Chapter V,

Efficacy of Artemisinin in experimental VL
Introduction:
Host parasite interaction:

The digenetic life cycle of the protozoan parasite *Leishmania* consists of free living, motile flagellated promastigotes residing within the sandfly gut and as aflagellated amastigotes residing within the mammalian host macrophages causing Leishmaniasis, affecting millions of people around the world. Leishmaniasis is one of the most diverse and complex of all vector borne diseases. In order to develop a successful parasitic relationship with the host, the parasite cleverly evades both the innate and adaptive immune responses. With respect to *Leishmania*, the infection process begins with the bite of the infected sandfly resulting in the inoculation of promastigotes into the dermis of the secondary host. Within the mammalian host, *Leishmania* parasite resides as amastigotes in phagocytic cells such as macrophages, dendritic cells and neutrophils. During infection, promastigotes are readily engulfed by the macrophages but become resistant towards degradation in phagosomes. Macrophages are present in all tissues in the body and carry out fundamental protective functions against invading pathogens through phagocytosis, destruction of microorganisms as a part of innate immunity. Macrophages also play an essential role in acquired immunity to infectious organisms through presenting antigen to lymphocytes and secreting a variety of cytokines during development of the cell mediated immune response. These promastigotes attached to the recruited macrophages through a variety of receptors, the major ones being complement receptor type 1 and type III [Matlashewski, 2002]. A surface glycoprotein, gp63 present in *Leishmania* converts C3b, a complement protein and one of the potent immune opsonins to iC3b [Hermoso et al., 1991] favouring phagocytic clearance rather than a lytic clearance of intracellular parasites and this point of intervention is critical for survival of parasite. After being engulfed, the parasite experiences the macrophages harsh environment within the phagosomes, like an acidic environment of pH 4 and an oxidative burst to kill foreign pathogens. Macrophages play a primary role in the host defense and regulation of immune responses via activation of superoxides, hydroxyl radicals and also via acidic enzymes towards parasitic degradation especially when lysosomes fuse with phagosomes [Sharma et al., 2009a]. Within the parasitophorous vacuole, the metacyclic form of promastigotes transforms into intracellular amastigotes shedding off the promastigote LPG (lipophosphoglycan) that consequently inhibits the respiratory burst, a natural process that
occurs after phagocytosis [Awasthi et al., 2004]. The parasites perform a complex host-parasite interaction inside the harsh milieu of the phagolysosomes and effectively evade and influence the host immune system.

**Host responses in Leishmaniasis:**

**Humoral response-**

*Leishmania* infection is characterized by the appearance of anti-leishmanial antibodies and the presence of elevated anti-leishmanial antibody titres in the sera of infected individuals. In CL, usually titres are at low levels during active phase of the disease contrasting with strong anti-leishmanial titres, in VL [Saha et al., 2006] although its role towards protection or pathogenesis in Kala-azar is still not clear [Neogy et al., 1987]. *Leishmania* antigen specific immunoglobulin (Ig) isotypes revealed elevated levels of IgG, IgM and IgE during disease. To establish a correlation if any, between production of these isotypes and progression and resolution of infection has been studied. A marked elevation of IgG1 in patients with VL was evidenced. Passive administration of antileishmanial IgG also resulted in larger lesions in BALB/c mice, with greater amounts of IL-10 production which correlated with high level of antileishmanial antibodies during active phase of the disease and a consecutive fall in antibody titre following successful cure [Saha et al., 2006].

**Cell mediated immune response-**

In Leishmaniasis, immunological dysfunctions are associated with T cells, Natural Killer cells causing in particular, incapacitation of host macrophages as only after macrophages are activated, can parasites be killed. [Kaye et al., 2004]. T cells play a major role in generating specific and memory T-cell responses to intracellular parasitic infections and these have been extensively characterized in *Leishmania* infection. The outcome of leishmanial infection is associated with two functionally distinct T-helper cells (Th), namely Th1 and Th2. Gamma-interferon (IFN-γ) interleukin-2 (IL-2) and interleukin-12 (IL-12) are classical cytokines for Th1 responses and cause increase in nitric oxide production, and importantly are impaired during acute VL [Figure 5.1, Ahmed et al., 2003, Ganguly et al., 2008]. This is accompanied by up regulation of Th2 responses, so as to achieve macrophage dysfunction, resulting in disease progression. Accordingly, cure and control of *Leishmania* infection is generally associated with recovery of the Th1-type immune response.
Experimental model of Visceral Leishmaniasis:

Several studies made on mouse model to represent the response in human VL with species, *L. donovani, L. infantum/chaghasi* have underscored the fact that host responses to these parasites differ significantly from *L. major* infection. In murine models of experimental CL, a clear-cut dichotomy between Th1-mediated protection and Th2-mediated disease susceptibility is established; however in VL, the immune response evident in disease vs. protection is not so clearly demarcated [Tripathi et al., 2007]. The Th1/Th2 paradigm of resistance/susceptibility to intracellular infection is largely based on investigations using *L. major*. Most strain of mice (C57BL/6, C3H, and CBA) develop a self limiting cutaneous disease when infected with the same. In these mice, resolution of infection is mediated by Th1 cells which produce IFN-γ that induces production of nitric oxide in infected macrophages leading to destruction of parasites. T-cell differentiation into Th1 or Th2 effector cells depends chiefly upon priming during differentiation as IL-4 induces Th2 whereas IL-12 induces Th1 differentiation (Figure 5.1). The BALB/c mouse model meets the eligibility requirements as the chronic infection pattern resembles human VL [Ahmed et al., 2003] and also, as its innate susceptibility to numerous intracellular pathogens has been linked to hypoproduction of IFN-γ along with a preferential increase in Th2-type cytokine responses. Therefore, protective immunity is achieved by upregulation of Th1 responses after successful chemotherapy.

Immunomodulation:

New antileishmanial drugs are urgently needed to minimise the disease burden. Leishmaniasis is associated with immunological dysfunction of T cells, natural killer cells and in particular, incapacitation of macrophages, established cellular harbors of the parasite cure and control of Leishmaniasis by chemotherapy appears to be dependent upon development of effective immune responses, that can activate macrophages to produce toxic nitrogen and oxygen intermediates and kill intracellular amastigotes [Crofts and Coombs, 2003]. Indeed, novel experimental approaches using antileishmanial compounds with microbicidal and immunomodulatory action have utilized a macrophage-activated drug delivery system aimed at recovering the Th1 immune response [Murray, 2001, Figure 5.1]. Thus, we selected BALB/c mice model to evaluate the immunomodulatory role of Artemisinin and confirm the *in vivo* antileishmanial activity of Artemisinin and its analogs.
Efficacy of Artemisinin in experimental VL

**Figure 5.1: Th1 and Th2 responses in Leishmaniasis**
Adapted from Tripathi et al., 2007. The switch over of T cells either to Th1 or Th2 depends upon priming with IL-12 towards Th1 or IL-4 towards Th2 cell responses. In human VL a mixed Th1/Th2 response occurs whereas in animal models a clear cut Th1 and Th2 dichotomy exists.
Materials and Methods:

Measurement of nitric oxide (NO):

Levels of nitrite in macrophages were measured using Griess assay as described in Materials and Methods.

Reverse transcriptase polymerase chain reaction (RT-PCR):

RNA was isolated from BALB/c mice macrophages treated with Artemisinin (10 and 25 µM) for 18 h, using the RNAqueous® Kit and subsequently, RT-PCR was carried out using one-step RT-PCR kit as described in Materials and Methods.

Experimental model of VL in BALB/c mice:

BALB/c mice were chosen since their infection pattern closely resembles human VL. Briefly, late log phase *L. donovani* promastigotes were injected i.p. into 4-6 weeks old BALB/c mice and anti-leishmanial antibody titre was monitored (approx. 80 days) and expressed with respect to normal uninfected mice.

Subsequently efficacy of Artemisinin and its analogs were studied in *in vivo* model (Figure 5.2).

Preparation of Leishmania donovani antigen (LDA):

Crude *Leishmania* antigen was prepared from a *L. donovani* strain as described in Materials and Methods.

Indirect ELISA:

IgM (for primary response) and IgG (for secondary response) was measured to monitor *Leishmania* infection by ELISA as described in Materials and Methods.

