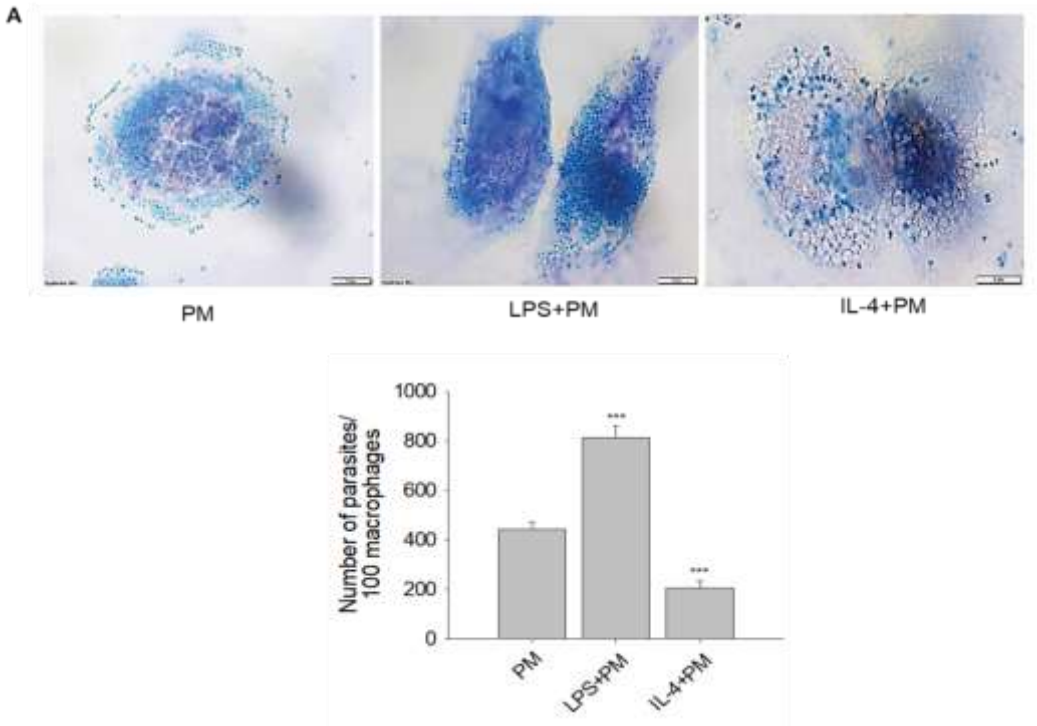
CHAPTER- IV

Investigating the mechanism of *L. donovani* promastigotes internalization in macrophages

4.1 Results

4.1.1. *L. donovani* infection is associated with macrophage inflammation status:

To investigate the role of macrophage TLR4 on *L. donovani* entry inside the macrophages, we treated THP-1 macrophage cell line or human peripheral blood mononuclear cells (PBMC) with LPS (100 ng/ml) or IL-4 (10 ng/ml), as LPS and IL-4 are known to induce or repress [1,2] the TLR4 expression and activation, respectively, followed by the exposure of *L. donovani* for 4 h. On termination of incubations, cells were thoroughly washed to remove the free parasites, and then counted the parasite entry inside the macrophages under the microscope. LPS-stimulated macrophages exhibited profound parasite burden which was considerably reduced in IL-4 treated macrophages without any significant alteration of cell viability **(Fig. 4.1 A,B).** We also determined the infection rate in human PBMC by incubating the LPS or IL-4-treated cells with FITC-labeled *L. donovani* followed by the flow cytometric analysis. These results indicate that the macrophage TLR4 and its inflammatory condition associated with the promastigote burden in macrophages **(Fig.4.1C)**.



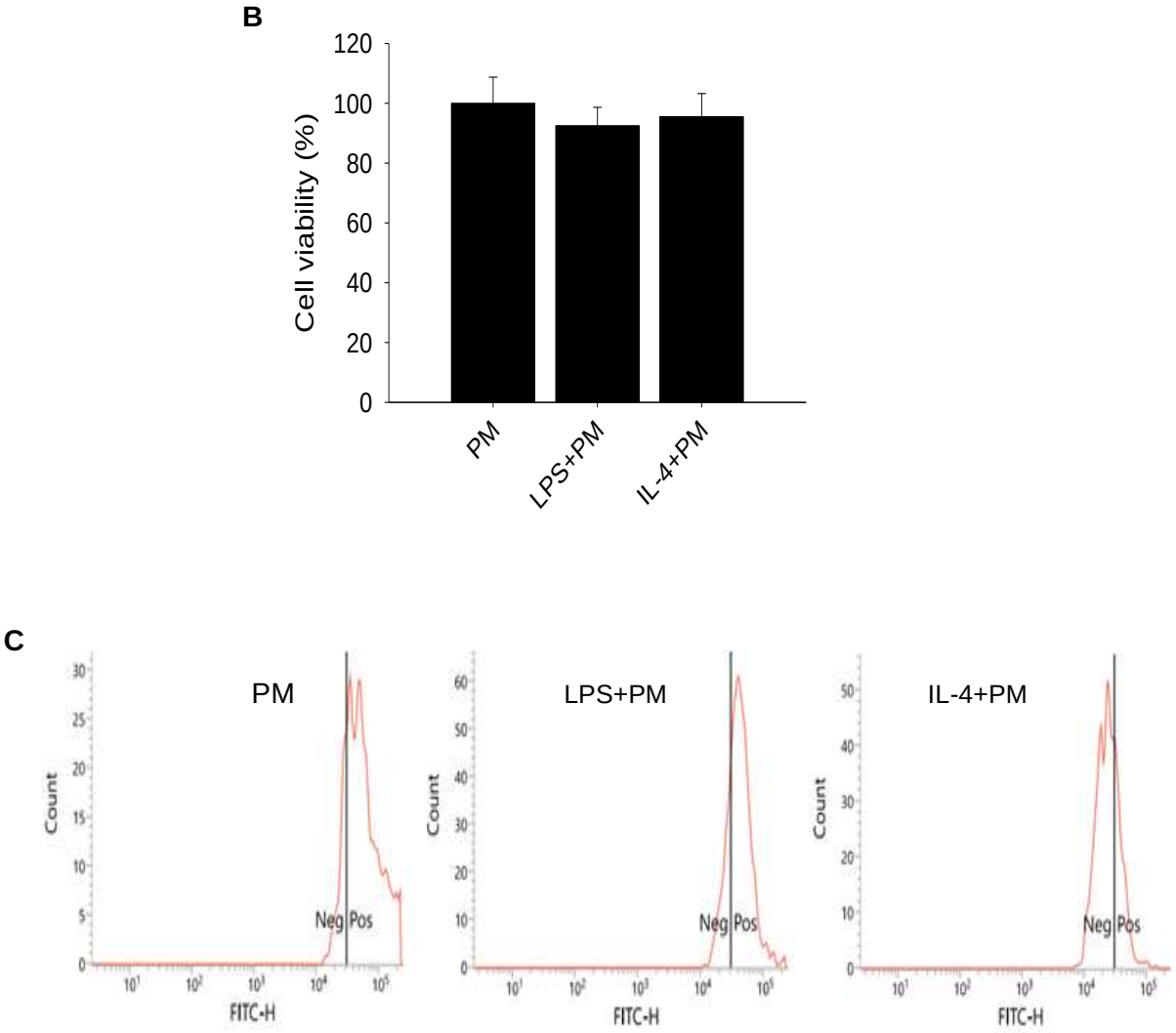
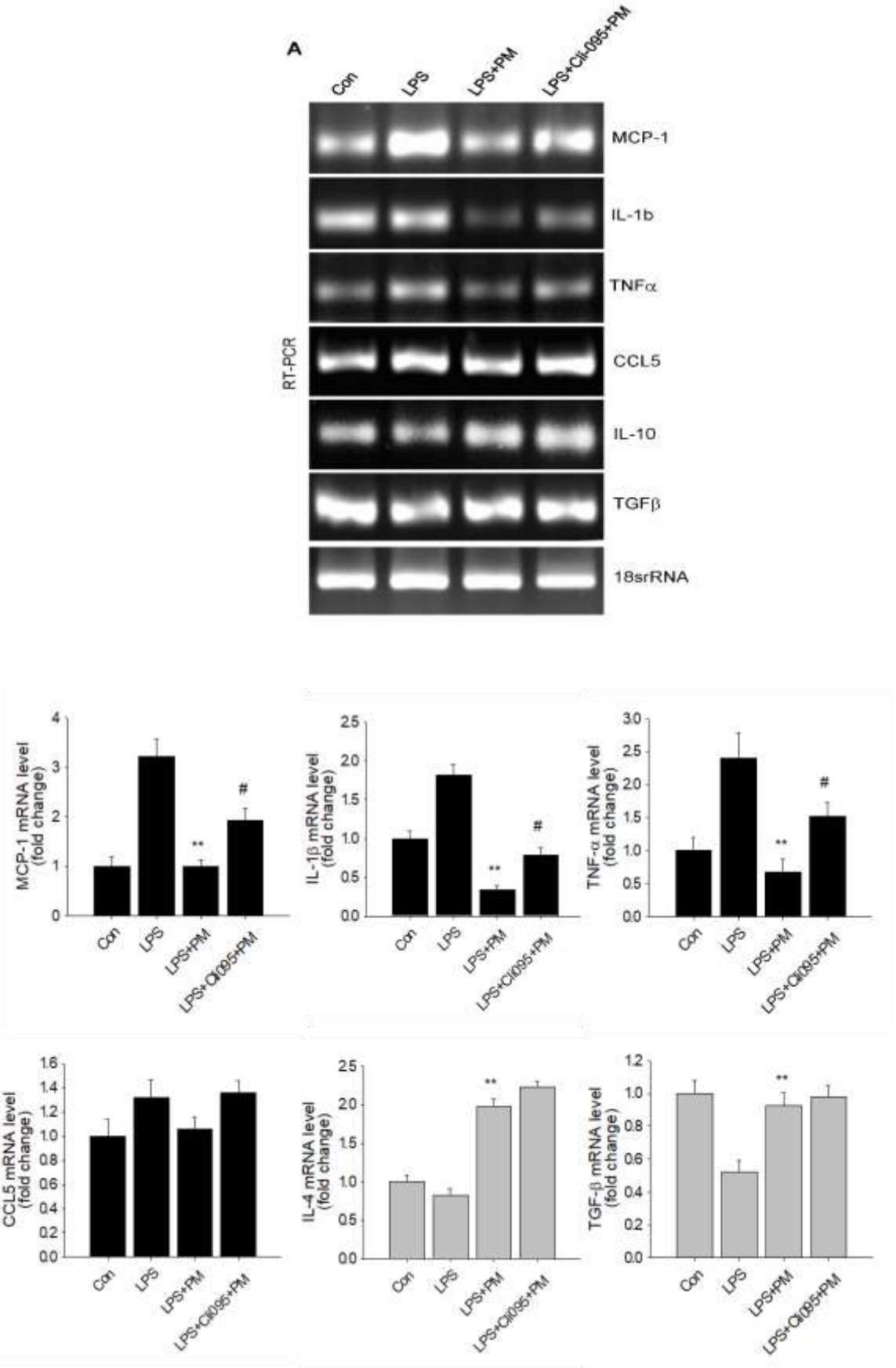


Fig 4.1: Inflammatory status of macrophages regulates *L. donovani* burden.

