CHAPTER- V

Modulation of TLR4 expression and activity govern *L. donovani* infection in liver by regulating AHSG expression

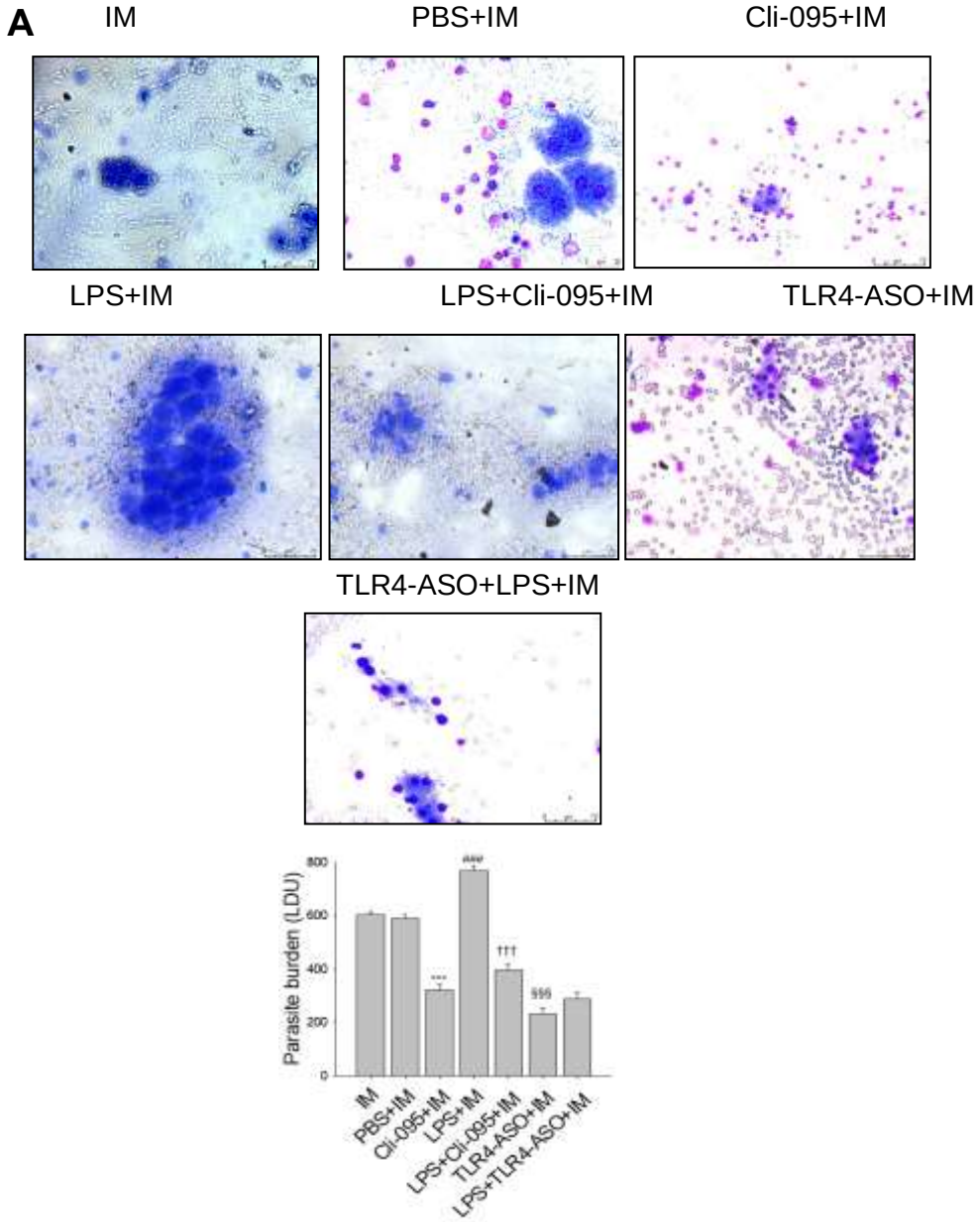
5.1 Results

5.1.1 Effect of TLR4 silencing and the inhibition of TLR4 signaling on the *L. donovani* infection in the liver of in-vivo mice model of VL

Visceral leishmaniasis is one of the major vector-borne diseases, typically occurred in tropical and subtropical regions of the world, caused by the intracellular pathogenic protozoa of *Leishmania* genus (1), affecting over 12 million populations worldwide (2). The main clinical manifestations of leishmaniasis include cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). Among these, VL is the most severe and fatal type of leishmaniasis, if untreated (3). *Leishmania donovani* serves as a causative agent for the infection and pathogenesis of VL (3). The complex interplay of factors associated with parasite and host interactions resulted in clinical variations that manifested severe challenge in understanding of disease pathophysiology and the novel treatment options. Thus, a better understanding of disease pathology and the mechanisms of organ specific host defense need to be explored for the effective management of VL.

To understand the disease pathology in the context of liver-specific host defense mechanism in response to LPS or Cli-095 stimulation and the role of TLR4 therein, we infected in BALB/c mice with *L. donovani* along with the delivery of TLR4 antisense oligonucleotides (ASO) or pharmacological inhibitor of TLR4, Cli095, for 4 weeks. The BALB/c mouse model is the most widely used experimental animal model to study the VL pathophysiology as it resembles the clinical feature of human VL (4). After 4 weeks, we sacrificed the mice and parasite burden was evaluated in impression smears of liver by Giema staining and parasite amastigote-specific A2 immunofluorescence staining of liver sections. While stimulation of TLR4 by LPS significantly promoted parasite load, the TLR4 activation inhibitor Cli-095 markedly reduced the parasite burden in the impression smears of liver (**Fig. 5.1 A**). Suppression of parasite burden in response to the systemic delivery of TLR4 antisense oligonucleotide was also evident in the impression smears of liver (**Fig. 5.1 A**). Next,

we examined the amastigote population in the liver sections of these mice and found that LPS stimulation increased the number of amastigotes in the liver as indicated by the A2 immunostaining (**Fig. 5.1 B**). Similar to the observation of impression smears of liver, silencing of TLR4 expression or its activity notably reduced the amastigote population in the liver sections (**Fig. 5.1 B**). Moreover, H&E staining demonstrated that the alteration of liver architecture in response to *L. donovani* infection was considerably reduced in TLR4-ASO or Cli-095 delivered mice (**Fig. 5.1 B**). These studies highlighted the importance of liver-specific TLR4 in parasite burden and amastigote proliferation in VL.



