EVALUATION OF CRUDE *LEISHMANIA DONOVANI* ANTIGEN CO-ADMINISTERED WITH TH1 ADJUVANTS AS A POTENTIAL VACCINE FOR LEISHMANIASIS IN VERVET MONKEY MODEL

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DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other university.

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A thesis dedicated to my family and all those involved in the fight against leishmaniasis
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<td>Antigen</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>BCG</td>
<td>Bacille Calmette Guérin</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CD4+</td>
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<td>VL</td>
<td>Visceral leishmaniasis</td>
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<td>CL</td>
<td>Cutaneous <em>Leishmaniasis</em></td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<td>DCL</td>
<td>Diffuse cutaneous leishmaniasis</td>
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<td>DHFR-TS</td>
<td>Dihydrofolate reductase-thymidylate synthetase</td>
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<td>DNA</td>
<td>Deoxy-ribonucleic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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Visceral leishmaniasis (VL) or kala-azar is the most dreaded and devastating form of leishmaniasis, causing high mortality rate, mainly in children. A vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccines that can protect experimental animals against challenge with different *Leishmania* species. However, to date, there is no effective vaccine against any form of leishmaniasis for general human use. Efforts to develop an effective vaccine so far have been limited due to lack of an appropriate adjuvant. A mixture of safe *Leishmania* antigens and an adjuvant that preferentially stimulates cellular immune response presents a rational option for a vaccine against leishmaniasis. A vaccine for man needs to be tested in suitable primate models such as the vervet monkey due to their close phylogenetic relation to humans. This study used the vervet monkey model of visceral leishmaniasis to evaluate the safety, immunogenicity and efficacy of *Leishmania donovani* sonicate antigen (Ag) delivered alone or in conjunction with alum-BCG (AIBC), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) as adjuvants. Following vaccinations of groups of vervet monkeys at days 0, 28, and 42, safety was assessed by observation of inductions and erythema at sites of vaccination. Antibody responses and cytokines were quantified by enzyme linked immunosorbent assay (ELISA). Antigen recall lymphoproliferation was measured by blast assay while interferon gamma (IFN-γ)-producing CD4+ and CD8+ T cell responses were measured by intracellular cytokine staining and quantification using flow cytometer. Animals were challenged with *L. donovani* parasites and efficacy evaluated by parasite quantification in splenic impression smears. Data were analyzed using CellQuest, one way analysis of variance (ANOVA), Tukey-Kramer test, Spearman rank correlation analysis or Wilcoxon matched-paired sign-rank test. A P value < 0.05 was considered significant. Results indicated that vaccinations with MPL+Ag or MISA+Ag were safe while animals vaccinated with AIBC+Ag developed erythematous ulcerative inductions at sites of vaccination. Delayed-type hypersensitivity (DTH) responses were significantly higher in the MISA+Ag group than in other vaccinated groups (P < 0.001) while both the AIBC+Ag and MISA+Ag groups induced the highest total IgG and IgG2 subclass antibody responses as compared to vaccinations with MPL+Ag. Interleukin-4 and IL-10 cytokine responses were relatively higher in the MPL+Ag group than in other groups while vaccinations with AIBC+Ag and MISA+Ag induced comparable IFN-γ or TNF-α levels which were significantly higher than levels induced by MPL+Ag vaccination (P < 0.001). Interestingly, significantly higher IFN-γ producing CD4+ or CD8+ T cells were induced in AIBC+Ag and MISA+Ag vaccinated animal groups as compared to other experimental groups (P < 0.001). There was a positive and significant correlation between IFN-γ producing CD4+ and CD8+ T cell populations in both experimental and control groups (r = 1.000; P = 0.0167). Positive significant correlations were also observed between either CD4+ or CD8+ T cells with IFN-γ, TNF-α cytokines or DTH responses (r = 1.000; P < 0.0167). Significant reductions in parasitic loads were associated with vaccinations with AIBC+Ag or MISA+Ag as compared to other study groups (P < 0.001). All Th1 immune response parameters including DTH, IFN-γ, TNF-α, and IFN-γ-producing CD4+ or CD8+ T cells correlated negatively and significantly with parasitic loads. The findings from this study conclude that, MISA 720 is an appropriate adjuvant in terms of safety and immunogenicity and can be effectively used in the formulation of *Leishmania* vaccines for clinical applications. The study recommends the use of MISA 720 in the development of a *Leishmania* vaccine for clinical trials in humans. Data generated in this study is useful in *Leishmania* vaccine development.
CHAPTER 1: INTRODUCTION

1.1 General introduction

Leishmaniasis is a zoonotic infection that is caused by obligate intracellular protozoa of the genus *Leishmania*. Natural transmission of *Leishmania* parasites is carried out by sandflies of the genus *Lutzomyia* in America or *Phlebotomus*, in the rest of the world (Mandell *et al.*, 2005).

Leishmaniasis is endemic in 88 countries in the world and 350 million people are considered at risk of infection. An estimated 14 million people are infected, and each year, about two million new cases occur (WHO, 2007). The disease is endemic throughout parts of Africa, India, the Middle East, southern Europe, and Central and South America (Figure 1.1) and epidemics are also well recognized (Handman, 2001). With the advent of the human immunodeficiency virus (HIV) epidemic, leishmaniasis has surged as a reactivating infection in AIDS patients in many parts of the world (WHO, 1994). Even when coinfected patients receive proper treatment, they relapse repeatedly and the outcome frequently is fatal (WHO, 2007).

Expression of the two basic forms of the disease, namely cutaneous and visceral leishmaniasis, depends on the species of *Leishmania* responsible and the immune response to infection. Cutaneous leishmaniasis (CL) produces a skin ulcer that heals spontaneously in most cases, leaving an unsightly scar (Sohrabi *et al.*, 2005). Visceral leishmaniasis, the most devastating and severe form, is fatal in almost all cases if left untreated. It may cause epidemic outbreaks with high mortality (WHO, 2007).
Leishmaniasis has been known to be endemic in many parts of Kenya from as back as early 19th century. Nine sporadic cases of leishmaniasis were reported between 1911 and 1939 (Fendall, 1961). The main endemic foci of visceral leishmaniasis in Kenya are Eastern and the Rift valley provinces (Muigai et al., 1987). Cutaneous leishmaniasis occurs on the eastern slopes of Mount Elgon in Western Kenya, parts of the Rift valley and some parts of Central Kenya among other areas (Mutinga et al., 1975). Some of the districts affected by
Leishmaniasis include: Baringo, Isiolo, Meru, Turkana, Laikipia, Kajiado, Machakos, Kitui and Wajir districts (Figure 1.2). Improved control will reduce both mortality and morbidity (WHO, 2007). Current control measures rely on chemotherapy to alleviate disease and on vector control to reduce transmission (Handman, 2001). Developing an effective vaccine is a better option in the control of leishmaniasis (Reed and Scott, 2000).

To date, there are no vaccines against leishmaniasis (Handman, 2001; Garg and Dube, 2006; Mutiso et al., 2010). It has been indicated that, efforts to develop Leishmania vaccine have been limited due to lack of an appropriate adjuvant (Sohrabi et al., 2005). Immunity against leishmaniasis requires Th 1-induced IFN-γ for activation of infected macrophages to produce leishmanicidal nitric oxide. A mixture of safe Leishmania antigens together with an adjuvant that preferentially stimulates specific IFN-γ-secreting helper type 1 CD4+ cells (Th 1 cells) presents a rational option for a vaccine against leishmaniasis (Aebischer et al., 2000). The number of adjuvants currently approved for use in humans is quite limited (Mutiso et al., 2010), and of those adjuvants that have been tested in humans, alum has had the greatest clinical use and is relatively nonreactogenic (Kaslow et al., 1994). The use of BCG as an adjuvant is regarded as an acceptable practice in man, and at present this adjuvant is routinely used in vaccination and immunotherapy trials against leishmaniasis (Khalil et al., 2000).
There has been a considerable effort in identifying suitable vaccine adjuvants in order to improve immunogenicity. When alum is used in combination with other selected adjuvants, it
can induce strong protective Th1 response against intracellular pathogens including HIV and leishmaniasis (Jankovic et al., 1997; Ahlers et al., 1997; Kenney et al., 1999). In leishmaniasis vaccine studies, BCG combined with alum and Leishmania major antigen was shown to successfully protect Leishmania donovani-challenged Indian langur (Misra et al., 2001), with the vaccine inducing protective Th1 immune response. Other adjuvants that have shown high promise for an effective vaccine against leishmaniasis include Montanide ISA 720 (Masina et al., 2003) and Monophosphoryl lipid A (Skeiky et al., 2002; Coler et al., 2007). In the development of an effective vaccine these potential adjuvants need to be tested in the most relevant animal model. The vervet monkey offers the most suitable model for leishmaniasis studies (Gicheru et al., 1995).

