CHAPTER- I

INTRODUCTION

1. Introduction

1.1 Leishmaniasis:

Leishmania belongs to Trypanosomatidae family, is an obligate protozoan parasite that transmitted to humans by sandflies *Phlebotomus* in the Old World and *Lutzomyia* in the New World [1]. Leishmaniasis is considered as the second most fatal and chronic parasitic disease after malaria and is classified by the World Health Organization as a neglected tropical disease [2]. Leishmaniasis geographically divided into the Old World and New World. The Old World, refers to the Eastern Hemisphere, includes Asia, the Middle East, Africa, and Southern Europe and the New World refers to the Western Hemisphere specifically Mexico, Central America, South America, and the USA [1]. The symptom of the disease has been characterized by irregular and repetitive bouts of fever, severe anaemia, muscular atrophy and excessive swelling of the spleen and liver [3]. Major Ronald Ross, who coined the term *Leishmania donovani*, eventually discovered the link between this organism and kala-azar [4]. Difference in *Leishmania* species can cause disease severity which ranges from self-healing cutaneous infection (Cutaneous Leishmaniasis) to a lethal visceral infection (Visceral Leishmaniasis) **(Table. 1.1)** [5].

Disease pattern	Old World species	New World species	Symptoms, exam, lab findings
Visceral leishmaniasis	*L. donovoni, L. infantum, L. tropica*	*L. chagasi* (identical species to *L. infantum* but in New World), *L. amazonensis*	Fever, weight loss, fatigue, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia
Post-kala azar dermal leishmaniasis	*L. donovoni, L. infantum*	*L. chagasi* (identical species to *L. infantum*)	Skin lesions (always starts on face) 6 months following VL
Cutaneous leishmaniasis	*L. tropica, L. major, L. aethiopica, L. infantum, L. donovani*	*L. mexicana* species complex, *L. mexicana, L. amazonensis, L. venezuelensis, L. Vianna (V) subgenus, L. (V) braziliensis, L. (V) panamensis, L. (V) guyanesis, L. (V) peruviana, L. major* like organisms, *L. chagasi*	Skin lesions on extremities and face:Painless papules which progress to nodules then ulcers
Leishmaniasis recidivans	*L. tropica, L. major*	*N/A*	Satellite lesions around prior ulcer site difficult to treat and may relapse
Diffuse cutaneous leishmaniasis	*L. aethiopica*	*L. mexicana, L. amazonensis, L. panamensis (rarely)*	Diffuse, anergic skin lesions with non-ulcerative nodules and plaques progressing from primary lesion. Rare but more common in immunocompromised individuals
Disseminated leishmaniasis	N/A	*L. Vianna subgenus, L. (V) braziliensis* (most common), *L. amazonensis*	Noncontiguous pleomorphic lesions in immunocompetent hosts. Difficult to treat
Mucosal leishmaniasis	*L. tropica, L. major, L. donovani, L. infantum*	*L. Vianna (V)* subgenus, *L. (V) brazilensis, L. (V) panamensis, L. (V) guyanesis, L. (V) peruviana, L. amazonensis*	Nasal secretions, nasal obstruction, pain, epistaxis. Destructive lesions in nose, oropharynx. Initially involves nose and mouth, can progress to include pharynx and larynx

Table 1.1: Clinical forms of different *Leishmania* species and their geographical distribution. Adapted from John E. Bennett, RD MBJB. Mandell, Douglas, and Bennett's principles and practice of infectious diseases: 8th ed. Philadelphia: Elsevier/Saunders, 2015.

1.2 Epidemiology of visceral leishmaniasis:

Visceral leishmaniasis (VL) also known as "Kala-azar" is one of the fatal forms of leishmaniasis. Causative agent of VL is primarily *Leishmania donovani*, which mainly affects the mononuclear phagocytic cells of visceral organs like spleen, liver and bone marrow cells in human. It is still treated as a "neglected tropical diseases" because of lacking interest in bringing therapeutic interventions by pharmaceutical companies and researchers globally and it is prevalent in developing countries till date [6]. Moreover, in most affected regions inaccessibility of health care center and the most expensive nature of recommended drugs lead to more fatality from this disease [7]. Reports of the World Health Organization (WHO), displayed that almost 13,000 cases of VL occurred in 2020. It is endemic in 70 countries which spread in almost all continents, except Antarctica and Australia, with an estimated at-risk population of 200 million people. However, VL is widely disbursed in seven countries, such as, Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan, where more than 90% of the worldwide VL cases are reported. Death rate due to VL is near about 50,000 annually and more than 500,000 new cases reported annually, which is a matter of concern globally [8]. In three main endemic areas, the number of cases in Eastern Africa showed increased pattern from 2015 to 2016,and in the Indian subcontinent it showed a gradual decreasing pattern in this two-year period, while VL cases in Brazil remained unchanged in 2015 and 2016 [9]. Since 1992, after years of accelerated programme implementation, the number of kala-azar cases in India has dropped by 97%. Fatalities have fallen from 1419 in 1992 to 58 in 2018.The four states of India that is endemic for kala-azar are: Bihar (33 districts, 458 blocks), Jharkhand (4 districts, 33 blocks), West Bengal (11 districts, 120 blocks) [2].