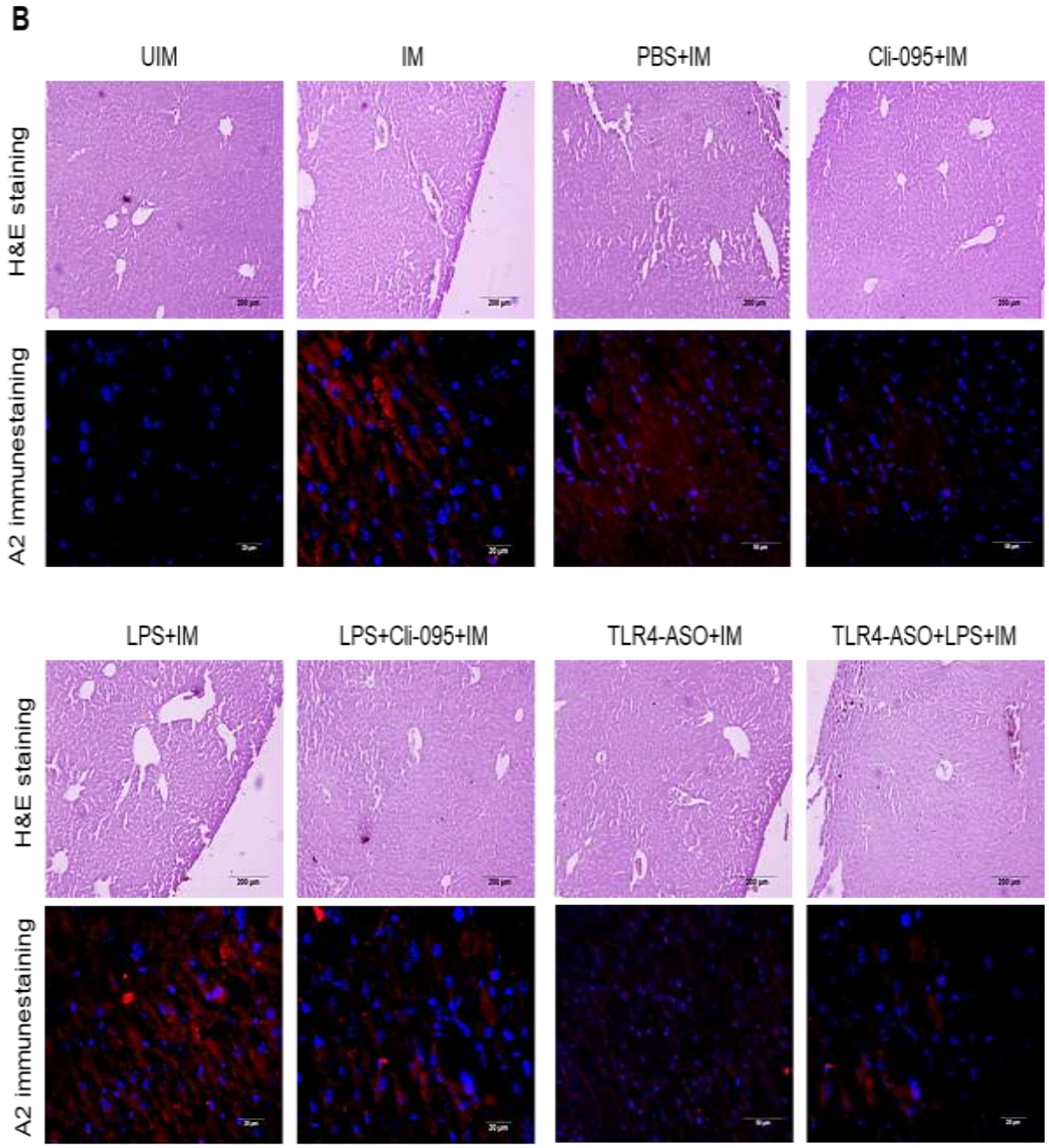
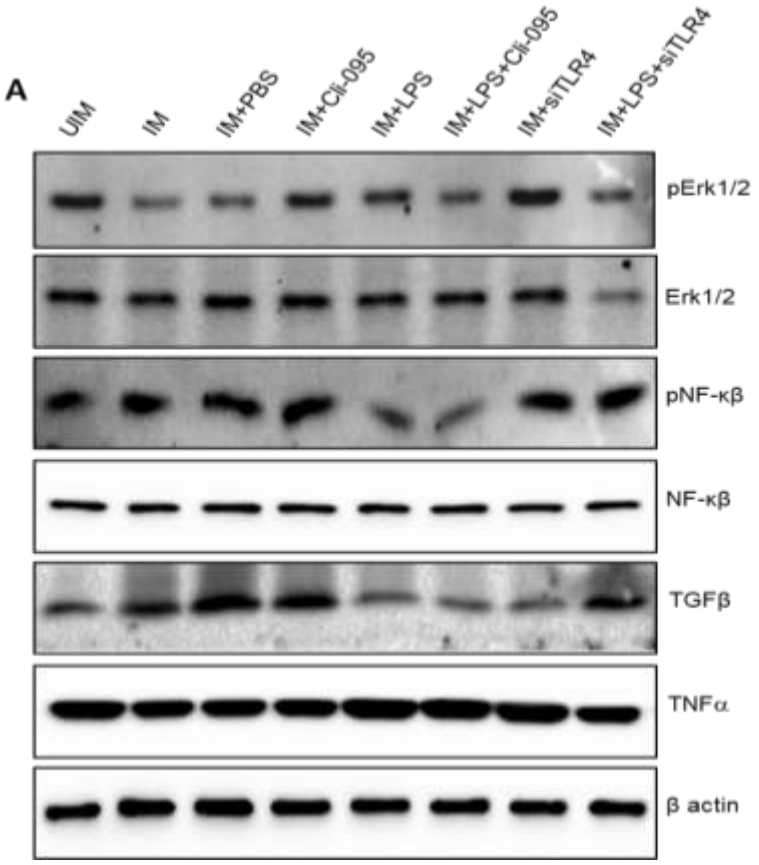
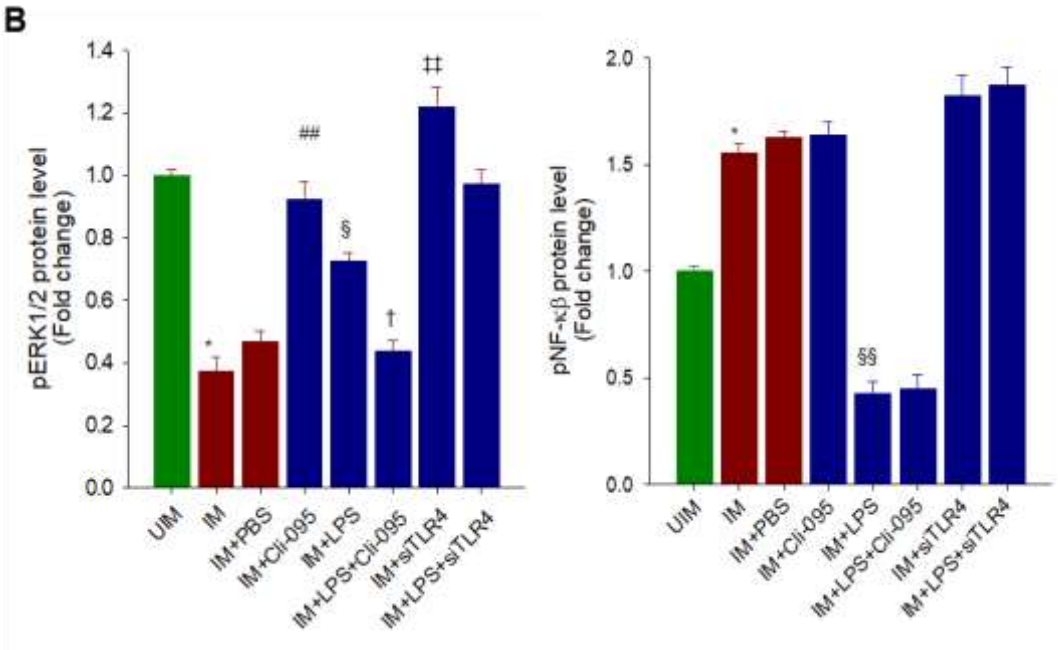