The aim of this study was to describe vaccination experiments in vervet monkeys against visceral leishmaniasis caused by Leishmania donovani, strain NLB-065, which causes visceral leishmaniasis in Kenya. Three adjuvants: alum-BCG, Monophosphoryl lipid A or Montanide ISA 720 were delivered with L. donovani whole cell sonicate antigen. The selected adjuvants, Montanide ISA 720, alum-BCG and Monophosphoryl lipid A, elicited cellular, Th1 biased immune responses and are in principle, applicable for use in humans. Central to the objectives of the study, was the identification of the most superior adjuvant for development of an effective vaccine against human leishmaniasis.

1.2 Problem statement

Presently, 88 countries including Kenya suffer huge economic losses from infections of Leishmania that currently has a prevalence of 14 million clinical cases worldwide, 2 million new infections annually and 350 million people at risk of infection (WHO, 2007). Of the various forms of leishmaniasis, visceral leishmaniasis (VL) or kala-azar is the most dreaded and devastating, causing high mortality rates, mainly in children (Saha et al., 2006). A
vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccines that can protect experimental animals against challenge with different *Leishmania* species. However, there is no vaccine against any form of leishmaniasis for general human use. The lack of an appropriate adjuvant has frustrated efforts to develop an effective human *Leishmania* vaccine. Although promising adjuvants including alum-BCG, monophosphoryl lipid A and montanide ISA 720 are available, no study has compared the safety, immunogenicity or efficacy of these adjuvants in a suitable animal model such as the vervet monkey to identify the most appropriate adjuvant for use in development of an effective human *Leishmania* vaccine. There is therefore, an urgent and great need to carry out feasible *Leishmania* vaccination studies in order to develop an effective vaccine against human leishmaniasis.

1.3 Study justification

Leishmaniasis remains a public health problem with over 350 million people at risk of infection (Alvar *et al.*, 2006; WHO, 2007). In Kenya, the disease is found mainly in the Rift Valley, Western, North Eastern, Eastern and parts of the coastal regions of the country (Tonui, 2006). In the search for a leishmaniasis vaccine, extensive studies of cutaneous leishmaniasis have been carried out. Investigations in this regard with the visceral form are limited (Afrin and Ali, 1997). There is therefore, a great need for vaccine studies against this deadly parasite as there is currently no vaccine against any form of leishmaniasis for general human use (Khamesipour *et al.*, 2006).

Vector control measures are poorly implemented in many endemic countries and are beyond the means of many families in endemic villages (Alvar *et al.*, 2006). Chemotherapy against
Leishmaniasis is very expensive and therefore inaccessible to many people and the drugs are highly toxic, causing severe reactions (Sharifi et al., 1998; WHO, 2007), including death in 10% of treated patients (Handman, 2001). There is also increasing prevalence of drug-resistant Leishmania parasites and tendency for patients to relapse after an initially successful regimen of chemotherapy (WHO, 2007). Developing an effective vaccine is a better option in the control of leishmaniasis (Reed and Scott, 2000).

Successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunologic vaccines (Reed et al., 2009). Killed Leishmania parasites are poorly immunogenic and generally need to be administered together with an adjuvant to improve immunogenicity (Kenney et al., 1999). The number of adjuvants currently approved for human use is quite limited (Mutiso et al., 2010) and hence the need to evaluate adjuvants with high potential for vaccines to identify the most appropriate adjuvant for Leishmania vaccines. This study used Th-1 adjuvants delivered with whole promastigotes sonicate antigen in a new immunization protocol that presents a feasible approach to effective immunization against leishmaniasis.

A vaccine for man needs to be tested in primates due to their close phylogenetic relation to humans in the evolutionary tree (Garg and Dube, 2006). For visceral leishmaniasis, the availability of a non-human primate model would increase the understanding of various aspects of host parasite interactions. The vervet monkey offers the most relevant animal model for vaccination studies against leishmaniasis. Disease outcome and immunological responses in the vervet monkeys have been shown to be similar to that in humans (Gicheru et al., 1995). Given all the above factors, it is plausible to conduct studies that may contribute to the platform of information that will form clinical studies. To achieve this, the current study
utilized vervet monkeys to evaluate the safety, immunogenicity and efficacy of alum-BCG, Monophosphoryl lipid A, and Montanide ISA 720 as adjuvants delivered with *L. donovani* sonicate antigen. The study identifies the most appropriate adjuvant for development of *Leishmania* vaccine for clinical trials in humans.

1.4 Research questions

a) What is the safety level of the sonicate vaccine when delivered with either alum-BCG, Monophosphoryl lipid A or Montanide ISA 720 in vervet monkeys?

b) What are the immune responses induced by sonicated whole promastigotes of *L. donovani* delivered with either, alum-BCG, Monophosphoryl lipid A or Montanide ISA 720 in vervet monkeys?

c) What is the efficacy of the sonicate vaccine when used with any of the three different adjuvants in vervet monkeys following vaccination and challenge?

1.5 Hypothesis

Alum-Bacille Calmette Guérin, Monophosphoryl lipid A and Montanide ISA 720 adjuvants co-administered with *Leishmania donovani* sonicate antigen are not safe and do not induce comparable protective immune responses in vervet monkeys following vaccination and challenge.
1.6 Objectives

1.6.1 General objective

To evaluate crude *Leishmania donovani* antigen co-administered with Th1 adjuvants as a potential vaccine for leishmaniasis in vervet monkey model.

1.6.2 Specific objectives

a) To assess the safety of *Leishmania donovani* sonicate antigen (Ag) delivered alone or in conjunction with alum-BCG (AlBCG), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) in the vervet monkey model.

b) To determine the level of antileishmanial total IgG and IgG1-4 subclasses antibody and cellular immune responses in the vervet monkey model following vaccination with sonicate antigen alone or combination with AlBCG, MPL or MISA.

c) To determine the efficacy of *Leishmania donovani* sonicate antigen delivered alone or in conjunction with alum-BCG (AlBCG), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) in the vervet monkey model following vaccination and challenge.

1.7 Significance and limitations of the study

The lack of cheaper and safe antileishmanial drugs coupled with the poorly implemented and inaccessible vector control measures and the fact that an effective vaccine against leishmaniasis is feasible prompted this study. The study was relevant as it utilized readily available and cheap adjuvants in the search for an effective *Leishmania* vaccine. The identification of an adjuvant with potential for effective *Leishmania* vaccine brings closer, the realization of a cheaper universal control method for leishmaniasis.
While this study is significant, several limitations have to be considered: Only three animals per study group were used in this study and this number of study subjects is low. Although higher numbers of study subjects are recommended, this study was dictated by resources which could only provide three animals per group; The animals were only kept for 103 days after challenge but it would have been appropriate to keep challenged animals for a long period of time so that observations could be made as to whether animals are completely cured or are going to die of disease; Limitations of funds also allowed sampling to be done only at one time point per parameters for immunology and efficacy and not several time points including after each vaccination time point which would have given a complete picture of all immune responses and efficacy at different durations and it would also have been important to measure more immunological parameters and toxicity tests were it not for limited resources.
CHAPTER 2: LITERATURE REVIEW

2.1 Life cycle of *Leishmania*

In nature, leishmaniae are alternately hosted by the insect (flagellated promastigote; Figure 2.1(a)) and by mammals (intracellular amastigote stage; Figure 2.1(b)). When a female sandfly takes a blood meal from a *Leishmania* infected mammal, intracellular amastigotes are ingested by the insect (Figure 2.2). Inside the blood meal, amastigotes transform into motile promastigotes, which escape through the peritrophic membrane enveloping the blood meal (Roberts, 2006). The promastigotes multiply intensively inside the intestinal tract of the sandfly, successively as free elongated promastigotes (nectomonads) or as attached pro- and paramastigotes (haptomonads) (Walters, 1993).

(a) (b)

![Magnification X1000](image1.png) ![Magnification X400](image2.png)

Figure 2.1. Giemsa stained smears showing *Leishmania* amastigotes (a) and promastigotes (b).
This intraluminal development occurs in the midgut. Whatever the multiplication site, the parasites subsequently migrate to the anterior part of the sandfly midgut, where they change into free swimming metacyclic promastigotes, the stage infective for the vertebrate host (Roberts, 2006). The bite of an infected sandfly deposits infective metacyclic promastigotes in the mammalian skin which are rapidly phagocytosed by cells of the mononuclear phagocyte system. The intracellular parasites change into amastigotes, which multiply by simple mitosis.

Figure 2.2. Schematic diagram of the Leishmania digenetic life cycle (Handman, 2001).
2.2 Clinical presentations of leishmaniasis

Leishmaniasis is a disease attributed to any of a number of protozoan species of the genus Leishmania (Table 2.1). It is transmitted by the bite of an infected female Phlebotomus sandfly. There are four major clinico-pathological categories of leishmaniasis: cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), muco-cutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) each caused by distinct species (Enrique et al., 2005). Following treatment of VL, the skin can become the focus of infection with a condition termed ‘post-kala-azar dermal leishmaniasis’. The disease causes splenomegaly, recurring and irregular fever, anaemia, pancytopenia, weight loss, and general weakness. Visceral leishmaniasis is a silent killer, invariably killing almost all untreated patients (Boelaert et al., 2000). Mucocutaneous leishmaniasis (ML) is a severe disfiguring disease that usually evolves chronically and is extremely difficult to treat (Marsden, 1986).

