Immunomodulation of the STAT6 pathway for treatment of Visceral Leishmaniasis

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ABSTRACT

Visceral Leishmaniasis (VL) is a chronic, life-threatening parasitic disease that mostly affects the impoverished populations of the world. Currently, there are no licensed antileishmanial vaccines for human use and existing therapies are quite toxic. Miltefosine is the only oral antileishmanial drug but its long half-life makes it vulnerable to the development of resistance. Combining an immunomodulatory agent with miltefosine could potentially help to alleviate this problem. For this study, we employed an immunomodulatory peptide called signal transducer and activator of transcription 6 inhibitory peptide (STAT6-IP). The STAT6 signalling pathway is essential for the development of Th2-type immunity and STAT6-IP has been shown to have significant benefit in allergic and infectious conditions associated with aberrant Th2 responses. In these studies, we combined STAT6-IP with a sub-curative dose of miltefosine for treatment of *L. donovani*-infected BALB/c mice. Since this parasite induces a Th2 pattern response to ensure its survival, our hypothesis was that inhibition of the parasite-driven Th2 response cytokines would permit a more effective (Th1-type) anti-parasitic response.

Although infected animals treated with STAT6-IP alone had reduced parasite burden comparable to that observed in the group treated with sub-curative miltefosine, there was no synergistic effect in animals treated with both STAT6-IP and miltefosine. Anti-leishmanial antibody titres were similar in all groups but IgG2a concentrations were higher in the combined treatment group suggesting at least some degree of modulation towards a more balanced Th1/Th2 response. However, this difference was not statistically significant and did not translate into a reduction in parasite burden. Surprisingly, the groups that received either STAT6-IP or miltefosine alone had similar levels of IgG1 and IgG2a. These results suggest that the immunomodulatory effects of STAT6-IP are insufficient to influence the Th1/Th2 balance in a clinically-significant way in the murine model of *L. donovani* infection.

RÉSUMÉ

La leishmaniose viscérale (LV) est une maladie parasitaire chronique menaçant le pronostic vital, qui touche principalement les populations pauvres du monde. À l'heure actuelle, il n'existe aucun vaccin antileishmanien homologué à usage humain et les traitements existants sont assez toxiques. La miltéfosine est le seul médicament antileishmanien oral, mais sa longue demi-vie la rend vulnérable au développement de résistances. La combinaison d'un agent immunomodulateur avec de la miltéfosine pourrait potentiellement contribuer à atténuer ce problème. Pour cette étude, nous avons utilisé un peptide immunomodulateur appelé transducteur de signal et activateur du peptide inhibiteur de la transcription 6 (STAT6-IP). La voie de signalisation STAT6 est essentielle au développement de l'immunité de type Th2 et il a été démontré que STAT6-IP avait un effet bénéfique significatif sur les affections allergiques et infectieuses associées aux réponses Th2 aberrantes. Dans ces études, nous avons associé STAT6-IP à une dose sous-curative de miltéfosine pour le traitement des souris BALB / c infectées par *L. donovani*. Comme ce parasite induit une réponse de type Th2 pour assurer sa survie, notre hypothèse était que l'inhibition des cytokines à réponse Th2 induite par le parasite permettrait une réponse antiparasitaire plus efficace (de type Th1).

Bien que les animaux infectés traités avec STAT6-IP seul aient une charge parasitaire réduite comparable à celle observée dans le groupe traité avec de la miltéfosine sous-curative, il n'y a pas eu d'effet synergique chez les animaux traités à la fois avec STAT6-IP et la miltefosine. Les titres en anticorps anti-leishmaniens étaient similaires dans tous les groupes, mais les concentrations en IgG2a étaient plus élevées dans le groupe sous traitement combiné, suggérant au moins un certain degré de modulation vers une réponse Th1 / Th2 plus équilibrée. Cependant, cette différence n'était pas statistiquement significative et ne s'est pas traduite par une réduction de la charge parasitaire. De manière surprenante, les groupes recevant soit STAT6-IP, soit la miltéfosine seule avaient des taux similaires d'IgG1 et d'IgG2a. Ces résultats

suggèrent que les effets immunomodulateurs de STAT6-IP ne suffisent pas pour influencer la balance Th1 / Th2 de manière cliniquement significative dans le modèle murin d'infection à *L. donovani*.

DEDICATION

This thesis is dedicated to my family and friends for their constant support and encouragement through the challenges of this program. You all remain dear in my heart.

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CONTRIBUTION OF CANDIDATE

All experiments were designed and performed by the author under the supervision of Dr. Brian Ward and co-supervision of Dr. Momar Ndao. The author also performed all data analysis and wrote this thesis with editorial contributions from Dr. Brian Ward.

1 CHAPTER 1: INTRODUCTION

Visceral Leishmaniasis (VL; commonly known as kala-azar) is a vector-borne chronic infectious disease caused principally by *Leishmania donovani* and *L. infantum* (synonym *L. chagasi* in South America)[1]. In natural vertebrate hosts (e.g. humans, dogs, some rodents), these *Leishmania* species spread systemically to propagate in macrophage reservoirs distributed in tissues of internal organs, primarily the liver, spleen, bone marrow and lymph nodes[2]. The clinical presentation of VL typically involves long-term, low-grade fever, enlarged spleen and liver, anaemia, weight loss, pancytopenia and hypergammaglobulinemia. Hypoalbuminemia seen in VL is associated with oedema and other features of malnutrition. Diarrhoea may also occur due to intestinal parasitization and ulceration. With time, untreated VL can cause severe cachexia and bleeding due to thrombocytopenia[3]. In the absence of treatment, more than 95% of VL cases leads to death[4].

Control of VL relies almost exclusively on chemotherapy as no antileishmanial vaccines in clinical use[5]. Few treatment options are available (pentavalent antimonials, amphotericin B and its lipid formulations, oral miltefosine, pentamidine and paromomycin) but most of them have serious shortcomings, including toxic side effects, parenteral administration, hospitalization, length of treatment (weeks to months), high cost and susceptibility to the development of resistance[6]. As a result, attention has more recently turned towards the use of combination therapy for VL. Combination therapy has the potential to shorten treatment duration, reduce cost and preserve the therapeutic efficacy of the respective drugs as has been demonstrated for diseases like malaria, HIV and tuberculosis[7]. Although there are no reports of resistance to antileishmanial drug combinations in human studies, recent animal studies raise the possibility that even combination therapies can select for resistant *L. donovani* strains under experimental conditions[8,9]. In this regard, efforts are underway to develop novel antileishmanial therapies by combining immunomodulatory agents that boost host immune responses with leishmanicidal

drugs[2]. Employing immunotherapeutic agents may be particularly useful in VL because these patients often have depressed immune function and any agent that augments the immune responses may be of clinical importance.

Leishmania parasites are able to survive in their mammalian hosts by manipulating key signalling pathways involved in the ability of macrophages to kill pathogens or to engage with the adaptive immune system[10]. One of such pathways is the Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT). The JAK-STAT signalling pathways are major mediators of the effects of cytokines on immune cells and therefore play a large role in the orchestration of immune responses to infectious challenges. The STAT family is comprised of seven genes that code for STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, many of which have been shown to play a role in immunity to/or pathogenesis of various species of *Leishmania*[11].

Among the STAT molecules, STAT6 may play a particularly important role in *Leishmania* infection since STAT6 signalling mediates the biological activities of IL-4 and IL-13, Th2-type cytokines that have been associated with disease progression in leishmaniasis[12]. Macrophages exposed to IL-4 and IL-13 fail to produce the microbicidal molecules required for parasite killing[13]. Elevated levels of IL-4 and IL-13 has been observed in the serum of VL patients[14]. Experimental studies in hamsters have also reported increased concentrations of IL-4 and IL-13 in the serum of hamsters with active VL disease[15]. In the same study, miRNAi-mediated knockdown of STAT6 in BHK cells controlled parasite replication, indicating a clear role for this signalling pathway in the pathogenesis of VL. In light of these observations, we considered that immunological interventions designed to inhibit STAT6 signalling pathway might be effective in VL.

STAT6-inhibitory peptide (STAT6-IP) is a chimeric peptide developed in the Fixman laboratory at McGill University that has been used to modulate both allergic and infectious conditions associated with aberrant Th2-type immune responses (eg: RSV challenge-rechallenge, formalin-inactivated RSV vaccination, house dust mite-induced allergy)[16,17]. This inhibitory peptide was designed to act as a dominant negative inhibitor of STAT6 by binding to its SH2 domain and preventing dimerization of STAT6, thereby reducing the production of Th2-type cytokines like IL-4 and IL-13[18,19]. Since IL-4 and IL-13 also play a role in the immunopathogenesis of human VL, we decided to investigate the therapeutic potential of STAT6-IP alone and in combination with an antileishmanial drug for treatment of *L. donovani* infection in susceptible BALB/c mice.

As the only oral drug available for treatment of VL, miltefosine was the obvious choice for this study due to its ease of administration and minimal side effects[20]. To date, its long half-life and long treatment course, potential teratogenic effect in pregnant women and susceptibility to the development of resistance have limited the use of miltefosine as monotherapy[21].

In the present study, we explored the potential combination of STAT6-IP and low-dose of miltefosine for treatment of visceral *L. donovani* infection.

2 CHAPTER 2: MATERIALS AND METHOD

2.1 Animals

Female BALB/c mice were obtained from Charles River Laboratories (Senneville, QC). The mice were bred and maintained at the Glen Animal Facility, Montreal according to the guidelines for animal research. Animal procedures were performed in accordance with institutional Animal Care and Use guidelines and were approved by the Animal Care and Use committee at McGill University.

2.2 Parasites

L. donovani 2134 parasites were obtained from the World Health Organization, Geneva, Switzerland. Parasites were cryopreserved at -80^{0}C and taken out when needed. Parasites were grown in non-vented T75 flasks containing 5 mL RPMI media supplemented with 20 % fetal bovine serum (Sigma Aldrich, Oakville, ON) non-essential amino acids (Wisent, St-Bruno, QC), MEM amino acids (Wisent), 1 mM sodium pyruvate, 2 mg/ml dextrose, 2 mM L-glutamine, 100 u/ml penicillin/streptomycin, and 25 mM HEPES for 7 days at 27 ^{0}C. Parasites were cultured for seven days to obtain infective stage stationary-phase promastigotes required for infection.

