2c ii, *lane 3*) and untreated (Fig. 2c ii, *lane 4*) infected $IL\text{-}10^{-/-}$ sACs showed comparable NF- B nuclear translocation and DNA binding activity (10.5- and 12.3- fold increase, respectively) when compared to NF- B DNA binding activity in *LD*-infected sACs from control WT mice (Fig. 2c ii, *lane 4* and *5*). This indicated that *LD*-mediated IL-10 production prevented NF- B activation and GP29-mediated IL-10 suppression induced NF- B activation.

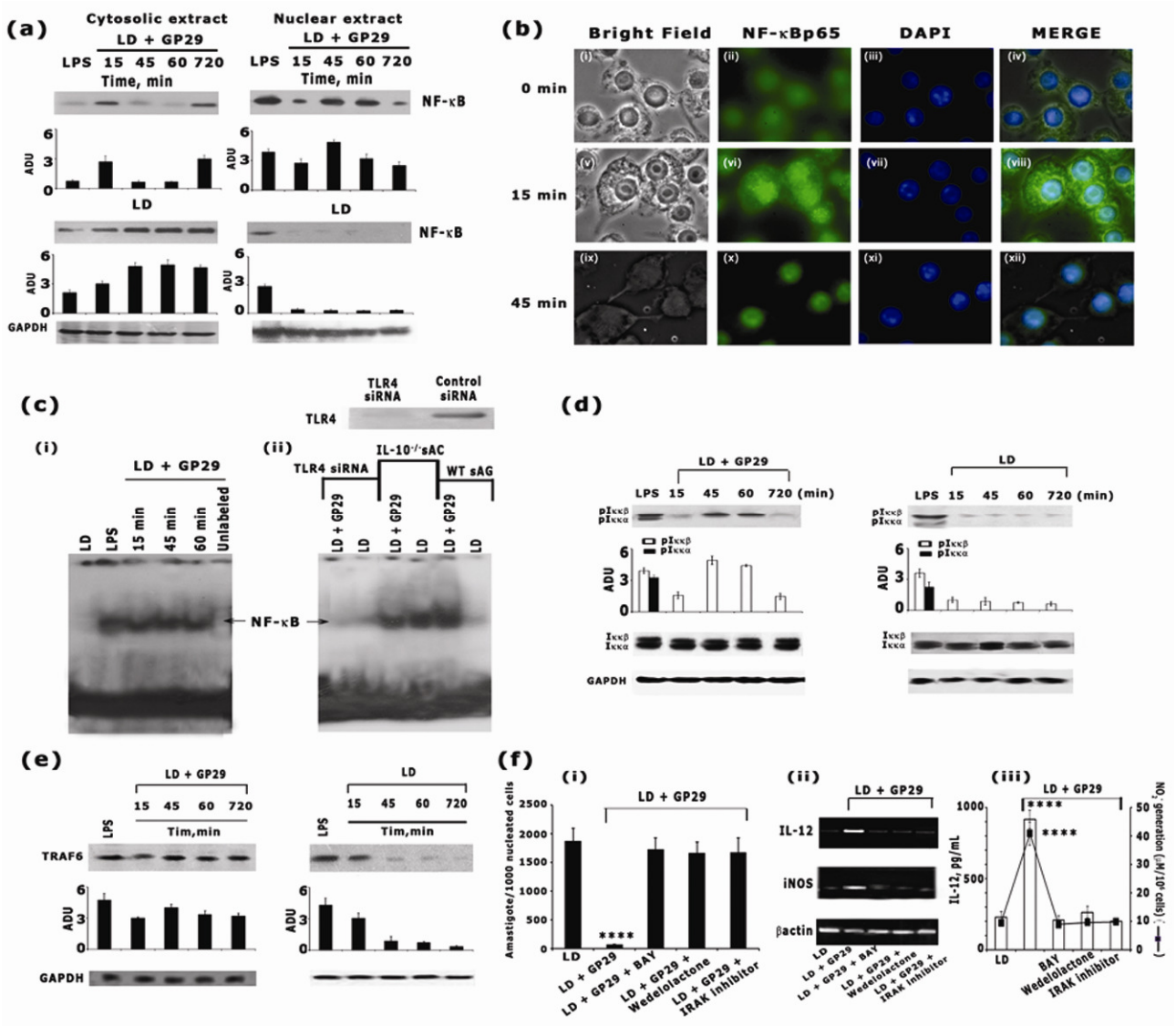

Fig. 2—Effect of GP29 on TLR4-MyD88-NF- B signalling, parasite survival and anti-leishmanial immune response [Splenic adherent cells were infected with *L. donovani* (*LD*) promastigotes (for various times as indicated or for 24 h for GP29 treatment) at a parasite/Mφ ratio of 20:1. 24 h parasitized cells were exposed to GP29 for various times as indicated (a). Cytosolic and nuclear distribution of NF-κB was assessed by immunoblotting. GAPDH was used as the loading control; (b**)** RAW 264.7 monolayers (1×10^5 cells/coverslip) were infected with *LD* promastigotes at a parasite to Mφ ratio of 20:1 and treated with 5 µg/mL GP29 for the indicated times. Cells were fixed with cold-methanol and visualized by fluorescence microscopy. NF- B was stained with anti-p65 mAb and secondary FITC-conjugated Ab (FITC, green) and