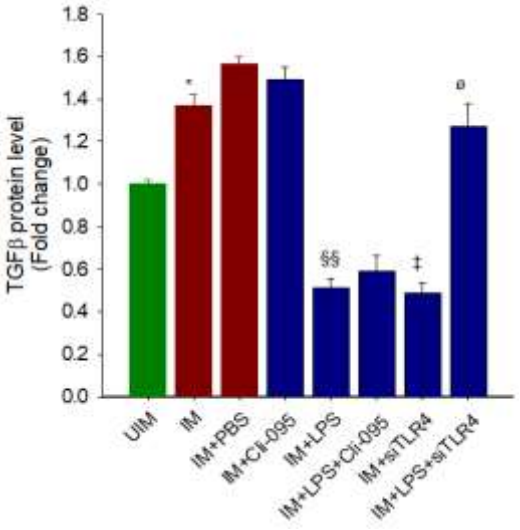
Fig 5.1: TLR4 abundance and inflammatory status governs the parasite load in the liver. (A) Giemsa staining images of liver impression smears (upper) and their quantification (lower) of mice delivered with LPS (100 µg/mice), or Cli-095 (1.5 mg/kg/bw), or both or administered with TLR4-ASO (1 µM/mice) without or with LPS (100 µg/mice), once in a week for 28 days and infected with *L. donovani* promastigotes. (B) Histological examination of mouse liver sections by H&E staining (upper) and amastigote-specific A2 immunostaining (lower) of the above-mentioned mice. ***p< 0.001 vs PBS+IM; ###p< 0.001 vs PBS+IM, †††p< 0.001 vs LPS+IM; §§§ p< 0.001 vs IM.

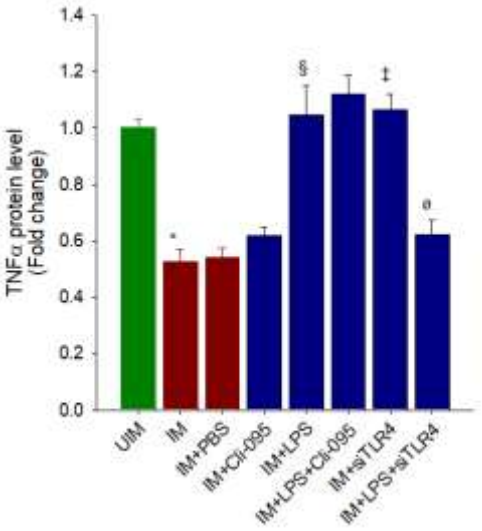
5.1.2 Modulation of ERK and NF-κB activations, inflammatory cytokines expression, and AHSG levels in the liver of TLR4 knockdown and suppressed mice model of VL

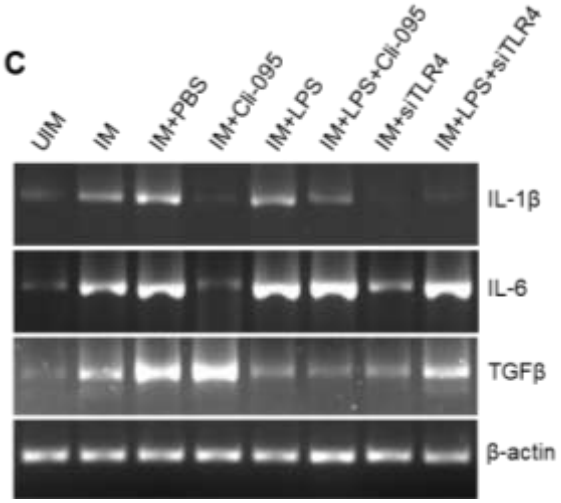
Since ERK and NF-κB plays a pivotal role in the modulation of host response to *L. donovani* infection by modulating the expression of pro-inflammatory and anti-inflammatory cytokines which governs the disease outcome against VL (5), we therefore examined the activation status of ERK and NF-κB along with the expression levels of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine TGF-β in liver samples of these mice. While, ERK activation was significantly reduced in response to *L. donovani* infection, the activation of NF-kB was notably increased upon infection of this parasite (**Fig. 5.2 A, B**). Interestingly, both silencing of TLR4 and the pharmacological inhibition of TLR4 by Cli-095 strikingly increased the ERK phosphorylation, however, such effect was not evident with NF-κB phosphorylation status in the liver (**Fig. 5.2 A, B**). Moreover, it is evident that *L. donovani* infection caused a notable alteration of TNF-α and TGF-β expression. We noticed that parasite infection significantly upregulated the TGF-β expression and downregulated the TNF-α expression in the liver (**Fig. 5.2 A, B**). Intriguingly, while, LPS stimulation or TLR4 silencing notably reduced the TGF-β upon infection, the induction of TNF-α in response to these conditions was noticed in the liver (**Fig. 5.2 A, B**). These observations indicated the opposite role of TGF-β and TNF-α upon *L. donovani* infection in liver. Although a similar observation has been made in investigating the TGF-β gene expression in response to the above -mentioned conditions, however, the expression profile of IL-1β and IL-6 pro-inflammatory cytokines were strikingly reduced in TLR4-ASO-treated parasite infected mice (**Fig. 5.2 C, D**).

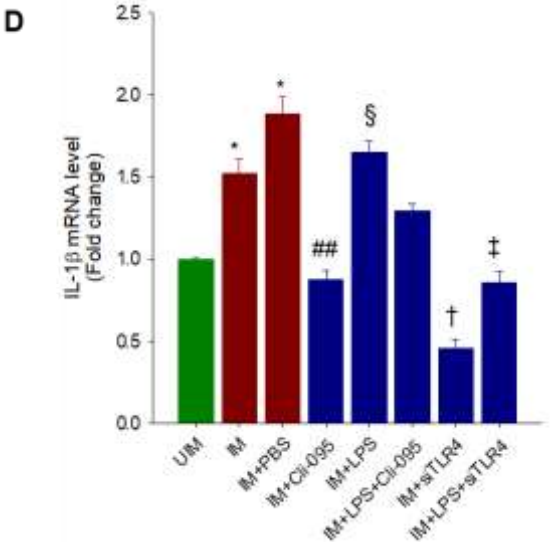

A
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4
pErk1/2
Erk1/2
pNF-κβ
NF-κβ
TGFβ
TNFα
β actin