In preparing the parasites for infection, seven-day parasite cultures were centrifuged at 400 xg for 10 min at 25 ^{0}C to remove media and then washed twice with phosphate-buffered saline (PBS). A 1:50 dilution was made with formalin and parasites were counted with a haemocytometer under a light microscope at x40 magnification. Parasites were resuspended as 5 x 10^7 *L. donovani* 2134 stationary-phase promastigotes in 100 µL PBS for infection per mouse.

2.3 Preparation of STAT6-IP and Miltefosine

STAT6-IP was a kind gift from Dr. Fixman (McGill University, Canada) while miltefosine was purchased from Sigma-Aldrich, Saint Louis, USA. For *in vivo* treatment, STAT6-IP and miltefosine were dissolved in PBS at 10 mg/mL.

2.4 Mice Challenge and Treatment

For the first drug combination study, 20 BALB/c mice (5 mice/group) were infected through the tail vein with 5 x 10^7 *L. donovani* 2134 stationary-phase promastigotes. One week after infection, treatment was administered. Animals in the uninfected and the infected untreated group were given PBS alone and served as controls. Animals in the monotherapy groups received either 100 µg of STAT-IP intraperitoneally (IP) or a curative dose of miltefosine (20 mg/kg, oral gavage) while animals in the combined therapy group received both STAT6-IP (100 µg) IP and miltefosine (20 mg/kg). All drugs were prepared in PBS and administered as a single dose daily for five days. All animals were sacrificed one week after treatment.

In a second study, 20 BALB/c mice (4 mice/group) were infected through the tail vein with 5 x 10^7 *L. donovani* 2134 stationary phase promastigotes. One week after infection, drugs were administered as in the first study. Animals in the uninfected and the infected untreated group received PBS alone while animals in the monotherapy groups received either 100 µg of STAT-IP, IP or the sub-curative dose of miltefosine (5 mg/kg) by oral gavage. Animals in the combined therapy group received both STAT6-IP (100 µg) and miltefosine (5 mg/kg). Drugs were prepared and administered as in the first study and animals were sacrificed one week after treatment. In both studies, livers were collected to quantify parasite burden by qPCR.

2.5 *Leishmania* DNA extraction and qPCR for quantification of parasite burden

Prior to DNA extraction, a 25 mg sample was cut from whole livers for quantification of parasite burden. The cut samples were crushed and proteinase K (QIAGEN, Hilden, Germany) was added to lyse the samples overnight at 70^0C. *Leishmania* kinetoplast DNA (kDNA) was extracted from the lysed samples using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Following centrifugation and washing steps, DNA was eluted from the spin columns in a 200 µL elution buffer and stored at -20 °C until use. Real-time PCR reactions were performed using the Light Cycler Fast Start DNA Master HybProbe kit (Roche, Mannheim, Germany). The PCR master mix comprised of 5 µL PCR-grade water, 0.2 µM of each primer, 0.04 µM Leish-P1 TaqMan probe and 4 µL DNA TaqMan Master. 5 µL template DNA (from each sample) was added to 15 µL of the master mix for amplification of the kDNA. Real-time PCR cycling was performed on the Light Cycler 1.5 (Roche) with amplification at 95 °C for 10 min followed by 40 cycles of 95 °C for 5 s, 53 °C for 8 s, and 72 °C for 9 s, with single fluorescence acquisition at the end of each annealing step. An infected mouse DNA sample acted as a positive control while PCR-grade water and an uninfected mouse DNA sample acted as non-template and negative controls, respectively. All controls were included in each run.

2.6 Preparation of soluble *L. donovani* promastigote antigen (SLA)

To prepare soluble *L. donovani* promastigote antigen (SLA), stationary-phase promastigotes were harvested from 7-day parasite cultures, centrifuged at 400 xg for 10 min at 25 °C to remove media and then resuspended in endotoxin-free PBS. Resuspended parasites were subjected to 5 cycles of rapid freeze-thawing (-80 °C and 37 °C) that lasted for 10 min each. Lysed promastigotes were centrifuged at 22 000 xg for 15 min at 4 °C. The protein content of

the supernatant containing soluble antigen was estimated by Bradford assay and stored at -80 °C.

2.7 Measurement of serum antibody levels by ELISA

At sacrifice, blood was collected and serum was obtained from untreated and treated mice. Anti-*Leishmania*-specific antibody IgG1 and IgG2a titers were determined by enzyme-linked immunosorbent assay (ELISA). Serial dilutions of IgG1 and IgG2a standard antibodies (Sigma) were coated on 96-microtiter plates. The remaining wells of the 96-microtiter plates were coated with 500 ng/mL SLA and plates were incubated overnight at 4°C. The next day, coated plates were washed with washing buffer (0.05% Tween 20 (Sigma-Aldrich) in PBS at pH 7.4) and then incubated with blocking buffer (2% bovine serum albumin (Sigma-Aldrich) in washing buffer) for 1 hr at 37°C to block nonspecific binding sites. Serum samples were heat-inactivated by incubating at 56°C for 1 hr, diluted with blocking buffer at 1:50 and incubated at 37°C for 1 hr. Plates were washed four times and then incubated either with Anti-mouse IgG1(Fc specific)- Peroxidase (Sigma) at 1:10000 or with Anti-mouse IgG2a (Fc specific)-Peroxidase (Sigma) at 1:20000 for 30 min at 37°C. After incubation, plates were washed and 3,3,'5,5'-tetramethylbenzidine (Millipore, Billerica, MA) was added before plates incubated again for 15 min at room temperature. Reaction was stopped with sulphuric acid Sigma-Aldrich) and plates were read (using the ELx800 microplate reader, software version 2.04.11) at an absorbance of 450 nm. IgG1 and IgG2a are expressed as concentrations (ng/mL).

2.8 Statistical analysis

Graphs and data comparisons were obtained using GraphPad Prism® Version 8.0 (GraphPad Inc. San Diego, CA) and a value of $P < 0.05$ was considered statistically significant. Results are represented as mean ± SEM (standard error of mean).

3 CHAPTER 3: RESULTS

3.1 Efficacy of Curative Miltefosine combined with STAT6-IP On Parasite Burden

3.1.1 Hypothesis

Combining the curative dose of miltefosine with STAT6-IP will induce leishmanicidal activity and provide an effective treatment option against visceral leishmaniasis.

3.1.2 Objectives

a) Establish an appropriate infection model for visceral leishmaniasis using *L. donovani*
b) Determine if the curative dose of miltefosine is effective in the treatment model
c) Evaluate the combination of curative miltefosine and STAT6-IP for treatment of visceral leishmaniasis caused by *L. donovani*

3.1.3 Results

3.1.3.1 Combination of curative dose of miltefosine with STAT6-IP

Mice infected with *L. donovani* parasites were placed into three treatment groups consisting of five mice receiving 100 µg of STAT-IP intraperitoneally, 20 mg/kg of miltefosine (orally) or a combination of STAT6-IP (100 µg) and miltefosine (20 mg/kg). The curative and sub-curative doses of miltefosine were obtained from previous dose optimization studies carried out in mice[22,23]. Combining STAT6-IP with the curative dose of miltefosine was carried out as a proof-of-concept study to establish and confirm the treatment model. Parasite burden was quantified using q-PCR and the number of parasites per liver was extrapolated from a standard curve. Results are displayed in Figure 3.1. As expected, STAT6-IP alone had no effect and there was a 95% reduction in parasite burden in mice treated with curative miltefosine alone compared to untreated control. Having confirmed efficacy in treatment model, we proceeded to the main study to investigate the effect of combining STAT6-IP with a sub-curative miltefosine dose on parasite burden.

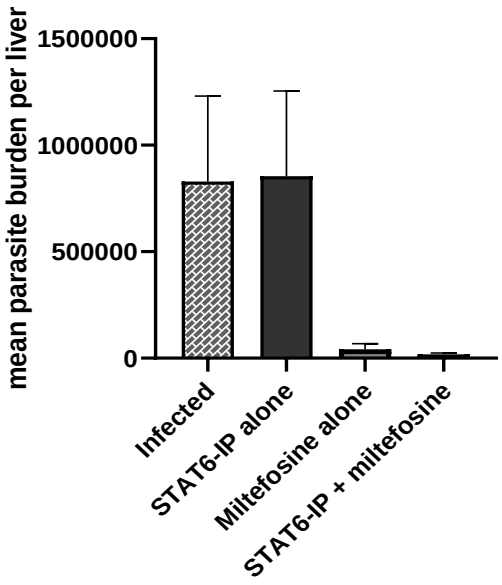

Figure 3.1. Combination therapy with curative miltefosine and STAT6-IP. BALB/c mice were infected with *L. donovani* promastigotes through the tail vein with 5 x 10^7 parasites/animal. One week after infection, mice were treated for 5 consecutive days. Mice were sacrificed one week after treatment was completed and livers were collected to determine parasite burden by qPCR. Percent parasite reduction was calculated by comparing the mean number of parasites in treatment groups to those in infected control animals. Results were analysed by one-way ANOVA.

3.2 Efficacy of Sub-Curative Miltefosine combined with STAT6-IP On Parasite Burden

3.2.1 Hypothesis

The antileishmanial capacity of the sub-curative dose of miltefosine will improve when combined with STAT6-IP.

3.2.2 Objectives

a) Evaluate the effect of combining a sub-curative miltefosine dose with STAT6-IP on parasite burden compared to monotherapy with sub-curative miltefosine

b) Effect of the combination therapy in modulating *Leishmania*-specific antibody responses

c) Determine the immunomodulatory effects of STAT6-IP in visceral leishmaniasis

3.2.3 Results

3.2.3.1 Antileishmanial effect of combination of a sub-curative dose of miltefosine with STAT6-IP

Using the sub-curative dose of miltefosine at 5 mg/kg, this study had the same pattern as the pilot. Results are presented in Figure 3.2. A 53% decrease in parasite burden was observed in the miltefosine group compared to the infected control. The combination group maintained high parasite burden, indicating that combination with STAT6-IP failed to decrease the parasite load. Interestingly, the group treated with STAT6-IP alone showed a considerable decrease in parasite burden that was not observed in the first study.