cell nuclei were stained with DAPI (blue) (orginal magnification X1000). (c) RAW 264.7 cells, TLR4 siRNA transfected RAW cells (TLRr-siRNA RAW), C57BL/6 splenic adherent cells (BL6 sAC), and IL-$10^{-/-}$ sAC were infected with LD promastigotes and then treated with GP29 (for various times as indicated for RAW cells or for 45 min for BL6 sAC, IL-$10^{-/-}$,ˈsAC or TLR4-siRNA cells. Cells were lysed and EMSA of NF-κB was performed using nuclear extracts. Competition experiments were performed using a 200-fold excess of unlabeled NF-κB consensus (*i, lane 6*). RAW cells were infected with *LD* promastigotes and then treated with GP29 for various time periods as indicated. Levels of total and phosphorylated Ikk /β (d) and levels of TRAF6 (e) were measured by immunoblotting. RAW cells were infected with *LD* promastigotes for 24 h followed by treatment with wedelolactone (20 µM) & BAY11-7082 (5 µM) or IRAK 1/4 inhibitor (20 µM) for 1 h prior to treatment with GP29 for 24 h. (f) Intracellular parasite number was determined by Giemsa staining and expressed as amastigotes/1000 nucleated cells (i). IL-12, IL-10 and iNOS mRNA levels were measured by RT-PCR analysis (ii). Production of IL-12, IL-10 in the cell culture supernatant was measured by ELISA and nitrite production was measured by the Griess reagent (iii).