(A) Giemsa staining images of parasites numbers (Up) and their quantification (Down) in THP-1 macrophages treated without or with LPS (100 ng/ml) or IL-4 (10 ng/ml) for 4 h and infected with *L. donovani* promastigote infection at 1:10 ratio (macrophage to parasite) for 4 h. (B) Determination of THP-1 cell viability (%) in response to indicated treatment conditions. (C) Human PBMC-derived macrophages treated without or with LPS or IL-4 were infected with FITC-labelled parasites at 1:10 ratio (macrophage to parasite) for 4 h. After infection, cells were washed and incubated with 0.02% trypan blue solution for 5 min to quench fluorescence associated with membrane bound uninternalized promastigotes. Cells were then washed, analyzed in flow cytometry. All experiments were performed in triplicate. Each value represents as mean ± SEM, ***$p < 0.001$ vs PM.

4.1.2. *L. donovani* infection downregulates the LPS-induced inflammation in macrophages:

Since we noticed a considerable increase in parasite internalization in LPS treated macrophages, we therefore intended to explore the effect of *L. donovani* infection on various inflammatory cytokines gene expression. We observed a notable suppression of LPS-induced MCP-1, IL-1β, TNF-α, and CCL5 pro-inflammatory cytokines gene expression along with the upregulation of IL-10 and TGF-β anti-inflammatory cytokines gene expression in response to parasite infection **(Fig. 4.2A)**. This result suggests that LPS-induced TLR4 activation effectively promotes the expression of inflammatory molecules but were unable to demonstrate the microbicidal mechanisms in levels to kill the parasites. Moreover, parasite infection in LPS-treated macrophages demonstrated a notable suppression of proinflammatory cytokines expression. Next, we examined the macrophage cell surface TLR4 level in response to parasite infection. Interestingly, *L. donovani* incubation in THP-1 macrophages significantly increased TLR4 abundance in macrophage cell membrane **(Fig. 4.2B)**. Moreover, incubation of parasites with THP-1 macrophages transfected with κB-luc, AP1-luc, and IRF3-luc plasmids notably inhibits κB-luciferase activity without any significant alteration of AP-1- and IRF3- luciferase activities **(Fig. 4.2C)**. In addition, incubation of TLR4 signaling inhibitor Cli-095 or NF-κB inhibitor parthenolide in mouse PBMC-derived macrophages followed by the exposure of *L. donovani* promastigotes considerably reduced parasite numbers in macrophages **(Fig. 4.2D)**. All these observations indicate that parasite incubation significantly upregulates the abundance of surface TLR4 levels and pro-inflammatory state in macrophages favoring increased numbers of parasites in macrophages.


A
Con
LPS
LPS+PM
LPS+Cli-095+PM
MCP-1
IL-1b
TNFα
CCL5
IL-10
TGFβ
18srRNA
RT-PCR
MCP-1 mRNA level (fold change)
IL-1β mRNA level (fold change)
TNF-α mRNA level (fold change)
CCL5 mRNA level (fold change)
IL-4 mRNA level (fold change)
TGF-β mRNA level (fold change)
Con
LPS
LPS+PM
LPS+Cli095+PM

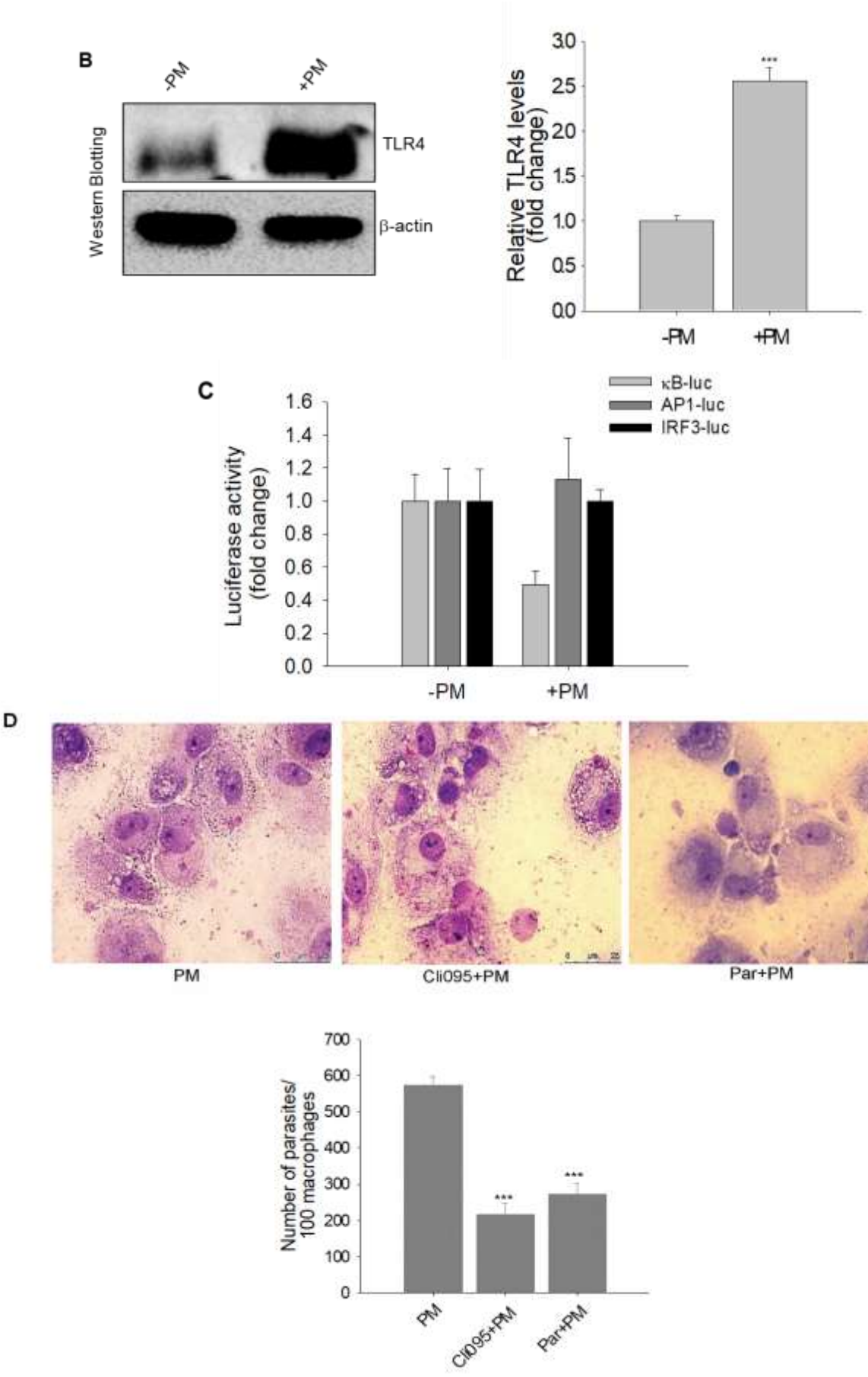
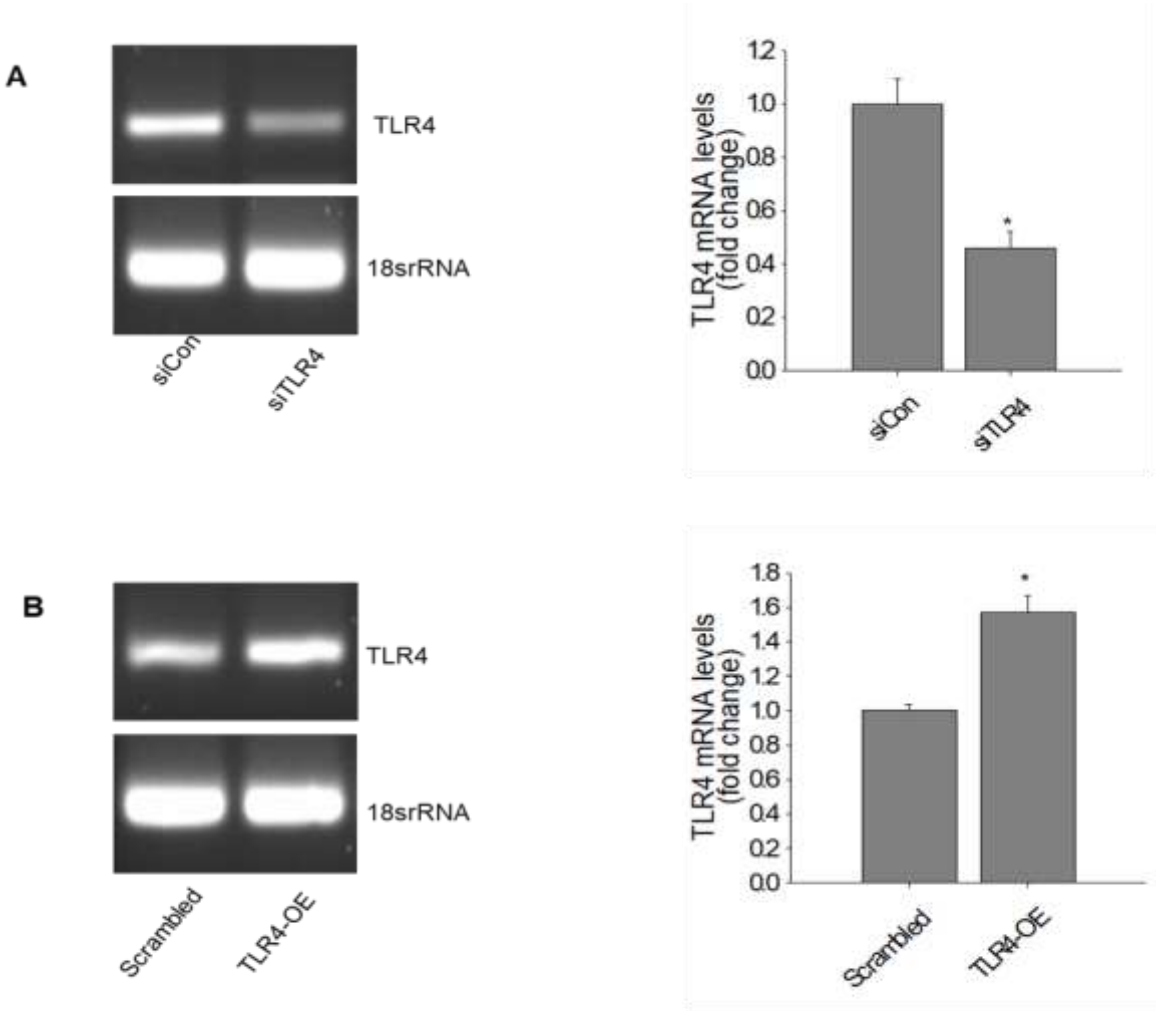