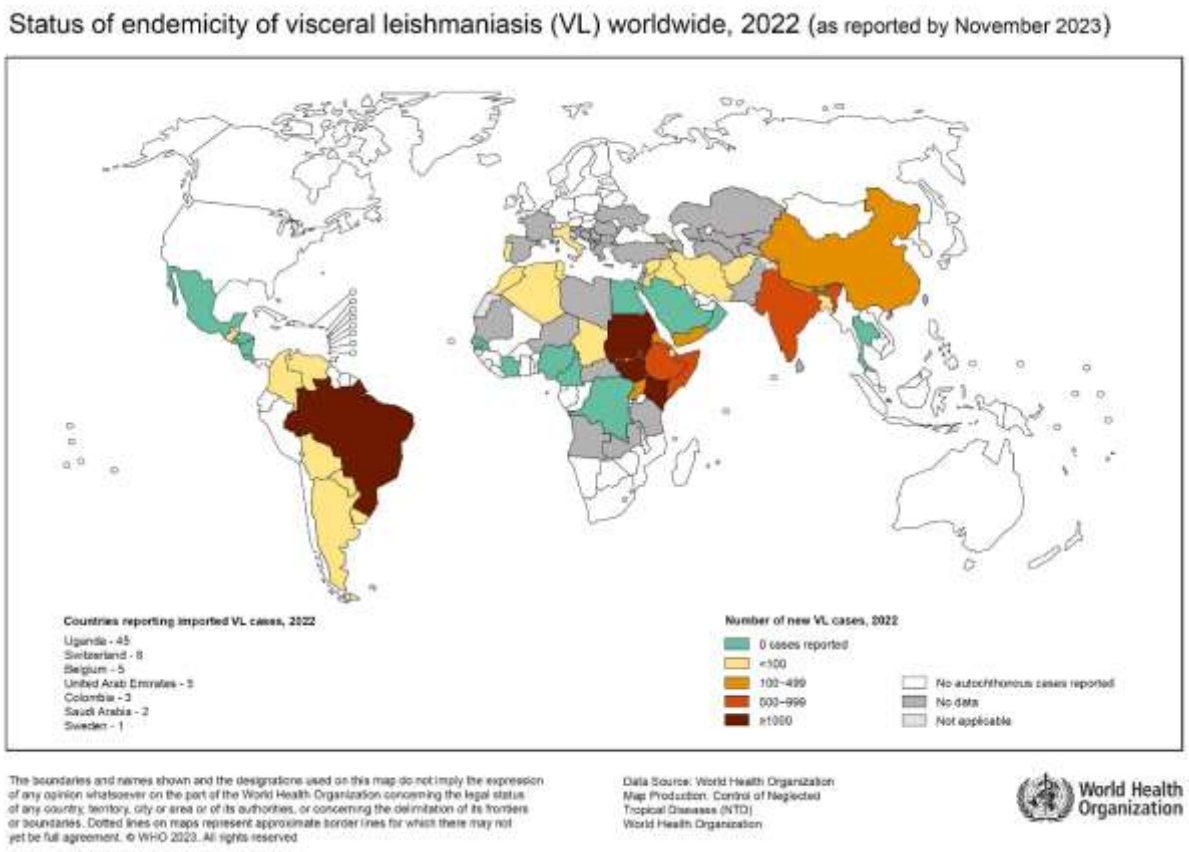

Fig. 1.1: World map showing VL endemicity globally. Source (WHO, 2023)

1.3 Transmission biology and life cycle of *Leishmania donovani*:

Among the different species of sand fly responsible for the transmission of leishmaniasis, *Phlebotomus argentipes* is the responsible vector for transmitting VL or "kala-azar". The sand fly *Phlebotomus argentipes* is found in the Indian sub-continent and is known for carrying protozoan parasite *Leishmania donovani*, causative agent for VL, is a major public health concern in Bangladesh, India, and Nepal [10]. The female sandfly is a haematophagous, silent and 2-3mm long arthropod that effectively transmits *L. donovani*. There are two major morphological forms of *L. donovani*, one is extracellular form which resides in the sandfly midgut as promastigotes, and the other form is intracellular amastigote form which harbors in macrophage cells of the mammalian host [11]. *L. donovani*, transmission in the Asiatic and African regions occurs via anthroponotic cycles, in which infected humans represented as reservoirs [12].