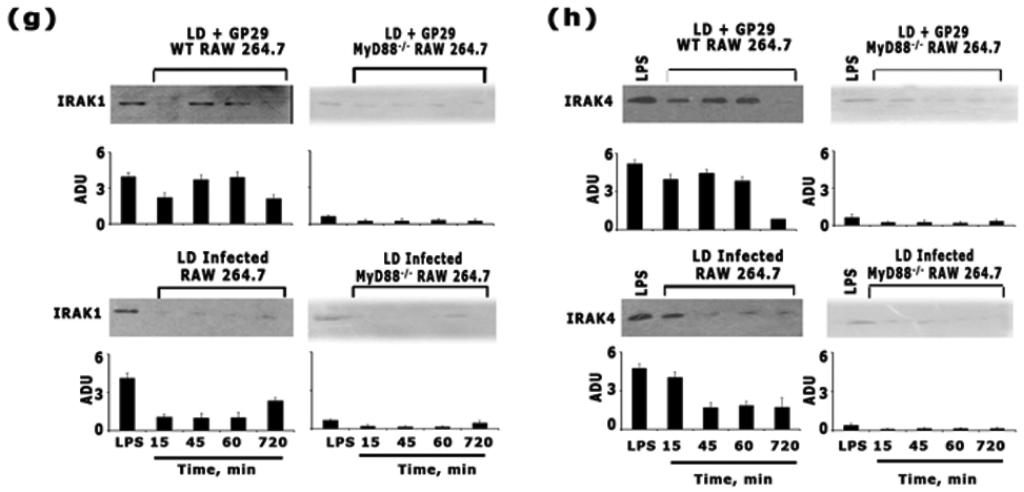

Fig. 2—(g, h) RAW 264.7 macrophages or MyD88 siRNA transfected RAW cells (MyD88$^{-/-}$RAW 264.7) were either infected with *LD* promastigotes for various time periods as indicated or were infected with LD promastigotes for 24 h prior to treatment with GP29 for the indicated time periods and levels of (g) IRAK1 and (h) IRAK4 were measured by immunoblotting. Results were representative of three independent experiments and expressed as mean ± SD. ****p< 0.0001 *versus* corresponding infected control; paired two-tailed Student's t-test. The corresponding band intensities of the immunoblots were quantified by densitometry and are shown as bar graphs below each blot; ADU = arbitrary densitometry unit]

Activation of IKKα/β and TRAF6

As TLR-mediated NF-κB activation is relayed through TRAF6 and a succession of kinases, including IKKα/β, we treated *LD*-infected-RAW cells with GP29 and examined the expressions of IKKα/β and TRAF-6 by Western blot analysis. Results shown in Fig. 2d indicated that there was a time-dependent up-regulation of IKKβ in GP29-treated *LD*-infected RAW cells. Maximal expression was observed at 45 min of GP29 treatment (4.9-fold over corresponding infected cells). TRAF6 expression was also induced by GP29 (Fig. 2e).

To assess whether GP29-induced anti-leishmanial response involved the IKKα/β-NF- B signaling cascade, the effects of specific inhibitors was examined. As shown in Fig. 2f, incubation of infected cells with NF- B (BAY 11-7082, 5 μM) and IKK inhibitor (Wedelolactone), prior to GP29 treatment favored intracellular growth of *LD* parasites (Fig. 2f i) and inhibited the induction of IL-12 and iNOS transcripts in GP29 treated *LD*-RAW cells (Fig. 2f ii). In parallel, GP29 treatment in presence of BAY and Wedelolactone also resulted in a substantial decrease in IL-12 (67.50 ± 6.68% and 72.91 ± 4.537% respectively) and NO (77.75 ± 6.67% and 74.05 ± 3.42%, respectively) production, compared to control treated cells (Fig. 2f iii). These results suggested a probable role of the NF-κB signaling pathway in GP29-mediated anti-leishmanial immune response.

MyD88 mediated activation of IRAK1/4

Initiation of MyD88-mediated TLR signaling results in the recruitment of protein kinases IRAK1 and IRAK4[16]. To assess the molecular mechanism of unresponsiveness to GP29 in MyD88 KD cells, we examined whether or not GP29-mediated signaling cascades were impaired. *LD*-infected control siRNA transfected-RAW cells and MyD88$^{-/-}$-RAW cells were treated with GP29 (5 μg/mL) for the indicated times and IRAK1 and IRAK4 expressions were examined by Western blot analysis. There was a time-dependent increase in IRAK1 and IRAK4 expressions in GP29-treated *LD*-infected control siRNA-RAW cells, but not treated MyD88$^{-/-}$-RAW cells. Maximal expression of IRAK 1 and IRAK4 (4- and 2.7- fold respectively, compared with control siRNA-infected cells) was observed in GP29-treated infected control siRNA-RAW cells at 45 min (Fig. 2g, h). This indicated that MyD88 was a critical molecule for the activation of IRAK in response to GP29. Treatment of *LD*-RAW cells with IRAK1/4 inhibitor prior to GP29 stimulation abrogated the GP29-mediated protective immune response (Fig. 2f i-iii). These findings demonstrated that GP29-mediated protective immune response depended on IRAK1/4 kinase activity that transduces signals from MyD88 to promote parasite killing.