Cutaneous leishmaniasis is caused by different species of the hemoflagellate protozoan Leishmania and affects approximately 1.5 million individuals per year. Old World CL is separated into four distinct types (Table 2.1): L. aethiopica, L. major, L. tropica and L. infantum (Enrique et al., 2005). Leishmania aethiopica in eastern Africa and the L. mexicana species complex in the Americas are also the aetiological agents of DCL. Unlike CL, DCL is difficult to treat due to disseminated cutaneous lesions that resemble leprosy and which do not heal spontaneously. It is associated with a defective immune system, with most patients failing to display Leishmania specific cell-mediated immunity (Castes et al., 1984).
Table 2.1 Spectrum of leishmaniases, aetiological agents and worldwide distribution (Tonui, 2006)

<table>
<thead>
<tr>
<th>Type of leishmaniasis</th>
<th>Causative organism</th>
<th>Worldwide distribution</th>
</tr>
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<tbody>
<tr>
<td>Visceral leishmaniasis</td>
<td><em>L. donovani</em></td>
<td>China, India, Iran, Sudan and Kenya.</td>
</tr>
<tr>
<td>Post kala-azar dermal leishmaniasis</td>
<td><em>L. infantum</em></td>
<td>Ethiopia and the Mediterranean basin.</td>
</tr>
<tr>
<td></td>
<td><em>L. chagasi</em></td>
<td>Brazil, Colombia, Venezuela and Argentina.</td>
</tr>
<tr>
<td>Cutaneous leishmaniasis</td>
<td><em>L. tropica</em></td>
<td>Mediterranean basin, Afghanistan, Middle East, W. and N. Africa.</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>East Africa.</td>
</tr>
<tr>
<td></td>
<td><em>L. aethiopica</em></td>
<td>Kenya and Ethiopia.</td>
</tr>
<tr>
<td></td>
<td><em>L. mexicana</em></td>
<td>Central America and Amazon basin</td>
</tr>
<tr>
<td>Mucocutaneous leishmaniasis</td>
<td><em>L. braziliensis complex</em></td>
<td>Brazil, Peru, Ecuador, Columbia and Venezuela</td>
</tr>
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</table>

Cutaneous leishmaniasis or oriental sores, produces disfiguring skin lesions on the face, arms and legs, and is often self-healing (Ashford and Bates, 1998). The initial papule rapidly gives rise to an ulcer. Most patients have one or two lesions but the lesions may be multiple and occur in groups. Lesions are usually present on the exposed sites. Their sizes vary from 0.5 cm to 3 cm in diameter (Shiraz and Syed, 2007). Some lesions do not ulcerate at all and remain as bluish papules; others develop sporotrichoid nodular lymphangitis. Secondary bacterial infection is common and must always be suspected if the painless lesion becomes painful. Most lesions heal over months or years, leaving an atrophic scar. In general 50 percent of those lesions associated with *Leishmania major* will have healed in 3 months; those associated with *Leishmania tropica* take about a year, and those associated with *Leishmania braziliensis* persist much longer (Shiraz and Syed, 2007). Cutaneous leishmaniasis infection
induces immunity to re-infection by the particular species that caused the disease, upon recovery or successful treatment (Ashford and Bates, 1998).

2.3 Immunology of leishmaniasis

2.3.1 Mammalian immune responses to leishmaniasis

Evidence for the critical role for IFN-γ in the control of Leishmania infection comes from the demonstration that IFN-γ knockout (KO) mice fail to cure infection (Wang et al., 1994). Furthermore, in experimental L. major infections genetically resistant mice develop a T-cell response dominated by a CD4+ T helper 1 (Th1) phenotype characterized by IFN-γ secretion, whilst in susceptible mice the dominant response is a CD4+ T helper 2 (Th2) phenotype characterized by interleukin (IL)-4, IL-5 and IL-13 secretion (Roberts, 2006).

The correlation between a polarized immune response and outcome to infection led to the concept that the balance of Th1 to Th2 responses determines the outcome (Scott et al., 1988). These observations of L. Major in mice led to the emergence of the Th1/Th2 paradigm as opposing cytokine responses in the control of infections. Hence, the quest to discover how naïve T cells, with the potential for differentiation to either Th1 or Th2, are directed towards one of these opposing extremes. Studies on the early immune response to high-dose infection with L. major in mice on resistant C57BL/6 or C3H backgrounds or a susceptible BALB/c background revealed three distinct patterns. Infection in C3H mice was dominated by an IL-12-driven, CD4+ Th1 response with high IFN-γ levels secreted by natural killer cells and no IL-4 (Scharton and Scott, 1993). In contrast, progressive disease in susceptible BALB/c mice was characterized by early IL-4 synthesis in the absence of IL-12 and a bias towards a Th2 response (Scott, 1991).
Evidence that early IL-4 synthesis drives this Th2 response came from experiments in IL-4 KO BALB/c mice and mice treated with anti-IL-4 antibody, demonstrating that they heal infection (Kopf et al., 1996). The cellular origin of IL-4 in BALB/c mice is confined to an oligoclonal CD4+ T-cell population with a Vβ4Va8 T-cell receptor, recognizing the *Leishmania* homologue of the receptor for activated C kinase (LACK) (Julia et al., 1996). The critical importance of IL-12 in mediating a Th1 response and resistance is demonstrated by IL-12 depletion experiments leading to susceptibility in naturally resistant mice (Mattner et al., 1996) and the conversion of susceptible BALB/c mice to a resistant phenotype by treatment with IL-12 (Heinzel et al., 1993).

Dendritic cells are the source of IL-12 (von Stebut et al., 1998). The antigens responsible for the IL-12 response and the exact mechanisms are not defined. Despite a wealth of evidence for IL-4 in the development of a nonhealing phenotype, virulent strains of *L. major* can lead to susceptibility in BALB/c IL-4 KO mice (Noben-Trauth et al., 1996). In a search for an explanation to this, it was observed that IL-4/IL-13 KO BALB/c mice exhibited greater resistance than single KO strains and additionally, IL-4Ra mice displayed greater resistance than IL-4 KO mice, indicating that IL-13 can substitute for IL-4 in promoting Th2 differentiation (Mathews et al., 2000). However, the discovery that IL-4Ra/IL-10 KO BALB/c mice and IL-4Ra mice treated with anti-IL10R antibody became highly resistant identified IL-10 as a key cytokine (Noben-Trauth et al., 2003).

There are three potential sources of IL-10: (1) Th2 cells of the lineage that produce IL-4 as described; (2) a discrete subpopulation of CD4+ T cells termed ‘T regulatory cells’ and (3) dendritic cells (DCs) and macrophages. In a physiological, low-dose ($10^2 - 10^3$) model of infection in C57BL/6 mice, a clear role for CD8+ T cells in primary immunity was defined in
the control of *L. major* infection in resistant mice (Belkaid *et al*., 2002). However, parasites persist even in resistant mice. Using this low-dose model, it was demonstrated that IL-10 played an essential role in parasite persistence. Only IL-10 KO and IL-4/IL-10 KO mice achieved sterile cure demonstrating the requirement for IL-10 in establishing latency (Belkaid *et al*., 2001). A role for IL-10 was confirmed when C57/BL/6 mice treated with anti-IL-10R antibody transiently during the chronic phase of infection achieved sterile cure (Belkaid *et al*., 2001).

A key study determined that an endogenous, naturally occurring population of CD4+CD25+ T regulatory cells (Treg), expressing high CTLA-4, are the source of IL-10 controlling *L. major* persistence and immunity in C57BL/6 mice (Belkaid *et al*., 2002). Treg constitute 5-10% of CD4+ T cells in normal mice and humans, developing in the thymus where, following high-affinity recognition of self peptides, they up-regulate the transcription factor FoxP3 and the expression of the cell surface marker CD25, essential for their survival and a constitutive marker of Treg in the periphery. Two distinct sub-populations of Treg have been described: naturally occurring Treg, involved in the maintenance of peripheral tolerance, and antigen-specific T regulatory cells (Tr1) that encounter pathogen-derived foreign antigen in the periphery. In a model of *Bordetella pertussis* infection, pathogen-specific Tr1 were demonstrated for the first time (McGuirk and Mills, 2002). A possible mechanism of increased IL-10 production by macrophages involves antibodies via ligation of Fcy receptors (Kane and Mosser, 2001).

A study in *L. mexicana* suggests antibody responses block Th1 development (Kima *et al*., 2000). This highlights the popular view that innate immunity drives adaptive immunity and also indicates that antibody responses may be a further critical component of the immune
response against these pathogens. Another important component of the immune response are natural killer cells acting primarily through their ability to produce IFN-γ, which can optimize the production of IL-12 by DCs and the expression of IL-12R by activated T cells (Akuffo et al., 1999). Finally, production of IFN-γ leads to intracellular death of amastigotes through a common effector mechanism. Macrophage activation is associated with induction of nitric oxide (NO) synthase, which in turn leads to NO-mediated killing. Tumour necrosis factor (TNF)-α is a co-factor with NO. Two other mechanisms of intracellular killing have been proposed involving destruction of infected macrophages by cytotoxic T lymphocytes, CTL (Muller et al., 1991) and FasL-mediated macrophage apoptosis (Huang et al., 1998).