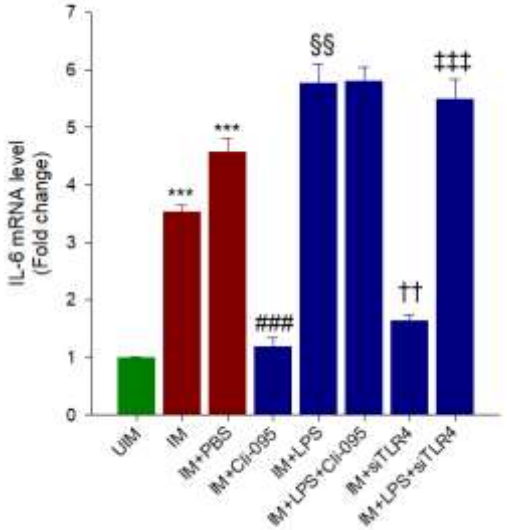

B
pERK1/2 protein level (Fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
##
§
†
‡‡
pNF-κβ protein level (Fold change)
2.0
1.5
1.0
0.5
0.0
*
§§
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4

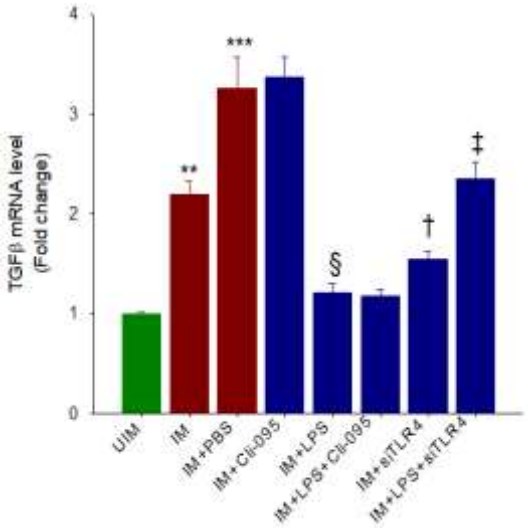

TGFβ protein level
(Fold change)
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4


TNFα protein level
(Fold change)
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4


C
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4
IL-1β
IL-6
TGFβ
β-actin


D
IL-1β mRNA level
(Fold change)
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4


IL-6 mRNA level
(Fold change)
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4


TGFβ mRNA level
(Fold change)
UIM
IM
IM+PBS
IM+Cli-095
IM+LPS
IM+LPS+Cli-095
IM+siTLR4
IM+LPS+siTLR4