Measurement of Gr1+ve surface staining in mouse blood:

To check the infectivity in BALB/c mice, blood was collected from the orbital plexus and surface stained with Gr1 antibody (marker of polymorphonuclear cells or PMNs) as described in Materials and Methods.
**In vivo evaluation of anti amastigote activity:**

Two weeks after completion of treatment, all animals were sacrificed, splenic weight measured and parasitic burden estimated in terms of Leishman-Donovan units (LDU) as described in Materials and Methods.

**Intracellular cytokine analysis:**

Splenocytes were obtained by macerating the spleen of infected BALB/c mice and intracellular cytokines were measured as described in Materials and Methods.
Results:
*Artemisinin restored production of NO in L. donovani infected macrophages:*

Impairment of macrophage oxidative metabolism by intracellular *Leishmania* species plays a pivotal role in their survival [Bhattacharjee et al., 2009]. *Leishmania* achieve this by deviously impairing activation of host macrophages, thereby preventing production of lethal nitric oxide [NO, Murray and Nathan, 1999]. In our study, *Leishmania* infection translated into a decrease in NO production as compared to uninfected macrophages, mean ± SEM being 4.48 ± 0.25 vs. 6.51 ± 0.23 μM (p < 0.001). The addition of Artemisinin (10 μM) to uninfected macrophages caused no alteration in generation of NO, whereas the higher conc. of 25 μM induced a 1.3 fold (Figure 5.3a). In infected macrophages, Artemisinin (10 μM) increased levels of NO to 5.52 ± 0.45 μM and with 25 μM, the levels increased significantly to 6.78 ± 0.43 μM (p < 0.01); importantly, the levels of NO achieved were comparable with NO generated by uninfected macrophages (Figure 5.3a).

Figure 5.3: Effect of Artemisinin upon generation of NO and mRNA expression of iNOS in normal and parasitized peritoneal macrophages
(a) Levels of nitrite, as a measure of NO production, in uninfected untreated murine peritoneal macrophages (1×10⁶/mL) (1) and uninfected macrophages treated with artemisinin [10μM (2) and 25μM (3)], or macrophages infected with stationary-phase *L. donovani* promastigotes (4) and subsequently incubated with artemisinin [10 μM (5) and 25μM (6)] at 37 °C in 5% CO2. Supernatants were collected and measured for NO using Griess reagent as described in M &M. Each point represents the mean ± standard error of the mean of at least three experiments in duplicate. *p < 0.05 compared with infected macrophages; **p < 0.01 compared with uninfected macrophages.
(b) Representative profile of mRNA expression of iNOS and β-actin in murine peritoneal macrophages of the same treatment for 18 h as described in part (a).RNA were isolated and subjected to reverse transcriptase polymerase chain reaction (RT-PCR). The RT-PCR products of iNOS and β-actin were resolved on an agarose gel (1.5%), visualised by Etbr and their expression quantified densitometrically.
As generation of NO is dependent on the activation of iNOS, we studied its expression to evaluate the effect of Artemisinin. In uninfected macrophages, that expressed basal levels of iNOS, addition of Artemisinin (10 μM, 18 h) showed no changes whereas 25 μM evoked a 1.4 fold increase (Figure 5.3b). In untreated, Leishmania-infected macrophages, the expression of iNOS decreased, corroborating with published data [Bhattacharjee et al., 2009]. Notably, following treatment with Artemisinin (10 and 25 μM, 18 h), the mRNA expression increased by 2.3 and 2.9 fold respectively, the levels being comparable with uninfected macrophages (Figure 5.3b). In terms of infectivity, Artemisinin (10 and 25 μM) decreased the % infectivity to 57.1 ± 5.42 % and 49.3 ± 3.12 % respectively, with reference to parasitized macrophages, whose infectivity was considered as 100 % [Sen et al., 2010a].