2.3.2 Human immune responses to leishmaniasis

Cutaneous leishmaniasis usually leads to self-healing disease with lifelong immunity against re-infection. Resolution is characterized by induction of specific IFN-γ releasing CD4+ T cells (Kemp et al., 1994). Failure to cure is associated with elevated levels of IL-4 with low IFN-γ responses from Leishmania-specific CD4+ T cells (Ajdary et al., 2000). Increased expression of IL-10 in L. major lesions was found to be associated with progressive disease (Louzir et al., 1998). Studies have also highlighted a dichotomy between Th1 versus Th2 responses in simple versus diffuse CL in humans (Convit et al., 1993).

Patients with active VL usually demonstrate anergy with a negative skin test to Leishmania antigens. Peripheral blood mononuclear cells from such individuals fail to proliferate or to produce IFN-γ when exposed to specific antigen in vitro (Ghalib et al., 1993). Addition of anti-IL-10R antibody to T cells harvested from these patients restores cytokine responses, indicating a role for IL-10 in suppressing T-cell responses in active disease (Ghalib et al., 1993). Further evidence of a role for IL-10 comes from studies demonstrating increased IL-10
mRNA expression in bone marrow (Karp et al., 1993), lymph nodes (Ghalib et al., 1993) and spleen (Kenney et al., 1998). Cure from disease was associated with a fall in IL-10 mRNA levels (Ghalib et al., 1993; Karp et al., 1993). Imbalanced IL-10 production may play a role in progression of disease to PKDL (Ghalib et al., 1993). Increased expression of classical Th2 cytokines has been reported in VL with elevated IL-4 particularly associated with treatment failure (Sundar et al., 1997). Elevated levels of IL-13 have been observed in active disease that returned to normal following successful treatment (Babaloo et al., 2001). In these studies IL-10 and not IL-13 was associated with disease relapse. Investigating the potential sources of immunoregulatory cytokines, investigators have found a population of antigen-specific T cells co-producing IL-10 and IFN-γ which expand in response to L. donovani infection in humans (Kemp et al., 1999). However, a role for antigen-specific Tr1 in humans has not been reported. Effector mechanisms are also important in determining the outcome of infection in humans with evidence that host genetic factors play a crucial role (Karplus et al., 2002; Mohamed et al., 2003).

2.4 Experimental leishmaniasis

Many experimental models of leishmaniasis have been developed. These models have the major attraction of allowing control over the genetics of both the parasite and the host, but none entirely reproduces the disease in humans (Handman, 2001). One of the factors contributing to differences between humans and animal models is the size and nature of the parasite inoculum. In natural infections, the sandfly introduces into the skin a very small number (possibly as few as 100 to 1,000) metacyclic promastigotes together with strongly bioactive saliva, whereas in laboratory infections thousands to millions of culture-derived promastigotes or tissue-derived amastigotes are injected (Handman, 2001).
The sandfly is a blood pool feeder, using its mandibles to cut a wound in the skin and sucking up the blood that accumulates. It is in this superficial pool that the infective parasite inoculum is deposited, most probably in a very small volume. In contrast, the laboratory infection is commonly done in relatively large volumes of 50 µl or more. In addition, in the laboratory the syringe-delivered parasites are deposited mostly subcutaneously or, in visceral leishmaniasis models, intravenously. A better understanding of the molecular mechanisms involved in parasite maturation in the sandfly and the ability to mimic some of these in the laboratory are leading to much improved protocols for infection (Dekrey and Titus, 1999). Investigators are now using small numbers of in vitro-derived metacyclic promastigotes and intradermal rather than subcutaneous infection into the ears of mice (Dekrey and Titus, 1999).

2.4.1 Experimental models of cutaneous leishmaniasis

*Leishmania enriettii* infection of guinea pigs was the first model to be well characterized. It established the requirement for cell-mediated immunity for recovery from cutaneous disease. Guinea pigs develop T-cell responses to parasite antigens within 2 weeks of infection, and the lesions heal within about 10 weeks (Mann *et al.*, 1998). A major attraction of this animal model is the fact that the host-parasite combination is a natural one and that the disease pattern is similar to that observed in human cutaneous leishmaniasis caused by *L. major*. The *L. enriettii* guinea pig model has now been superseded by infection of inbred mice with *Leishmania* species pathogenic for humans. Although not perfect, the spectrum of disease manifestations observed in human leishmaniasis can be mimicked in the laboratory by infection of different inbred strains of mice with *L. major*.

The mouse model reproduces many aspects of the human disease, including a range of susceptibility states depending on the strain of mouse used. In this animal model, the use of a
clonal parasite population eliminates the contribution of the genetic diversity of the parasites and allows analysis of the host factors, which determine disease manifestations. BALB/c mice are highly susceptible; upon infection they develop large skin ulcers, which expand and metastasize, leading to death. On the other hand, C57BL/6 and CBA/N mice are resistant and develop small lesions which cure in 10 to 12 weeks, and are resistant to reinfection. Most other strains of mice are intermediate in susceptibility (Preston and Dumode, 1976). In mice, the outcome of infection depends on the polarized activation of one of two subsets of CD4+ T cells, Th1 or Th2. The subdivision into Th1 and Th2 cells is based on the pattern of cytokines that they produce. Th1 cells produce gamma interferon (IFN-γ) and interleukin-2 (IL-2), whereas Th2 cells produce IL-4, IL-5, and IL-10. Protective immunity depends on the induction of T cells producing Th1 cytokines, which activate macrophages to kill the intracellular organisms primarily through a nitric oxide-mediated mechanism (Liew et al., 1990).

BALB/c mice produce mainly Th2 cytokines, in particular IL-4, and this pattern is established within hours of infection (Bogdan, 1998; Solbach and Laskay, 2000). However, during the period of active lesion development, both susceptible and resistant mice produce a wave of Th2 cytokines (Morris et al., 1992; Morris et al., 1993). An important difference between susceptible and resistant mice is that the resistant mice are able to switch to a Th1 profile and control the disease (Heinzel et al., 1991; Solbach and Laskay, 2000). An important factor in the “decision” to form a Th1 or Th2 phenotype is the early cytokine environment, and IL-12 is one of the cytokines that contributes significantly to the establishment of the Th1 phenotype (Solbach and Laskay, 2000). While it is useful in many ways, one must remember that the mouse model for leishmaniasis is just a model and that the mechanisms of pathogenesis and
immunity may be a little different in humans. Extrapolation from mouse to human requires much care (Kelso, 1995; Kelso, 1998).

2.4.2 Experimental models of visceral leishmaniasis

Visceral leishmaniasis in humans has generally been ascribed to members of the *L. donovani* species, *L. donovani, L. infantum*, and *L. chagasi*, although some dermatotropic strains such as *L. tropica* and *L. aethiopica* strains may also visceralize, presumably depending on host factors. The golden hamster was used in one of the early animal models for the study of visceral leishmaniasis. Infection with *L. donovani* leads to visceral disease and death. Anemia, hyperglobulinemia, and cachexia are aspects of the human disease mimicked in the hamster, making it a useful tool for the characterization of molecules and mechanisms involved in pathogenesis (Hommel et al., 1995). However, in recent years, interest in it has waned and the hamster is now used primarily as a source of *L. donovani* amastigotes, which seem to be the required life cycle stage for infection of mice, the currently preferred model animal for visceral leishmaniasis. Outbred mice are generally resistant to infection with *L. donovani*, but inbred strains display marked differences in susceptibility, which led, in the early 1970s, to the isolation of the *Lsh* susceptibility gene (subsequently designated *NRAMP1*) (Bradley, 1974). *NRAMP1* determines the degree of early expansion of the parasites in the liver and spleen (Bradley, 1989; Blackwell, 1996).

Studies with the mouse model also led to the characterization of the immune mechanisms important for the development of organ-specific immune responses which cause the clearance of the parasites from the liver but not the spleen (Kaye et al., 1995; Engwerda and Kaye, 2000). Another important contribution of the mouse model has been the discovery that
Chemotherapy is ineffective in the absence of intact T-cell mediated responses (Kaye et al., 1995). These experimental studies pointed to the need to activate the immune system for successful chemotherapy and led to the successful trial in India which combined IFN-γ and antimony treatment (Sundar et al., 1995; Sundar and Murray, 1995). One of the difficulties with the mouse as a model for human disease is the need to inject amastigotes intravenously in order to induce a reproducible pattern of colonization of the liver and spleen. This route of administration does not mimic the natural infection by the sandfly. In addition, there is no evidence of wasting, as in the human disease, and the infection is chronic but not fatal. As mentioned above, the outcome (cutaneous or visceral) may depend on interactions between the genotype of the parasite and that of the host rather than exclusively on the parasite. Because of the limitations of the mouse experimental system using human visceralizing strains such as *L. donovani* and *L. infantum*, it has been proposed that visceral leishmaniasis induced in BALB/c mice by the otherwise “dermatotropic” *L. major* may be a better model of human visceral leishmaniasis (Handman, 2001).