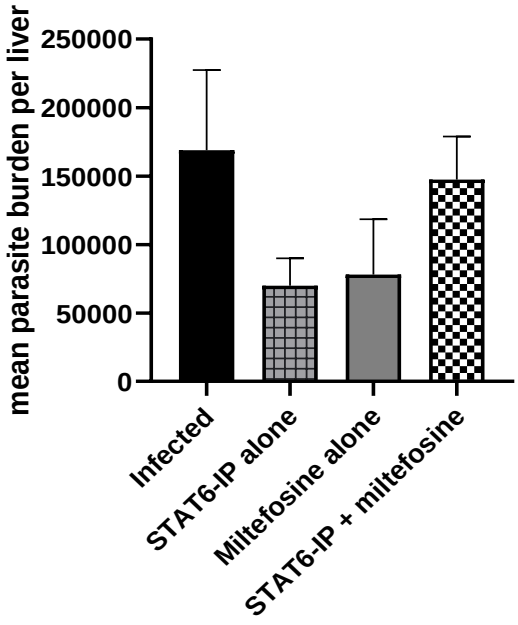

Figure 3.2. Effect of combination of sub curative miltefosine with STAT6-IP on parasite burden. Percent parasite reduction was calculated by comparing the mean number of parasites in treatment groups to those in control animals. Results were analysed by one-way ANOVA.

3.2.3.2 Outcome of Combination Therapy on *Leishmania*-specific humoral immune response

The progression of the immunomodulatory effects of the parasite in VL can be monitored by the production of *Leishmania*-specific antibodies. In mouse model, IgG1 and IgG2a isotypes act as useful surrogate markers for Th2 and Th1-type immune responses, respectively[24]. IFN-γ production by Th1-type cells promotes IgG2a production while IL-4 from Th2-type cells promotes IgG1 production[25]. As expected in the untreated group, there was a higher concentration of IgG1 compared to IgG2a, indicating a Th2-shift that was reflected in the elevated parasite load observed in this group. In the combined therapy group, the IgG1:IgG2a ratio was completely reversed, suggesting that at least one aspect of the parasite-driven Th2-shift was potentially reversible despite the failure to translate into reduced parasite burden. The STAT6-IP alone and miltefosine alone groups that had reduced parasite burden in this study had a lower or equal IgG1:IgG2 ratio, respectively, which is consistent with the literature supporting the idea that a mixed Th1/Th2 response rather than a dominant Th1 response may be required for protection against VL[26–30].

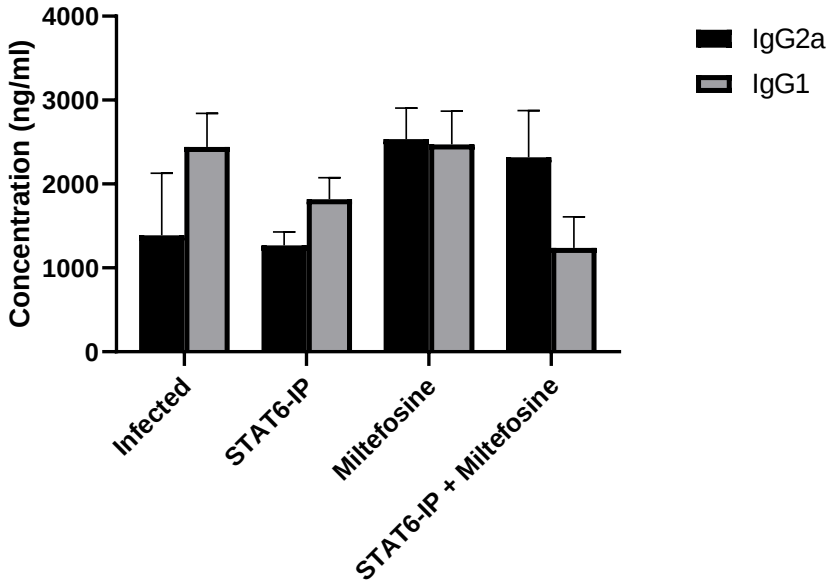

Figure 3.3. Antibody responses to combination treatment. IgG2a and IgG1 levels in the serum were determined by ELISA. Results were analysed by two-way ANOVA.

4 CHAPTER 4: DISCUSSION

Leishmaniasis: Worldwide incidence, Global estimates and Classification

Leishmaniasis encompasses a spectrum of neglected tropical diseases caused by intracellular protozoan parasites in the genus *Leishmania* that are transmitted by infected female phlebotomine sand flies[31]. The digenetic lifecycle of the parasite includes flagellated procyclic promastigotes that differentiate into non-dividing, infective metacyclic forms upon entry into the sandfly gut. When the fly takes a bloodmeal, these metacyclic parasites are taken up by professional phagocytic cells where they transform into aflagellated, replicative amastigotes[32] (Figure 1).

Leishmaniasis is endemic in 98 countries, with over 12 million infected people worldwide and more than 350 million at risk[33]. Ranking second only to malaria in parasite-related deaths, leishmaniasis is a public health issue with significant social stigma[34]. This disease is mostly associated with developing countries as it affects some of the most underprivileged and poverty-stricken people across the globe[35]. In the last two decades, there has been a worrisome increase in the incidence of leishmaniasis in some parts of the world. This has been attributed to multiple factors, including increased international travel, migration of people from rural to urban areas seeking work opportunities, migration as a consequence of war and civil unrest, disturbances in microenvironments due to climate change, deterioration of socioeconomic conditions and the presence of HIV/*Leishmania* coinfection[32,36].

Leishmaniasis has several clinical manifestations ranging from self-healing skin lesions to deadly systemic complications. The two main forms are cutaneous and visceral leishmaniases[37]. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and is characterized by the presence of skin ulcers. These ulcers usually heal spontaneously after several months but can leave disfiguring scars. Alternatively, non-healing ulcers can develop

and lead to long-term, chronic infections as seen in diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL), and leishmaniasis recidivans (also called chronic relapsing cutaneous leishmaniasis)[37]. Visceral leishmaniasis (VL) is the most severe form and has a high mortality rate if not treated. It is characterized by dissemination of parasites throughout the reticuloendothelial system, and presents with symptoms such as prolonged fever, anorexia, weakness, weight loss, pancytopenia, hepatosplenomegaly and hypergammaglobulinemia[38].

VL has an estimated global incidence of 0.5 million new cases with close to 60, 000 deaths every year[39]. Ninety percent of these cases occur in seven countries: India, Bangladesh, Nepal, Ethiopia, Sudan, South Sudan and Brazil[40]. The actual number of VL cases is likely underestimated due to underreporting and scarcity of epidemiological data in remote endemic locations[33]. VL may be zoonotic or anthroponotic depending on the presence of animal reservoir hosts in its transmission cycle[41]. Zoonotic VL is caused by *L. infantum* and is prevalent in the Americas, Central Asia, China, the Middle East and the Mediterranean while anthroponotic VL is restricted to the Indian subcontinent and East Africa and is caused by *L. donovani*[42]. Dogs are the main reservoirs for *L. infantum* and infection results in a multisystemic disease known as canine visceral leishmaniasis (CVL). CVL is present in 50 countries with reports of emergence in new locations such as the United States, Canada and Europe[43].

Immunopathology of Leishmaniasis

Clinical outcomes of *Leishmania* infection are largely dependent on the infecting species and host immune status. Interestingly, not all humans exposed to *Leishmania* develop overt disease[44]. Understanding the immunological events that underly resistance and susceptibility

to infection is paramount for designing more effective therapies which are needed in leishmaniasis[45]. Because using humans as experimental disease models is unethical, animal models remain the best choice for disease characterization[45,46]. The two most commonly used animal models are mice and hamsters and each model has specific features. It is noteworthy to mention that neither of these models can accurately reproduce what happens in humans, but they are very useful in giving insight into the pathogenesis of leishmaniasis and they provide a platform for testing novel therapeutics[47].

Mice (BALB/c, NMRI, DBA/1 and C57BL/6) are the preferred models of experimental VL due to their high availability, easy handling and initial susceptibility, although not all laboratory mice are susceptible to *Leishmania* infection[46,48]. In the mouse model, genetic background, inoculation route, parasite strain and dose of parasites injected all influence the outcome of infection. Outbred mice are generally resistant to *L. donovani* infection while inbred mice display variable susceptibility[48]. Susceptibility to infection has been associated with mutation of the *Slc11a1* gene encoding a phagosomal component, solute carrier 11a1 (also known as Nramp1) which makes susceptible mice strains (BALB/c and C57BL/6) unable to control early parasite growth[49]. C57BL/6 mice are able to cure disease by developing a self-healing phenotype that prevents further growth of the parasite, whereas BALB/c strains are characterized by progressive, non-healing lesions in *L. major* cutaneous infection[50]. Disease progression in *L. donovani*- and *L. infantum*-infected BALB/c mice is organ specific as the responding tissues (liver and spleen) present varying patterns of immune response within the same animal[51]. While the liver is able to resolve VL infection and control parasite burden at a later stage, infection in the spleen remains throughout the entire course of disease. Because there is no progression to the chronic disease observed in humans, BALB/c mice can only serve as models of subclinical infection[47]. Hamsters (Syrian, Chinese and European) are considered more suitable models for chronic disease as they closely mimic the clinicopathologic features

of human VL, with gradual progression to fatal disease. Although efficient and highly relevant, hamsters are not frequently used because of the relative lack of reagents for immunological analyses[46–48].

Evidence from murine models shows marked differences in the immune responses generated between experimental VL and CL. While the immune mechanisms underlying susceptibility to VL remain poorly understood, extensive investigations in murine CL using *L. major* has helped in defining the role of $CD4^+$ T cell subsets in determining the outcome of disease[52]. Protection against leishmaniasis is predominantly mediated by cell-mediated immunity in which T helper-1 (Th1) $CD4^+$ T cells producing interferon-gamma (IFN-γ) confers disease resistance. In contrast, Th2-type $CD4^+$ T cell proliferation with production of interleukin-4 (IL-4), IL-10 and IL-13 is associated with susceptibility to and progression of disease[52,53].

In experimental VL, the Th1/Th2 dichotomy is not as clearly defined. A mixed T-cell cytokine profile with expression of both Th1 (IFN- γ, IL-2) and Th2 (IL-4, IL-10) cytokines following *L. donovani* infection has been reported[51,54]. Infected mice can show initial susceptibility to VL infection that is eventually overshadowed by a healing response mediated by granuloma formation and development of acquired resistance[54]. The ability to mount a dominant Th1 response with production of IL-2, IFN-γ and IL-12 is essential for resolving VL disease[55]. IL-12 is an important cytokine that promotes development of Th1 cells for production of IFN-γ that activates macrophages to produce nitric oxide (NO), the key effector molecule for parasite elimination[56,57]. Natural killer cells and cytotoxic $CD8^+$ T cells are also important sources of IFN-γ, with the latter involved in direct killing of *Leishmania* via cytolytic activity[58–61].