B
-PM
+PM
Western Blotting
TLR4
β-actin
Relative TLR4 levels (fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
-PM
+PM
C
κB-luc
AP1-luc
IRF3-luc
Luciferase activity (fold change)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-PM
+PM
D
PM
Cli095+PM
Par+PM
Number of parasites/ 100 macrophages
700
600
500
400
300
200
100
0
***
***
PM
Cli095+PM
Par+PM

Fig 4.2: *L. donovani* infection downregulates LPS-induced inflammation in macrophages. (A) RT-PCR images (Up) and their quantifications (Down) showing proinflammatory markers (MCP-1, IL-1β, TNF-α, CCL5) and anti-inflammatory markers (IL-10, TGF-β) gene expression in LPS-stimulated THP-1 macrophages in absence or presence of Cli-095 followed by the infection without or with *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h. Each value is the mean ± SEM of three independent experiments, **p < 0.01 vs LPS; #p < 0.05 vs LPS+PM. (B) Western blot image (left) and its quantification (right) showing abundance of TLR4 in the plasma membrane fraction of THP-1 macrophages infected without or with *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h. Each value is the mean ± SEM of three independent experiments, ***p < 0.001 vs –PM. (C) THP-1 macrophages transfected with κB promoter-luciferase or AP1 promoter-luciferase or IRF3 promoter-luciferase plasmids were incubated without or with *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h. On termination of incubations, cells were washed, lysed and luciferase activity was measured by multimode reader. Each value is the mean ± SEM of three independent experiments, *p < 0.05 vs –PM. (D) Giemsa staining images (Up) and their quantifications (Down) of mouse PBMC-derived macrophages treated without or with Cli-095 (3 μM) or Parthenolide (Par, 10 μM) for 1 h followed by the infection of *L. donvani* promastigotes (PM) at 1:10 (macrophage to parasite) for 4 h. Each value is the mean ± SEM of three independent experiments, ***p < 0.001 vs PM.

4.1.3. *L. donovani* LPG and macrophage TLR4 favor parasite entry:

To study the role of macrophage TLR4 on the recognition of *L. donovani* and its accumulation inside the macrophages necessary for establishing the infection, we silenced or forcedly expressed TLR4 **(Fig. 4.3 A, B**) in THP-1 macrophages and human PBMC-derived macrophages and then analyzed the rate of infection upon 4 h exposure to *L. donovani* promastigotes, *in-vitro*. Intriguingly, while TLR4 knockdown significantly reduced the parasite numbers in macrophages, the overexpression of TLR4 considerably increased the parasite numbers in macrophages (**Fig. 4.3 C, D**). These results suggest that TLR4 crucially involved in parasite recognition and entry in macrophages.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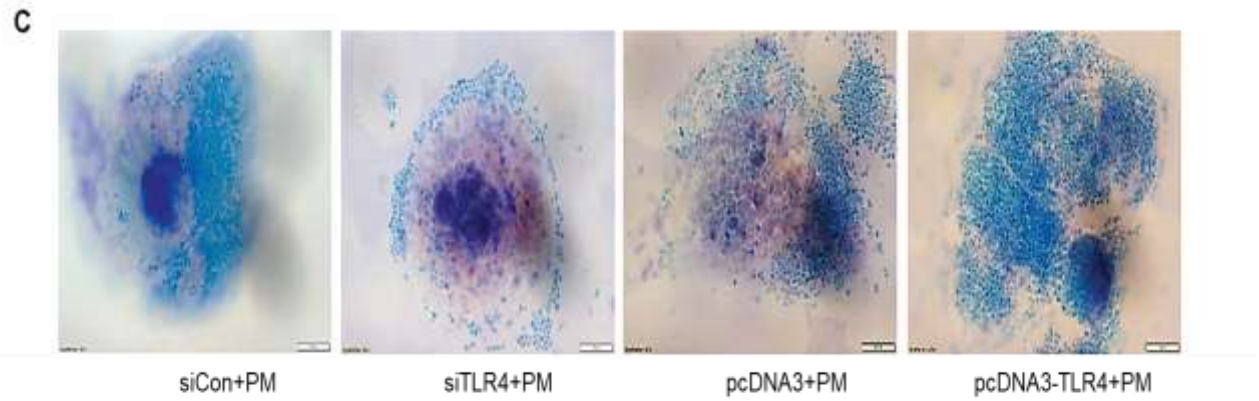
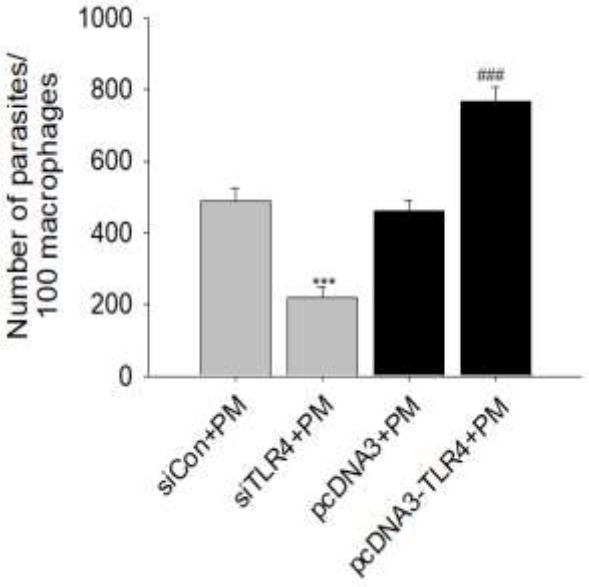
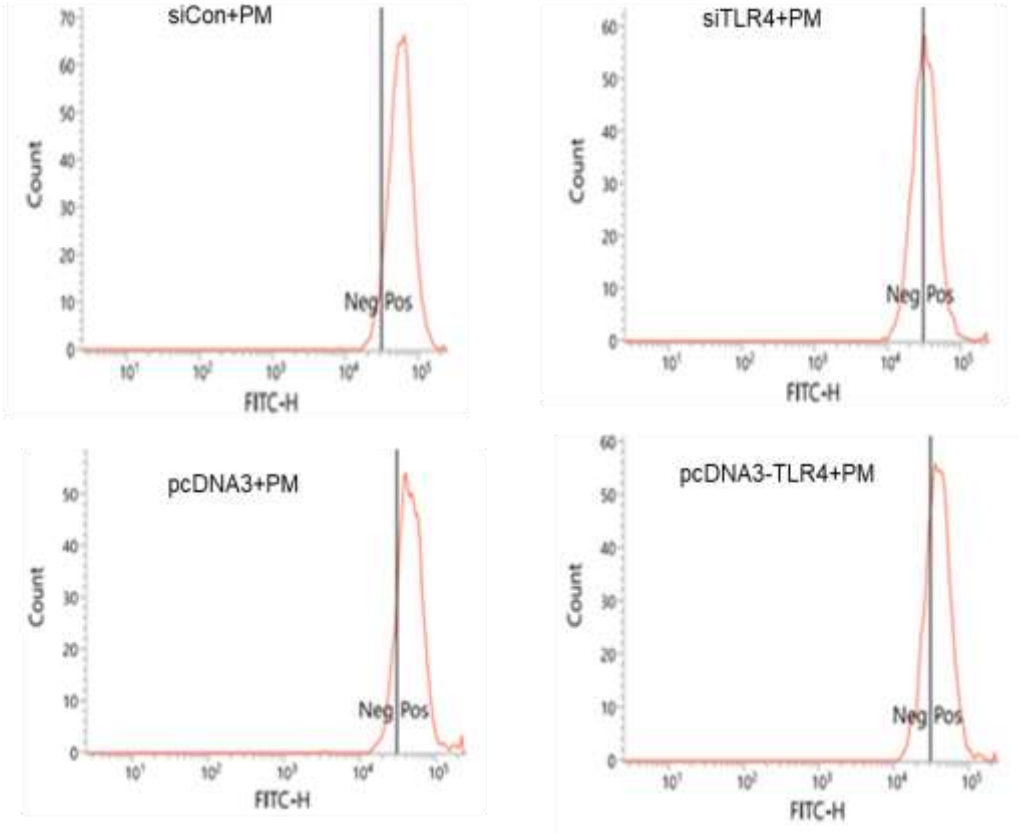
TLR4
18srRNA
siCon
siTLR4
TLR4 mRNA levels (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
siCon
siTLR4
B
TLR4
18srRNA
Scrambled
TLR4-OE
TLR4 mRNA levels (fold change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Scrambled
TLR4-OE


C
siCon+PM
siTLR4+PM
pcDNA3+PM
pcDNA3-TLR4+PM


Number of parasites/ 100 macrophages
1000
800
600
400
200
0
***
###
siCon+PM
siTLR4+PM
pcDNA3+PM
pcDNA3-TLR4+PM