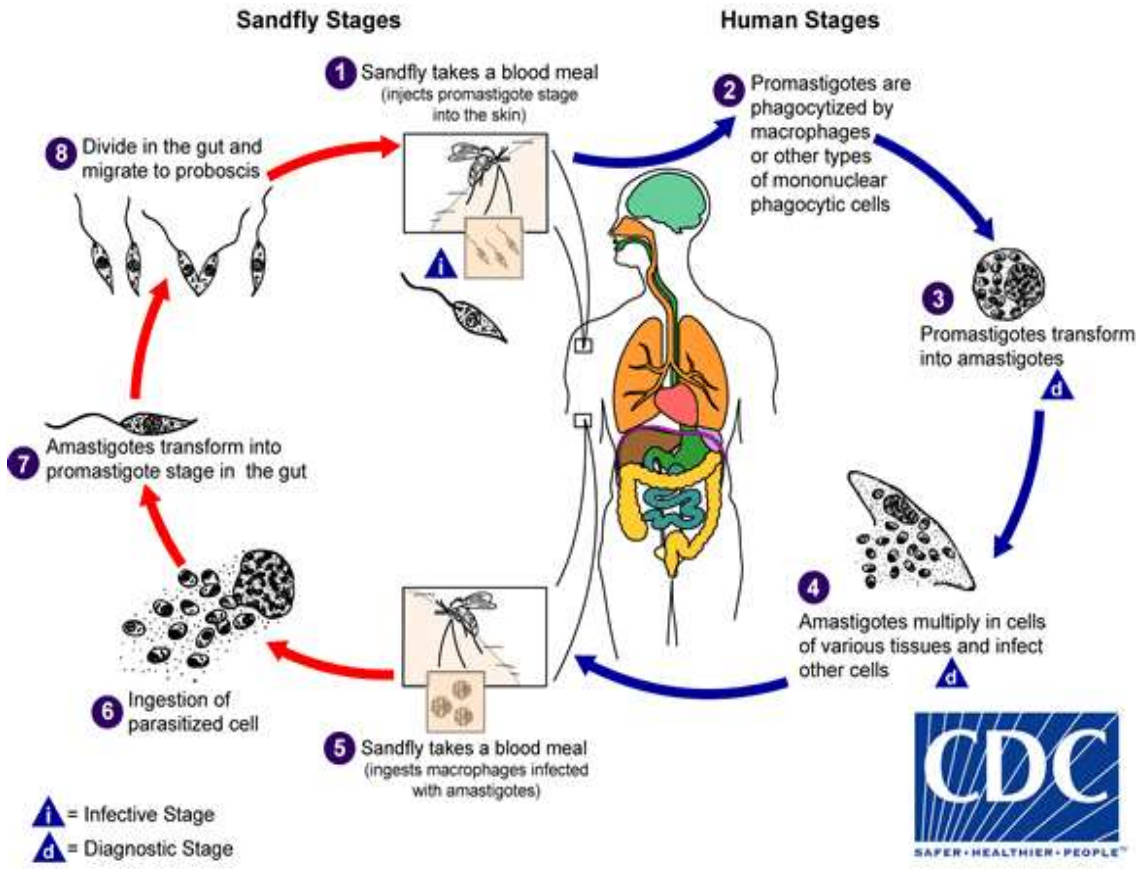

Fig 1.2: Infection and life cycle of *Leishmania donovani.* Source: CDC.

1.4 Role of macrophage inflammatory status in visceral leishmaniasis:

Macrophage cells are the sentinel of innate immune system and acts as first line of defense to the body against intruding pathogens [13]. Macrophages are considered as phagocytic cells which are mainly responsible for the management of pathogens, and in case of adaptive immunity these cells are key regulators of recognition, processing, and antigen presentations to T cells [14]. The M1 state of macrophage termed as classically activated macrophage is a pro-inflammatory subtype that displays microbicidal properties [15]. Whereas, M2 state of macrophage, termed as alternatively activated macrophage, is an anti-inflammatory/regulatory subtype that helps in the resolution of inflammation and tissue repair [16]. The polarization of macrophages of M1 and M2 phenotypes in leishmaniasis is dependent on the signals provided by the microenvironment [17]. Switching between M1 and M2 phenotypes of macrophages and their plasticity decides the fate of *Leishmania* infection. Macrophage plays dual role in leishmaniasis, while it is responsible for

the clearance of internalized parasites but also acts as a protective niche for *Leishmania* replication, which is typically an outcome of Th1/Th2 paradigm dependent upon species of *Leishmania* and immune response magnitude of host [18,19].