Leishmania-infected macrophages secrete several anti-inflammatory cytokines including IL-4, IL-5 and IL-10 [Tripathi et al., 2007]. It is firmly established that sustained levels of IL-10, a counter-regulatory immunosuppressive cytokine, fosters progression of infection [Ukil et al., 2005]. Therefore, it is logical to anticipate that inhibition of IL-10 can enhance Th1-cell-associated responses, promote IFN-γ secretion, enhance macrophage activation and collectively, ensure effective parasite killing [Murray et al., 2005]. In Leishmania-parasitized macrophages, elevated mRNA expression of IL-10 was evident which, following addition of Artemisinin (10 and 25 μM), decreased in a dose-dependent manner (Figure 5.4).

**Figure 5.4: A representative profile of mRNA expression of IL-10 and β-actin in murine macrophages**

Untreated macrophages (1), following infection with stationary phase *L. donovani* promastigotes (2) were incubated with Artemisinin [10 μM, (3) and 25 μM (4)] at 37°C, 5% CO₂ for 18 h; RNA was isolated and subjected to RT-PCR as described in Materials and Methods. The RT-PCR products of IL-10 and β-actin were resolved on an agarose gel (1.5%), visualized by ethidium bromide and their expression quantified densitometrically.
Development of experimental model of VL using BALB/c mice:

In India, patients with VL have shown elevated levels of *L. donovani* specific antibodies (Saha et al., 2006). In our study, *Leishmania* specific IgM and IgG levels were screened to confirm infectivity in BALB/c mice. IgM levels were used to check the primary response while levels of IgG level were measured to check the secondary response. It is questionable whether these elevated levels of *Leishmania* specific antibodies contribute in any way to protection against VL. Miles et al., (2005) sought to answer this question with a study on IgG deficient BALB/c mice wherein resistance to infection with *L. major* occurred as compared to normal BALB/c mice, indicating that anti-leishmanial IgG actually leads towards disease progression possibly by virtue of its capacity to increase IL-10 production in macrophages. In our data, both IgM and IgG of ten randomly selected *Leishmania* infected BALB/c mice showed a moderate increase in levels as compared to normal uninfected mice, specific OD_{405} being 0.23 ± 0.024 and 0.29 ± 0.04 respectively; Figure 5.5a. Additionally, kinetics of anti-leishmanial IgM and IgG showed an increase in IgM up to 60 days and a drop by 80 days (Figure 5.5b, 5c), while anti-leishmanial IgG levels showed successive increase up to 80 days. Thus we considered > 80 days of infection in BALB/c mice as a standard experimental model of VL.

![Graph showing levels of IgG and IgM in an experimental model of VL](image)

**Figure 5.5:** Levels of IgG and IgM in an experimental model of VL.

- **a:** Fold changes in anti-leishmanial IgM and IgG levels of *L. donovani* infected mice.
- **b:** Kinetics of anti-leishmanial IgM of infected mice.
- **c:** Kinetics of anti-leishmanial IgG of infected mice. Anti-leishmanial IgM and IgG were measured using ELISA as described in Materials and Methods.
**Efficacy of Artemisinin in experimental VL**

In **vivo activity of Artemisinin**:

In chronic VL, parasite load and cellular infiltration in the spleen and liver results in hepatosplenomegaly [Kaye et al., 2004]. With infection, the splenic weight significantly increased from 107.0 ± 8.4 mg to 220.0 ± 26.0 mg (p<0.01), which following effective parasitic elimination with SAG, significantly reduced to 105.0 ± 26.0 mg, p<0.05. Similarly, treatment with Artemisinin (10 and 25 mg/kg b.w, p.o) as compared to infected mice, also significantly decreased splenic weight to 94.0 ± 7.7 mg (p<0.01) and 110.0 ± 2.7 mg (p<0.01), respectively [Sen et al., 2010a].

The efficacy of Artemisinin in murine experimental visceral leishmaniasis was also evaluated in splenocytes in terms of LDU. In infected mice, the mean ± SEM of LDU was 1176.0 ± 180.0, which upon treatment with SAG demonstrated a significant decrease of 85% to 187.5 ± 21.25 (p<0.001) as compared to the untreated, infected group. Similarly, in the Artemisinin (A10 and A25) treated groups, the LDU decreased to 210.0 ± 27.0 (p <0.01) and 168.0 ± 5.0 (p <0.001) respectively which translated into a parasite removal of 82.6% and 86.0% respectively (Figure 5.6).