D


siCon+PM
siTLR4+PM
pcDNA3+PM
pcDNA3-TLR4+PM
Count
FITC-H
Neg
Pos

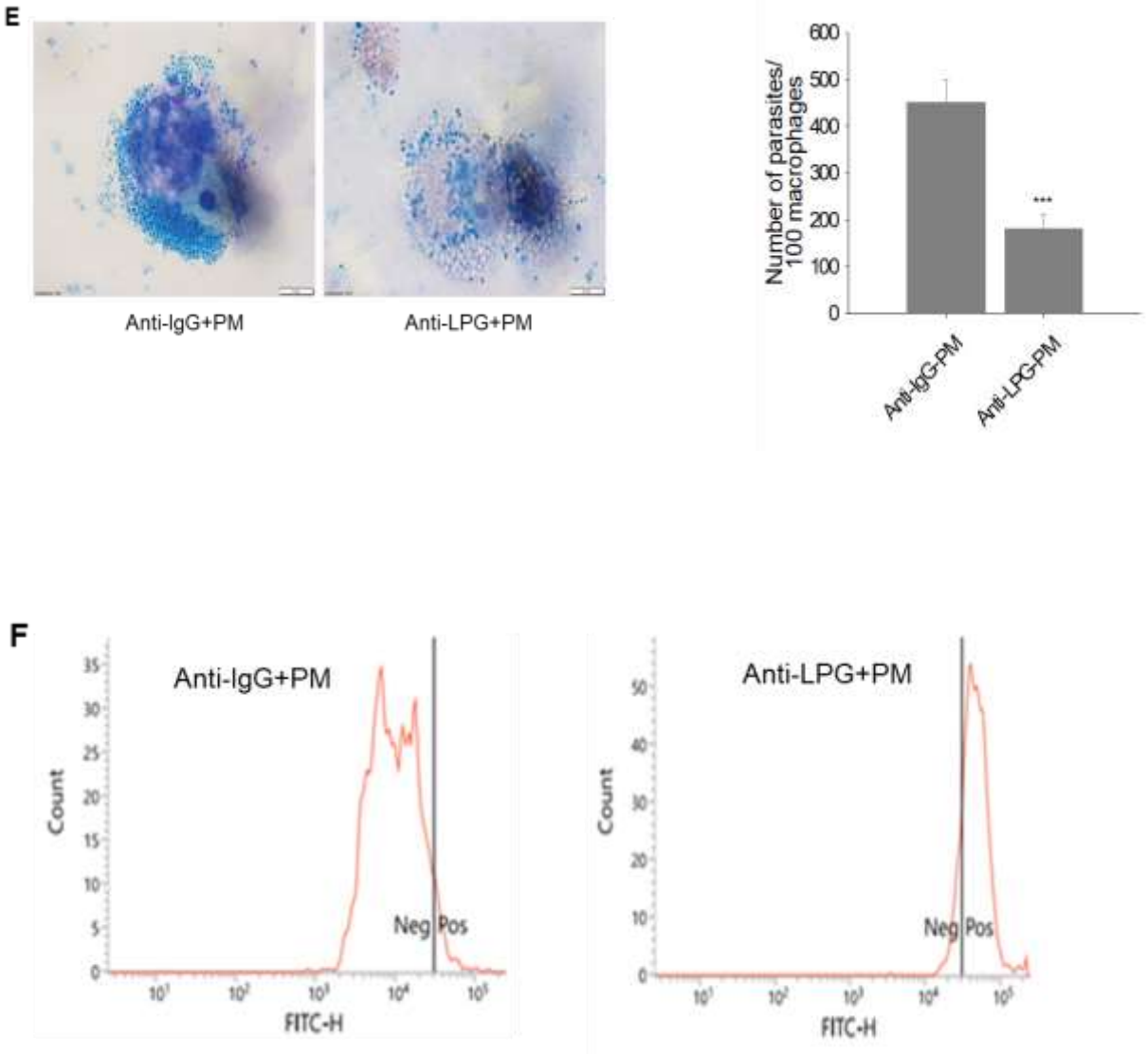


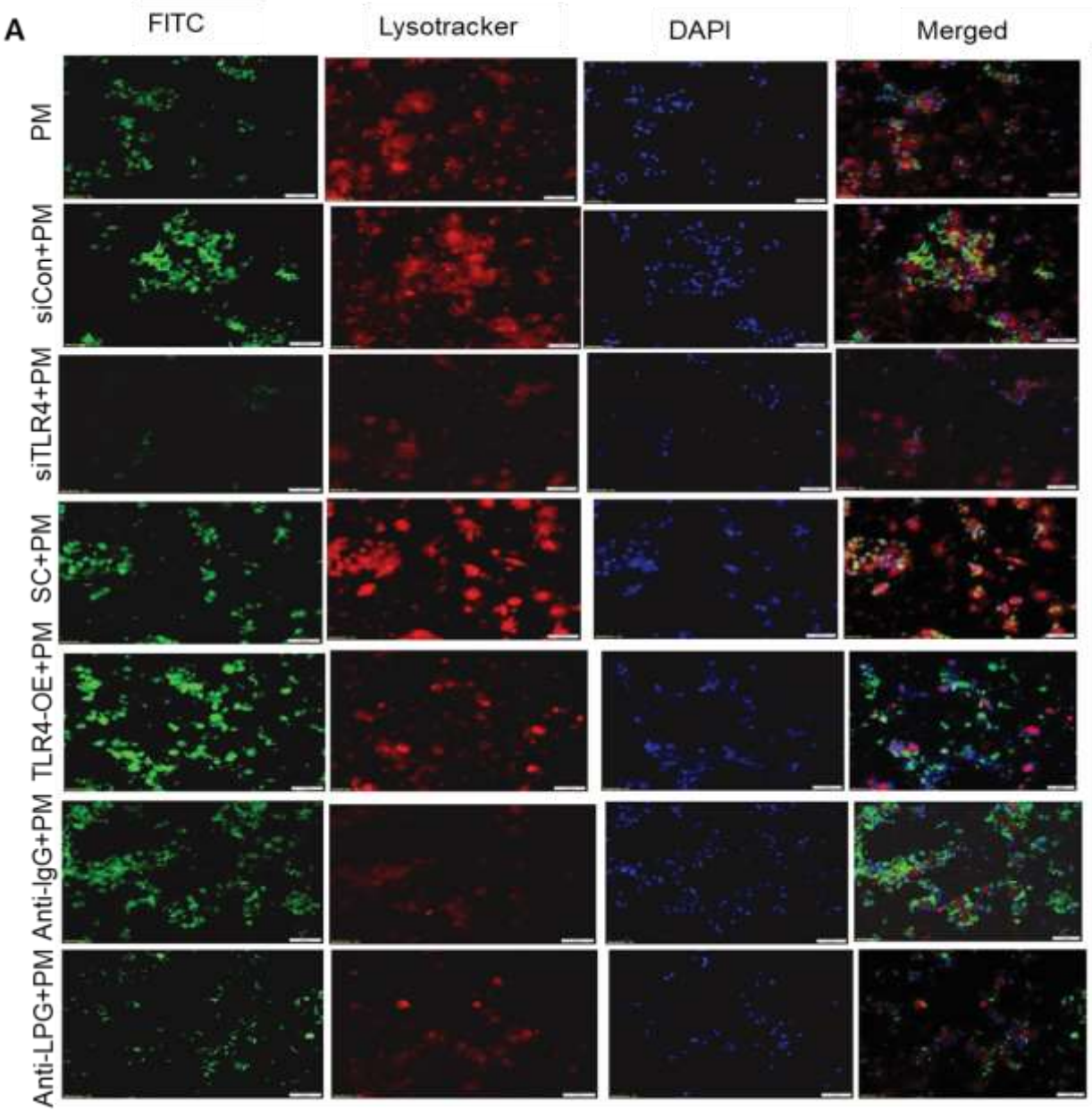
Fig 4.3: Macrophage TLR4 and promastigote LPG governs intracellular *L. donovani* burden. (A, B) RT-PCR analysis (left) and their quantification (right) showing TLR4 mRNA level in control siRNA and TLR4 siRNA transfected THP-1 macrophages (A) and scrambled plasmid and TLR4 overexpression plasmid transfected THP-1 macrophages (B). 18srRNA was served as loading controls. Each value is the mean ± SEM of three independent experiments, *p < 0.05 vs siCon or scrambled plasmid. (C) THP-1 macrophages transfected with control siRNA (siCon)/ TLR4 siRNA (siTLR4) or control plasmid (pcDNA3)/ TLR4 overexpression plasmid (pcDNA3-TLR4) were infected without or with *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h. Subsequent to infection, Giemsa staining images (Up) and their quantification (Down) showing parasite burden in THP-1 macrophages. Each value is the mean ± SEM of three independent experiments, ***p < 0.001 vs siCon; ###p < 0.001 vs pcDNA3. (D) Human PBMC-derived macrophages transfected with above mentioned siRNAs or plasmids were incubated with

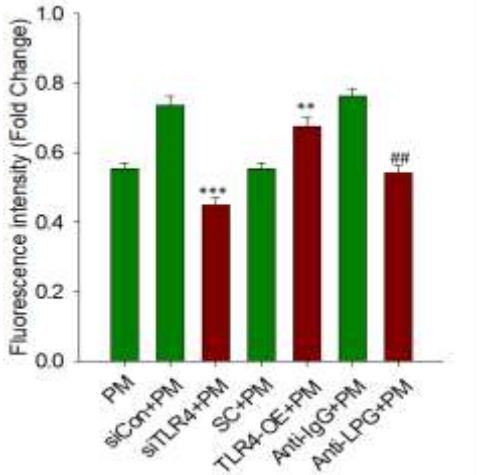
FITC-labeled *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h followed by the flow cytometric analysis. (E) *L. donovani* promastigotes incubated with anti-IgG or anti-LPG antibodies were infected with THP-1 macrophages at 1:10 ratio (macrophage to parasite) for 4 h. Subsequent to infection, Giemsa staining images (left) and their quantification (right) showing parasite load in THP-1 macrophages. Each value is the mean ± SEM of three independent experiments, ***$p < 0.001$ vs anti-IgG. (F) Human PBMC-derived macrophages infected with FITC-labeled and anti-IgG or anti-LPG antibodies treated *L. donovani* promastigotes at 1:10 ratio (macrophage to parasite) for 4 h. Subsequent to infection, flow cytometric analysis exhibited parasite numbers in macrophages.

However, to investigate the molecular signature of *L. donovani* that facilitates its interaction and accumulation inside the macrophages, we examined the role of *L. donovani* surface molecules Lipophosphoglycan (LPG) as it has been reported to play a critical role in establishing parasite infection [2,5]. For this, we incubated *L. donovani* promastigotes with anti-LPG antibodies and then exposed them to THP-1 macrophages and human PBMC. It could be seen from the microscopic imaging **(Fig. 4.3E)**, a marked reduction of parasite numbers in macrophages when promastigotes were pre-treated with anti-LPG antibodies. It could also be evident from the flow cytometric analysis of THP-1 macrophages infected with FITC-labelled promastigotes **(Fig. 4.3F)**. Together, these results suggest that silencing of macrophage TLR4 or masking of promastigote LPG notably reduced parasite burden, whereas, the TLR4 overexpression considerably increased promastigote load in macrophages.

4.1.4. Involvement of *L. donovani* LPG and macrophage TLR4 on infection-induced macrophage dysfunction:

To examine the involvement of *L. donovani* LPG and macrophage TLR4 on infection-induced macrophage dysfunction, it was observed that silencing of TLR4 in macrophages or masking of promastigote LPG notably reduced parasite infection, whereas, TLR4 overexpression considerably increased parasite numbers in macrophages **(Fig. 4.4A)**. This result confirms the importance of macrophage TLR4 and promastigote LPG in parasite entry into the macrophages.


A
FITC
Lysotracker
DAPI
Merged
PM
siCon+PM
siTLR4+PM
SC+PM
TLR4-OE+PM
Anti-IgG+PM
Anti-LPG+PM


Fluorescence intensity (Fold Change)
1.0
0.8
0.6
0.4
0.2
0.0
***
**
##
PM
siCon+PM
siTLR4+PM
SC+PM
TLR4-OE+PM
Anti-IgG+PM
Anti-LPG+PM