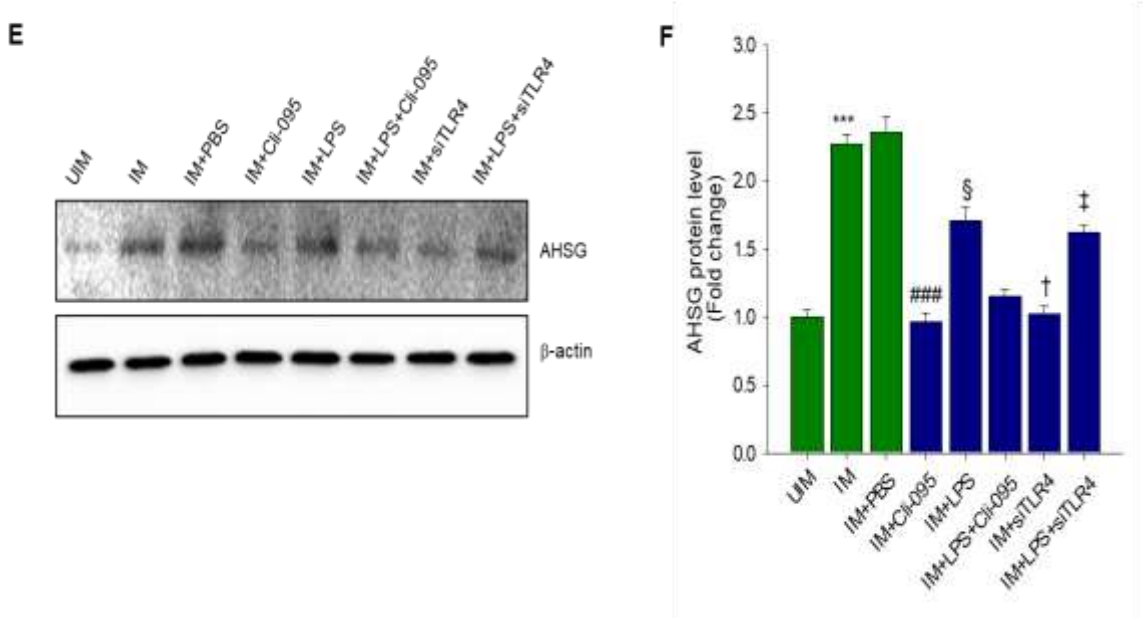


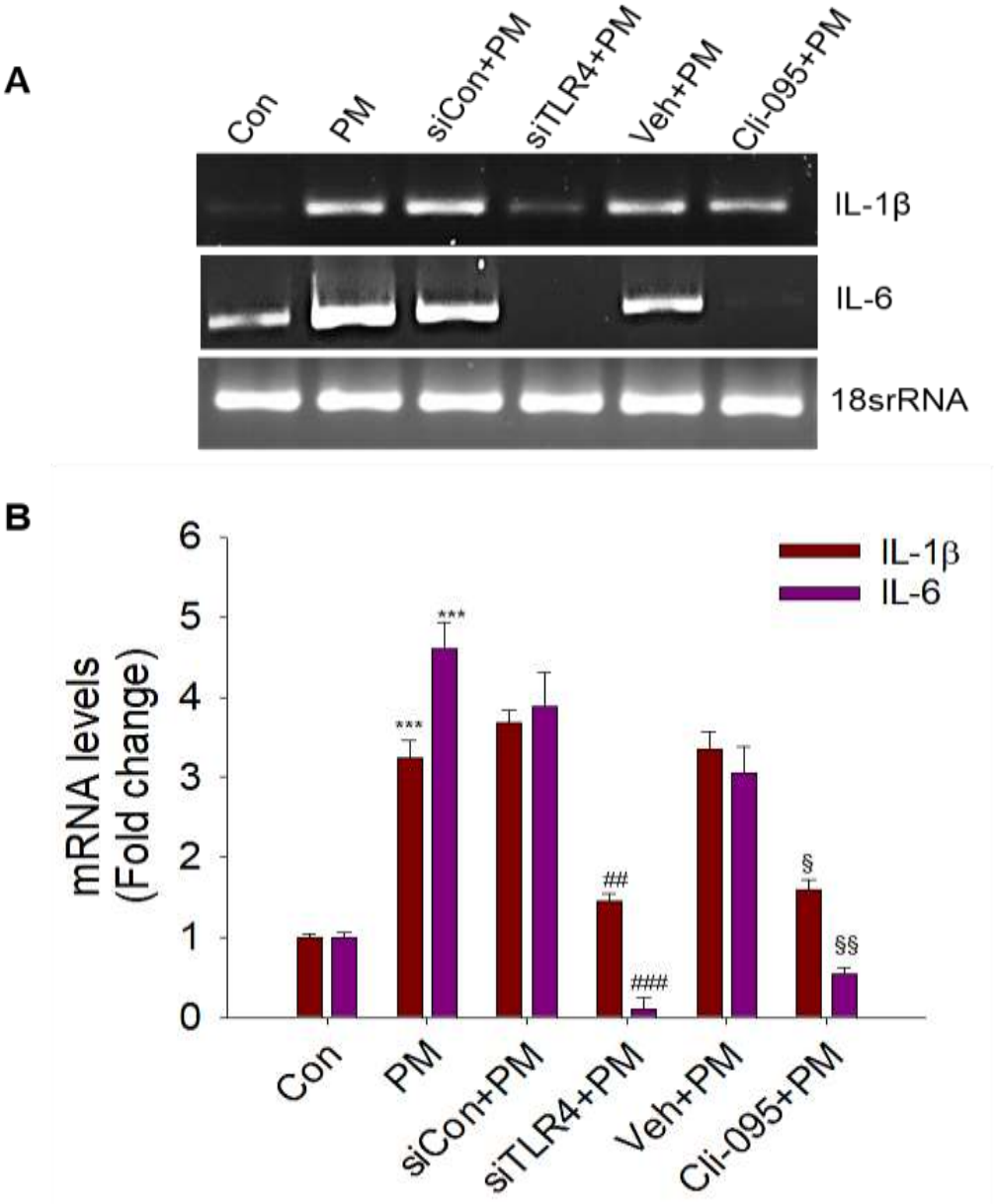
Fig 5.2: LPS stimulation and TLR4 silencing modulates ERK/NF-κB activation, inflammatory cytokines expression, and AHSG levels in the liver. (A, B) Western blot analysis (A) and their quantifications (B) showing abundance of ERK1/2 and NF-κB phosphorylation status, and inflammatory cytokines TNF-α and TGF-β protein expression in the liver samples of mice delivered with LPS (100 µg/mice), or Cli-095 (1.5 mg/kg/bw), or both or administered with TLR4-ASO (1 µM/mice) without or with LPS (100 µg/mice), once in a week for 28 days and infected with *L. donovani* promastigotes. *p< 0.05 vs UIM (uninfected control); ##p< 0.01 vs IM+PBS; §p< 0.05, §§p <0.01 vs IM+PBS; †p<0.05 vs IM+LPS; ‡p<0.05, ‡‡p <0.01 vs IM; ø p<0.05 vs IM+siTLR4. (C, D) RT-PCR analysis (C) and their quantifications (D) demonstrated abundance of IL-1β, IL-6, and TGF-β gene expression in the liver samples of above- mentioned mice. *p< 0.05, **p< 0.01, ***p< 0.001 vs UIM (uninfected control); ##p< 0.01 vs IM+PBS; §p< 0.05, §§p <0.01 vs IM+PBS; †p<0.05, ††p<0.01 vs IM+LPS; ‡p<0.05, ‡‡‡p <0.001 vs IM. (E, F) Western blot analysis (E) and its quantification (F) of AHSG protein levels in the liver of the above- mentioned mice. ***p< 0.001 vs UIM (uninfected control); ###p< 0.001 vs IM+PBS; §p< 0.05 vs IM+PBS; †p<0.05 vs IM+LPS; ‡p<0.05 vs IM.

Moreover, parasite infection in the absence or presence of LPS, notably upregulated the IL-1β and IL-6 gene expression in liver, whereas, parasite infection although increased the abundance of TGF-β but their expression was considerably decreased in

presence of LPS stimulation (**Fig. 5.2 C, D**). These results suggest that the liver inflammatory status in response to *L. donovani* infection was considerable altered and influenced by the TLR4 expression and its activation states. However, it would be interesting to note that while parasite infection significantly upregulated the Alpha 2-HS Glycoprotein (AHSG) levels in liver, the suppression of TLR4 expression or activation by TLR4-ASO or Cli-095, respectively, markedly decreased the abundance of AHSG in the infected liver (**Fig. 5.2 E, F**). This finding indicates a possible involvement of liver AHSG in the establishment of VL. Previous report suggests that AHSG regulates the cellular inflammatory state by acting as a positive or negative acute phase protein in injury and infection (6). However, the role of AHSG in VL has not yet been explored.

5.1.3 Effect of TLR4 silencing and its inhibition on the ARG activity and NO production in the *L. donovani* infected THP-1 macrophages

We then explored the role of TLR4 silencing and inhibition of TLR4 signaling in macrophages on the production of inflammatory cytokines, arginase (ARG) activity, and the generation of NO in response to *L. donovani* incubations. Interestingly, we have found that incubation of *L. donovani* promastigotes with THP-1 macrophages significantly upregulates the gene expression of pro-inflammatory cytokines IL-1β and IL-6 in macrophages (**Fig. 5.3 A, B**). Moreover, both knockdown of TLR4 and the Cli-095-mediated inhibition of TLR4 signaling markedly reduced the IL-1β and IL-6 gene expression in infected macrophages (**Fig. 5.3 A, B**). The fate of *Leishmania* parasite infection is critically regulated by the activation state of macrophages, while, classically activated macrophages promotes the activation of inducible NO synthase (iNOS), the alternatively activated macrophages induces the arginase (ARG) activity (7,8). The THP-1 macrophages when infected with *L. donovani* promastigotes, it exhibited a significant induction of ARG activity along with reduction of NO production (**Fig. 5.3 C, D**). However, TLR4 ablation or its signalling inhibition notably diminished ARG activity and considerably elevated NO levels in infected macrophages as compared to their respective controls (**Fig. 5.3 C, D**).


A
Con
PM
siCon+PM
siTLR4+PM
Veh+PM
Cli-095+PM
IL-1β
IL-6
18srRNA
B
mRNA levels (Fold change)
IL-1β
IL-6
***
***
##
###
§
§§
Con
PM
siCon+PM
siTLR4+PM
Veh+PM
Cli-095+PM

