ABSTRACT

Abstract

Visceral Leishmaniasis (VL) is a severe form of leishmaniasis and one of the world's most neglected diseases, primarily affecting the poor in developing countries. Macrophages not only function as a replicative niche during the infection, but also operate as anti-parasitic activator cells, immunoregulators and protective host cells for long term survival of persistent parasites. In VL, *L. donovani* mediated establishment of infection requires a protective niche for their internalizationand survival, wherein macrophages are the perfect place for harboring the parasites. As a principal host cells for *Leishmania* infection, the inflammatory and activation states of macrophages such as M1 classically activated proinflammatory phenotype or M2 alternate activated anti-inflammatory phenotype are key determinant of disease outcome which either mediate the killing through the induction of oxidative stress or survival of parasites by developing the evasion mechanism to avoid anti-parasitic defense mechanism of host cells. The homeostasis between the pro-inflammatory and anti-inflammatory cytokines of host macrophages potentially decides the fate of infection; however, the macrophage polarizations as well as the resistance and susceptibility effects are not fully understood in human VL. Although previous reports suggested that in experimental VL model and in human VL, the upregulation of Th2 cytokines such as IL-10,1L-13 and IL-4 significantly promoted infection with parasite burden and their proliferation in host microenvironment; whereas, the severe downregulation of Th1 cytokines such as TNF-α, IL-1β, and IL-6 favoring the increased burden and susceptibility to infection. However, the exact molecular mechanisms involved in the parasite entry or uptake by macrophages and associated downregulation of pro-inflammatory cytokines along with the upregulation of anti-inflammatory cytokines are still an unsolved puzzle in VL. Parasite entry into the macrophage is a multi-step process that requires interaction of membrane-associated receptors in macrophage and surface molecules of *L. donovani* promastigote for successful parasite internalization and establishment of infection. Although several studies highlighted the importance of toll-like receptors (TLR), particularly TLR2 and TLR4, in the pathophysiology of leishmaniasis, however, their role and specific

molecular signatures involved in *L. donovani* uptake and establishment of infection in macrophages are still elusive. Several reports indicated that *L. donovani* contains several surface molecules including lipophosphoglycan (LPG) and gp63 in their surface which facilitates their internalization in macrophages during VL.

Although plethora of studies highlighted the various molecular cues responsible for the immune evasion strategies by *L. donovani* promastigotes and amastigotes, however the mechanisms of parasite entry into macrophages and associated inflammatory status activation in host macrophages is still a subject of intense investigation to find out molecular targets for mitigating VL. Here we found that the interaction between macrophage TLR4 receptor and *L. donovani* promastigote LPG mediates the entry of parasites into the macrophages which downregulated the pro-inflammatory signalling cascade through the inhibition of NF-κB. Parasite uptake mediates the modulation of host immune activation of macrophages through TLR4-LPG interaction. Studies suggested that the extracellular repeated segments of leucine-rich repeats (LRRs) of TLR4 serves as key determinants for recognition of its ligands. We, therefore, interested to find out the specific LRR regions that could mediate macrophage/TLR4 and parasite/LPG interaction. Cumulative experimental evidences exhibited that LRR5 and LRR6 of TLR4 specifically involved in recognition of parasite LPG that facilitate its entry into the macrophages. Therefore, targeting LPG-TLR4 interaction and its signaling could provide a potential therapeutic option for the management of VL.

To explore the organ specific role of TLR4 on establishment of *L. donovani* infection in liver, we prepared BALB/c mice model and found that LPS stimulation markedly increased parasitic burden in liver, whereas, depletion of TLR4 expression and activity by TLR4 antisense oligonucleotide (ASO) and Cli-095, respectively, notably modulates the parasite burden in liver which associated with alteration of inflammatory cytokines expression and signaling pathways. Interestingly, we have found that increased expression of AHSG in the liver upon *L donovani* infection. While, systemic silencing of TLR4 or the inhibition of TLR4 signaling considerably reduced AHSG expression, the stimulation of TLR4 by LPS

notably increased AHSG levels in the liver. These observations suggest that hepatic AHSG expression possibly associated with the establishment of *L. donovani* infection in liver. To examine further, we noticed that knockdown of AHSG in THP-1 macrophages significantly suppressed parasite number with increased ROS levels in macrophages. All these results indicate that macrophage AHSG could able to modulate the survival status of *L. donovani*. However, future study in this direction is necessary to establish the role of AHSG in *L. donovani* infection and associated pathophysiology of VL.

Drugs available in the market to mitigate or combat a complex and critical disease like VL generally associated with severe side effects like drug resistance and organ toxicity. Heterocyclic organic compounds were considered as potential therapeutic choice as drug candidates in recent years due to their anti-leishmanial potencyover conventional drugs. Among the heterocyclics, imidazoles particularly gained researchers interest as a therapeutic choice to prevent leishmaniasis with lesser side effects. It has been well established that imidazopyridine have its medicinal implications for containing biologically active heterocycle. Therefore, it can be elucidated that imidazo[1,2-α] pyridine as a potential core pharmacophore for mitigating leishmaniasis. Particularly, amino pyran belongs to pyran family of heterocycles gained more insight for its cytotoxic and microbicidal potential and thus serve as a suitable candidate for synthesizing imidazo[1,2-α] pyridine (IMPA) derivatives. Among the different IMPA derivatives such as IMPA-2,-5,-6,-8, and -12, particularly IMPA-2 and IMPA-12 notably induced oxidative stress and apoptosis hindering promastigote growth inside host macrophages. Moreover, IMPA-2 is more proficient in inhibiting parasite growth, ROS induction, mitochondrial dysfunction, cell cycle inhibition at G0/G1 phase and promoting apoptosis as compared to IMPA-12. Thus, IMPA-2 and IMPA-12 could be used as potential therapeutics against VL.