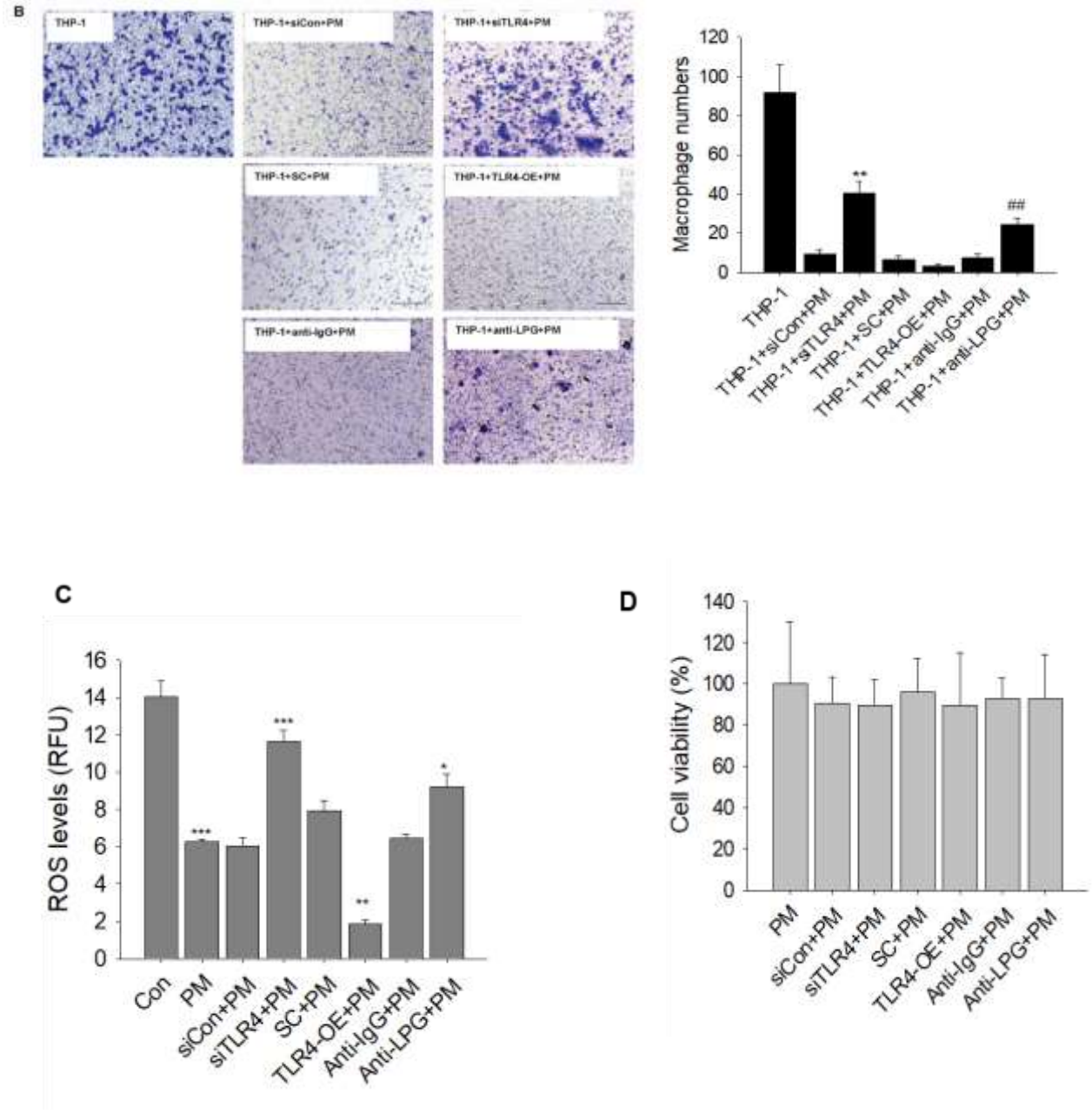


Figure 4.4: ***L. donovani*** **LPG and macrophage TLR4 favouring parasites burden and macrophage dysfunction. (**A) Fluorescence microscopic images showing abundance of FITC-labelled parasite in TLR4 silenced or overexpressed THP-1 macrophages incubated with anti-IgG or anti-LPG antibodies followed by staining with lysotracker red. DAPI was used as nuclear counterstaining. Scale bar, 50 µm. Each value is the mean ± SEM of three independent experiments. **p< 0.01 vs SC+PM; ##p< 0.01 vs anti-IgG; ***p< 0.001 vs siCon. (B) Representative images (Up) and its quantification (Down) of trans-well migration of TLR4 silenced or overexpressed THP-1 monocytes incubated with *L. donovani* promastigotes treated with anti-IgG or anti-LPG antibodies in response to the media

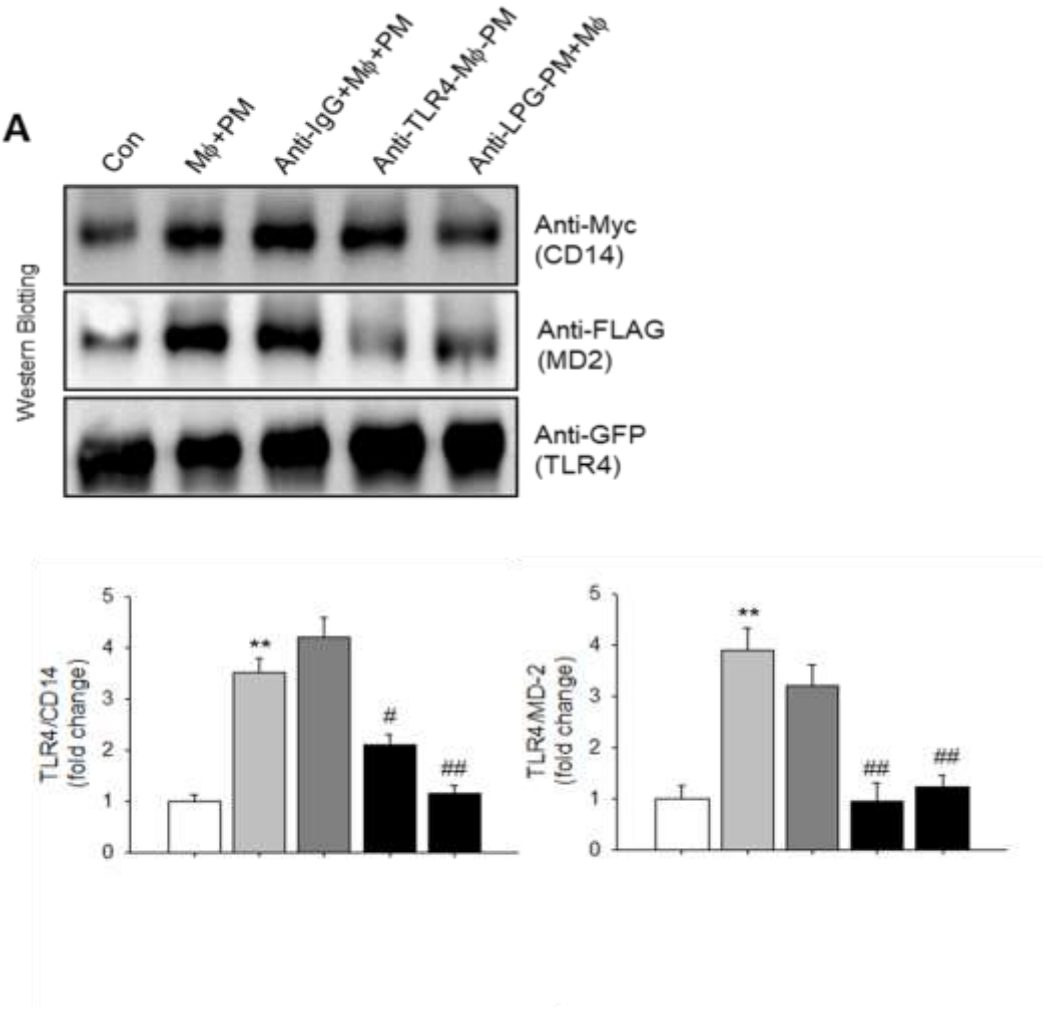
containing 10% FBS. Scale bar, 250 μm. Each value is the mean ± SEM of three independent experiments. **p < 0.01 vs siCon; ##p < 0.01 vs anti-IgG. (C) THP-1 macrophages transfected with control siRNA/TLR4 siRNA or scrambled/TLR4 overexpression plasmids were incubated with promastigotes treated with anti-IgG or anti-LPG antibodies followed by the addition of H2DCFDA. On termination of incubations, cells were lysed and ROS production was measured by Varioskan LUX multimode reader. Each value is the mean ± SEM of three independent experiments. ***p < 0.001, **p < 0.01, *p < 0.05 vs respective controls. (D) Determination of THP-1 cell viability (%) in response to indicated treatment conditions. All experiment was performed in triplicate.

To examine the macrophage functions in parasite infection and the involvement of TLR4 therein, TLR4 silenced or overexpressed THP-1 macrophages were infected with *L. donovani* promastigotes followed by the analysis of macrophage migration, cellular ROS levels and cell viability. TLR4 deficient macrophages and LPG-masked promastigotes exhibited significant improvement in the parasite-induced attenuation of macrophage migration **(Fig. 4.4B)**, and cellular ROS generation **(Fig. 4.4C)** without any significant alteration of macrophage cell viability **(Fig. 4.4D)**. All these results indicate a possible involvement of LPG-TLR4 interaction in parasite burden and macrophage dysfunction.

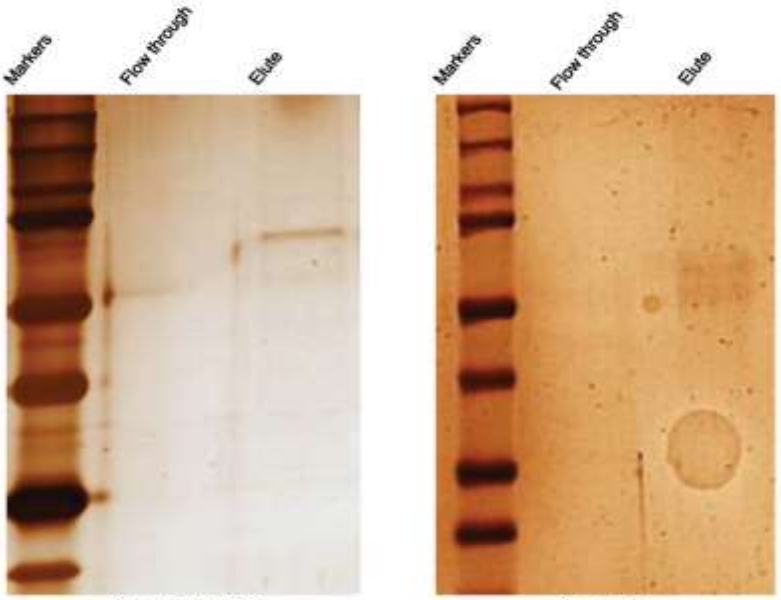
4.1.5. Direct interaction of LPG-TLR4 enhances intracellular amastigote burden:

Since ligand-dependent TLR4 stimulation leads to the increased association of TLR4 with its co-receptor proteins CD14 and MD2, we, therefore, analyzed the TLR4 interaction with both CD14 and MD2 in absence or presence of *L. donovani* promastigotes. Similar to known TLR4 agonist LPS, promastigotes incubation with macrophages markedly enhanced TLR4-CD14 and TLR4-MD2 associations as indicated by the co-immunoprecipitation study **(Fig. 4.5A)**. Moreover, blocking of macrophage TLR4 by anti-TLR4 antibodies or masking the promastigote LPG with anti-LPG antibodies significantly reduced the association of TLR4 with CD14 and MD2 **(Fig. 4.5A)**. To gain more insight about the interaction between LPG of *L. donovani* with macrophage TLR4, purified LPG and TLR4 **(Fig. 4.5B)** were used to perform isothermal titration calorimetry (ITC) analysis. A high-affinity binding of

TLR4 with LPG (K_D= 35.8 µM) suggests a direct interaction between them **(Fig. 4.5C)**. Moreover, HEK293 cells transfected with TLR4 expression plasmid followed by the infection with FITC-labeled promastigotes display notable accumulation of parasite in macrophages **(Fig. 4.5D)**. Further, to explore the specific molecular signature of TLR4 that involved in its recognition with LPG, we focused our study on analyzing the leucine-rich repeats (LRRs) in TLR4 as these are reported to play a pivotal role in ligand recognition [3,6].