GP29 mediated IL-12 and NO release depends on TLR4 mediated activation of JNK and p^{38} MAPKs

The MAPK signaling pathway is known to regulate a number of cytokine productions[17]. *LD* parasites have been reported to modulate the TLR-stimulated MAPK pathway[6]. GP29-treated *LD*-RAW, but not *LD*-TLR4 siRNA transfected RAW (TLR4 siRNA-RAW) showed a time-dependent up-regulation of phosphorylated p^{38} (p-p^{38}) MAPK and c-Jun NH_2-terminal kinase (pJNK) protein expression, while phosphorylated extra-cellular signal-regulated kinases (pERK) expression was associated with *LD*-RAW (Fig. 3a-c). Lipopolysaccharide (LPS) (10 ng/mL, 15 min) treated RAW264.7 cells served as controls. In GP29-treated *LD*-RAW cells, p^{38} MAP kinase phosphorylation peaked at 45 min and remained elevated up to 1 h, but returned to baseline within 12 h (Fig. 3a). High SAPK/JNK phosphorylation was sustained for up to 12 h (Fig. 3b). In the

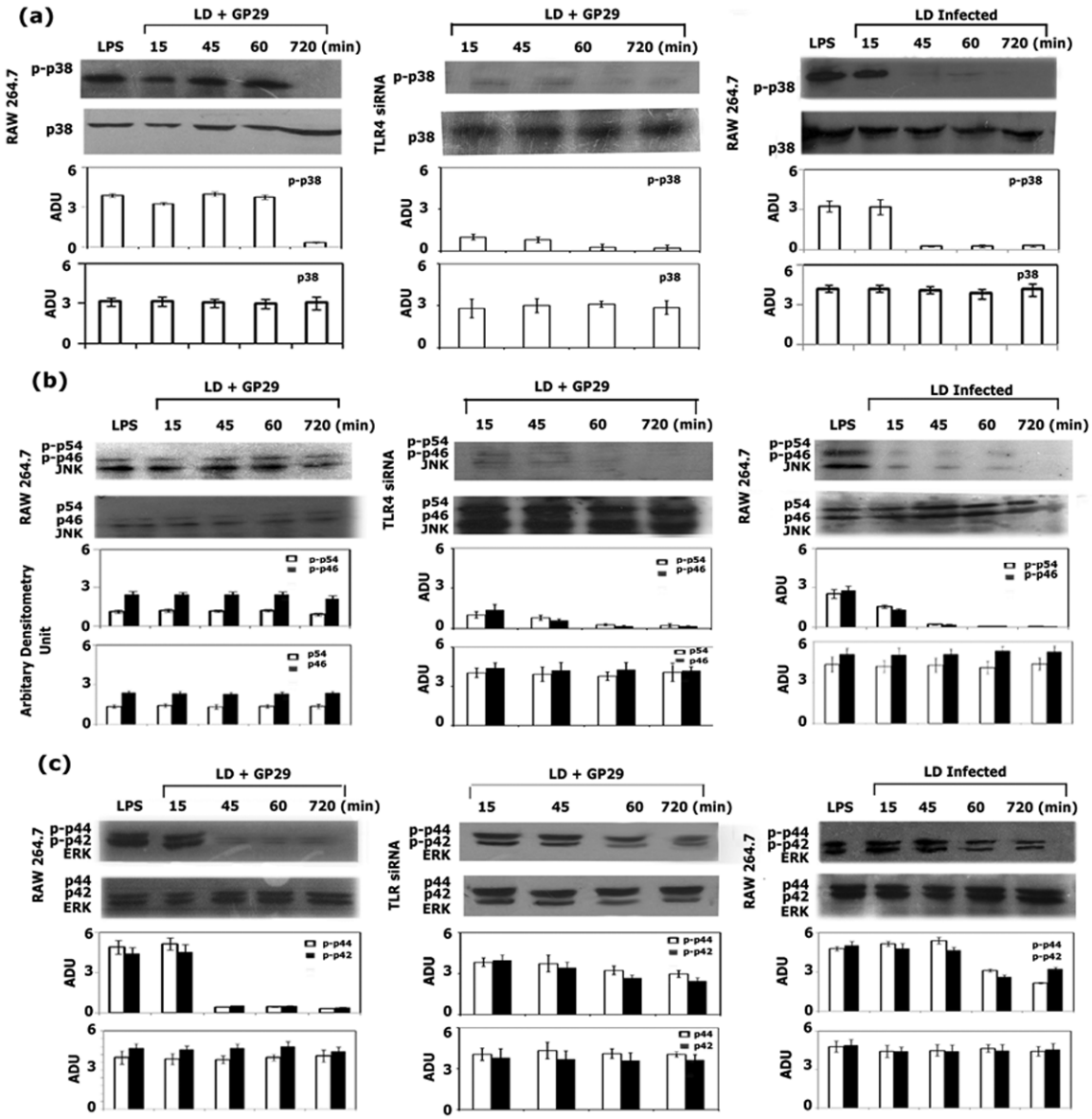

Fig. 3—Effect of GP29 on TLR4-MAPK signaling, parasite survival and anti-leishmanial effector responses [RAW 264.7 macrophages were infected with *LD* promastigotes for various time periods as indicated. In parallel sets, RAW 264.7 or TLR4 siRNA transfected RAW cells were infected with *LD* promastigotes for 24 h prior to treatment with GP29 for the