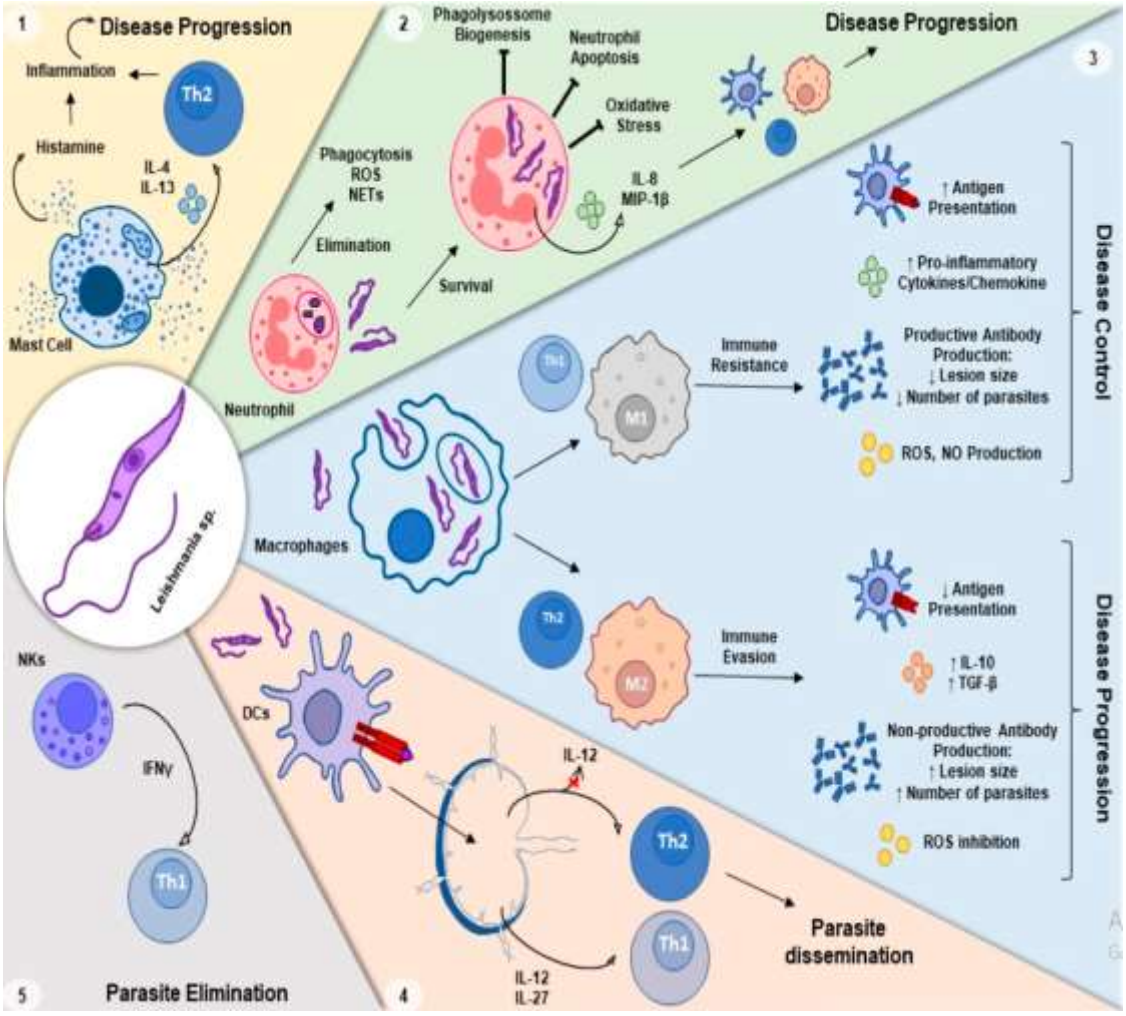

Fig 1.3: Macrophage activation in response to leishmaniasis. Source: The figure was adopted from Costa-da-Silva, A.C., et al, Immune responses in leishmaniasis: an overview. *Tropical medicine and infectious disease*, *7*(4): 54, 2022.