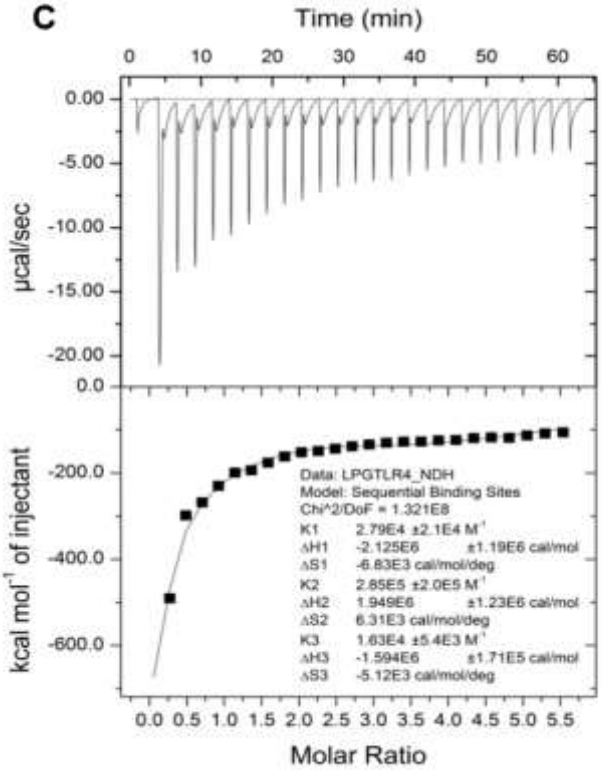
B



IP: Anti-GFP (TLR4)
(HEK293)

IP: Anti-LPG
(*L. donovani*)

C



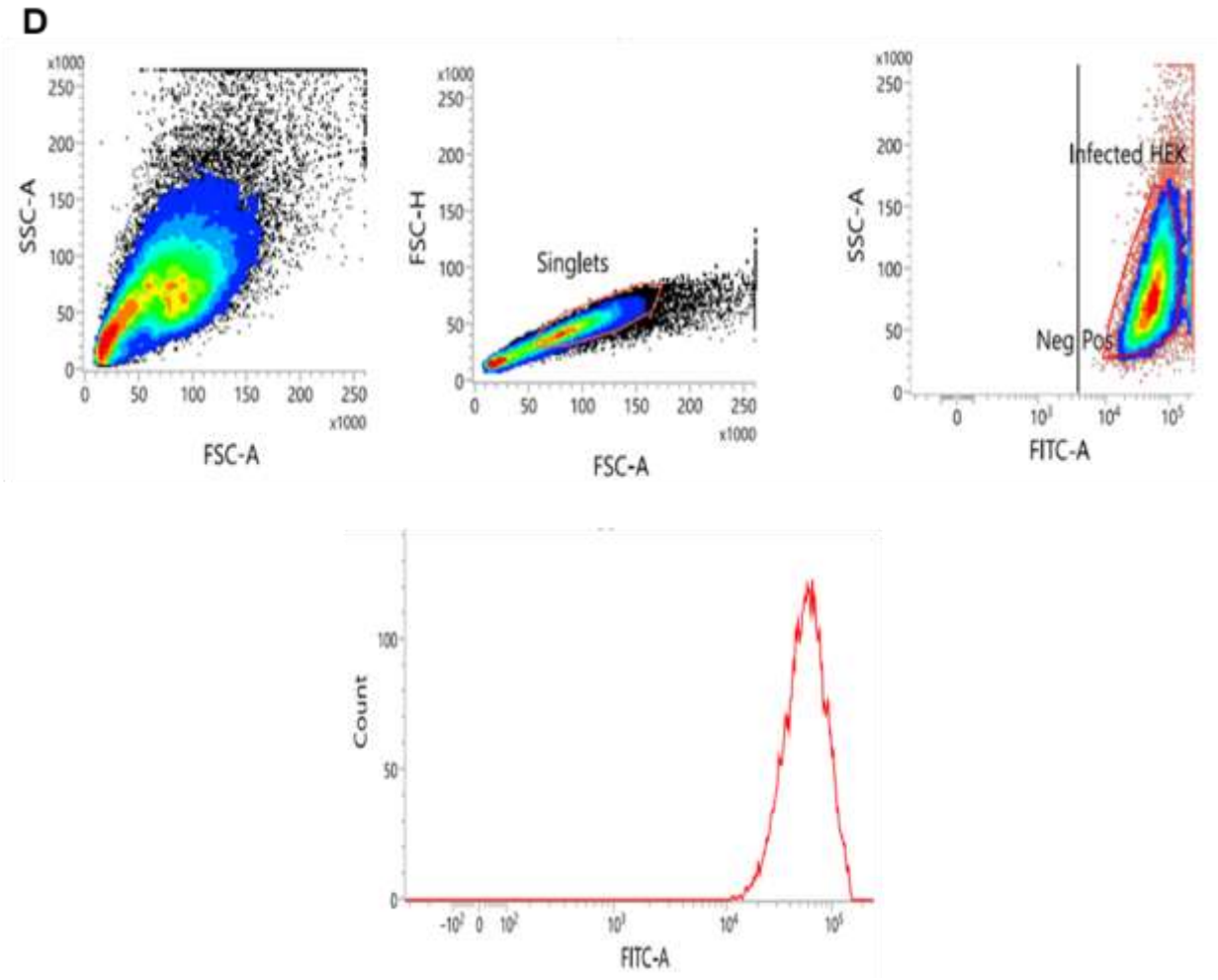
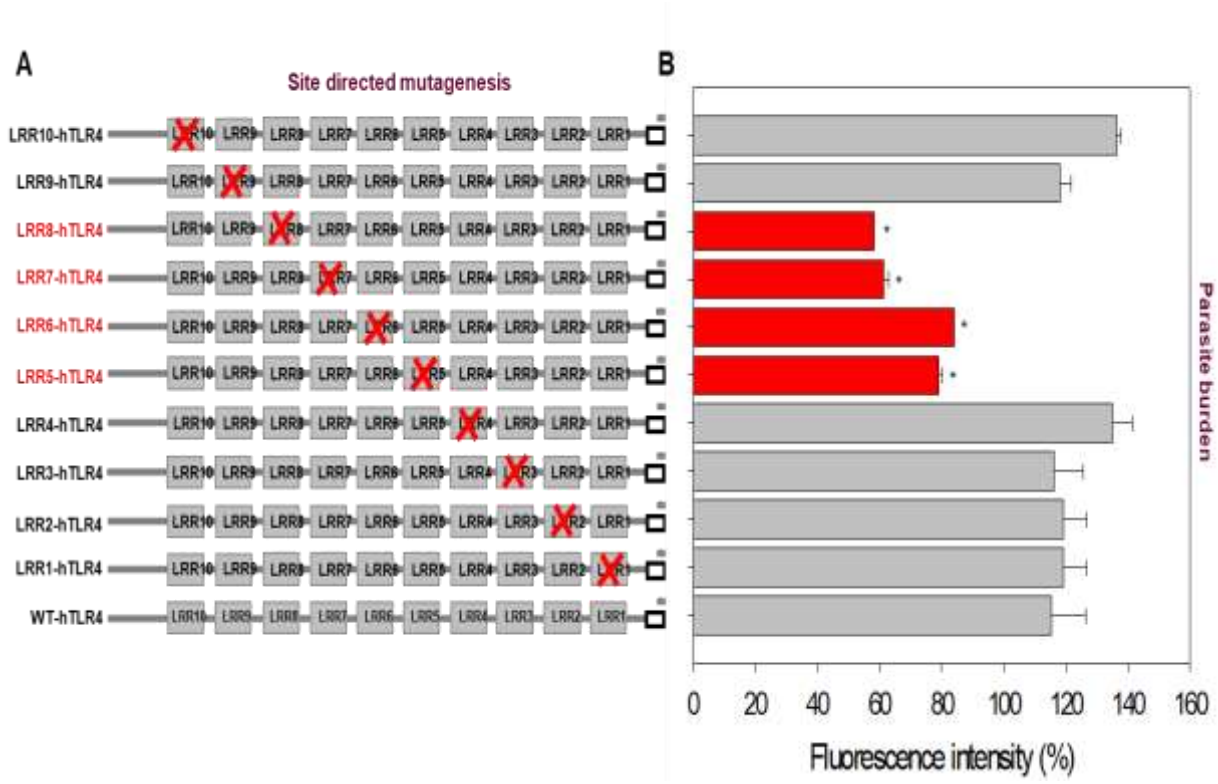


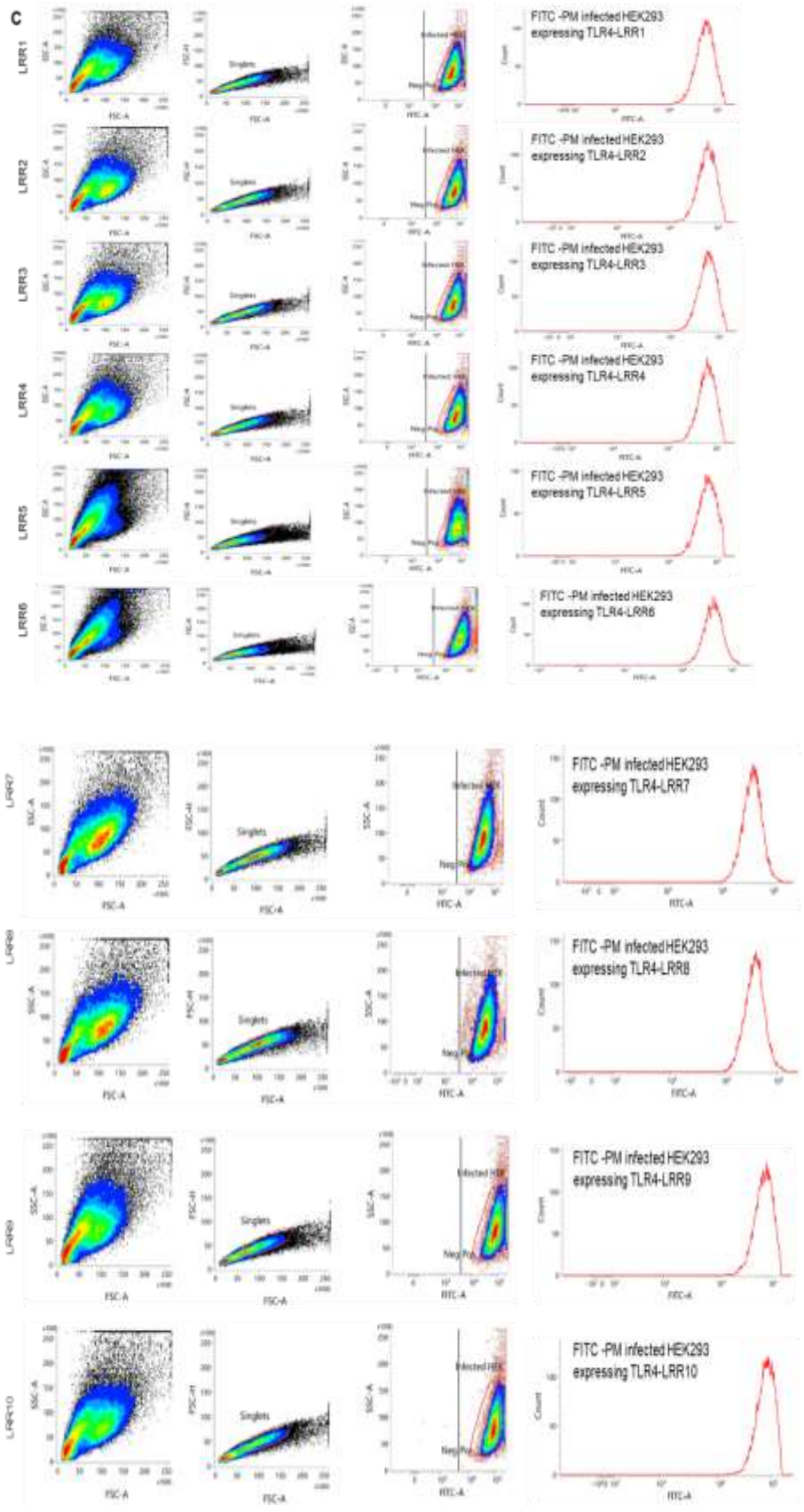
Fig 4.5: *L. donovani* LPG directly interacts with macrophage TLR4. (A) THP-1 macrophages transfected with TLR4-GFP and CD14-Myc or MD2-FLAG plasmids were incubated in absence or presence of anti-TLR4 antibodies and infected without or with *L. donovani* promastigotes treated without or with anti-LPG antibodies. On termination of incubations, cell lysates were prepared and used for immunoprecipitation with anti-GFP antibodies followed by the immunoblotting with anti-Myc or anti-FLAG antibodies showing molecular interactions (left) and their quantifications (right) in these treatment conditions. Each value is the mean ± SEM of three independent experiments, **p< 0.01 vs Con, ##p< 0.01, #p< 0.05 vs anti-IgG+Mϕ+PM. (B) HEK293 cell lysate overexpressing TLR4-YFP fusion protein was subjected to immunoprecipitation with GFP-Trap agarose followed by silver staining (left) and promastigote cell lysate was subjected to immunoaffinity chromatography using anti-LPG antibody tagged beads followed by the isolation of LPG and silver staining (right). (C) Isothermal Titration