**Figure 5.6: Anti-amastigote activity of Artemisinin**

Mice infected with *L. donovani* were treated with Artemisinin [10 and 25 mg/kg b.w. in 1% DMSO, oral, A10 and A25 respectively] or SAG [20 mg/kg b.w. in 0.9% normal saline, s.c., SAG] for 5 alternate days while control infected animals received normal saline. Two weeks post treatment, mice were sacrificed and levels of parasite burden in spleen quantified in terms of Leishman Donovan units (LDU) as described in Materials and Methods. Values represent the mean ± SEM of five animals per group. *p< 0.01 and **p<0.001 as compared to infected group.

**Expression of granulocytes in mice blood:**

Gr1 is a surface antigen marker expressed on myeloid cells whose expression level increases with maturation of PMNs. A balanced neutrophil response may at the expense of maximal anti-leishmanial activity, represent a mechanism for protecting against excess pathology [Smelt et al., 2000]. A study by Rousseau et al., (2001) revealed that during early
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infection, in the spleen, neutrophils play a role in controlling *L. infantum* burden. As neutrophils possibly play a role in *Leishmania* entry into macrophages, they are considered as a perfect temporary shelter to preserve *Leishmania* from the hostile extracellular milieu before entering their final host cells, the macrophages. The ability of parasites to survive and maintain infectivity in PMN enables these organisms subsequently to establish a productive infection in macrophages. It has therefore been proposed that these intracellular parasites possibly use neutrophils as “Trojan horses” to invade their definitive host cells, the macrophage [van Zandbergen et al., 2004]. Therefore, an increase in Gr1 expression can be used as a marker for disease progression and vice versa. We demonstrated that upon infection, Gr1 expression increased significantly as compared to normal mice, the mean % positivity being 54.57 ± 11.97 % vs. 22.21 ± 9.12 %, in control, uninfected mice. Upon treatment with SAG, the % positivity decreased to 33.89 ± 14.66 % and in Artemisinin (10 and 25 mg/kg b.w) treated groups, the expression also decreased to 39.48 ± 10.92 and 34.12 ± 12.39 % respectively (Figure 5.7).

![Figure 5.7: Changes in Gr1 expression following *Leishmania* infection](image)

Mice infected with *L. donovani* were treated with Artemisinin [10 and 25 mg/kg b.w. in 1% DMSO, oral, A10 and A25 respectively] or SAG [20 mg/kg b.w. in 0.9% normal saline, s.c., SAG] for 5 alternate days while control infected animals received normal saline. Two weeks post treatment, mice were sacrificed and blood collected and stained for Gr1 expression as described in Materials and methods. *p< 0.05 as compared to normal (N) group.

**Artemisinin restored production of Th1 cytokines:**

It is well established that during leishmanial infection, critical skewing from a pro-inflammatory to an anti-inflammatory response results in sustained intracellular survival of parasites. As cure is primarily associated with production of Th1 cytokines that aids parasite
elimination [Halder et al., 2009], we evaluated the efficacy of Artemisinin upon Th1 cytokine responses in infected BALB/c mice. Accordingly, the intracellular profiles of IFN-γ and IL-2 were studied in CD3+ gated T lymphocytes. Following infection, the % of IFN-γ and IL-2 decreased significantly as compared to untreated mice (Table 5.1). The addition of SAG or Artemisinin (A10 and A25), effected a surge in both IFN-γ and IL-2 bringing their levels comparable to the control, uninfected group (Table 5.1). Treatment with 1% DMSO (vehicle) showed no effect upon the release of IFN-γ and IL-2, confirming its immunological inertness (data not shown). The intracellular levels of IL-4 and IL-10 were low in uninfected mice and minimal changes occurred following infection; treatment with SAG or Artemisinin also caused no appreciable changes in levels of IL-4 and IL-10 (Table 5.1, Figure 5.8).