Activation of M1 macrophages by Th1 lymphocytes mainly produced interferon gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). These two primary cytokines plays a crucial role in intracellular parasite clearance and oxidative stress mediated killing of parasites in macrophages as a protective and inflammatory measure [14,19]. Conversely, Th2 lymphocytes activation produces two key

cytokine modulators, IL-4 and IL-13, which induces the M2 profile determined by synthesis of polyamines via arginase activation and production of urea and L-ornithine, which favours intra-macrophagic parasite survival and proliferation leading to systematic disease progression [19,20]. Pathogenic role of IL-4 is widely studied in *L. donovani* and *L. major* mediated manifestation and establishment of leishmaniasis in both *in-vivo* and *in-vitro* [21]. Previous reports denoted that, IL-4 pre-incubation leads to switching of macrophage phenotype from efficient phagocytosis to reduced phagocytic activity in *N meningitidis* infection via downregulated Akt phosphorylation and MAPK signaling pathway [22]. In *Trypanosoma cruzi,* IL-4 treatment elevated the uptake and induced intracellular parasite killing [23]. Research evidences already showed that, IL-4 neutralization during early period of infection resulted into complete abolition of IL-4 producing CD4+ T cells and decrease of IFN-γ producing CD8+ T cells in *L. major* infected BALB/c mice. Altogether, these T cells mediated responses induce *L. major* parasite clearance and enhancement of immunopathology [22].

Lipopolysaccharide (LPS) is widely known as the ligand for TLR4 receptor activation and its downstream pro-inflammatory signaling cascade activation [24]. TLR4 receptor governs the intracellular proliferation of *L. donovani* promastigotes. LPS mediated activation of TLR4 through NF-κB transcription factor stimulation upregulates the expression of various defensive pro-inflammatory mediators like TNF-α and IL-1β, but parasites can evade this killing potential of pro-inflammatory Th1 cytokines by giving an upper hand to Th2 cytokines such as IL-4, IL-13 and T_{reg} cytokines IL-10 and TGF-β for successful propagation and infection establishment of parasite through TLR4 receptor mediated entry [25]. Research reports displayed that, type I interferon signaling plays a crucial role in regulating parasite growth in host macrophage cells by balancing the CD4+ T cells population in immunopathological scenario of VL [26]. Thus, priming effect of LPS induces primary tolerance to TLR4 receptor and facilitates enhancement of intracellular parasite load through secondary effects of TLR4 stimulation through induction of type I interferons [27]. Interplay between type I interferons, IFN-α, IFN-β and IFN-γ decides the fate of parasite clearance or parasite growth in macrophages. Reports suggested that in *L. infantum* mediated VL, TLR4 signaling pathway negatively regulates anti-parasitic immune response through IRF1 promoting IFNβ production

which hinders Th1 pro-inflammatory response favoring IL-10 upregulation and disease progression [28]. However, infection-mediated by this parasite did not prevent M1 polarization, as evidenced by the ability of infected macrophages to produce inflammatory cytokines and glycolysis in response to IFN-γ and LPS stimulation. This may imply that macrophages may still actively react to an external stimulus even when they are infected. As a result, inhibiting macrophage cytokine responses aids in the establishment of a chronic infection, providing new opportunities for further research for developing newer therapeutic approaches [29]. In case of *T. brucei* infection, type I IFNs plays a significant role in controlling parasite in early phase but it results into the downregulation of IFNγ production leads to the chronicity of infection [30]. *L. donovani* infection is known to produce micro-vesicles, called exosomes, which attenuates the production of pro-inflammatory cytokine TNF-α and induces the generation of anti-inflammatory cytokine IL-10, that resulted into the impaired IFN-γ signaling [31]. Homeostasis between the Th1 and Th2 cytokines mainly controls the whole scenario of *L. donovani* mediated leishmaniasis progression or its elimination. However, the *L. donovani* parasites entry into the macrophages in its inflammatory state is still a mystery to solve as species-specific variation of virulence proteins and their role in impairment of parasite killing mechanism and macrophage polarization plays crucial aspects in determining the fate in VL disease manifestation.

1.5 Role of TLR4 receptor in visceral leishmaniasis:

Till date, there are 13 different mammalian TLRs identified and among them TLRs 1–9 are conserved between humans and mice [32-35]. Interestingly, TLR10 found to be active in humans but different at the C-terminus in the mouserendering it inactive. Whereas, TLR11 is operative in mouse but dysfunctional in human. TLRs can be divided into extracellular: TLR1-2, TLR4-6, and TLR11 or intracellular: TLR3, TLR7-9 and TLR13, and these receptors recognize specific family of ligands either at the cell surface or in the endosomal compartment, consequently [32-35]. TLRsare belongs to the type I integral membrane receptors, consisting of N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain [36]. Extracellular domains of all TLRs copies of repeated motif known as the leucine-rich repeats (LRR), consisting of 22–

29 residues in length and hydrophobic residues spaced at intervals [37,38]. A specific TLR such as TLR4 mainly involved in the recognition of bacterial lipopolysaccharide (LPS). Several LRRs present in different TLRs contain extracellular domain which encapsulated by LRR-NT and LRR-CT motifs [39].