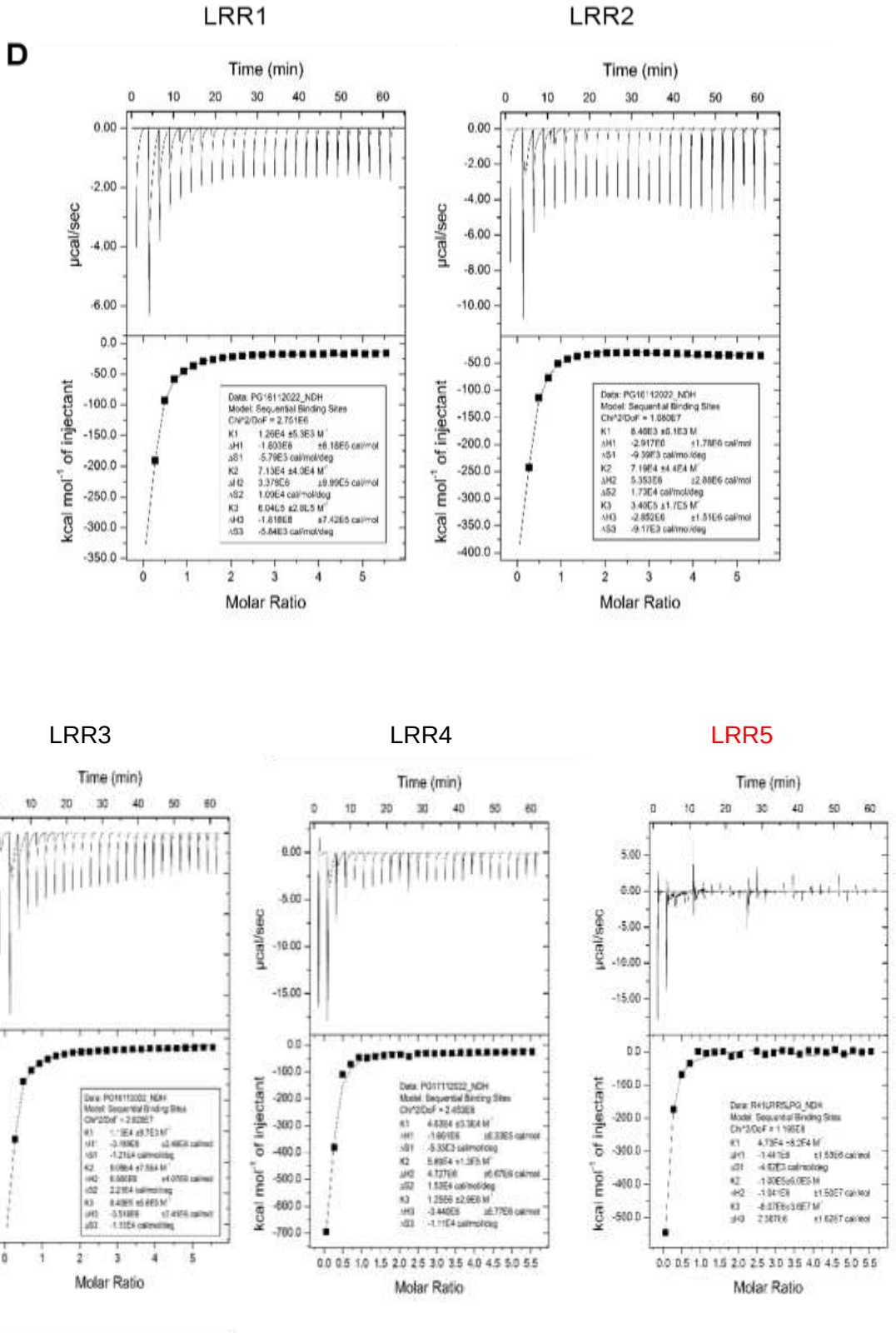
Calorimetry (ITC) assay with TLR4 as titrate and LPG as titrant showing their interaction (K_D = 35.8 μM) as heat change on injection of titrant (upper) and heat change per mole of titrant was plotted against the molar ratio of titrant to titrate (lower). (D) HEK293 cells transfected with WT-TLR4 plasmid was infected without or with FITC-labeled *L. donovani* promastigotes. Subsequent to infection, cells were washed and incubated with 0.02% trypan blue solution for 5 min to quench fluorescence associated with membrane bound uninternalized promastigotes. Cells were then washed, analyzed in flow cytometry. All experiments were performed in triplicate.

4.1.6. *L.donovani* Lipophosphoglycan (LPG) recognition is mediated by macrophage TLR4 leucine rich repeats (LRR):

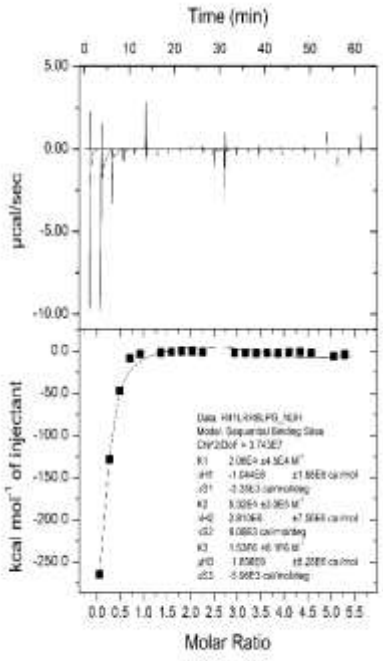
To examine LPG recognition of specific LRR sites, we mutated these sites in TLR4 by site-directed mutagenesis **(Fig. 4.6A)**. Deletion mutation of LRRs in the extracellular domain of TLR4 considerably alters the parasite burden, while, LRR5, LRR6, LRR7 and LRR8 deletion notably reduced the intracellular parasites, the deletion of LRR4 and LRR10 did not significantly increased the parasite numbers inside the cells **(Fig. 4.6B & Fig. 4.6C)**.



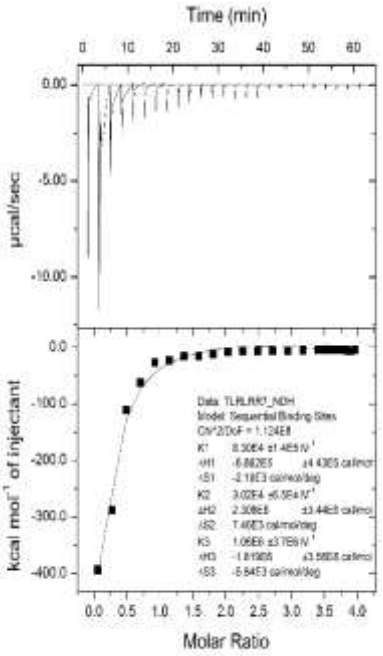

c
LRR1
LRR2
LRR3
LRR4
LRR5
LRR6
LRR7
LRR8
LRR9
LRR10
FSC-A
SSC-A
FSC-H
Singlets
FITC-A
Count
FITC -PM infected HEK293 expressing TLR4-LRR1
FITC -PM infected HEK293 expressing TLR4-LRR2
FITC -PM infected HEK293 expressing TLR4-LRR3
FITC -PM infected HEK293 expressing TLR4-LRR4
FITC -PM infected HEK293 expressing TLR4-LRR5
FITC -PM infected HEK293 expressing TLR4-LRR6
FITC -PM infected HEK293 expressing TLR4-LRR7
FITC -PM infected HEK293 expressing TLR4-LRR8
FITC -PM infected HEK293 expressing TLR4-LRR9
FITC -PM infected HEK293 expressing TLR4-LRR10


D
LRR1
LRR2
LRR3
LRR4
LRR5
Time (min)
µcal/sec
kcal mol⁻¹ of injectant
Molar Ratio