Akin to murine visceral leishmaniasis, human VL does not closely follow the classical Th1-Th2 paradigm of resistance and susceptibility. Instead, the outcome of infection is determined by the balance between the contrasting effects of protective (IL-2 and IFN-γ) and non-

protective (IL-4 and IL-10) cytokines[62–65]. During active disease, VL patients display depressed cell-mediated immune response and lack reactivity to the Leishmanin skin test (LST; a measure of delayed type hypersensitivity). Their PBMCs also fail to proliferate or produce IFN-γ in response to *Leishmania* antigens[3]. Conversely, high levels of IFN-γ and TNF-α are detected in the blood, along with elevated expressions of IFN-γ mRNA in aspirates of the lymph node, spleen and bone marrow, indicating the presence of a theoretically protective Th1 response[66–68].

The presence of IL-4, IL-13 and IL-10 in these same patient sera suggests the possibility of compartmentalization of the immune response with Th2 immunomodulatory effects that prevent effective Th1-mediated control of parasite growth in some tissues[14,69,70]. Although IL-4 and IL-13 are generated during disease, their role in VL pathogenesis is not clear. While some reports implicate IL-4 and IL-13 in progressive infection, others do not[71–73]. In contrast, the immunodeactivating cytokine, IL-10, appears to be the primary suppressive factor in visceral infection[74]. It has been shown that IL-10 has antagonistic effects on IFN-γ that prevent IFN-γ-mediated activation of macrophages for intracellular parasite destruction[75]. IL-10 has emerged as the major regulatory cytokine that can suppress T-cell activation in murine and human VL[76,77,75]. IL-10 has also been incriminated in the promotion of disease and persistence of parasites in cutaneous *L. major* infection[78–80]. IL-10 dampens the production of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) and downregulates major histocompatibility complex (MHC) class II expression, leading to reduced parasite clearance and suppressed Th1 activation[81].

The role of IL-10 in experimental and human VL pathogenesis has also been well documented[82–85]. Humans with active disease have elevated plasma concentrations of IL-10, increased expression of IL-10 mRNA in lesional tissues and IL-10 production is readily detected from antigen-stimulated whole blood cells[86]. $CD4^+$ $FoxP3^-$ cells are an important

source of IL-10[82,102]. Data from murine VL studies have shown that secretion of IL-10 by $CD4^+$ $FoxP3^-$ correlates with disease severity[68]. IL-27 and IL-21 are also important factors that promotes disease progression via their promotion of IL-10 production in both human and experimental VL[89–92].

TGF-β may be another important cytokine involved in human VL pathology, although its precise function is still unknown. Enhanced TGF-β levels have been detected in plasma, splenic aspirates and antigen-stimulated PBMCs of VL patients during active disease[65,68,69]. Like IL-10, TGF-β is a potent inhibitor of macrophage leishmanicidal activity and its blockade limits parasite replication in these cells[3]. $CD4^+CD25^+FoxP3^+$ Regulatory T (Tregs) cells are an important source of TGFβ (and IL-10) and as such, have been implicated in the pathogenesis of VL[65,93–95]. A positive correlation between the production IL-10 and TGFβ by Treg cells and parasite burden has been demonstrated, suggesting a pivotal role for Tregs in secretion of these cytokines[87].

Diagnosis of VL

Definite diagnosis of VL is demonstration of parasites by light microscopic examination of tissue aspirates from spleen, bone marrow, or lymph nodes. The specificity of this technique is high, but its sensitivity varies depending on the tissue sampled. The challenges of this procedure is that sample collection is invasive and technical expertise is required for parasite detection under the microscope. Polymerase chain reaction (PCR) remains the most sensitive and reliable technique for VL diagnosis. This method utilizes different primer sequences that target several multicopy sequences like *Leishmania* kinetoplast DNA (kDNA) for detection of parasites. The principal drawbacks of PCR-based assays are that they are expensive, cumbersome to perform and unsuitable outside the laboratory. Serological analysis with FD-

DAT (freeze-dried direct agglutination test), rK39 plasma strip tests and KAtex urine dipstick are non-invasive and more field-adaptable for VL diagnosis. These tests also have some significant drawbacks, one of which is the inability to distinguish between past and recent infections[96–98].

Treatment strategies in VL

Chemotherapy

Visceral Leishmaniasis is a chronic disease that poses an enormous burden on world health and the most affected are children, young adults and women[99]. No human vaccine is available against leishmaniasis and chemotherapy remains the primary tool for VL control[100]. Conventional drugs commonly used for treatment of VL are shown in Table 1. The pentavalent antimonial compounds, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are the recommended first-line drugs for treatment of all forms of leishmaniasis worldwide except in Bihar, India, where widespread treatment failure due to drug resistance has been reported[101,102]. Development of resistance in this region was linked to drug misuse due to unrestricted availability[101,103]. Antimony use is also associated with perilous side effects such as organ (liver, heart and kidney) toxicity and clinical pancreatitis[104,105]. Other antileishmanials such as amphotericin B (deoxycholate and its lipid formulations [liposomal amphotericin B also known as AmBisome®), miltefosine, paromomycin (an aminoglycoside) and pentamidine are not without limitations. Most of them are toxic and require long term treatment. The less toxic drugs are expensive and there are fears of resistance development[106].

Clinical studies have shown that ineffective treatment with current antileishmanials can lead to the development of a dermatological condition known as post-kala-azar dermal leishmaniasis (PKDL). PKDL is characterized by heavily parasitized skin lesions which may present as

nodules, macules or papules[107,108]. Because skin lesions are parasite-rich, individuals with PKDL play a pivotal role in the transmission of VL. HIV-*leishmania* coinfected patients have also been implicated in the transmission of VL[109]. The presence of HIV in many countries and the overlap in transmission areas between *Leishmania* and HIV has resulted in an increasing global incidence of coinfection[40]. HIV coinfection with *Leishmania* augments the severity of VL, reduces the therapeutic response to treatment and increases the risk of relapse[43]. Monotherapy with current VL drugs has yielded consistently poor results in those with HIV coinfection. Taken together, the current arsenal for treatment of VL is unsatisfactory. There is urgent need for alternative approaches to therapy.

Preventive chemotherapy via mass drug administration (MDA) has been introduced in several disease control programs for elimination of neglected tropical diseases including schistosomiasis and lymphatic filariasis. Toxicity issues and parenteral administration of antileishmanials are some of the problems that preclude such programs in leishmaniasis[110]. Moreover, MDA has the potential to speed up development of drug resistance especially in the anthroponotic foci (Indian subcontinent and East Africa) where there are no animal reservoirs to dilute resistant genotypes[110,111]. Because human beings are the reservoir of infection in anthroponotic cycle, when resistance emerges, it can spread rapidly since drug-sensitive parasites are eliminated quickly whereas those that are resistant to drugs continue to circulate in the community[112]. This was presumably what led to the high incidence of antimonial resistance in Bihar, India. As a result of these events, the use of pentavalent antimonials was eventually suspended for use in India; however, they remain the first-line choice in other parts of the world including Latin America and East Arica where there is no evidence for resistance yet[5]. Pentamidine, a second-line drug that was initially used for antimony-resistant patients in Bihar, has also been abandoned because of serious toxicity and poor efficacy[111]. Paromomycin, (aminoglycoside antibiotic), an affordable drug of moderate toxicity has been shown to have

good efficacy in the treatment of Indian VL at a dose of 15 mg/kg/day[113]. The main drawback with this drug is that it needs to be injected over 21 days which can affect patients' compliance[114]. Lately, there have been serious concerns with paromomycin monotherapy as recent studies have reported high susceptibility to resistance under laboratory conditions[115].

Miltefosine is the first and only oral agent available for the treatment of VL. Approved in 2002, the Indian-subcontinent initiative for kala-azar elimination adopted miltefosine as its first-line alternative to pentavalent antimonials[116]. Miltefosine is easy-to-administer and reasonably-priced. It has only mild side-effects (except in pregnant women in whom it is potentially teratogenic). At a recommended dose of 2.5 mg/kg/day for 28 days, miltefosine has high efficacy against Indian visceral leishmaniasis[117–119]. Unfortunately, there are now reports of treatment failure in the same region which is not surprising as miltefosine has a narrow therapeutic index and long half-life which makes it particularly vulnerable to the development of resistance[120–123]. Hence, further use of miltefosine monotherapy has been discontinued in this region and a single dose of liposomal amphotericin B is now advocated as the drug of choice for VL[115]. Liposomal amphotericin B is similar to amphotericin B deoxycholate but with minimum toxicity[7,124]. Before lipid formulations of amphotericin B were developed, amphotericin B deoxycholate was used predominantly in areas of antimony resistance. Although effective, amphotericin B deoxycholate required prolonged hospitalization and infusion-related adverse effects were quite common. Lipid-based formulations have the double advantages of less toxicity and shorter treatment[111].

Liposomal amphotericin B (AmBisome, AmB; Gilead) is currently the safest and most effective antileishmanial drug. At a single dose of 5 mg/kg, high cure rates have been reported in India, with even better efficacy at 10 mg/kg[124,125]. Clinical resistance to AmB is rather unlikely due to its high therapeutic index and short half-life but with increasing use, the possibility cannot be ignored[7,112]. AmB is expensive however and as such, is more realistic as

the first treatment choice in developed countries. In most poor endemic countries, even short courses of AmB treatment are unaffordable and this makes selection of antileishmanial treatment a question of cost rather than of efficacy[5].

Unlike in Asia, very few treatment options are available for management of VL in Africa. A 30-day course of 20 mg/kg antimonial SSG remains the standard monotherapy in most African countries. Although effective, SSG is very toxic and requires 4 weeks of hospitalization. Liposomal amphotericin B at the recommended dose of 20-30 mg/kg is mainly administered as second-line treatment due to high cost and reliance on cold chain storage (exposure to temperatures $>25^0$C or $<0^0$C enhances toxicity and reduces efficacy)[114,124]. Miltefosine and paromomycin monotherapy have had poor efficacy in clinical trials with African patients despite their success in India[114,126].