The dog is the major reservoir of *L. infantum* in the Middle East and the Mediterranean region and *L. donovani chagasi* in South America. The disease pattern in dogs and humans is similar, with a long period of asymptomatic infection followed by wasting, anemia, enlarged lymph nodes, and fever. As in humans, the infection remains asymptomatic in some dogs (Pinelli et al., 1995). One of the few differences is the presence of skin lesions in the dogs, rarely detected in humans (Hommel et al., 1995). The dog may be the best animal model for visceral leishmaniasis in which relevant immunological studies and vaccine development could be performed (Mendonca et al., 1995; Moody et al., 2000). With the recent cloning of several dog genes encoding cytokines and immunologically important cell markers, as well as the
development of monoclonal antibodies to these molecules, there is hope for a more sustained exploitation of this excellent animal model (Handman, 2001).

A vaccine for man needs to be tested in primates due to their close phylogenetic relation to humans in the evolutionary tree. For VL, the availability of a non-human primate model would increase the understanding of various aspects of host parasite interactions (Garg and Dube, 2006). Earlier efforts in establishing VL in New and Old World monkeys demonstrated that *Aotus trivirgatus* (owl monkeys) and *Saimiri sciureus* (Squirrel monkeys) developed fulminating but short lived infection. Antileishmanial screening was performed in owl monkey (Chapman et al., 1983) and squirrel monkey (Chapman and Hanson, 1981). Old World monkeys such as Macaca spp. viz., *M. mulatta*, *M. fascicularis* and *M. nemestrina* developed low and/or inconsistent infections (Dube et al., 2004). Attempts to establish VL in *Presbytis entellus* showed that this species was highly susceptible to single intravenous inoculation of hamster spleen-derived *L. donovani* amastigotes which invariably produced consistent and progressive acute fatal infection, leading to death between 110 to 150 days post infection. The infected animals presented all the clinicoimmunopathological features as observed in human kala-azar (Anuradha et al., 1990; Dube et al., 1999).

The Indian langurs have also been used for preclinical evaluation of potential antileishmanial drugs (Sharma et al., 2004) and vaccine (Dube et al., 1998; Misra et al., 2001). The vervet monkey (*Chlorocebus aethiops*) has also been demonstrated to be a natural host for *L. major* in Kenya (Binhazim et al., 1987). Disease outcome and immunological responses in the vervet monkey have been shown to be similar to that in humans and the animal has been characterized as a model for both CL and VL that is very ideal for use in vaccine and drug development (Githure et al., 1987; Gicheru et al., 1995). The model has been used in several
Leishmania vaccine studies (Gicheru et al., 1997; Olobo et al., 2001; Gicheru et al., 2001; Masina et al., 2003).

2.5 Control of leishmaniasis
Improved control reduces both mortality and morbidity. It also reduces the role of humans as a reservoir in anthroponotic cycles and makes it possible to avert progression of the disease to complicated forms (WHO, 2007). Proper species and strain identification is important before the correct medication can be instituted. This diagnosis may involve direct sample observation from patients or samples may be cultured for identification of isolates. Control measures aim at eradicating the parasite reservoir in anthroponotic transmission cycles and using control strategies against the vector and zoonotic reservoir in order to break transmission in zoonotic cycles (Lacerda, 1994).

2.5.1 Diagnosis of leishmaniasis

2.5.1.1 Visceral leishmaniasis
The gold standard for diagnosing visceral leishmaniasis is parasite identification in tissue smears, with splenic aspirate being more sensitive than bone marrow or lymph node aspirates. However, difficulties in obtaining and examining tissues mean that serological methods are increasingly being used. The direct agglutination test, in which stained parasites are agglutinated by serum antibodies, is popular in Iran and Africa, but variation between batches and the high cost of commercially available antigen are limiting factors. In the Indian subcontinent (Sundar et al., 1998) but less so in Europe and Africa (Zijlstra et al., 2001) a rapid strip test is used to detect antibody to rK39 (a conserved antigen of L. infantum) and is both sensitive (67-100%) and specific (93-100%). The use of enzyme linked immunosorbent assay (ELISA) using serum has been evaluated for recombinant gp63, the major Leishmania
surface glycoprotein, as a diagnostic molecule for leishmaniasis in vervet monkeys (Gicheru et al., 1994).

Weak responses in some patients, persistence of antibodies after cure, and presence of antibodies in some healthy individuals are inherent limitations with antibody based diagnostics. Detection of leishmanial antigen in urine through a latex agglutination test (Katex) seems to be promising for both diagnosis and prognosis (Attar et al., 2001). Techniques based on polymerase chain reaction are potentially highly sensitive and specific (Martin-Sanchez et al., 2001) but they need to be made more suitable for field use in terms of cost and user skills required. In patients co-infected with HIV and visceral leishmaniasis, blood smears and culture might yield good results (Davies et al., 2003).

2.5.1.2 Cutaneous and mucocutaneous leishmaniasis

Touch smears or culture of exudates or scrapings yield good results in the diagnosis of cutaneous leishmaniasis. From a nodule, slit skin smears are often rewarding. Tissue biopsy can be used for impression smears, culture, or animal inoculation, especially for mucocutaneous leishmaniasis. Although multiple Leishmania species sometimes coexist, species identification is unlikely to be cost effective in the field unless major treatment decisions for cutaneous leishmaniasis become species specific (Davies et al., 2003).

2.5.2 Vector and reservoir control

Vector control using indoor spraying of insecticides is always determined by the behaviour of the species of sandfly present in each area: whether it is endophilic or exophilic and endophagous or exophagous (WHO, 2007). Spraying houses with insecticide is the most widely used intervention for controlling sandflies that are endophilic. Where sandflies are
endophagic (mainly feed indoors) and most active when people are asleep, bed nets provide considerable protection (Davies et al., 2003). Whatever the case, logistics and costs limit the sustainability of periodic spraying of walls. Sustained vector control utilizing large-scale insecticide spraying (Dye, 1996) in developing countries is costly and not feasible. In Brazil, about 200,000 houses are sprayed and 20,000 dogs are culled each year to prevent zoonotic visceral leishmaniasis. After annual surveys in endemic regions, dogs are culled if their blood samples are diagnosed positive by immunofluorescence (Davies et al., 2003). Combined campaigns targeting Anopheles mosquitoes and sandflies, however, are more cost effective. A suitable alternative, at an estimated cost of US$ 5 per unit, is the use of bed nets impregnated with long-lasting insecticide; on average, the nets last for five years (WHO, 2007).

2.5.3 Chemotherapy

The first line approach for the treatment of leishmaniasis is administration of pentavalent antimony compounds (Herwaldt, 1999). Often, serious adverse reactions occur such as cardiac arrhythmias, severe arthritis, liver dysfunction, lethargy and, eventually, sudden death (Pearson et al., 1999). Historically, thousands of leishmaniasis patients treated with antimonials are successfully cured, but always with the danger of well-documented side effects of heavy metal poisoning (WHO, 1984). In addition, emergence of leishmanial resistant organisms to the pentavalent antimony is well documented and, in some endemic areas of the world, treatment failure has reached a level of 60% to 80% (Grogl et al., 1992). Unfortunately, second line alternative drugs are more toxic than antimonial compounds (Sampaio et al., 1971).

Amphotericin B and Pentamidine have shown reasonably good efficacy results in a series of cases reported, but both have been associated with severe, life threatening organ dysfunction.
and death (Herwaldt, 1999; Pearson et al., 1999). During the last decade, new formulations of Amphotericin B in a liposome or other lipid-complex drug delivery system have significantly decreased the side effects of Amphotericin based therapy (Thakur et al., 1996). However, the price of the liposome-Amphotericin B preparation is prohibitive for most of the millions of people with leishmaniasis in the tropics (Davidson et al., 1996). In addition, many reports of dramatic resistant or refractory cases of leishmaniasis leave unsolved challenge (Lira et al., 1999). The most recent invention, Miltefosine, the only medicine administered orally, is to date licensed only in Colombia, Germany and India. As the possibility of its being teratogenic has not been excluded, it should be used under direct observation. Also, to avert the emergence of resistance, it should be given in combination with other antileishmanial drugs (WHO, 2007). In an attempt to develop new safer and cheap Leishmania drugs, diminazene diaceturate has been tested both in vitro and in the murine model of cutaneous (Macharia et al., 2004) and visceral leishmaniasis (Mutiso et al., 2011). In the latter study, diminazene diaceturate was tested in combination with artemesunate. The efficacy of this diminazene based drug was low.