TLRs are considered as a class of pattern recognition receptors (PRR) predominantly expressed in cells of the innate immune system and are crucial for recognition of motifs displayed by pathogens called pathogen associated molecular pattern (PAMPs). The specific PAMP present in the surface of *Leishmania* plays a pivotal role for their entry and internalization-mediated altered macrophage functions in leishmaniasis [40]. In context of importance of TLR4in VL, previous findings denoted that TLR4 facilitates TGF-β1-regulated induction of SHP-1 and ubiquitin-edited enzyme A20 which promotes *L. donovani* infection. Human THP-1 derived macrophages when infected with *L. donovani* promastigotes, suppression of TLR2-stimulated IL-12 release was evident with increased production of IL-10, resulted from the inhibition of p38 MAPK phosphorylation and activation of ERK1/2 phosphorylation, favoring intracellular proliferation of parasite and disease establishment. Additionally, it was found that *L. donovani* and *L. major* infection modulated macrophage SHP-1 to attenuate different kinases including IRAK, that involved in downstream TLR signaling [41]. In *L. major* mediated leishmaniasis, TLR4 displayed a protective role for the control of infection, and this findings was validated by in-vivo studies in TLR4−/− mice suggesting that TLR4 govern the fate of infection [42,43]. *Leishmania* developed strategies to amend TLR4 signaling pathways in favor of infection establishment. *L. major* attenuates TLR4 activation through its inhibitors of serine protease (ISP) to hinder NE regulated, inhibition of *Leishmania* uptake and killing by macrophages [44,45]. 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 [46]. In contrast, TLR4-deficient mice of C57BL/6 mice are resistant to *L.*

panamensis infection than wild-type counterparts [47]. 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-κB, AP1, and IRF3 which upregulate the expressions of various pro-inflammatory cytokines and type I interferons which crucially linked in controlling bacterial infection [48]. In *L. donovani* mediated visceral leishmaniasis, the mechanisms involved in parasite internalization and macrophage dysfunction through TLR4 receptor is still unclear and needs to be investigated for better therapeutic insight for the identification of novel therapeutic target against *L. donovani* mediated VL.