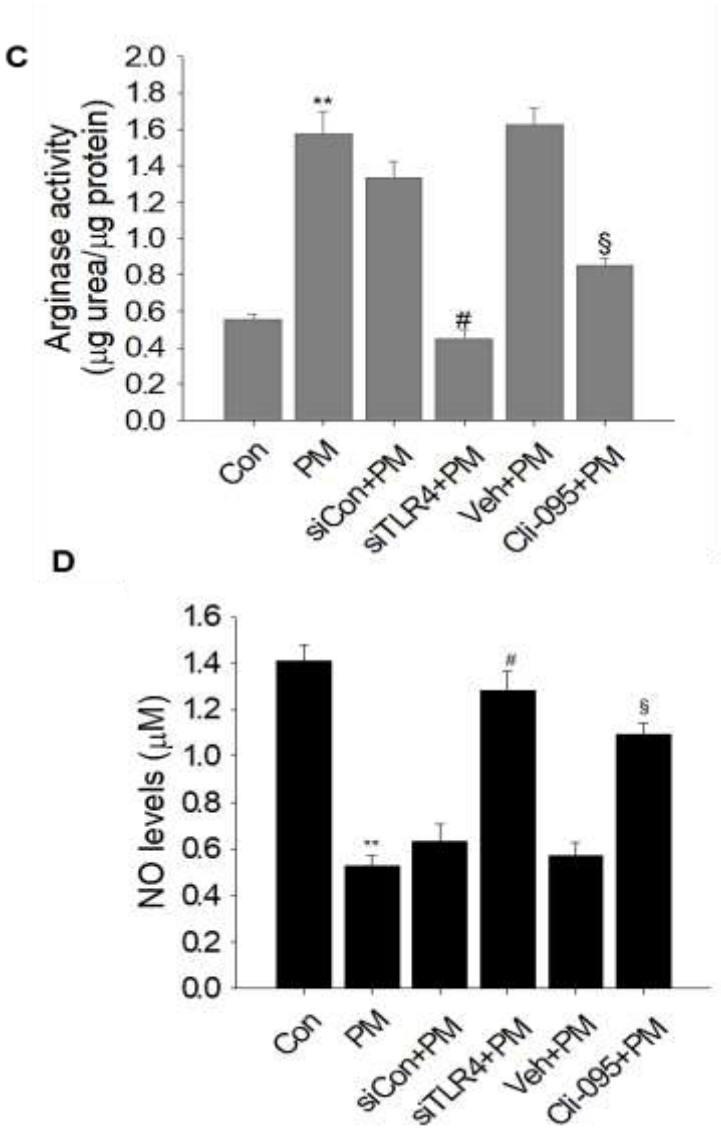
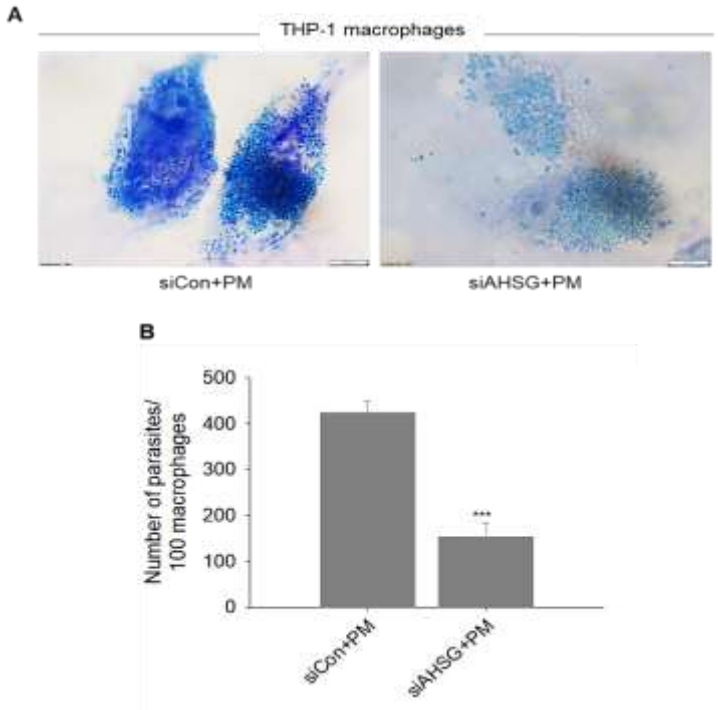


Fig 5.3: TLR4 mediated inflammatory state in *L. donovani* infected THP-1 macrophages. (A,B) RT-PCR analysis (A) and their quantifications (B) showing abundance of pro-inflammatory cytokines mRNA levels in THP-1 macrophages treated with or without TLR4 inhibitor Cli-095 or control siRNA or TLR4 siRNA transfected cells infected with *L. donovani* promastigotes Each value is the mean ± SEM of three independent experiments, ***p< 0.001 vs Con; ##p< 0.01, ###p< 0.001 vs siCon+PM; §p< 0.05, §§p<0.01 vs Veh+PM. (C) THP-1 macrophages transfected with siCon or siTLR4 or treated with vehicle or Cli-095 were subjected to *L. donovani* infection. On completion of experiment, cells were lysed and then supplemented with addition of L-Arginine, and incubated further with alpha-iso-nitroso-

propiophenone. The concentration of urea was quantified at 540 nm. Each value is the mean ± SEM of three independent experiments, **p< 0.01 vs Con; #p< 0.05 vs siCon+PM; §p< 0.05 vs Veh+PM. (D) Nitric oxide assay demonstrated NOS production level in response to THP-1 macrophages transfected with siCon or siTLR4 or treated with vehicle or Cli-095 and then infected with *L. donovani* promastigotes. Each value is the mean ± SEM of three independent experiments, **p< 0.01 vs Con; #p< 0.05 vs siCon+PM; §p< 0.05 vs Veh+PM.

5.1.4 Involvement of macrophage AHSG on parasite burden and oxidative stress status of the *L. donovani* infected THP-1 macrophages

Since involvement of macrophage AHSG in *L. donovani* infection is yet to be established, we, therefore, interested to investigate the effect of AHSG silencing on the parasite burden and macrophage function. For this reason, we transfected THP-1 macrophages with either control siRNA (siCon) or AHSG siRNA (siAHSG) for 48 h. After 48 h, cells were exposed to *L. donovani* infection, in vitro, for 6 h. On the termination of incubations, cells were thoroughly washed to remove the free parasites, and then rate of parasite internalization was evaluated under the microscope using Giemsa staining. AHSG silencing exhibited striking reduction of parasites number in infected THP-1 macrophages as compared to control (**Fig. 5.4 A,B**).