2.5.4 Development of vaccines against visceral leishmaniasis

There is currently no vaccine available for any form of leishmaniasis, including visceral leishmaniasis (VL), which if left untreated is almost always fatal (Mutiso et al., 2010). Vaccination against VL has received limited attention compared with cutaneous leishmaniasis (CL). Historically CL has been the focus of vaccination attempts, as it has been known for centuries that people who resolve a primary CL skin lesion are protected from further infections. It is generally acknowledged that human VL trials will follow on from any successful CL immunization programme. Ideally a vaccine would provide cross-protection against multiple Leishmania species. The recent comparative genomic analysis of three
*Leishmania* species, which cause distinct disease pathologies, showed that *L. major*, *L. braziliensis* and *L. infantum* genomes are highly conserved and have very few species-specific genes (Peacock *et al.*, 2007). The level of amino acid conservation within coding regions is also high between species, suggesting that the major *Leishmania* antigens are conserved and that a pan species vaccine may be achievable. However there is a high degree of variability in the cross-protective immunity induced by infection with different *Leishmania* species (Lainson and Shaw, 1977; Gicheru *et al.*, 1997; Porrozzi *et al.*, 2004) and VL specific vaccines may provide a more successful intervention.

Experimental infection models are used to screen and evaluate VL vaccines and several animal species have been used including mice, hamsters, monkeys and dogs (Garg and Dube, 2006). However no single *in vivo* model accurately reflects all aspects of human VL disease, which has been a major limitation in the development of VL vaccines. The precise immune mechanisms underlying human VL are still not fully understood, and the responses necessary for protection by vaccination in experimental infection models may not reflect those required for efficacy in endemic areas (Nylen and Gautam, 2010).

The profile of an antileishmanial vaccine would need to incorporate several important features, such as safety, ease of production at a low cost in endemic countries, the induction of successful intervention against the diversity of inoculation routes, the induction of a robust, long-term T cell responses, and both prophylactic and therapeutic efficacy (Mutiso *et al.*, 2010). Ideally, such a vaccine would offer cross-species effectiveness against CL and VL. As this might not be feasible, the development of a VL specific vaccine remains an important global health priority.
2.5.4.1 Vaccines against leishmaniasis

To achieve sustainable control of human leishmaniasis, it would be necessary to develop safe and effective vaccines. The natural history of leishmanial infections demonstrate that resolution can lead to development of resistance, which forms the basis for vaccine development studies (Modabber, 1990). Two approaches are used in the development of Leishmania vaccines: pragmatic and systematic methods (Modabber, 1990). The pragmatic approach involves trials of crude Leishmania components in animals and humans with or without Bacille Calmette Guerin (BCG). The systematic approach requires identification, production and purification of protective immunogens, usage of adjuvants, carriers, mode of presentation and determination of protective immunogen responses (Modabber, 1990). Both approaches have made considerable progress towards making anti-leishmanial vaccines (Sharifi et al., 1998). Definition of the nature of the protective Th1 response, in particular, the cytokine mediators of such a response, has been fundamental to the process of vaccine development (Scott et al., 1988). It is now well established that an effective vaccine should and must induce a Th1 response, providing an essential criterion for both antigen and adjuvant selection (Afonso et al., 1994).

Different antileishmanial vaccines have been tried with some degree of success. The only successful intervention against leishmaniasis is inoculation using virulent parasites, a process known as leishmanization (LZ) (Handman, 2001). This ancient practice involves the administration of cutaneous Leishmania parasites to a discrete skin location, allowing a self-healing lesion to form. Initial immunological exposure then protects the individual from further infection and lesion development. Leishmanization was traditionally practised by directly transferring infectious material from cutaneous lesions to uninfected individuals. However the establishment of an in vitro culture system in the early 20th century led to the
large-scale production of promastigote forms of *Leishmania* for expanded clinical use. Leishmanization induces a controlled, but full infection, and was successfully used as a prophylaxis throughout the Soviet Union, Asia and the Middle East, with reported efficacy levels up to 100% (Nadim *et al.*, 1983; Khamesipour *et al.*, 2005). However LZ was largely abandoned due to safety issues associated with the use of live vaccines. Also, standardisation of the inoculum proved difficult as parasites used for LZ experience a dramatic loss of infectivity when subject to repeated subculturings (Modabber, 2010).

Infection with live *Leishmania* also causes immunosuppression, which resulted in reduced immune responses to childhood vaccines and threatened the efficacy of immunization programmes (Handman, 2001; Serebriakov *et al.*, 1972). Currently only one country, Uzbekistan, employs the use of LZ, where a mixture of live and dead *L. major* is licensed as a vaccine for high-risk populations (Gafurov, 1999). As LZ is the only vaccine strategy against *Leishmania* with proven efficacy in humans, efforts are being made to improve the safety of this practise. The inclusion of killed parasites in the inoculum and the use of adjuvants that promote rapid immune responses reduce the severity of primary lesions and accelerates wound healing during LZ (Khamesipour *et al.*, 2005; Tabbara *et al.*, 2005).

The relative merits of live-attenuated vaccines versus killed vaccines have been a constant subject of debate in relation to many antimicrobial and viral vaccines. Most notable arguments have been those concerned with immunogenicity, efficacy, safety, ease of production and distribution, and stability. Early studies indicated (surprisingly) that most parasites cloned directly from a skin lesion in mice were avirulent (Handman *et al.*, 1983). This suggested that the parasite population present in the lesion may be heterogeneous and that the avirulent organisms (which are rapidly killed by the host and provide antigens), rather
than the virulent organisms, contribute most to the immune response observed in the infected mice.

More recent data indicate that, indeed, *L. mexicana* antigens can be presented to T cells by macrophages harboring dead organisms but not by cells harboring live parasites (Overath and Aebischer, 1999). In line with this argument, mice injected with cloned avirulent lines were protected from challenge infection with a virulent clone derived from the same lesion (Handman *et al.*, 1983). However, in the absence of a clear genetic profile of any avirulent cloned organisms available at the time, their use for human vaccination would have been unacceptable because of the risk of reversion to a virulent phenotype. Other data showed that mice injected with irradiated parasites were also protected from infection (Rivier *et al.*, 1993). Taken together, these data strongly supported prophylactic vaccination with attenuated organisms as a useful approach to human vaccine development.

Recent advances in the ability to manipulate the *Leishmania* genome by introducing or eliminating genes has the potential to make live-attenuated vaccines much more feasible. It is now possible to generate parasites lacking genes essential for long-term survival in the mammalian host, such as the gene encoding the enzyme dihydrofolate reductase-thymidylate synthetase, DHFR-TS (Titus *et al.*, 1995). These organisms can invade and undergo a limited number of replications in macrophages without producing disease. In a mouse model, *L. major* parasites lacking DHFR-TS induced protection against infection with either *L. major* or *L. amazonensis* (Titus *et al.*, 1995; Veras *et al.*, 1999). An attenuated line of *L. mexicana* was also used successfully to protect against homologous infection. This mutant lacked two genes encoding the cysteine proteases (cp), cpa and cpb (Alexander *et al.*, 1998; Russel and Alexander, 1988).
In summary, the use of attenuated organisms is very attractive because they are the closest mimic to the natural course of infection and may therefore lead to similar immune responses. Moreover, because of the small load of antigen delivered by the transient infection, the immune responses may be skewed even more towards a Th1 protective response than in natural infection (Constant et al., 1995; Metz and Bottomly, 1999). Such immunization will also deliver many more parasite antigens than the limited number possible with subunit or recombinant antigens. Summarizing a large amount of experimental evidence, Rivier et al. (1999) concluded that, injection of attenuated organisms achieved better protection than any method involving recombinant gp63 as test antigen delivered with a variety of adjuvants and delivery systems. If this conclusion is shown to be generally applicable to other vaccine candidates, the prospect of using attenuated Leishmania vaccines in preference to subunit or recombinant approaches will gain favor. The disadvantages of such vaccines are the logistics of their large-scale production and distribution in the field.

The newer vaccines under consideration comprise recombinant DNA-derived antigens and peptides. Manipulations now allow targeting of the antigen to specific locations or to particular antigen-presenting cells, such as dendritic cells or langerhans cells, which are considered essential for the initiation of primary T-cell responses. Injection of bacteria or naked DNA may have the added advantage of providing an adjuvant effect, which may “activate” or “licence” these antigen-presenting cells (Matzinger, 1998).

The first recombinant antigen used to vaccinate against leishmaniasis was leishmaniolysin or gp63. This 65,000 Da membrane protease present in promastigotes and amastigotes of all species. gp63 is one of the parasite receptors for host macrophages, and parasite mutants lacking the protein are avirulent (Chang et al., 1990). gp63 belongs to a multigene family,
with different members being expressed in promastigotes and amastigotes. Interestingly, both the recombinant and native proteins seem to protect better against infection with \textit{L. amazonensis} than against infection with \textit{L. major}, suggesting species-specific epitopes, at least in animal models (Olobo \textit{et al.}, 1995; Russel and Alexander, 1988). It is unfortunate that in humans and animal models the T-cell responses to gp63 have been variable. However, when detected, they appeared to be of the Th1 type (Jaffe \textit{et al.}, 1990; Mendonca \textit{et al.}, 1991; Russo \textit{et al.}, 1991). Overall, gp63 is still considered a promising vaccine candidate. The gene has been engineered in a number of delivery systems (BCG, vaccinia virus, and \textit{S. enterica} serovar \textit{Typhimurium}) in the hope of inducing the appropriate Th1 immune response.