indicated time periods. Cells treated with LPS (10 ng/mL) for 15 min were used as positive control. Levels of phosphorylated and total (a) p^{38}, (b) SAPK/JNK and (c) ERK were measured by immunoblotting. RAW 264.7 macrophages were infected with *LD* promastigotes for 24 h followed by treatment with inhibitors of p^{38} (SB202190), JNK (SP600125) and ERK (U0126) for 1 h followed by the treatment with GP29 (5 µg/mL) for 24 h.

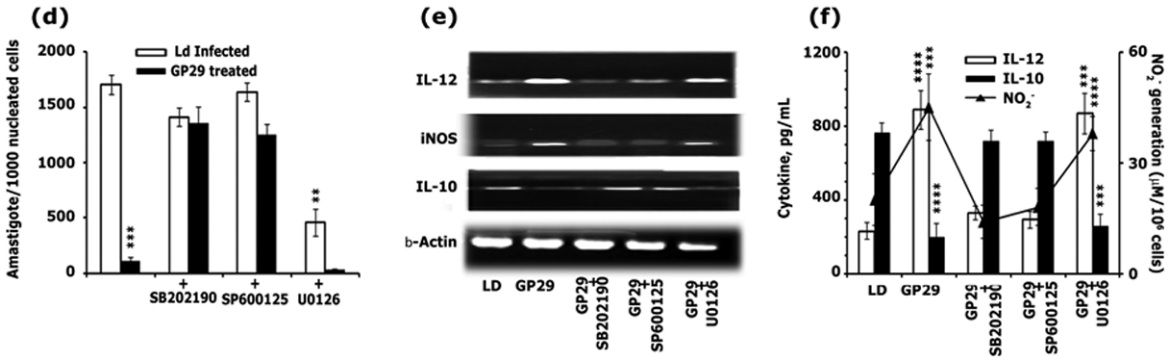

Fig. 3—(d) Intracellular parasite number was determined by Giemsa staining and expressed as amastigotes/1000 nucleated cells. (e) IL-12, IL-10 and iNOS mRNA levels were measured by RT-PCR analysis. (f) Production of IL-12, IL-10 in the cell culture supernatant was measured by ELISA and nitrite production was measured by the Griess reagent. Results were representative of three independent experiments and expressed as mean ± SD. ****p< 0.0001; ***p<0.001;**p<0.01 *versus* corresponding infected control; paired two-tailed Student's t-test. The corresponding band intensities of immunoblots were quantified by densitometry and are shown as bar graphs below each blot; ADU = arbitrary densitometry unit]

infected-RAW cells, there was a transient p^{38} and SAPK/JNK activation that peaked at 15 min but came back to baseline within 45 min (Fig. 3a, b).