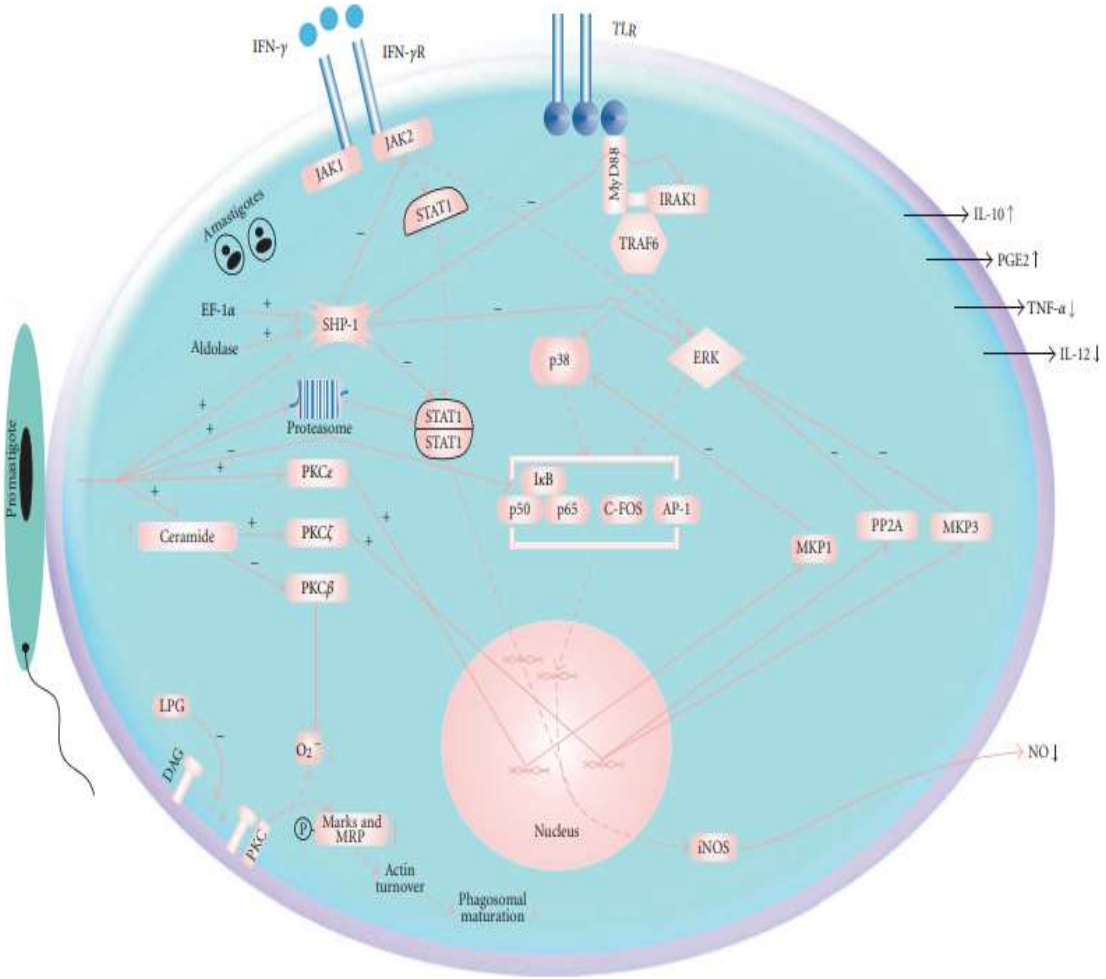

Fig 1.4: Alteration of host macrophage defense signaling pathways by *L. donovani*. Source: Image adopted from Shadab, M., et al, Evasion of host defense by *Leishmania donovani*: subversion of signaling pathways. *Molecular biology international*, 2011.

1.6 Involvement of *L. donovani* lipophosphoglycan (LPG) in visceral leishmaniasis:

L. donovani promastigote contains stage-specific surface glycoconjugate such as lipophosphoglycan (LPG), which plays an essential role for the establishment of infection inside host macrophages [49]. LPG consists of a repeating polymer of Gal1,4Man-PO4 that attached to a membrane anchor glycophosphatidylinositol. During phagocytosis, LPG is transported from the parasite to the host macrophage membrane and causes accumulation of peri-phagosomal F-actin that prevents phagosome-lysosome fusion. Reports suggested that, LPG incorporates into the membrane lipid rafts of host macrophage cell membrane attenuating phagosomal maturation [49]. LPG, in accordance with the gp63, activates the complement system which facilitates the generation of the C3b and C3bi opsonins. The C3b and C3bi are main components that attaches to the parasite surface and mediate the parasite phagocytosis by complement receptor (CR)-1 and CR-3 [50]. Phagocytosis of *Leishmania* mediated by CR1 and CR3 receptors is considered as "silent" mechanism in the host macrophages as it has no effect on the activation of host oxidative burst mechanism and thus IL-12 production [51-54]. LPG of *L. donovani* metacyclic promastigote is known to mediate phagocytosis through its interaction with CRP receptor of host macrophages [55]. Studies also displayed that, CRP receptor mediated *L. donovani* promastigote phagocytosis facilitates defective activation of macrophages, resulting into parasite survival and replication by modulation of host defensive attributes [56]. Moreover, it has also been highlighted that LPG is crucially responsible for the alteration of host immune activation by inhibiting phagosome-lysosome maturation, NADPH oxidase assembly and upregulating IL-10 and TGF-β expression which ultimately leads to the persistence of parasite survival through arginase-1 induction without hampering host microenvironment. It has been implicated that the LPG molecule in promastigote plays a pivotal role in the establishment of macrophage infection as *lpgl* defective mutant *Leishmania* was unable to infect mice and establish the infection in macrophages [57]. *L. donovani* LPG evidently showed intra-macrophagic parasite survival by altering activation of defensive pro-inflammatory cytokines via TLR4 or TLR2 receptor binding on the host macrophages. The LPG-TLR interactions upregulates the ERK phosphorylation with concomitant

downregulation of p38 MAPK phosphorylation, that resulted the induction of reactive oxygen species (ROS) production and generation of nitric oxide preventing pro-inflammatory cytokines secretion [58-63]. Research studies in this direction intrigued us to examine the exact role of *L. donovani* lipophosphoglycan (LPG) for parasite internalization and alteration of macrophage function and the involvement of macrophage TLR4 therein, as their involvement of both LPG and TLR4 in *L. donovani* mediated VL is still elusive due to the polymorphisms, species diversity, and lack of information related to specific molecular motifs involved in parasite uptake in macrophages. Therefore, detailed investigation in this direction could lead us to the identification of specific molecular motifs present in TLR4 of host macrophage and the LPG of *L. donovani* parasite. Moreover, LPG and their involvement in parasite uptake and immune inactivation of host macrophages could provide a novel therapeutic target to mitigate *L. donovani* mediated VL.