**Table 5.1: Effect of Artemisinin upon intracellular cytokine expression in T lymphocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N)</td>
<td>6.59 ± 0.45</td>
<td>5.77 ± 0.67</td>
<td>1.29 ± 0.63</td>
<td>1.55 ± 0.28</td>
</tr>
<tr>
<td>Infected (Inf)</td>
<td>1.16 ± 0.27*</td>
<td>1.13 ± 0.40*</td>
<td>0.84 ± 0.06</td>
<td>1.15 ± 0.31</td>
</tr>
<tr>
<td>SAG (20 mg/kg b.w., s.c.)</td>
<td>8.59 ± 0.26®</td>
<td>8.28 ± 0.17®</td>
<td>0.58 ± 0.01</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>Artemisinin (A10, 10 mg/kg b.w., oral)</td>
<td>5.80 ± 0.44#</td>
<td>8.92 ± 0.87®</td>
<td>1.14 ± 0.17</td>
<td>1.19 ± 0.16</td>
</tr>
<tr>
<td>Artemisinin (A25, 25 mg/kg b.w., oral)</td>
<td>7.36 ±1.16®</td>
<td>6.7 ± 0.32#</td>
<td>0.77 ± 0.06</td>
<td>0.88 ± 0.07</td>
</tr>
</tbody>
</table>

Splenocytes from BALB/c mice isolated from different groups (n = 5) were incubated with PMA (5 ng/mL), Ionomycin (1 μM) and Brefeldin A (10 μg/mL), surface-stained with anti-mouse CD3- FITC, permeabilized and stained with PE-conjugated IFN-γ, IL-2, IL-4 or IL-10, as described in Materials and Methods. The data are expressed as mean % positivity ± SEM. *p<0.01 as compared to normal mice; ®p<0.01 and #p< 0.05 as compared to infected mice.

-98-
Figure 5.8: Dot plot analysis of intracellular interferon-gamma (IFN-γ) or interleukin (IL)-2 from murine T-lymphocytes. A representative profile of IFN-γ or IL-2 present within CD3+ T-lymphocytes of BALB/c mice infected with *L. donovani*. Mouse splenocytes were isolated from untreated normal (N) or infected (Inf) mice as well as mice that received either sodium antimony gluconate (SAG) [20 mg/kg body weight (b.w), subcutaneous) or artemisinin [10 mg/kg b.w (A10) or 25 mg/kg b.w (A25), oral]. Cells were stained using phycoerythrin (PE)-conjugated anti-cytokine antibodies as described in Materials and Methods.
Efficacy of Artemisinin in experimental VL.

Artemisinin demonstrated decrease levels of serum IgM and IgG in infected mice as compared with normal mice:

Infection of *Leishmania* is often characterized by the appearance of anti-leishmanial antibodies in the sera of Leishmaniasis patients. The presence of antibodies against *L. braziliensis* infection in the sera of infected patients has been critically monitored and utilized for diagnosis and prognosis of the diseases [Valli et al., 1999]. In the same way, in VL the antibody response is well documented [Neogy et al., 1987] and critical analysis of *Leishmania* antigen-specific Ig isotypes revealed elevated levels of IgM, IgG and IgE at presentation and decreased levels of IgG upon cure [Saha et al., 2006]. In our results we demonstrated a significant increase in IgM in the infected group (the specific OD\textsubscript{405} being 1.2 ± 0.05) with respect to normal group (0.42 ± 0.02) and upon treatment with Artemisinin (10 and 25 mg/kg, b.w) it showed a significant decrease to 0.58 ± 0.05 and 0.59 ± 0.07 respectively as compared to infected group (Figure 5.9a). In case of IgG it significantly increased the specific OD\textsubscript{405} in the infected group being 1.12 ± 0.12 with respect to normal (0.49 ± 0.07); upon treatment with Artemisinin (10 and 25 mg/kg, b.w), it significantly decreased to 0.54 ± 0.11 and 0.76 ± 0.09 respectively with respect to the infected group (Figure 5.9b). This data also corroborated the anti-leishmanial activity of Artemisinin in BALB/c mice.

![Figure 5.9: Decreased levels of IgM (a) and IgG (b) in Artemisinin treated infected mice](image)

Anti-leishmanial IgM and IgG were measured using ELISA as described in Materials and Methods. Mouse sera were collected from untreated normal (NMS) or infected (Inf) mice as well as mice that received either artemisinin [10 mg/kg bw (A10) or 25 mg/kg bw (A25), oral]. Anti-leishmanial IgM and IgG were measured using ELISA as described in Materials and Methods. *p*<0.05 as compared to infected mice.