In contrast, sustained p^{42}/p^{44} ERK activity (phosphorylated up to 12 h) became inactivated (presumably by de-phosphylation) within 45 min on GP29 treatment (Fig. 3c). MAPK phosphorylation kinetics in GP29-treated *LD*-TLR4 siRNA-RAW was similar to the MAPK phosphorylation kinetics of control-infected cells (Fig 3a-c). This indicated that in absence of TLR4 signaling, GP29 failed to induce the p^{38} and SAPK/JNK pathway.

Pre-incubation of infected RAW-cells with inhibitors of p-p^{38} (SB202190) and pJNK (SP600125), but not of pERK (U0126) prior to GP29 treatment selectively impaired GP29-mediated effector responses. p^{38} MAPK or JNK inhibition induced an increase in intracellular parasite number (90.31 ±1.77% and 89.89 ± 1.25%, respectively) in GP29-treated parasitized RAW cells (Fig. 3d). In contrast, pre-incubation of cells with ERK inhibitor prior to GP29 treatment resulted in a slight decrease (27.99 ± 10.79%) in intracellular parasite number.

SB202190 and SP600125 treatment of infected macrophages reduced GP29-induced generation of IL-12 and NO, whilst enhancing IL-10 production both at the mRNA (Fig. 3e) and protein (Fig. 3f) level, resulting in drastic increase in intracellular parasite load (Fig. 3d). Pre-incubation of cells with ERK inhibitor prior to GP29 treatment did not affect IL-12 or NO production. On the other hand, though p^{38} MAPK and JNK inactivation correlated with increased IL-10 production in GP29-treated infected RAW cells, ERK inhibition decreased (66.42 ± 6.24%) IL-10 production in infected cells (Fig. 3f). Consistent with a reduction in IL-10 production, intracellular parasite number was reduced (93.48 ± 1.50%) in infected cells (Fig. 3d) in presence of ERK inhibitor. This suggested that ERK activation augmented IL-10 production. In contrast, treatment of *LD*-infected cells with SB202190 and SP600125 post-GP29 treatment did not affect GP29-mediated effector responses (data not shown).

Discussion

TLR ligand recognition and binding leading to signaling responses can be programmed to drive specific adaptive immune responses. We have earlier identified a *Leishmania*-derived TLR4 agonist GP29[5] that induces a TLR4-mediated pro-inflammatory response. In the present study, we identified the cellular mechanisms that regulate the inflammatory response after GP29-mediated TLR4 stimulation. We showed that GP29 induced signaling through a MyD88-dependant pathway, resulting in host-protective responses.

Leishmania parasites have evolved elegant strategies to abate the host innate immune machinery and create a safe environment to survive within the host cells. Numerous escape mechanisms are employed by the parasite. To survive within the hostile environment of their host cells, *Leishmania* parasites suppress macrophage microbicidal activities and prevent activation of an effective immune response. In order to do this, it has evolved strategies to alter host inflamatory cytokine response and host cell signaling cascade[18]. Engagement of an innate receptor like the TLRs expressed primarily by cells of the innate immune compartment by pathogen-specific ligands results in the

production of cytokines typically via host cell-signaling cascades. Suppression or activation of pro-inflamatory cytokine production by *Leishmania* parasites have been linked to the down-regulation of the innate MAPK- NF-κB signaling pathway[19-22]. Study of the *Leishmania*-TLR interaction as an experimental inflammatory regulatory system possibly open up new avenues for therapeutic intervention of this dreadful disease.