Combined chemotherapy

Since current VL drugs are sub-par and threatened by emerging resistance, there is pressing need for new and better therapeutics. While research is underway to identify and evaluate novel drug candidates, a short term strategy that is safe, effective and inexpensive is urgently required for controlling VL in endemic regions[126]. Over the past decade, the use of combination therapy for VL has attracted much attention because of the substantial benefits it might offer. Combining drugs with different mechanisms of action could theoretically reduce both cost and treatment duration, resulting in fewer toxic side effects and higher patient compliance[7,110]. It could also delay the emergence of resistance in VL drugs thereby increasing their therapeutic life span and preserving their efficacy[7].

Specific multidrug regimens have been suggested by the World Health Organisation (WHO) for treatment of VL and they differ from region to region due to geographical variability in

drug efficacy. In eastern African countries, co-administration of SSG with paromomycin for 17 days is currently the recommended mainstay while on the Indian subcontinent, a combination of liposomal amphotericin B with either miltefosine or paromomycin is the recommended therapy[127–130].

Combination therapy is a valuable strategy that helps to avoid some of the problems associated with monotherapy, including the development of drug resistance. Although many clinical trials have highlighted the efficacy and safety of antileishmanial drug combinations, there are still concerns that in the long run, even combination therapy may select for drug resistant strains. In (rural) areas where intravenous infusion or cold-chain problems make AmB impossible, a multidrug regimen comprising of miltefosine and paromomycin has been proposed as an alternative treatment option[129]. Under experimental conditions however, *L. donovani* parasites were recently shown to become resistant to miltefosine and paromomycin combinations[9,131]. Metabolomic analysis of the combination-resistant lines has shown metabolic changes in multiple pathways which may have permitted these parasites to become more tolerant to ATP loss, resist depolarization of the mitochondrial membrane and sustain membrane integrity, among other advantages[8,132]. These reports and observations clearly indicate the possibility of resistance development to multi-drug combinations. Because of this danger, the exploration of immune-based therapy (either alone or combined with antiparasitic drugs) is becoming an attractive approach for the treatment of leishmaniasis.

Immunotherapy and Immunochemotherapy

It is general consensus that drug therapy works most efficiently with help from host immune system, and in particular, the cell-mediated immune response[93]. Hence, immunotherapeutic agents that stimulate host immunity have enormous potential for therapeutic success. In

addition, these agents, when combined with chemotherapeutic drugs (immunochemotherapy) can result in synergism of immune activation and direct action of drugs against the parasite[43]. In leishmaniasis, immunomodulatory molecules that have the ability to tilt the Th1-Th2 imbalance in favour of Th1 are of immense clinical benefit[133].

Cytokines have been assessed both experimentally and clinically as immunomodulators for treatment of VL. Due to their involvement in protective immunity (through upregulation of Th1 immune responses), IL-12 and IFN-γ have been employed as therapeutic agents. Treatment with either recombinant IFN-γ (rIFN-γ) or rIL-12 halts hepatic parasite replication in *L. donovani*-infected BALB/c mice[134,135]. Both cytokines have also shown additive effects in combined therapy. In murine VL, rIL-12 given in combination with pentavalent antimony cures mice from *L. donovani* infection[136]. IFN-γ and its well-known ability to enhance macrophage antimicrobial activity motivated the evaluation of IFN-γ combination with Pentostam in *L. donovani*-infected mice. The addition of rIFN-γ to Pentostam substantially augmented the antileishmanial effect of Pentostam, resulting in reduced parasite burden[137,138]. Combination of IFN-γ and antimony is clinically well-tolerated. In Kenya, Brazil and India, human rIFN-γ administered in combination with pentavalent antimony showed higher parasitological and clinical efficacy in VL patients compared to pentavalent antimony alone but results were not statistically significant[139,140].

Suppression of Th1-deactivating cytokines IL-10, IL-4 and IL-13 and TGF-β has been evaluated for VL therapy. It has been reported that monoclonal antibody blockade (mAb) of IL-10 restores T cell proliferation and IFN-γ production in the PBMCs of VL patients[70,141]. In mouse models of VL, neutralization of IL-10 or blockade of its receptor leads to IFN-γ secretion, granuloma formation, macrophage activation and parasite killing. These effects are even further enhanced when IL-10 inhibitors are combined with antimonials[142]. In contrast, blockade of other immunosuppressive cytokines IL-4, IL-13 and TGF-β via receptor fusion

antagonists has demonstrated only marginal parasite elimination in *L. donovani*-infected mice with no synergistic effect when combined with pentavalent antimonials[142].

Despite the promising effects of cytokines as immunotherapeutic agents, certain problems limit their full therapeutic potential. Administering high dose of cytokines can result in side effects characterized by malaise and influenza-like syndromes. Also, cytokines have a short half-life which means multiple doses are needed and this can further increase the side effects. Finally, the production of recombinant cytokines in quantities sufficient for therapy is very expensive: for cytokine therapy to be practical, it must be cost-effective compared to conventional treatments[143].

In view of the above challenges, examining other potential compounds with immunomodulatory activity against leishmaniasis should be focused on for developing better therapeutics in VL. The following section explores a few small molecules that have been used to target phagocytic cells that participate in host immunity against *Leishmania* parasites. Compounds that target intracellular signalling pathways involved in parasite survival in mammalian host are also examined. Identifying these immunomodulatory molecules could encourage future combinations with antileishmanial drugs and the outcomes may improve the efficacy of current treatment protocols.

Targeting phagocytic cells

Compounds that target host immune cells is being explored for treatment of leishmaniasis. Phagocytic cells such as PMNs (polymorphonuclear neutrophil granulocytes), macrophages and dendritic cells are safe havens for *Leishmania* and participate in innate immunity against these parasites, making them potential targets for therapy (Figure 2). Following entry of *Leishmania* into mammalian host, neutrophils are rapidly recruited to the site of infection

where they become activated to ingest and kill parasites via production of proteolytic enzymes, reactive oxygen species (ROS) and neutrophil extracellular traps (NETs)[144–146]. Neutrophils are inherently short-lived cells after which they undergo spontaneous apoptosis using a mitogen activated protein kinase (MAPK) signalling pathway[147,148]. Since *Leishmania* parasites are known to delay neutrophil apoptosis, compounds capable of increasing apoptotic activity and generating oxidative burst within *Leishmania*-infected neutrophils are effective for eliminating parasites. Berberine chloride has been reported to mediate antileishmanial activity via enhancement of apoptosis in infected neutrophils, subsequent to modulation of MAPK pathway[149].

Leishmania parasites can enter macrophages via uptake of infected, apoptotic PMNs through a process known as phagocytosis. This process requires special recognition molecules on both parasite and macrophage surfaces to facilitate macrophage entry. Following internalization, *Leishmania* evades the microbicidal consequences of phagocytosis by secreting acid phosphatase on its surface to inhibit the oxidative burst within the macrophage. Compounds like tamoxifen have been employed to reduce acidification in macrophage intracellular compartments while CPG-ODN (synthetic unmethylated Cytosine-Guanine oligodeoxynucleotides) has been used to increase the phagocytic activity of macrophages[150,151]. As NO is the principal effector molecule critical for parasite elimination in macrophages, any NO-releasing agent may have great potential. An example of such an NO-inducing compound is diperoxovanadate. Along with sub-optimal doses of Sodium Antimony Gluconate (SAG), diperoxovanadate reduced parasite load in *L. donovani*-infected BALB/c mice by expanding the antileishmanial T-cell repertoire and increasing NO production[152]. Other compounds like trinitroglycerin, hydrolyzable tannins and 18 β-glycyrrhetinic acid can also upregulate NO production[153–155]. Many plant-based products also show similar NO-enhancing ability. This is no surprise as the use of natural plants for treatment of disease dates back many centuries and

indeed, many of them are known to be active against various forms of *Leishmania* parasites. Natural plant extracts with antileishmanial activity has recently been reviewed[156]. Despite their leishmanicidal potential, most natural plant products do not meet all of the requirements considered essential for their commercialization which include topical or oral administration, efficacy at moderate doses, and freedom from severe side effects[157].

Dendritic cells (DCs) are professional antigen presenting cells (APCs) specialized in antigen uptake, processing and presentation to T cells. They are involved in the initiation of immune response and secrete IL-12, which is critically important for the polarization of naïve T cells toward the Th1 phenotype. Infection of DCs with *Leishmania* results in functional IL-12p70 production and the subsequent release of interferon-gamma (IFN-γ) from activated Th1 cells[56]. To avoid this, parasites block maturation of dendritic cells and prevent production of IL-12[158]. It is therefore not surprising that pyrazinamide's anti-leishmanial effects are mediated by increasing the activation of infected DCs via enhanced secretion of proinflammatory cytokines and NO[159].

Targeting intracellular signalling pathways

Leishmania parasites reside mainly inside macrophages and to survive within their host cell, they interfere with macrophage signalling pathways by activating inhibitory protein tyrosine phosphatases (PTPs) and degrading key signalling molecules such as kinases, transcription factors and translation regulators[160]. Thus, blocking important intracellular signalling pathways that are indispensable for *Leishmania* parasite growth and survival shows potential in VL therapy. Since PTPs are negative regulators of cell signalling, their modulation by *Leishmania* is of critical importance in their establishment of infection and immune evasion[160]. Treatment

with PTP inhibitors bpV-phen and bpV-pic greatly diminishes parasite proliferation and aids in resolution of infection through NO production[161,162].

An important costimulatory molecule that determines the outcome of macrophage-*Leishmania* interactions is CD40. Binding of CD40 on macrophages to CD40L on T cells upregulates IL-12 expression via pathways, MAPK and nuclear factor-*kappa* B (NF-κB). Conversely, weaker interactions between these costimulatory molecules can downregulate IL-12 production and increase IL-10 production which occurs subsequent to enhanced phosphorylation of extracellular stress-related kinase-1/2 (ERK-1/2)[163]. It has been shown that *Leishmania* skews CD40 signalling towards ERK-1/2, inducing IL-10, which prevents activation of CD40-induced p38 MAPK and expression of inducible NO synthase. ERK-1/2 inhibition restores CD40-induced p38 MAPK activation and parasite killing in macrophages and *L. major*-infected mice[164]. Clearly, ERK and p38 MAPK are important regulators of macrophage effector molecules and can dictate the outcome of infection making them putative targets for treatment.