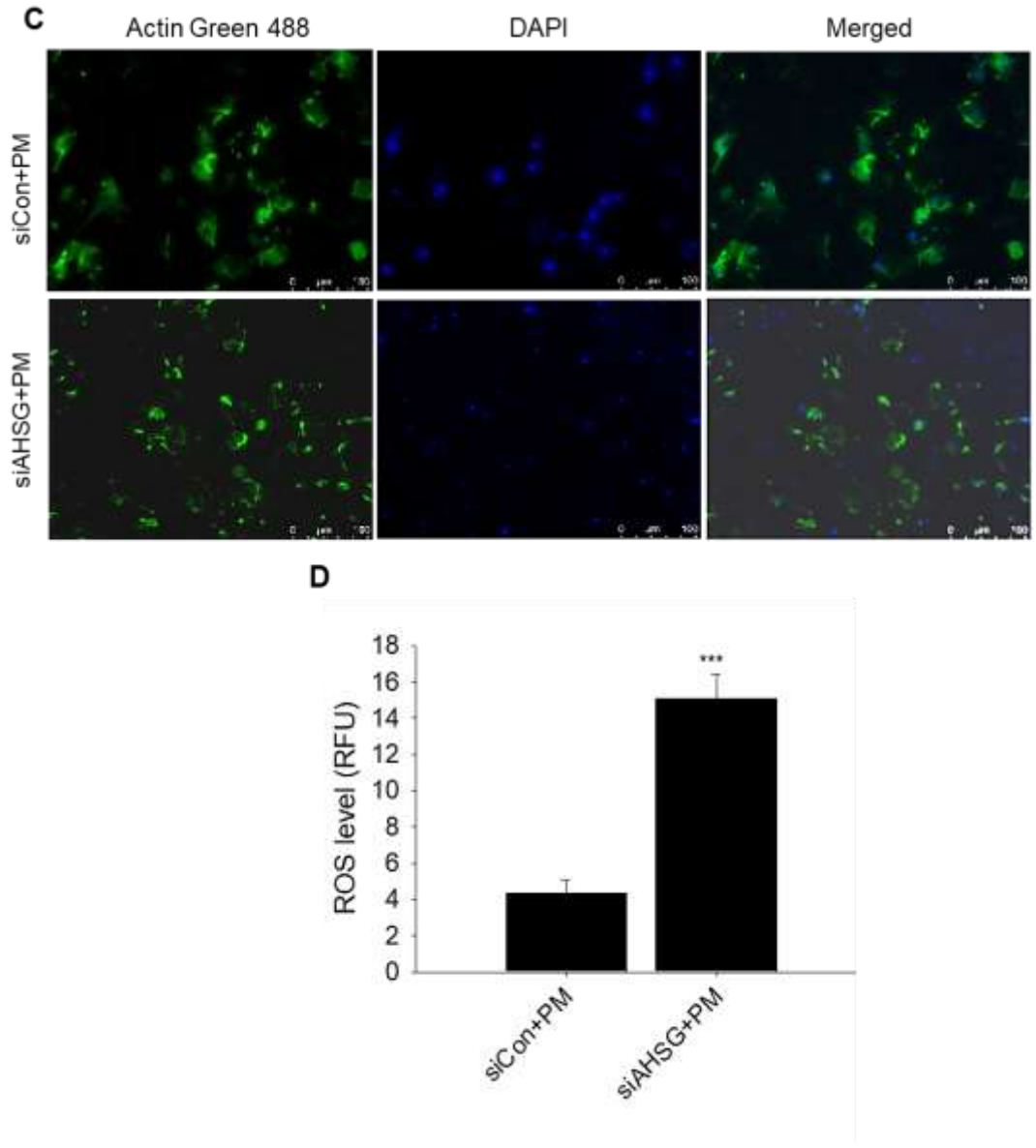


Fig 5.4: AHSG silencing causes reduction of parasite load and alteration of F-Actin accumulation in *L. donovani* infected THP-1 macrophages. (A, B) Giemsa staining images (A) and their quantifications (B) showing number of parasites in THP-1 macrophages transfected with control siRNA or AHSG siRNA followed by infection with *L. donovani* promastigotes. Each value is the mean ± SEM of three independent experiments, ***p< 0.001 vs siCon+PM. (C) Representative immunofluorescence images of F-Actin Green 488 staining of THP-1 macrophages transfected with control siRNA or AHSG siRNA followed by infection with *L. donovani* promastigotes. DAPI was used for nuclear counterstaining. Scale bar 100 µM. (D) Measurement of ROS level in THP-1 macrophages transfected with control siRNA or AHSG siRNA followed by infection with *L. donovani* promastigotes. Each value is

the mean ± SEM of three independent experiments, ***p < 0.001 vs siCon+PM.

The analysis of F-actin accumulation revealed that the impairment of F-actin arrangement in AHSG ablated macrophages infected with *L. donovani* promastigotes as indicated by Actin Green 488 immunostaining (**Fig. 5.4 C**). Moreover, in comparison to control, AHSG silenced macrophages demonstrated profound induction of ROS generation as indicated by the DCFDA staining (**Fig. 5.4 D**). Together, these results suggest that macrophage AHSG play a crucial role in parasite burden and oxidative stress in macrophages. However, detailed analysis of AHSG's involvement in parasite internalization and survival are need to be analysed both *in-vitro* infection model and *in-vivo* model of VL.

5.2 Discussion

Recent studies in VL advance our knowledge about the role of liver, spleen, and bone marrow microenvironments in shaping the host-parasite interactions and infection outcome (9,10). It has been shown that *L. donovani* parasite when reached to the spleen, it provoked activation of localized dendritic cells, severe loss of stromal cell population with complete splenic disorganization, and priming of macrophages polarization for establishing disease outcome (11-13). On contrary, the liver demonstrated effective granuloma formation in response to *L. donovani* infection for successful elimination of parasites (11,14). However, the clear distinction of these two organs in response to *L. donovani* infection has not yet been completely understood. Since, infection of *L. donovani* leads to the generation of inflammatory responses in the liver that critically linked with parasite elimination, we aimed to investigate the role of TLR4 and it's signaling on resolving the parasite infection in the liver. Studies have shown that, $TLR^{-/-}$ mice (C57BL/6 strain) developed higher liver parasite of *L. major* infection and *L. donovani* (LV9 and MW897 strain) infection [15,16]. Moreover, TLR4 knockout mice of C57BL/10ScCr strain are strongly susceptible to *L. major* infection exhibiting severe lesion and parasite burden than TLR4 competent wild type mice [17]. The outcome of Leishmania infections is complex, it not only depends on parasite species and strains but also depends upon the immune status of mouse strain. In our study we have

developed in-vivo BALB/c mouse model of VL by infecting with pathogenic morph of *L. donovani* (MHOM/IN/1983/AG-83) and this may be the probable reason that we noticed a significant reduction of liver parasite infection in TLR4 knockdown BALB/c mice.

We developed in-vivo BALB/c mouse model of VL by the intravenous administration of *L. donovani* parasites (10^7 stationary-phase of promastigotes). Mice infected with parasites were received either Cli-095 or LPS or both or administered with TLR4-ASO without or with LPS once in a week for 28 days and one week prior to infection. We then explored the liver-specific parasite burden, histopathological changes, and inflammatory cytokines gene expression. Both Giemsa staining of liver impression smears and A2 immunostaining of liver sections revealed that while LPS stimulation significantly increased parasite burden in liver smear and sections, the TLR4 activation inhibitor Cli-095 notably diminished the parasite burden in the liver. Interestingly, parasite burden in the liver was considerably reduced in infected mice where TLR4 antisense oligonucleotides were administered. Moreover, H&E staining also showed that the alteration of liver architecture in response to *L. donovani* infection was considerably reduced in TLR4-ASO or Cli-095 delivered mice. These findings indicated the importance of liver-specific TLR4 and its signaling in parasite burden and amastigote proliferation in VL.

We then examined the activation status of ERK and NF-κB along with the expression levels of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine TGF-β in liver samples of these mice. Results clearly demonstrated the alteration of ERK and NF-κB activation along with the expression profile of TNF-α and TGF-β which suggested differential regulation of these signature candidates and the role of TLR4 therein in the liver of *L. donovani* infected mice. These results indicate that the liver inflammatory status in response to *L. donovani* infection was considerable altered and was notably influenced by the TLR4 expression and its activation states. Interestingly, we noted that the parasite infection significantly upregulated the expression of AHSG in the liver, however, such induction was notably suppressed by TLR4 ablation or inactivation by TLR4-ASO or Cli-095, respectively. This finding indicates a possible involvement of liver AHSG in the

establishment of VL. AHSG have been implicated as a positive or negative acute phase protein in injury and infection (4), however, their involvement in VL has not yet been explored.