Macrophage response to parasitic infection is regulated through a delicate balance between phosphatases and their kinase counterparts. It is reported that MAPK-mediated LPS-induced iNOS expression[23,24] is counterbalanced by MAPK-phosphatase-1 (MPK-1)[25]. MPK-1 skews arginine metabolism from NO production to L-ornithine production. L-Arginine is metabolized to NO by iNOS or to urea and L-ornithine by arginase. We have recently shown that the TLR4 agonist GP29 down-regulates arginase expression in *LD*-infected mice[5]. Based on this observation, we have hypothesized that GP29 may activate the MAPK signaling cascade through TLR4. In support of our hypothesis, we have demonstrated that TLR4-MyD88-mediated signaling via the IRAK-1-TRAF6 pathway leads to GP29-mediated p^{38} MAPK, JNK and NF-κB activation.

The TLR4 ligand GP29 activates the TLR signaling pathway. TLR4 activation is associated with increased IL-12/iNOS induction, NF-κB transactivation and reduced IL-10 expression. Use of $TLR4^{-/-}$ RAW cells confirmed the importance of GP29-TLR4 interaction in GP29-mediated immune response. We and others have demonstrated the importance of TLR4 for efficient parasite control[5,26]. TLR4-mediated activation of iNOS leads to NO formation and parasite killing. In absence of TLR4, arginase-mediated urea formation increased while NO formation was decreased[27].

Leishmania parasites alter macrophage signaling mechanisms to their own advantage[28]. An important macrophage effector mechanism for host defense is the phosphorylation of specific proteins and *Leishmania* parasites are able to thwart this mechanism before it is activated. *LD* parasites impair macrophage MAP kinase pathway to survive within the host cells[29]. Though there are contradictory results regarding the role of MAP kinase ERK in *Leishmania* infection, the activation of p^{38} MAPK is important in controlling intracellular parasites[30]. In VL, IL-10 and IL-12 are the main regulatory cytokines. Activation of MAPKs, including ERK, JNK and p^{38} MAPK involves differential regulation of IL-12 and IL-10. Consistent with these findings, we observed that GP29-mediated increased IL-12 production was paralleled with increased p^{38} MAPK and JNK activation and decreased IL-10 production was associated with deactivated ERK expression.

The TLR4 signaling consists of a MyD88-dependent and a MyD88-independent TRIF-dependent pathway. Importance of MyD88 in fighting *Leishmania* infection has been reported[31]. Use of MyD88 gene silenced RAW cells and TRIF inhibitory peptide suggested that GP29 activated the MyD88-dependent pathway. The TIR domain containing molecule TIRAP is specifically involved in the MyD88 pathway. Use of TIRAP gene silenced RAW cells confirmed the involvement of TIRAP in the GP29-mediated anti-leishmanial effector mechanism. Activated MyD88 recruits IRAK and TRAF6 and induces MAPK and NF-κB activation[32]. Induction of GP29-mediated TLR4 signaling resulted in IRAK1/4, TRAF6 and NF-κB activation. Failure of IRAK1/4 activation in MyD88 gene silenced cells further confirmed the involvement of MyD88-dependent pathway in GP29-mediated protective immune responses.

MAPK activation leads to the activation of the transcription factor NF-κB, resulting in the production of pro-inflammatory cytokines, such as IL-12. Previous studies have indicated that NF-κB plays an important role in immunity to Leishmaniasis[19,33,34]. NF-κB is maintained in the cytoplasm in an inactivated form associated with IκB. Ikk-mediated degradation of IκB leads to the activation and nuclear translocation of NF-κB[35]. Ikkβ-mediated NF-κB activation indicated the likely involvement of the canonical pathway of NF-κB activation by GP29. Incubation of infected TLR4 gene silenced RAW cells with GP29 did not activate p^{38} MAPK and JNK, thus indicating the requirement of TLR4 for GP29-mediated activation of MAPK pathway. Specific inhibitors of TLR4-MAPK-NF-κB signaling pathway reversed the protective effect of GP29. Together these results indicated that GP29 triggered TLR4 signaling induced p^{38} MAP kinase and JNK phosphorylation, leading to NF-κB induced type 1 cytokine production.

In conclusion, the present study demonstrated that GP29-mediated TLR4 activation resulted in the production of NO and IL-12 through the activation of MyD88 signaling events that culminate the efficient clearance of intracellular parasites.

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