Another attractive option is targeting NF-κB signalling which upon activation by MAPKs, increases its expression of Th1 cytokines and iNOS[163]. Transcription factor NF-kB is usually held inactive in the cytoplasm via binding to Inhibitors of NF-κB (Iκ-B). Phosphorylation of Iκ-B by upstream signalling pathways dissociates them from NF-kB and allows for NF-kB dimerization and translocation into the nucleus for gene expression[160]. Preventing degradation of Iκ-B and its downstream effects is a strategy employed by *Leishmania* and its abrogation by antileishmanial compounds such as 18 β-glycyrrhetinic acid corroborates its potential as a putative target[155]. Similarly, cystatin (a natural cysteine protease inhibitor) with IFN-γ decreases Iκ-B levels with concomitant activation of NF-κB and upregulation of NO for parasite killing[165].

Protein Kinase C (PKC) is a family of serine-threonine kinases that play a key role in signalling related to many microbicidal functions including response to TNF-α and IFN-γ as well as NO and ROS production. Different *Leishmania* surface molecules such as lipophosphoglycan (LPG) and ceramide inhibit PKC[160]. While *Leishmania* surface sphingomyelinase activates the atypical PKC-ξ (calcium independent), LPG inhibits activation of classical PKC-β (calcium independent) resulting in disruption of the lipid rafts necessary for cytokine receptor assembly (especially that of IFN-γ); reversal of this phenomenon can be achieved with amphotericin B[166]. Similarly, the activation of phosphatidylinositol 3-kinase/Akt by *Leishmania* enables downregulation of IL-12 and blockade of this pathway with PI3K/Akt inhibitor wortmannin, reduced infection rates in *L. infantum*-infected bone marrow-derived dendritic cells (BMDDCs). Treatment with another PI3K/Akt inhibitor, AS-506240 significantly reduced entry of *L. mexicana* into neutrophils and macrophages resulting in reduced lesion growth and parasite load in infected C57BL/6 mice[167,168].

The Toll-like receptor (TLR) signalling pathway is one of the first defensive systems against invasive microorganisms. TLRs are transmembrane proteins that confer specificity to innate immune cells by recognition of pathogens that cause human disease. The TLR family consist of 11 members (TLR1 to TLR11) which are located on either the plasma membrane or the internal membrane of macrophages, DCs, NK cells as also T and B lymphocytes. Following recognition of a pathogen-associated molecular patterns, many TLRs trigger a series of cascades that eventually lead to nuclear translocation of NF-κB for synthesis of proinflammatory cytokines[169]. Upregulation of TLR signalling is required to overcome *Leishmania* infection as demonstrated by TLR agonists- imiquimod, resiquimod, CPG-ODN, Pam3Cys, monophosphoryl lipid A and arabinosylated lipoarabinomannan[23,170–175]. Imiquimod (imidazoquinoline) is a TLR 7 agonist and a potent inducer of cytokines such as IFN-α, a variety of interleukins and TNF[176]. It is widely used for treatment of genital warts caused by

human papilloma virus but shows leishmanicidal properties due to its ability to induce nitric oxide production in macrophages[177]. In *L. major*-infected BALB/c mice, treatment with imiquimod cream significantly reduced the severity of cutaneous leishmaniasis[178]. Imiquimod is also synergistic with meglumine antimonate as this combination was highly effective in treatment of CL in Peru but less so in Iran[179,180]. Combination of imiquimod ointment and Lescutan (15% paromomycin sulphate and 12% methylbenzethonium chloride) was not successful for treatment of CL in mice infected with *L.major*[181].

As mentioned earlier, cytokines play a critical role in determining the nature of host immune response to infection. They signal through a cascade of intracytoplasmic proteins known as JAK-STAT. The JAK-STAT pathway results in the transcription of many important inflammatory genes such as iNOS, IL-12 and major histocompatibility complex class II (MHC II), therefore its inhibition is of great importance for *Leishmania*. The STAT family is comprised of seven proteins: namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, many of which are important in the pathology and resolution for *Leishmania* infection as has been demonstrated[12]. As a result, targeting STAT signalling through the use of agonists, antagonists or inhibitors may have potential and could be useful in VL therapy. For our study, we decided to explore inhibition of STAT6 pathway.

STAT6 is involved in host immune response to *Leishmania* as it mediates the biological activities of IL-4 and IL-13, Th2 cytokines associated with non-healing forms of leishmaniasis[12]. Binding of Th2 cytokines, IL-4 and IL-13 to their respective receptors and to a common γc receptor they share, activates STAT6 leading to both the autocrine and paracrine induction of these cytokines (i.e. a positive feedback loop). To the best of our knowledge, there are no studies on the role of STAT6 in the pathogenesis of VL. Because IL-4 and IL-13 are upregulated during active human VL but significantly reduced after therapy, we decided to

exploit immunological interventions designed to inhibit the STAT6 pathway for treatment of VL[14].

STAT6-inhibitory peptide (STAT6-IP) is a chimeric peptide that has been used to inhibit STAT6 activity in murine models of aberrant Th2-type immunity associated with RSV vaccination/infection and allergic airways disease[16,17]. This inhibitory peptide comprises of a protein transduction domain (for penetrating into cells) coupled to eight amino acids that surround the phosphotyrosine (*Y) 641 of murine STAT6. The STAT6-IP was designed to act as a dominant negative inhibitor of STAT-6 by binding to its SH2 domain, preventing dimerization of STAT-6. STAT6-IP has previously been used to reduce airway inflammation in mice by inhibiting activation of Th2 cytokines including IL-4 and IL-13 which are implicated in pathogenesis of airway disease[18]. Because IL-4 and IL-13 are also implicated in VL, we decided to evaluate this peptide as part of a combination treatment.

Observations from our study

In the present study, we investigated the immunomodulatory potential of STAT6-IP alone and in combination with sub-curative dose of miltefosine for treatment of VL. We began with a pilot study in which the curative dose of miltefosine was combined with STAT6-IP for treatment of *L. donovani* infection in susceptible BALB/c mice. This was carried out as a proof-of-concept study to establish an infection and treatment model and to confirm the curative dose of miltefosine. Treatment with miltefosine at 20 mg/kg gave a 95% parasite clearance in infected mice which was well within the range of the degree of parasite reduction observed in other treatment studies that had employed this dose[22,23]. There was a 98% parasite reduction in the STAT6-IP + miltefosine group when compared with infected controls which was not significantly different from the miltefosine alone group. The group treated with STAT6-IP had

no reduction in parasite burden. This was not unexpected as many immunomodulators that have been employed in the past for treatment of leishmaniasis in mice did not have any effect on parasite burden when used alone[23,170,182]. Having validated the infection and treatment model, we proceeded to the main study.

In our next experiment, we combined the STAT6-IP with a sub-curative dose of miltefosine. The miltefosine group had a 53% reduction in parasite burden and we expected to be able to observe any additive activity of STAT6-IP. However, no synergistic effect was observed as the combination group still had high parasite load. Interestingly, STAT6-IP alone, which had no effect on parasite burden in the pilot study, seemed to reduce parasite load in the second study. Since the second study were carried out in a similar fashion to the pilot study and the same dose of STAT6-IP was used in both studies, we cannot conclude that STAT6-IP alone has an effect or not. This can only be resolved with further studies. However, the impact of STAT6-IP on the serologic response on infection (below) strongly suggests that this immunomodulator did indeed have physiologic effects in the infected mice.

Since VL progression is associated with high levels of *Leishmania*-specific antibody responses, we decided to measure the level of IgG isotypes-IgG1 and IgG2a in the sera of *Leishmania*-infected mice. IgG1 and IgG2a are often used as surrogate markers of Th2 and Th1 responses respectively as IgG2a production is dependent on IFN-γ, whereas IgG1 production is correlated with IL-4. As expected in the infected group, IgG1 was elevated compared to IgG2a (i.e. a high IgG1:IgG2a ratio) which was associated with a high parasite burden. The combined STAT6-IP + miltefosine group had increased IgG2a levels with a lower IgG1:IgG2a ratio suggesting some rebalancing of the parasite-skewed Th2 environment, but this did not translate into a reduction in parasite load. Monotherapy with either STAT6-IP or miltefosine reduced parasite burden in mice and also had impact on the balance of IgG1 and IgG2a production (reducing

the IgG1:IgG2a ratio) suggesting at least some enhancement in more effective Th1-type immunity.

It appears to be, from our findings in the second study, that STAT6-IP as adjunct had no synergistic effect with miltefosine. However, there seems to be good evidence of physiological effect, based on the IgG1:IgG2a ratio, in all treated groups. In the STAT6-IP group, the IgG1 levels appeared to be much lower compared to those in the infected and treated groups. This observation seemed to suggest that STAT6-IP may have been effective in reducing Th2 cytokine activation (similar to observations in RSV and allergy studies were this peptide was previously employed) even though it did not induce the upregulation of Th1 cytokines (low IgG1:IgG2a ratio) as was anticipated. The lack of a vigorous IFN-γ response (low IgG2a levels in the STAT6-IP group) in an environment with low IL-4 production should probably come as no surprise as early studies have reported that inhibiting IL-4 signalling does not necessarily equate to increased IFN-γ production[183]. There are speculations that IL-4 (and other Th2 cytokines) do not promote *Leishmania* infection by downregulating IFN-γ response but by interfering with macrophage activation or by recruitment (to the site of infection) of mononuclear phagocytes that permit parasite growth[183]. Cytokine quantification and measurement of NO production may have helped to shed more light on the immunologic effects of inhibiting IL-4 (and IL-13) signalling in this study.

Another important finding was the elevated concentrations of IgG2a antibodies in the miltefosine and combined treated groups. This elevation, known to be synonymous with upregulation of IFN-γ production, seems to back up previous hypothesis that miltefosine is capable of immunomodulation and Th1-cytokine induction[184]. Miltefosine belongs to a group of alkylphosphocholine compounds that are phosphocholine esters of aliphatic long chain alcohols. These alkylphosphocholine compounds are structurally related to alkyllysophospholipids which are synthetic analogues of the cell membrane lipid,

lysophosphatidylcholine[20]. Due to its similarity to lysophospholipids, miltefosine can interact with lipids in the parasite cell membrane thereby perturbing membrane integrity and interfering with signal transduction within the parasite[185–187]. Upon exposure to miltefosine, *Leishmania* parasites undergo nuclear condensation and DNA fragmentation that results in programmed cell death[188,189].