Examining the role of TLR4 on the stimulation of inflammatory cytokines expression, arginase (ARG) activity, and the production of NO in response to *L. donovani* exposure, it has been observed that silencing of TLR4 expression and inhibition of TLR4 signaling in infected macrophages markedly reduced the pro-inflammatory cytokines gene expression. *L. donovani* infection in macrophages is critically regulated by the activation state of macrophages. Macrophages can be activated to either classically activated state or alternatively activated state which metabolize L-arginine differentially (7,8). The classically activated macrophages induces the activation of an enzyme inducible NO synthase (iNOS), whereas, the alternatively activated macrophages stimulate the enzyme arginase (ARG). Both parasites and the mammalian hosts ARG are known to hydrolyze the L-arginine to L-ornithine and urea. The L-ornithine serves as a key component of polyamines biosynthesis that has been used by Leishmania parasites for their growth, differentiation, and proliferation inside the host cells. Leishmania parasites could able to modulate the cellular ARGs activity and suppress the production of NO for enhancing their survival by depleting iNOS substrate (L-arginine) and diminish the NO levels (7,8). We found that when THP-1 macrophages were infected with *L. donovani* promastigotes, it exhibited a significant induction of ARG activity along with reduction of NO production, however, such effects were compromised in TLR4 ablated cells or cells preincubated with TLR4 inhibitor Cli-095.

Further investigation on the role of macrophage AHSG in *L. donovani* infection, we explored the *L. donovani*-induced parasite burden and macrophage function in AHSG silenced macrophages. The siRNA-mediated knockdown of AHSG expression in macrophages demonstrated striking reduction of parasites number in infected THP-1 macrophages with impaired F-actin arrangement and striking induction of oxidative stress. Thus, present study poised macrophage AHSG as a key player in parasite burden and macrophage dysfunction. Future study

investigating the role of AHSG in parasite internalization and survival may provide novel therapeutic target for the management of VL.

Our study primarily focused on the role of TLR4 in the liver parasite load and therefore examined only this aspect. However, it would be interesting to investigate the participation of TLR4 in the spleen parasite load. Thus, future study in the area would be meaningful to delineate the differential role of TLR4, if any, in the persistence and clearance of parasite infection.

Bibliography:

[1] de Araújo, F.F., Costa-Silva, M.F., Pereira, A.A.S., Rêgo, F.D., Pereira, V.H.S., de Souza, J.P., Fernandes, L.O.B., Martins-Filho, O.A., Gontijo, C.M.F., Peruhype-Magalhães, V., and Teixeira-Carvalho, A. Chemokines in Leishmaniasis: Map of cell movements highlights the landscape of infection and pathogenesis. *Cytokine*, 155339, 2020.

[2] Organization W.H. Report on the interregional meeting on leishmaniasis among neighbouring endemic countries in the Eastern Mediterranean, African and European regions, Amman, Jordan 23–25 September 2018. World Health Organization, Regional Office for the Eastern Mediterranean, 2019.

[3] van Griensven, J., and Diro, E. Visceral leishmaniasis. *Infectious disease clinics of North America*, 26(2), 309-22, 2012.

[4] Loría-Cervera, E.N., and Andrade-Narváez, F.J. Animal models for the study of leishmania immunology. *Revista do Instituto de Medicina Tropical de São Paulo*, 56(1), 1-11, 2014.

[5] Kar, S., Ukil, A., and Das, P.K. Cystatin cures visceral leishmaniasis by NF-κB-mediated proinflammatory response through co-ordination of TLR/MyD88 signaling with p105-Tpl2-ERK pathway. *European journal of immunology*, 41(1), 116-27, 2011.

[6] Wang, H, and Sama, A.E. Anti-inflammatory role of fetuin-A in injury and infection. *Current molecular medicine*, 12(5), 625-33, 2012.

[7] Tomiotto-Pellissier, F., da Silva Bortoleti, B.T., Assolini, J.P., Gonçalves, M.D., Carloto, A.C.M., Miranda-Sapla, M.M., Conchon-Costa, I., Bordignon, J., and Pavanelli, W.R. Macrophage Polarization in Leishmaniasis: Broadening Horizons. *Frontiers in immunology*, 9, 2529, 2018.

[8] Bogdan, C. Macrophages as host, effector and immunoregulatory cells in leishmaniasis: Impact of tissue micro-environment and metabolism. *Cytokine X*, 2(4), 100041, 2020.

[9] Poulaki, A., Piperaki, E.T., and Voulgarelis, M. Effects of Visceralising Leishmania on the Spleen, Liver, and Bone Marrow: A Pathophysiological Perspective. *Microorganisms*, 9(4), 759, 2021.

[10] Rodrigues, A.V., Valério-Bolas, A., Alexandre-Pires, G., Pereira, M.A., Nunes, T., Ligeiro, D., da Fonseca, I.P., and Santos-Gomes, G. Zoonotic Visceral Leishmaniasis: New Insights on Innate Immune Response by Blood Macrophages and Liver Kupffer Cells to Leishmania infantum Parasites. *Biology (Basel)*, 11(1), 100, 2022.

[11] Desjeux, P. Leishmaniasis: current situation and new perspectives. *Comparative immunology, microbiology and infectious diseases*, 27(5), 305–18, 2004.

[12] Leta, S., Dao, T.H.T., Mesele, F., and Alemayehu, G. Visceral leishmaniasis in Ethiopia: an evolving disease. *PLoS neglected tropical diseases*, 8(9), e3131, 2014.

[13] Mukherjee, B., Mukherjee, K., Nanda, P., Mukhopadhayay, R., Ravichandiran, V., Bhattacharyya, S.N., and Roy, S. Probing the molecular mechanism of aggressive infection by antimony resistant Leishmania donovani. *Cytokine*, 145, 155245, 2021.

[14] Malafaia, G. Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review. *Parasite immunology*, 31(10), 587–96, 2009.

[15] Murray, H.W., Zhang, Y., Zhang, Y., Raman, V.S., Reed, S.G. and Ma, X. Regulatory actions of Toll-like receptor 2 (TLR2) and TLR4 in Leishmania donovani infection in the liver. *Infection and immunity*, 81(7), 2318-2326, 2013.

[16] Dias, B.T., Vivarini, A.C., Costa, T.F., Mottram, J.C., Lopes, U.G. and Lima, A.P.C. Toll-like receptor-and protein kinase R-induced type I interferon sustains infection of Leishmania donovani in macrophages. *Frontiers in immunology*, 13, 801182, 2022.

[17] Müller, I., Freudenberg, M., Kropf, P., Kiderlen, A., and Galanos, C. Leishmania major infection in C57BL/10 mice differing at the Lps locus: a new non-healing phenotype. *Medical Microbiology and Immunology.* 186, 75-81, 1997.