The studies presented in this thesis have been grouped into seven different chapters:

Chapter I delineates the introduction for the current study. This chapter introduces withthe background and epidemiology of visceral leishmaniasis, transmission biology and life cycle of *L. donovani* infection. Moreover, the role of macrophage inflammatory status, macrophage TLR4 receptor, role of *L. donovani* lipophosphoglycan (LPG) in parasite infection in host macrophages along with alteration of macrophage functions were discussed. This chapter also highlighted the importance of heterocyclic organic compounds such as imidazole and its imidazo[1,2-α] pyridine derivatives as potent anti-leishmanial agents.

Chapter II represents the review of literature and justified how the present topic is important for gaining a better understanding of *L. donavani* infection in VL and also discussed the role of macrophage in VL manifestation, which includes involvement of macrophage inflammatory status during parasite entry and infection, participation of different macrophage receptors including TLR4 and *L. donovani* surface molecules such as lipophosphoglycan (LPG) and gp63 for parasite internalization and alteration of macrophage functions for the establishment of infection. Moreover, this chapter also discussed about the potential of heterocyclic organic compounds such as imidazole and its imidazo[1,2-α] pyridine derivatives as potent anti-leishmanial agents.

Chapter III discussed elaborately about details of all biological reagents, cell lines, and animals used in this study. Further, this chapter also describes the experimental and statistical procedures that were employed in this study.

Chapter IV represents experimental evidences to explore the mechanism involved in *L.donovani* promastigote internalization through LPG-TLR4 interaction and the identification of specific molecular patterns which facilitates this process. To investigate the mechanism of *L. donovani* entry inside the macrophages, we have found that the parasites LPG interacts with the macrophage TLR4 leading to parasite uptake. Increased burden of parasites within the macrophages strikingly

inhibits LPS-mediated NF-κB activation and pro-inflammatory cytokines gene expression. Silencing of macrophage TLR4 or blocking of parasite LPG markedly prevents parasite load within the macrophages. Interestingly, we observed a significant enhancement of macrophage migration, and generation of reactive oxygen species (ROS) in the parasite-infected TLR4 silenced macrophages, whereas parasite infection in TLR4 overexpressed macrophages exhibited a notable reduction of macrophage migration and ROS generation. Moreover, mutations at the leucine-rich repeats (LRRs) particularly LRR5 and LRR6 significantly prevent TLR4 interaction with LPG thus inhibiting intra-macrophagic parasite load. Collectively, our study revealed that *L. donovani* through its LPG interacted with the LRR5/LRR6 of macrophage TLR4 that facilitates parasite entry and favors parasite burden in macrophages and promotes macrophage dysfunction.

Chapter V evaluated experimental evidences about the organ specific role of TLR4 on establishment of *L. donovani* infection in liver, and for this, we prepared BALB/c mice model and found that LPS stimulation markedly increased parasitic burden in liver, whereas, depletion of TLR4 expression and activity by TLR4 antisense oligonucleotide (ASO) and Cli-095, respectively, notably modulates parasite burden in liver which associated with the alteration of inflammatory cytokine expression and signaling pathways. Interestingly, we have found that increased expression of AHSG in the liver upon *L. donovani* infection. While systemic TLR4 silencing or inhibition of TLR4 activity considerably reduced AHSG expression, the stimulation of TLR4 by LPS notably increased AHSG levels in liver. The observation suggests that hepatic AHSG expression possibly associated with *L. donovani* infection establishment. To examine further, we noticed that knockdown of AHSG in macrophages significantly suppressed parasite number with increased ROS levels in macrophages. All these results indicate that macrophage AHSG could able to modulate the survival of *L. donovani* and establishment VL.

Chapter VI displayed the anti-leishmanial potential of imidazo[1,2-α] pyridine derivatives IMPA-2 and IMPA-12. In summary, our study unveiled the efficacy of

IMPA derivatives, particularly IMPA-2 and IMPA-12, for their anti-proliferative and pro-apoptotic properties against *L. donovani* promastigotes. Both IMPA-2 and IMPA-12 are significantly induced oxidative stress and apoptosis hindering promastigote growth inside host macrophage cells. Evidence also pointed that IMPA-2 is more proficient in inhibiting parasite growth, ROS induction, mitochondrial dysfunction, cell cycle inhibition at G0/G1 phase and promoting apoptosis as compared to IMPA-12. Thus, IMPA-2 and IMPA-12 could be used as potential therapeutics against visceral leishmaniasis.

Chapter VII presents the conclusion of the thesis work. The chapter also highlighted some future prospects of the present study.