-100-
Treatment with Artemisinin analogs decreased splenic weight and upregulated Th1 cytokines back to normal levels:

Like Artemisinin, we also screened the efficacy of promising Artemisinin analogs (orally treated) using in an experimental model of Leishmaniasis. The splenic weight in infected BALB/c mice, significantly increased from 116.0 ± 5.6 mg to 220.0 ± 67.7 mg (p<0.01); following treatment, with GC003 (10 mg/kg b.w) it decreased to 103.7 ± 23.18 mg (p< 0.05) as also with GC012 (1 and 5 mg/kg b.w), the splenic weight decreased to 146.0 ± 22.2 and 164.5 ± 39.5 mg respectively. ML86.1 (10 mg/kg b.w) too showed a decrease being 97.7 ± 31.6 mg (p< 0.05), suggesting disease cure. The status of Th 1 intracellular cytokines like IFN-γ and IL-2 in CD3+ gated T lymphocytes showed that in Leishmania infected mice, the % of IFN-γ and IL-2 decreased significantly as compared to untreated mice. Upon treatment with GC003, GC012 and ML 86-1, there was an increased proportion of IFN-γ and IL-2 (Table 5.2), suggesting efficacy of tested Artemisinin analogs in experimental VL.

Table 5.2: Effect of analogs of Artemisinin upon intracellular cytokine expression in T lymphocytes of mice infected with *L. donovani*

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ (% +ve)</th>
<th>IL2 (% +ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.00 ± 0.36</td>
<td>7.14 ± 0.5</td>
</tr>
<tr>
<td>Infected</td>
<td><strong>1.41 ± 0.29</strong></td>
<td><strong>2.6 ± 0.74</strong></td>
</tr>
<tr>
<td>GC003 (5 mg/kg b.w, p.o)</td>
<td>7.56 ± 0.23@</td>
<td>5.92 ± 0.44@</td>
</tr>
<tr>
<td>GC003 (10 mg/kg b.w, p.o)</td>
<td>6.32 ± 0.18@</td>
<td>8.90 ± 0.70@</td>
</tr>
<tr>
<td>GC012 (1 mg/kg b.w, p.o)</td>
<td>7.08 ± 0.74@</td>
<td>6.02 ± 0.38@</td>
</tr>
<tr>
<td>GC012 (5 mg/kg b.w, p.o)</td>
<td>7.54 ± 0.14@</td>
<td>8.94 ± 0.29@</td>
</tr>
<tr>
<td>ML86.1 (5 mg/kg b.w, p.o)</td>
<td>8.47 ± 1.10@</td>
<td>7.04 ± 0.05@</td>
</tr>
<tr>
<td>ML86.1 (10 mg/kg b.w, p.o)</td>
<td>4.44 ± 0.32</td>
<td>7.98 ± 0.33@</td>
</tr>
</tbody>
</table>

Splenocytes from BALB/c mice isolated from different groups (n = 5) were stimulated with PMA (5 ng/mL), Ionomycin (1 μM) and Brefeldin A (10 μg/mL), surface-stained with anti-mouse CD3- FITC, permeabilized and stained with PE-conjugated IFN-γ and IL-2 as described in Materials and Methods. The data are expressed as mean % positivity ± SEM. *p<0.01 as compared to normal mice; @p<0.01 as compared to infected mice.


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**Discussion:**

A major component of innate host defense includes reactive intermediates released from macrophages. Therefore, to enhance survivability within host macrophages, the *Leishmania* parasite deviously inhibits parasiticidal effector responses (Figure 5.1) that include decreased production of reactive oxygen intermediates [Bhattacharjee et al., 2009; Murray and Nathan 1999]. We have studied the host response *ex vivo* and in an *in vivo* model of Leishmaniasis (Figure 5.2). *Leishmania* infection decreases production of NO which following addition of Artemisinin (10 and 25 μM), translated into a significant increase, but importantly, only to levels comparable with uninfected macrophages (Figure 5.3a). In concordance, infection translated into a lowered mRNA expression of iNOS which was restored by Artemisinin (Figure 5.3b). Taken together, the observed 50% reduction in parasite burden by Artemisinin is achieved primarily by a direct parasiticidal effect, rather than a NO-dependent pathway.