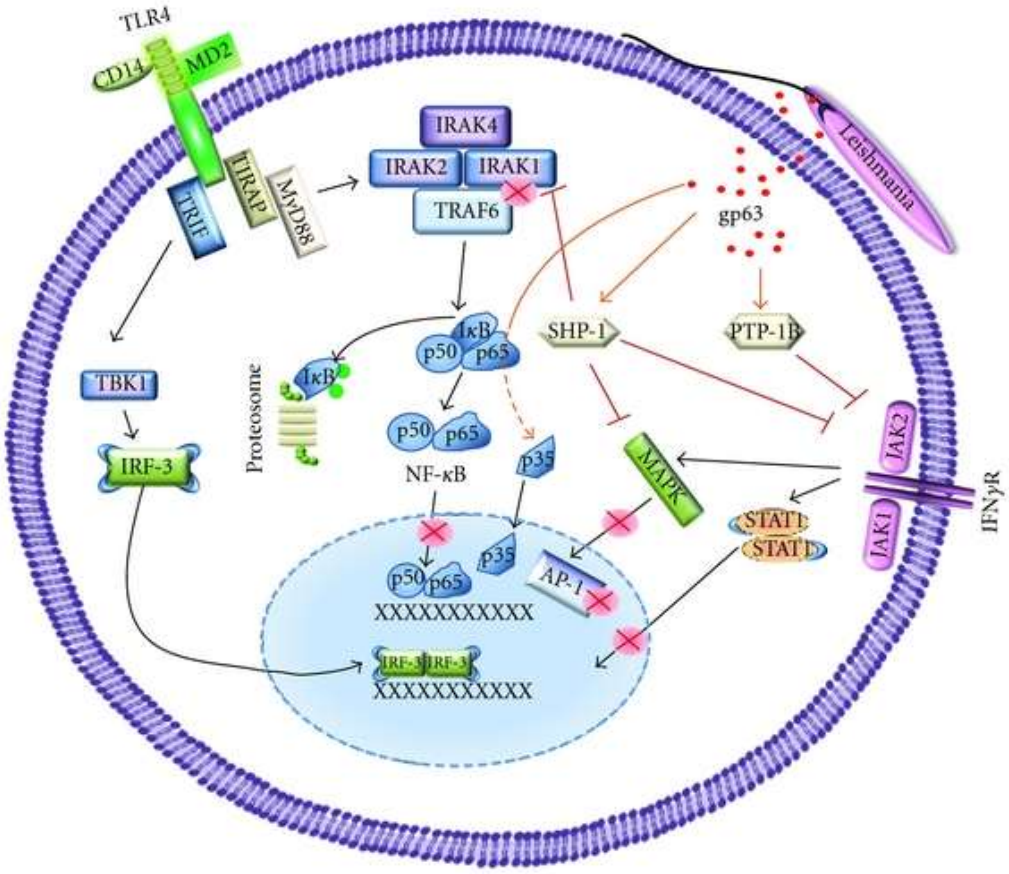

Fig 1.5: LPG mediated downregulation of macrophage signaling pathways in *Leishmania* infection. Source: Shio, M.T., et al, Host cell signaling and leishmania mechanisms of evasion. *Journal of tropical medicine,* 2012.

1.7 Therapeutic potential of imidazo[1,2-α] pyridine derivatives as anti-leishmanial agents:

The first-line and second-line of marketed anti-leishmanial drugs comes up with severe side effects like drug resistance, multiorgan toxicity and prolonged treatment regime.It has been well established that many commercially available anti-cancer drugs contain heterocycles as their key structural component [64]. In recent years, synthetic organic compounds such as, heterocyclics gained popularity among researchers for managing leishmaniasis as safer and improved therapeutically potent drug candidates. As potent anti-leishmanial agents, many heterocyclic motifs have been investigated in recent years for their therapeutic potential [65-70]. Imidazole moiety is a member of heterocyclic organic compound that already known for their medicinal applications as anti-microbial, anti-proliferative, anti-tumor, and anti-cancer properties [71,72]. The heterocyclic organic moieties such as triazoles, chalcones, chromone, thiazoles, thiosemicarbazones, indole, imidazole and quinolines are gained interest among the researchers for finding effective therapeutic alternatives with lesser side effects in managing VL [73]. Imidazopyridine moiety is well established for containing biologically functional nitrogen heterocycle in its structural component [74]. Among several imidazopyridine derivatives, particularly imidazo[1,2-α] pyridine already known for its anti-proliferative properties [75]. The coupling of imidazo[1,2-α] pyridine with 2-amino-4H-pyran moiety generates various derivatives which exhibited profound effect in inducing apoptosis in non-small cell lung cancer (NSCLC) [76]. Additionally, reports also denoted that imidazo[1,2-α] pyridine used as a potential core pharmacophore for mitigating leishmaniasis [77-80]. Precisely, 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 derivatives [81,82]. All these reports unveiled the therapeutic importance of heterocyclic compound imidazole and its imidazopyridine derivatives as these could provide us an improved therapeutic intervention to mitigate a complex disease like VL in near future.

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