Apart from direct killing of *Leishmania*, the proposed mechanisms of miltefosine may also involve several immunomodulatory effects such as inhibition of the P13K/Akt pathway (which is initially activated by the parasite for survival), macrophage activation for NO production and enhancement of IFN-γ receptor responsiveness (which can in turn lower the production of Th2 cytokines)[184,190,191]. A few studies have argued that miltefosine-mediated immunomodulatory effects are more advanced when miltefosine is combined with compounds that stimulate Th1 polarized cytokines[22,150,184]. Since the proposed mechanism of STAT6-IP involves inhibition of Th2 cytokines (and not enhancement of Th1 activation), this could be a reason as to why our peptide showed no additive activity with miltefosine. Another reason could also be the possible interference of STAT6-IP activity by combination with miltefosine. STAT6 pathway is activated by kinases and miltefosine has been reported to function through kinase inhibition[20,116]. For future studies, STAT6-IP should be tested for compatibility with other antileishmanial drugs.

In general, approaches applied in VL therapy have aimed at overcoming the overwhelming immunosuppressive environment via use of Th1 cytokine "upregulators" but there exists an underlying fear of generating an overdrive proinflammatory scenario[163]. In this context, compounds like STAT6-IP can be of advantage in terms of inhibiting Th2 cytokine production (that are responsible for immunosuppression) without creating a proinflammatory environment. Since STAT6-IP inhibits STAT6 signalling, it could be potentially used in identifying the role of IL-4, IL-13 and STAT6 in the pathogenesis of VL disease (which still

remains unclear). To the best of our knowledge, this is the first time that an inhibitory peptide is used for treatment in leishmaniasis. This peptide may also find therapeutic applications in other diseases that are associated with aberrant Th2 responses.

We do not dispute that further studies is required to determine how this inhibitory peptide induces antileishmanial activity since our observations and assumptions were mostly reliant on antibody characterization. Immunological analysis such as quantification of cytokines involved in VL progression and cure (IFN-γ, IL-12 and IL-4, IL-13, IL-10, TGF-β), measurement of iNOS levels/ NO production as well as determining if impaired T-lymphocyte proliferation is restored after treatment will be useful in making a more concrete report.

This study is the first time that STAT6-IP is employed systemically (IP administration). Intranasal injection in allergy studies and foot pad injections in our lab have shown that STAT6-IP can "safely" be delivered to the lungs and draining LNs respectively. Further analysis, through close observation of the liver and spleen, will be required to understand how this peptide elicits a systemic immune response.

Despite the contrasting reports on the effects of STAT6-IP on parasite burden in our two studies, it is still possible that STAT6-IP has antileishmanial properties that were not enhanced with miltefosine combination. In a study where Tucaresol (an orally bioavailable immunopotentiatory drug that enhances Th1 activity through increased production of IL-2 and IFN-γ) was used for treatment of murine *L. donovani* infection, results showed significant reduction of liver amastigotes with the immunomodulator when used alone but no synergistic effect when combination with Pentostam[192]. Also, in another study where the therapeutic efficacy of anti-IL-10 mAb and anti-GITR (glucocorticoid-induced TNF receptor-related protein) was tested in *L. donovani*-infected C57BL/6 mice, anti-IL-10 mAb reduced parasite burden but its combination with anti-GITR showed no antileishmanial effect despite significant

increase in IFN-γ and TNF-α production[193]. As with STAT6-IP, these observations seem to suggest that immunomodulators can have potential antileishmanial properties that are not enhanced when combined with specific compounds. The latter study (and ours as well) is also in agreement with previous reports that have shown that high levels of IFN-γ (which can drive IgG2a production) does not necessarily equate to protection against *Leishmania* infection[193–196]. In a study where SLA was used to treat *L. donovani* infection in mice, SLA favoured a mixed Th1/Th2 response (similar levels of IgG1 and IgG2a) and was still protective in mice[197]. A similar observation has also been reported with hydrophilic acylated surface protein B1 (HASPB1), thus supporting our preliminary observation that the coexistence of Th1/Th2 responses may be important for protection in VL[198].

Conclusion

The development of immunomodulatory strategies, whether as single or combined with antileishmanial drugs, appears to have real potential to improve VL therapy. The challenge now is identifying the best targets to achieve therapeutic success. In order to make optimal use of these new agents, a proper understanding of disease immunopathogenesis is paramount. BALB/c mice infected with *L. donovani*, because they differ from humans in many respects, may be reasonable experimental models for early parasite replication and the study of early immune responses[47]. Hamster models may be more suitable as they can mimic the clinical situation of human VL[199]. In times past, the lack of immunological reagents have limited usage of hamsters but with the availability of real time RT-PCR assays, quantification of cytokine replication in hamsters is now possible[200]. This development can facilitate better investigations into the mechanisms of visceral disease in hamsters and in the long run, results extrapolated

from studies in these models may guide advance in the use of these new approaches for VL control.

5 CHAPTER 5: SUMMARY

Extensive data from both experimental and clinical studies have shown the advantages of immune-based therapy for treatment of VL. Despite this observation, only few immunotherapeutic agents have been clinically tested and none has been introduced into routine use. This could be attributed to variations in the readouts obtained from different studies during evaluation of immunotherapeutic benefits. To resolve this problem, standardized protocols should be developed to determine what optimal conditions are required for the development of potent antileishmanial immunotherapeutics[201].

Another important question that needs to be addressed is why immunomodulatory compounds that induce Th1 response and protect mice from leishmaniasis have generally failed to induce significant protection in humans. There are several factors that could be responsible for this such as differences in mouse and human immune system, very low and/or non-stringent standards for what is considered protective in murine studies and the unrealistic treatment protocols employed in some murine studies. Treatment schedules are sometimes designed to maximize desirable results in mice that are completely unrealistic in clinical settings[202]. In this context, studies aimed at assessing the efficacy of immunotherapeutics should be accelerated in both mice and dogs before clinical trial evaluations.

Finally, efforts to eliminate VL cannot rely on treatment alone. Other interventions such as reducing human-vector contact via the use of treated bed nets and indoor residual spraying of insecticides will help to limit the spread of disease especially in endemic areas[99].

FIGURES

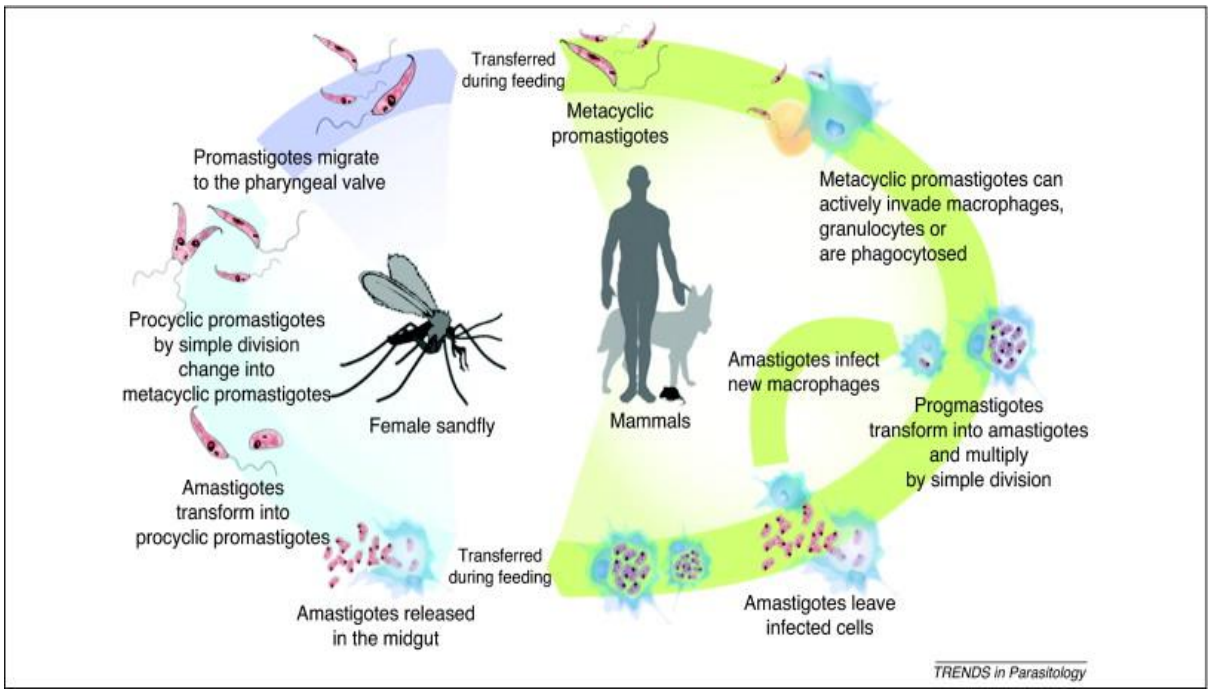

FIGURE 1. **Lifecycle of *Leishmania* parasite**. Leishmania is transmitted by the bite of infected female phlebotomine sand fly. Sand flies become infected by ingesting amastigotes while feeding from an infected host. The amastigotes are released into the sand fly gut where they transform into procyclic amastigotes and then to infective metacyclic promastigotes by binary fission. Infective metacyclic promastigotes migrate to pharyngeal valve of the sand fly where they are transferred to an uninfected host. In the new host, they invade macrophages and other phagocytic cells where they transform into amastigotes and multiply. Cells containing amastigotes eventually rupture and proceed to infect other cells, establishing infection.

Source: Harhay, M. O., Olliaro, P. L., Costa, D. L. & Costa, C. H. N. Urban parasitology: visceral leishmaniasis in Brazil. *Trends Parasitol.* **27,** 403–409 (2011).