In Leishmaniasis, the increased expression of IL-10 is directly linked with exacerbation of disease as it compromises antigen presentation and co-stimulatory mechanisms, downregulates T-cell proliferation and importantly, dampens macrophage responsiveness to Th1 cytokines [Figure 5.1, Miralles et al., 1994, Tripathi et al., 2007]. Therefore, as IL-10 can disable host anti-leishmanial defense, its inhibition would translate into effective elimination of *Leishmania* [Ukil et al., 2005]. Artemisinin caused a dose-dependent reduction of IL-10 expression in parasitized macrophages, thereby providing evidence of its immunomodulatory action playing an important role in parasite curtailment (Figure 5.4).

Miltefosine also exerts a direct microbicidal action as *in vivo* models have demonstrated effective parasite elimination in knock out mice devoid of T cells [Murray and Delph-Etienne, 1999]. Furthermore, Escobar et al., 2001 showed that Miltefosine remained effective in immunocompromised mice. Other drugs that similarly eliminate *Leishmania* in an immune independent manner include Cyclosporin A [Meissner et al., 2003], Nimodipine [Tempone et al., 2009], supercritical fraction isolated from *T. catarinensis* [Soares et al., 2007], (3S)-16 and 17-didehydrofalcarinol, an oxylipin isolated from *Tridax procumbens* [Martín-Quintal et al., 2010] suggesting that directly acting leishmanicidal drugs are equally beneficial.
Efficacy of Artemisinin in experimental VL

effective and in fact could well be a better option in immunocompromised individuals e.g. patients with Leishmania-HIV co-infection.

A suitable laboratory host for the target parasite, L. donovani, is vital for conducting research especially for studying host-parasite interactions, etiopathogenesis, biochemical changes and evaluation of anti-leishmanial compounds. The BALB/c mouse model meets the eligibility requirements as its chronic infection resembles human VL [Ahmed et al., 2003]. In our study, we have developed an in vivo model based on IgM and IgG levels as also parasitaemia as confirmed by parasite transformation. We found that >80 days can be considered as a standard infection model in BALB/c mice (Figure 5.5). Treatment with Artemisinin led to a significant reduction in splenic weight and Gr1 expression which was accompanied by significant inhibition of parasites and importantly, its efficacy was comparable with mice that received SAG (Figure 5.6, 5.7). Further, we corroborated the efficacy of Artemisinin by screening IgM and IgG levels and found a significant reduction (Figure 5.9). In Leishmania-infected BALB/c mice, markers of chemotherapeutic effectiveness include restoration of the Leishmania-specific T cell responses, which are severely impaired in VL [Ahmed et al., 2003, Ganguly et al., 2008]. Of the various cytokines, IL-12 is a key cytokine that drives Th1 cell development during host defense [Hailu et al., 2004] and the efficacy of anti-leishmanial therapy is consistently associated with restored expression of IL-2 and IFN-γ [Halder et al., 2009; Tripathi et al., 2007]. In infected mice, the pronounced impairment of Th1 responses (down-regulation of IFN-γ and IL-2) was restored by Artemisinin treatment suggesting that it directly killed the parasites and thereby corrected the skewed Th1/Th2 imbalance (Figure 5.8, Table 5.1). The minimal intracellular production of IL-4 and IL-10 could be attributed to their inherently lower production (Table 5.1). Potent Artemisinin analogs also showed good efficacy, upon splenic weight and intracellular cytokine expression (Table 5.2). In 1993, Yang and Liew demonstrated the parasiticidal activity of Artemisinin both in vitro and in vivo in an experimental model of CL. Our data herein suggests that administration of Artemisinin or its analogs to Leishmania-infected BALB/c mice eliminates intracellular amastigotes via generation of iron-Artemisinin adducts, that increases free radical generation, triggers parasite apoptosis, collectively contributing to the Artemisinin-induced protection observed in experimental VL, meriting further pharmacological investigations.