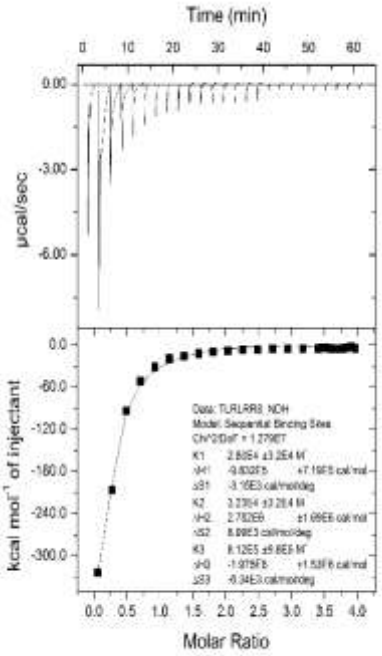
LRR6



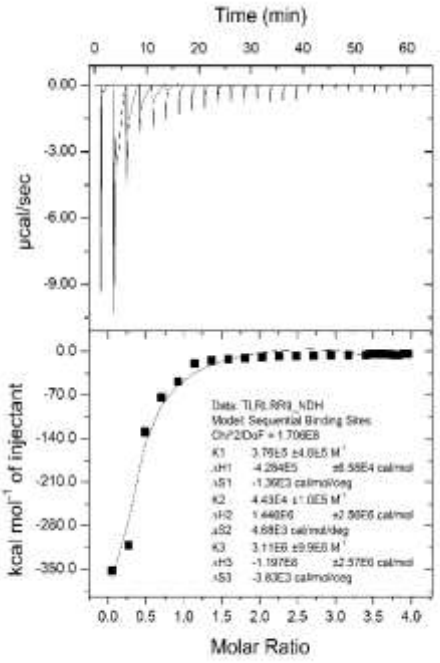
LRR7



LRR8



LRR9



LRR10

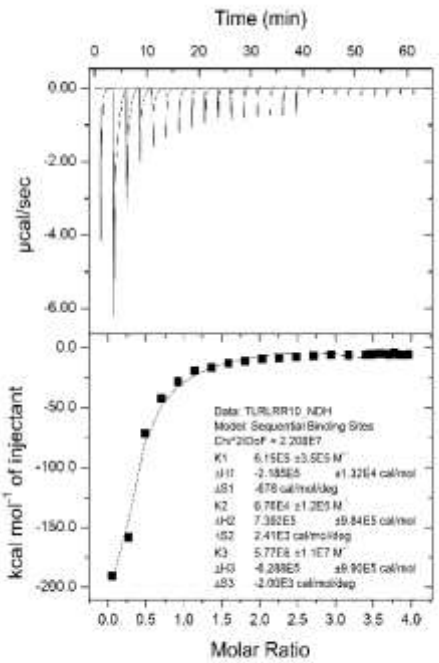


Figure 4.6: LRRs of TLR4 involved in the recognition of LPG and parasite entry in macrophages. (A) Schematic diagram showing the putative leucine-rich repeats (LRRs) regions in the extracellular domain of TLR4. These repeats were individually mutated in WT-TLR4 plasmid by site-directed mutagenesis. (B) HEK293 cells transfected with individual LRR mutant TLR4 plasmids were incubated or not with FITC-labeled L. donovani promastigotes for 4 h. Subsequent to infection, cells were washed and incubated with 0.02% trypan blue solution for 5 min to quench fluorescence associated with membrane bound un-internalized promastigotes. Cells were then washed, analyzed in flow cytometry. Each value is the mean ± SEM of three independent experiments, *p< 0.05 vs WT-hTLR4. (C) HEK293 cells transfected with individual LRR mutant-TLR4 plasmid and incubated or not with FITC-labelled *L. donovani* promastigotes (PM). Subsequent to infection, cells were washed and incubated with 0.02% trypan blue solution for 5 min to quench fluorescence associated with membrane bound un-internalized PM. Cells were then washed, analyzed in flow cytometry. All experiments were performed in triplicate. (D) Individual LRR mutant TLR4 protein was subjected to Isothermal Titration Calorimetry (ITC) assay using TLR4-LRR mutant protein as titrate and LPG as titrant showing their interactions as heat change on injection of titrant (upper panel) and heat change per mole of titrant was plotted against the molar ratio of titrant to titrate (lower panel).

Moreover, mutation at these sites were significantly altered its interaction with LPG, as indicated by ITC analysis, however, specific deletion of LRR5 and LRR6 in TLR4 considerably hinders its association with LPG **(Fig. 4.6D)**. Thus, mutational studies suggest that LRR5 and LRR6 of TLR4 are the critical sites for LPG-TLR4 interaction that govern *L. donovani* entry inside the macrophages.

4.2 Discussion:

In this study, we identified macrophage TLR4 as a novel receptor for the recognition of LPG of *L. donovani* promastigotes and such interaction play a pivotal role in parasite burden in macrophages. Leishmania infection could lead to the diseases ranging from mild to fetal depending on the parasite species and host factors. While *L. amazonensis* and *L. brazilliensis* infection primarily manifests localized cutaneous leishmaniasis, the infection of *L. donovani* and *L. infantum* causes life-threatening visceral leishmaniasis that seriously affecting liver, spleen, and bone marrow in human [7,8]. TLRs are recognized as a class of pattern recognition receptors (PRRs) and their activation

triggered by the engagement of pathogen-associated molecular pattern molecules (PAMPs). Activation of TLR4 signaling by LPS triggers MyD88 dependent or independent pathways that lead to the activation and nuclear localization of transcription factors such as NF-kB, AP1, and IRF3 that upregulate the expressions of various pro-inflammatory cytokines and type I interferons which crucially linked in controlling bacterial infection [10]. TLR4 along with several other isoforms of TLRs are known to play critical role in both leishmania pathology and clearance depending on the parasite types and host response [11]. Although previous studies have evaluated the importance of TLRs in mice infected with leishmania, however, the functional role of specific TLRs in the context of *L. donovani* internalization and infection in human macrophages has not been elucidated. Moreover, mice studies involving TLR4 are also elusive and controversial. TLR4 knockout mice of C57BL/10ScCr strain are strongly susceptible to *L major* infection exhibiting severe lesion and parasite burden than TLR4 competent mice [12]. In contrast, TLR4-deficient mice of C57BL/6 mice are resistant to *L. panamensis* infection than wild-type counterparts [13]. Since LPS and IL-4 stimulations are known to regulate the TLR4 expression and it's signaling associated with the inflammatory status of macrophages, we therefore examined the infection status of *L. donovani* promastigotes in LPS-induced pro-inflammatory and IL-4-stimulated anti-inflammatory macrophages. While LPS stimulated pro-inflammatory macrophages exhibits massive accumulation of parasites, the anti-inflammatory macrophages induced by IL-4 treatment notably reduced the parasite burden in macrophages. IL-4 induced alternatively activated macrophages play an important role in host defense in the context of a Th2 microenvironment such as parasitic infection. It has been shown that IL-4 pretreatment followed by parasitic infection markedly inhibits receptor-mediated phagocytosis [1].

However, interestingly, L. major infection followed by IL-4 treatment notably elevates the polyamine biosynthesis (via upregulation of arginase) that favors L. major parasite survival in macrophages [14]. These findings suggest that pre-treatment or post-treatment of IL-4 in parasitic infection play a crucial role in parasite infection and survival. We pretreated macrophages with IL-4 for 4 h and then infected with L. donovani for additional 4 h and observed that IL-4 pre-treatment markedly inhibits parasite burden in macrophages. Since, IL-4 treatment is known to downregulate the expression of TLR4, therefore, this could be a possible reason of reduced promastigote burden in IL-4 treated macrophages. We have found that macrophage stimulation with LPS significantly

induces a pro-inflammatory state,as indicated by the increased expression of IL-1b, TNF and MCP-1, however, no significant alteration in CCL5, IL-10 and TGFb gene expressions were observed. Thus, LPS treatment was unable to induce the levels of microbicidal action to kill the parasites. Interestingly, infection with *L. donovani* parasites in the LPS treated cells demonstrated inhibitory effect of LPS-mediated TLR4 stimulation as noted by the downregulation of IL-1b, TNF and MCP-1 gene expressions, and therefore we can suggest that the parasite use TLR4 to escape and establish the infection. Thus, initial LPS stimulation acted as priming effect which was followed by promastigote-mediated triggering of TLR4 activation. As LPS (first stimulus) and promastigote (second stimulus) both binds to TLR4 in consecutive manner, it could be possible that initial TLR4 stimulation by LPS may induce tolerance for the interaction of parasites with same TLR4 receptor. Alternatively, as TLR4 stimulation is known to induces the expression of interferon type I (IFN I) [15], therefore, LPS incubation could lead to an increase in parasite burden due to enhancement of IFN I production. The application of TLR4 signaling inhibitor Cli-095 confirmed that these effects were mediated by TLR4 priming, but only partial reversion of the LPS effect was observed after infection suggesting the possibility of other receptors may be involved in promastigote-mediated alteration of inflammatory status of the macrophages. It would be interesting to note that *L. donovani* infection strikingly reduced TLR4 activation-dependent pro-inflammatory cytokines (MCP-1, IL-1b, and TNF-a) gene expression and considerably increased the anti-inflammatory cytokines (IL-10 and TGF-b) gene expression. These results prominently suggest that although macrophage pro-inflammatory status decisively promoted *L. donovani* infection, however, their internalization succumb the MCP-1, IL-1b, and TNF-a pro-inflammatory cytokines expression and upregulation of IL-10 and TGF-b. Thus, LPS-induced tolerance could be responsible for the anti-inflammatory profile of macrophage when infected with parasite that utilize same receptor TLR4 for their entry. In addition, it could also serve as a counterstrategy of parasite evasion from host immune response. However, molecular mechanism associated with the downregulation of pro-inflammatory cytokines gene expression along with the upregulation of anti-inflammatory cytokines gene expression upon parasite internalization needs further investigation. Macrophage incubations with both IFN-g and LPS are known to promote the "classically activated macrophage" polarization exhibiting both pro-inflammatory and microbiocidal action in order to eliminate the pathogens [16]. Therefore, future study to examine the parasite burden in

macrophages treated with both IFN-g and LPS would help us to understand efficacy of 'classically-activated macrophages' polarization in parasite burden and intracellular amastigote proliferation. Interestingly, we have found that membrane localization of macrophage TLR4 was considerably increased when infected with *L. donovani* promastigotes. Moreover, significant reduction of parasite load was noted in macrophages when cells were pretreated with the pharmacological inhibitors of TLR4 or NF-κB signaling pathways, Cli-095 and parthenolide, respectively. Exploring the TLR4 involvement in macrophage parasite load, we noticed considerable reduction of parasite burden in TLR4 silenced macrophages, whereas, TLR4 overexpressed cells exhibits significant increase of parasite load in macrophages which coincides with the impairment of macrophage functions such as production of reactive oxygen species, macrophage migration, and macrophage polarizations states. It would also be evident that masking of promastigote LPG by anti-LPG antibodies significantly reduced macrophage amastigotes burden and modulates macrophage functions.

However, to examine the possibility of LPG-TLR4 interaction in regulating macrophage parasite load, we performed co-immunoprecipitation study and isothermal titration calorimetry analysis. Data from these studies clearly suggest a direct interaction between them. Further analysis of specific domain of TLR4 that involved in parasite LPG interaction and its uptake, we mutated individual leucine-rich repeats (LRRs) in TLR4 as these sites are previously reported in the recognition of ligands (repeats (LRRs) in TLR4 as these sites are previously reported in the recognition of ligands [3,6]. From our study, it has been clear that LRR5 and LRR6 of TLR4 play a pivotal role in LPG-TLR4 interaction which facilitates parasite burden in macrophages. Based on the evidence of this study, it can be concluded that *L. donovani* LPG through its interaction with macrophage TLR4 promotes parasite burden in macrophages. Moreover, promastigotes LPG upon binding with macrophage TLR4 markedly inhibits macrophage functions, which is partially dependent on LPG/parasites and TLR4/macrophage. Thus, present study strongly suggests the relevance of TLR4 in macrophage infection (recognition and entry) by *L. donovani* as well as in the downregulation of macrophage function. Therefore, targeting LPG-TLR4 pathway could provide a novel therapeutic option for the management of *L. donovani* infection.

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