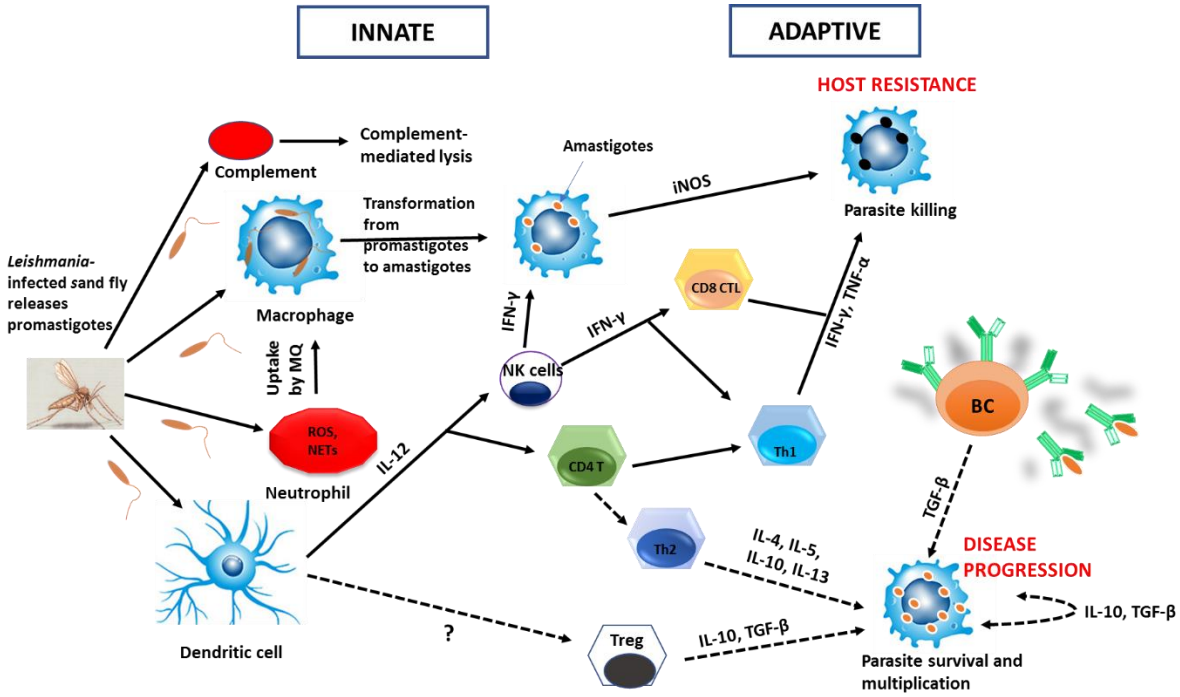

FIGURE 2. Interplay of Innate and Adaptive Immune System in Response to Leishmaniasis. Following infection of host, *Leishmania* metacyclic promastigotes are deposited into the skin by an infected sand fly. Majority of the parasites undergo killing by complement-mediated lysis while those that survive lysis are engulfed by macrophages, neutrophils (NTs) and dendritic cells (DCs). NTs carrying parasites become activated and release ROS and NETs to degrade parasites. They are also taken up by macrophages causing indirect macrophage infection. Infected DCs induce IL-12 production which activates NK and primes $CD4^+$ T cells to protective Th1 cells. Activated NK cells, $CD4^+$ Th1 and cytotoxic $CD8^+$ T cells release IFN- γ for upregulation of ROS and NO required for parasite killing and resistance. The mechanisms for susceptibility but an environment with low IL-12 can induce Th2 cells to release IL-4, IL-10 and IL-13 which enhances parasite survival. In addition, production of IL-10 and TGF-β by T reg cells contribute to the survival of parasites. The signals that promote differentiation to T regulatory (T reg) cells are still unknown. B cells are also activated during infection to produce anti-*Leishmania* antibodies. Depending on the predominant cytokine, IFN- γ and IL-4 can each stimulate B cells to undergo isotype switching to IgG2a and IgG1 respectively. *NETs: neutrophil extracellular traps; ROS: reactive oxygen species; NO: nitric oxide; IFN- γ: interferon-gamma, TGF-β: tumor growth factor.*

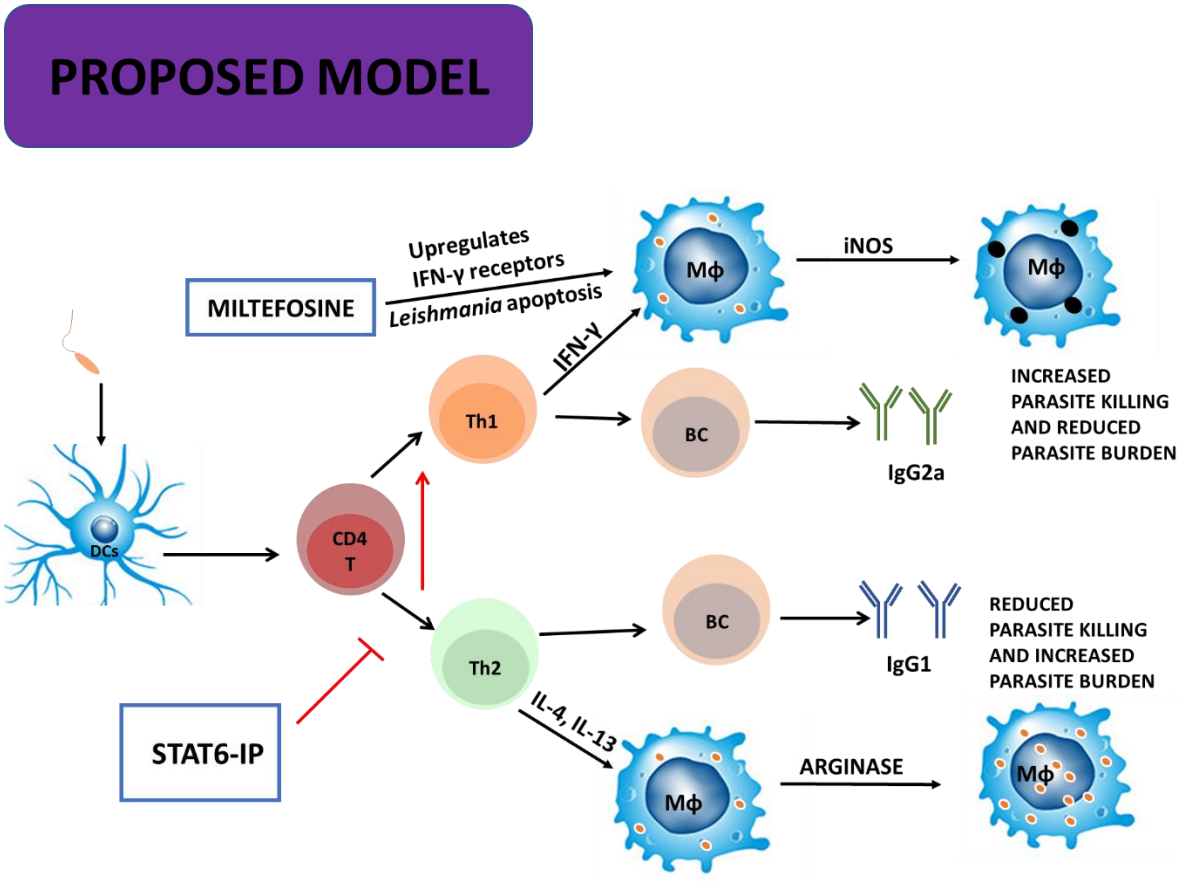

FIGURE 3. Combination of STAT6-IP and Miltefosine for treatment of *L. donovani* infection in BALB/c mice.

TABLES

TABLE 1. Currently available drugs for treatment of Visceral Leishmaniasis

Drugs	Sodium Stibogluconate	Pentamidine	Amphotericin B deoxycholate	Liposomal amphotericin B (Ambisome)	Miltefosine	Paromomycin
Mechanism of Action	Acts by inhibiting glycolytic enzymes and fatty acid oxidation in Leishmania amastigotes; inhibits the net formation of ATP and GTP in a dose-dependent manner	Acts on parasite genome by hampering replication and transcription at the mitochondrial level	Acts by inhibiting membrane lipid biosynthesis forming microspores, leading to increased membrane permeability and ultimate killing of leishmania	Same as conventional amphotericin but distribution of drug in the body is different	Acts by interfering with membrane synthesis and cell signalling pathways	Acts by interfering with initiation of protein synthesis by fixing the 30S-50S ribosomal complex at the start codon of mRNA, leading to accumulation of abnormal initiation complex
Regimen	20 mg/kg daily for 30 days	4 mg/kg thrice a week for 3-4 weeks (10-12 injections)	1 mg/kg on alternate days x 15 doses in 30 days	Total dose of 20 mg/kg split over several doses	2.5 mg/kg for 28 days (India only)	15 mg/kg for 21 days (India only)
Administration	intravenous or intramuscular	intravenous or intramuscular	intravenous	intravenous	oral	intramuscular
Toxicity	Arrhythmias, reversible pancreatitis, nephrotoxicity, hepatotoxicity, death	Myalgias, pain at the injection site, nausea, headache, metallic taste, burning sensation, numbness, hypotension, hypoglycaemia	Nephrotoxicity (in-patient care needed), infusion-related fever	Minor/no nephrotoxicity, mild infusion-related fever	Mild Gastrointestinal disturbances, nephrotoxicity hepatotoxicity possible teratogenicity	minor/no nephrotoxicity, reversible ototoxicity, hepatotoxicity (all relatively rare)

Source: Singh, P. & Kumar, M. Current treatment of visceral leishmaniasis (Kala-azar): an overview. *Int. J. Res. Med. Sci.* **2,** 810–817 (2014)

TABLE 2. Some immunomodulators that have been employed in VL therapy

IMMUNOMODULATOR	HOST	MODE OF ACTION	REFERENCES
Free and Liposomal Tuftsin	BALB/c mice	Augments the phagocytic activity of macrophages; activate macrophages to produce NO	203
Leptin (adipocyte-derived hormone)	BALB/c mice	Stimulates macrophages and NK cells for the production of proinflammatory cytokines with resultant reduction of Th2 cytokines	204
Picroliv (a purified iridoid glycoside from the roots of *Picrorhiza kurrooa*	Hamsters	Hepatoprotective, anti-inflammatory and antioxidant properties	182,205
Pam3Cys (synthetic bacterial peptide; TLR-2 agonist	BALB/c mice	Triggers Th1 effector functions	23,206
rHuGM-CSF	Human (HIV-VL coinfected patients)	Macrophage activator; mobilizes blood monocytes	207
Positively-charged liposomes containing SLA	BALB/c mice	Enhances uptake by APCs for efficient presentation by MHC class I and class II	197
Alum/ALM + BCG	Human (PKDL patients, healthy adults, children)	Extensive antigenic cross-reactivity between *Leishmania* species	208–210
Liposomal MDP analog Muramyl dipeptide	Hamsters and mice	Increases both cell-mediated and humoral immune responses	211
Quassin (an extract from the bark of the Bitter tree, *Quassia amara)*	Balb/c mice	Induces iNOS expression and Th1 cytokine production; suppresses Th2 cytokines	212
Polyinosinic-polycytidylic acid	Hamsters	Induces IFN- γ and NO	213
CP-46,665-1 (synthetic lipoidal amine)	C57BL/6 mice	Not fully evident, possibly through cytolytic activity	214
OX40L-Fc (chimeric fusion protein) and CTLA-4 inhibitor	Mice	Enhances granuloma maturation	215

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