A second vaccine candidate tested in animal models is a membrane antigen of unknown function, gp46/M2 or parasite surface antigen-2 (PSA-2) (McMahon-Pratt \textit{et al.}, 1993; Handman \textit{et al.}, 1995). As with gp63, PSA-2 belongs to a multigene family expressed in all \textit{Leishmania} species except \textit{L. braziliensis}. Similar but distinct gene products are found in amastigotes and promastigotes of \textit{L. major} and \textit{L. donovani}, but in \textit{L. mexicana} expression seems to be restricted to promastigotes (McMahon-pratt \textit{et al.}, 1992). Its presence in most species makes PSA-2 an attractive candidate for a pan-\textit{Leishmania} vaccine. PSA-2 protects against \textit{L. major} (Handman \textit{et al.}, 1995) as well as \textit{L. mexicana} when administered as purified protein or expressed in vaccinia virus (Mendonca \textit{et al.}, 1991).

Immunization with the \textit{L. donovani} PSA-2 protects mice against infection with \textit{L. major} and that, conversely, immunization with the \textit{L. major} proteins afforded partial protection against infection with \textit{L. donovani} (Handman, 2001). Recombinant DNA-derived PSA-2 protein was variable in its ability to confer protection, while the protein derived from the yeast \textit{Pichia pastoris} provided good protection. These data suggested that the native conformation of the
protein might be important for processing and presentation by antigen-presenting cells. These difficulties may be overcome by the development of a DNA-based vaccine (Handman, 2001).

The leishmanial eukaryotic ribosomal protein (LeIF), a homologue of the ribosomal protein cIF4A, is being considered as a vaccine candidate based on its ability to induce Th1-type cytokines in humans (Skeiky et al., 1998). This protein is highly conserved in evolution, but assuming that specific parasite epitopes will be used for vaccination such that autoimmune responses will be avoided, it may be useful as a component in a pan-Leishmania vaccine. A similarly conserved antigen, the Leishmania homologue of the receptor for activated C kinase (LACK), which is expressed by both promastigotes and amastigotes has been shown to protect mice from infection, in particular when administered with IL-12 as an adjuvant (Gurunathan et al., 1997; Mougneau et al., 1995). It is worth noting that, LACK is also the major target for Th2 responses in susceptible BALB/c mice, and BALB/c mice made tolerant to LACK are resistant to infection (Julia et al., 1996). The significance of this finding for the use of LACK as a vaccine in humans remains to be elucidated. Several other vaccine candidates identified in the last few years are in the process of being characterized. Some are amastigote specific, such as A2, P4, and P8 of L. Mexicana (Soong et al., 1995).

To date, only one multicomponent vaccine, Leish-111f, has been assessed in clinical trials (coler and Reed, 2005). Leish-111f is a single polyprotein composed of three molecules fused in tandem; the L. major homologue of eukaryotic thiol-specific antioxidant (TSA), the L. major stress-inducible protein-I (LmST11) and the L. braziliensis elongation and initiation factor (LeIF; Coler and Reed, 2005). There is some evidence that the Leish-111f vaccine can also induce partial protection against VL in animal models (Coler et al., 2007), however, it failed to protect dogs against infection and did not prevent disease development in a Phase III
vaccine trial in dogs (Gradoni et al., 2005). An optimized version, known as Leish-110f, has recently demonstrated strong immunogenicity and some protective efficacy against *L. infantum* in mice (Bertholet et al., 2009).

Another vaccine candidate is a flagellar antigen, lcr1, from *L. donovani* chagasi (Streit et al., 2000). In view of the fact that the target of host protection is the amastigote, which has only a rudimentary flagellum, the mechanism by which host protection is achieved with this antigen is not obvious. A most interesting approach to the identification of potential vaccine candidates has been the elution of antigenic peptides from antigen-presenting cells (Campos-Neto et al., 1995). Several peptides were identified, and the sequences were used to clone the cognate genes. One of these genes encodes a membrane polypeptide expressed in promastigotes and amastigotes. This polypeptide induced Th1-type responses in immunized mice (Campos-Neto et al., 1995). Surprisingly, in view of its potential, there have been few new data published since its discovery.

The 1980s were marked by a wave of enthusiasm concerning the use of peptide vaccines, in particular those considered to be T-cell epitopes (Jardim et al., 1990; Russo et al., 1993; Spitzer et al., 1999). This enthusiasm seems to have waned in recent times, and the focus appears to have moved to the use of recombinant DNA-produced polypeptides and to naked DNA. Several considerations make the peptide antigens less attractive: the magnitude of the T-cell memory induced, the inability of all individuals in the population to respond to the peptide, and the economics of production. Since the antigenic peptide is processed and presented to T cells in the context of MHC class I or class II and since not all peptides associate with all MHC types, some peptides will not be recognized by all individuals in the population. There are additional “holes” in the ability to respond to individual peptides due to
failure of processing, cleavage, transport or due to deletion of certain T-cell specificities due to self-tolerance (Howard, 1993). Despite these caveats, several Leishmania gp63 peptides have been tested successfully in animal models (Jardim et al., 1990; Spitzer et al., 1999). The results indicated long-lasting host protection, indicating the induction of long-term T-cell memory (Spitzer et al., 1999).

In general, the success of subunit vaccines based on recombinant proteins or peptides has been variable to poor. Several factors may account for this. Some polypeptides, such as PSA-2, need to be in their native conformation for antigen processing, and Escherichia coli-derived recombinant proteins may not fulfil this requirement (Sjolander et al., 1998a; Sjolander et al., 1998b). This problem may be overcome by exploitation of the parasites themselves by overexpression of parasite antigens in transfected nonpathogenic Leishmania strains or the related trypanosomatid Crithidia (Kelley, 1997). Presumably, polypeptides expressed in these systems will be abundant, correctly folded, and glycosylated (Constant et al., 1994; Moody et al., 2000). Another reason for the low success rate of subunit vaccines is that some polypeptides may be minor immunogens and so even though they may be excellent in a cocktail vaccine, individually they may provide only partial protection. The immune responses in leishmaniasis can range from protective to positively harmful, as described above. These differences in the quality of the response are at least partly due to predominance of Th1 or Th2 cytokines and may be greatly influenced by antigen dose (Bretscher et al., 1997). Accordingly, the amount of antigen and the route of administration and adjuvant used may be important issues (Hewlett and Cherry, 1990). Another thorny issue concerns adjuvants. The delivery system may be critical in biasing the type of T-cell responses induced, and this can determine whether protection is achieved or, indeed, whether immunization makes the disease worse (Hoskins et al., 1979; Hewlett and Cherry, 1990).
Nonprotein antigens have also been used against leishmaniasis. Early studies on vaccine development indicated that glycolipids such as the *Leishmania* lipophosphoglycan (LPG) provided excellent protection (McConville *et al.*, 1987; Russel and Alexander, 1988). Protection depended on the use of adjuvants such as liposomes or *Corynebacterium parvum* and on the integrity of the molecule. Not only was the water-soluble form of LPG lacking the glycosylphosphatidylinositol anchor not protective, but it exacerbated disease (Mitchell and Handman, 1986). At the time when that work was published, the immune mechanism leading to host protection by such a nonprotein molecule was totally mysterious. Immunity was known to be T-cell mediated, but T cells were not thought to recognize or present nonprotein antigens. Today, it is accepted that many novel and interesting microbial antigens including mycobacterial glycolipids can be recognized by T cells and that these antigens are presented to T cells by a special subset of MHC class I proteins known as CD1 (Sugita *et al.*, 1998; Moody *et al.*, 2000; Sieling *et al.*, 2000).

Immunization with naked DNA is a new approach, which promises to revolutionize the prevention and treatment of infectious diseases (Wahren, 1996; Seder and Gurunathan, 1999; Alarcon *et al.*, 1999; Gurunathan *et al.*, 2000). The gene encoding the vaccine candidate is cloned in a mammalian expression vector, and the DNA is injected directly into muscle or skin (Ulmer *et al.*, 1996; Wahren, 1996; Donnelly *et al.*, 1997; Walker *et al.*, 1998). The plasmid DNA is taken up by cells and translocated to the nucleus, where it is transcribed into RNA and then translated in the cytoplasm. The efficiency of uptake and the expression of plasmid DNA must be extremely low, but there is abundant evidence that it is sufficient to provoke immune responses in both T and B cells (Hasan *et al.*, 1999; Gurunathan *et al.*, 2000). The antigen encoded by the injected plasmid is presented via either class I or class II
MHC molecules on professional antigen-presenting cells (Donnelly et al., 1997; Hasan et al., 1999).

A large body of literature indicates that both CD4+ and CD8+-mediated responses are induced, making a DNA vaccine attractive for a Leishmania vaccine (Pardoll and Beckerleg, 1995). In addition to being able to induce the appropriate immune responses, DNA vaccines are attractive because they ensure appropriate folding of the polypeptide, produce the antigen over long periods, and do not require adjuvants. Another advantage is that the technology for production is very simple. DNA is stable, has a long shelf life, and does not require a strict cold chain for distribution. Concerns raised in relation to safety, such as integration of the DNA into the mammalian genome and induction of autoimmune disease or cancer, have not been substantiated to date.

Several DNA vaccines have undergone clinical trials; these include a malaria vaccine, a mycobacterial vaccine, and an HIV vaccine (Alarcon et al., 1999; Hasan et al., 1999). Vaccinations with DNA encoding gp63, LACK, and PSA-2 all protected both genetically resistant and susceptible mice from infection with L. major (Gurunathan et al., 1997; Walker et al., 1998). Protection was accompanied by Th1 immune responses. Unexpectedly, protection induced by LACK depended on CD8+ T cells, and depletion of this population abrogated protection (Gurunathan et al., 1998).

The concept of a Leishmania killed vaccine was neglected for many years, possibly because of conflicting results obtained in the 1940s. Vaccination with killed organisms failed to protect persons in the Middle East (Berberian, 1944), whereas a Brazilian trial showed excellent protection (Pesoa, 1941). The tide turned when studies performed in the 1980s...
showed that injection of irradiated parasites induced excellent protection in mice provided that they were injected intravenously or intraperitoneally but not subcutaneously. These experiments paved the way for a reassessment of the use of killed vaccines and led to the successful development and field trials of several formulations of killed vaccines (Mayrink et al., 1979; Alexander, 1982; Howard et al., 1982; Howard et al., 1984).

Extensive vaccination trials in Brazil and Ecuador have demonstrated that a cocktail of five killed *Leishmania* stocks or a single strain of *L. amazonensis* induces significant protection from natural infection (Modabber, 1995; Armijos et al., 1998; Marzochi et al., 1998; De Luca et al., 1999). In Iran, a mixed BCG-*L. major* killed vaccine has also undergone clinical trials for safety and efficacy. In one study there was little difference in disease incidence between the group vaccinated with BCG alone and the group given BCG and vaccine. A second study showed that in the longer term, the vaccine combination provided better protection than BCG alone, suggesting that BCG might have had only a transient immunostimulatory effect (Momeni et al., 1998; Sharifi et al., 1998). Vaccination with a single dose of 1 mg of *L. major* protein and BCG is the simplest vaccine tested so far. Although it proved safe, only about 35% of vaccinated individuals became skin test positive. If skin test conversion is a surrogate marker for protection, the efficacy of this vaccine is not remarkable. It may require multiple doses to increase immunogenicity. In a monkey model of cutaneous leishmaniasis, protective immunity was achieved using killed *L. amazonensis* coadministered with recombinant IL-12 as adjuvant (Kenney et al., 1999). In another unrelated study, killed *L. major* plus recombinant human IL-12 exhibited minimal protection in vervet monkey model of leishmaniasis (Gicheru et al., 2001).
2.5.4.2 The need for adjuvants in vaccines

Inactivated vaccines require adjuvants to stimulate an immune response. The choice of adjuvant or immune enhancer determines whether the immune response is effective, ineffective or damaging. Accordingly, there is need for search of new adjuvants that stimulate the appropriate immunity, for example, T cell immunity for intracellular pathogens and cancer vaccines (Marciani, 2003). The chemical nature of adjuvants, their mode of action and their reactions (side effect) are highly variable. According to Gupta et al. (1993), some of the side effects can be ascribed to an unintentional stimulation of different mechanisms of the immune system whereas others may reflect general adverse pharmacological reactions, which are less expected. Chemically, the adjuvants are a highly heterogenous group of compounds with only one thing in common: their ability to enhance the immune response—their adjuvanticity. They are highly variable in terms of how they affect the immune system and how serious their adverse effects are due to the resultant hyperactivation of the immune system.

The mode of action of adjuvants was described by Chedid (1985) as: the formation of a depot of antigen at the site of inoculation, with slow release; the presentation of antigen to immunocompetent cells; and the production of various and different lymphokines (interleukins and tumour necrosis factor). The choice of any of these adjuvants reflects a compromise between a requirement for adjuvanticity and an acceptable low level of adverse reactions. The discovery of adjuvants dates back to 1925 and 1926, when Ramon showed that the antitoxin response to tetanus and diphtheria was increased by injection of these vaccines, together with other compounds such as agar, tapioca, lecithin, starch oil, saponin or even breadcrumbs (Gupta et al., 1993).
The newly developed purified subunit or synthetic vaccines using biosynthetic, recombinant and other modern technology are poor immunogens and require adjuvants to evoke the immune response. The use of adjuvants enables the use of fewer antigens to achieve the desired immune response, and this reduces vaccine production costs (Mutiso et al., 2010). With a few exceptions, adjuvants are foreign to the body and cause adverse reactions. There are several types of adjuvants. Today the most common adjuvants for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate. However, there are a number of other adjuvants based on oil emulsions, products from bacteria (their synthetic derivatives as well as liposomes) or gram-negative bacteria, endotoxins, cholesterol, fatty acids, aliphatic amines, paraffinic and vegetable oils. Recently, monophosphoryl lipid A, immunostimulating complexes (ISCOMs) with Quil-A, and Syntex adjuvant formulations (SAFs) containing the threonyl derivative or muramyl dipeptide have been under consideration for use in human vaccines (Reed et al., 2009).

The use of adjuvants in *Leishmania* vaccines is a common practice. Although Mayrink et al. (1979) used the intramuscular route in man with killed parasites without an adjuvant, and successful protection without adjuvants has been achieved in a number of animal studies, it is probable that vaccines utilizing subcellular components or purified macromolecules may require the use of suitable adjuvants. Delivery of PSA-2 packaged in immunostimulating complexes induced a strong but mixed Th1/Th2 response and no protection, whereas its delivery as a DNA vaccine induced a low but exclusive Th1 response and protection (Sjolander et al., 1998a; Sjolander et al., 1998b).

Several studies have indicated that BCG, Freund’s incomplete adjuvant (FIA) and glucan utilized with killed parasites or with crude or semipurified fractions will protect or partially
protect against challenge (Bryceson et al., 1970; Smrkovski and Larson 1977; Cook et al. 1980). In the case of *Corynebacterium parvum* and Freund's complete adjuvant (FCA) used alone, there was actually enhancement of the infection (Bryceson et al., 1972). Cook et al. (1980) have found that glucan alone is protective in cross filial-1 (CF1) mice against *L. donovani*. The authors interpreted the protection as a stimulation of the non-specific activity of macrophages. In a second study (Holbrook et al., 1981), glucan served as an adjuvant for formalin-killed *L. donovani* promastigotes. When purified elongation factor (EF) of *L. major* bound to muramyl dipeptide (MDP), the material responsible for mycobacterial peptidoglycan adjuvant activity was used (Ellouz, et al., 1974); partial protection was obtained against challenge. Allison (1979) suggested that appropriate derivatives of MDP in conjunction with liposomes might meet the requirements for use in human vaccination.

Several attempts to treat MCL patients heavily exposed to antimonial therapy have been reported (Herwaldt, 1999). However, none of the alternatives have shown sufficient efficacy to recommend them as the solution for treatment of refractory MCL cases (Pearson et al., 1999). In contrast, reports of the efficacy of immunotherapy with crude *Leishmania* antigen preparations, in combinations with BCG, have indicated that there could be dramatic healing responses of the lesions in patients with CL and MCL by use of antigen vaccines (Convit et al., 1987; Convit et al., 1989).

The recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) has been documented to have a potent cytokine adjuvant effect based on its activity of inducing activation and migration of dendritic cells (Romani et al., 1994). It has been shown to be effective in doses of 25mg to 50mg as an adjuvant (Lin and Jones, 1997).
The adjuvant potential of IL-12 for a vaccine against leishmaniasis has been documented in the murine model (Afonso et al., 1994). Mice immunized with soluble *Leishmania* antigen (SLA) + IL-12 were completely protected against disease. IL-12 in the presence of SLA induces differentiation of CD4+ Th 1 cells in the lymph node and spleen (Afonso et al., 1994). Mice immunized with SLA+IL-12 produced little IL-4 but large amounts of IFN-γ that were comparable to those observed in resistant C3H/HeN mice. Lesions from unimmunized BALB/c mice or mice that had been immunized with SLA alone contained greater than $10^7$ parasites, whereas BALB/c mice immunized with SLA+IL-12 contained $10^3$ parasites (Afonso et al., 1994). In studies using the vervet monkey, low doses of a human recombinant IL-12 preparation induced a small increase in the parameters of cell-mediated immunity similar to animals that received antigen without IL-12 (Gicheru et al., 2001).

Bacille Calmette Guérin (BCG) is a weakened (attenuated) version of a bacterium called *Mycobacterium bovis*, which is closely related to *Mycobacterium tuberculosis*, the agent responsible for tuberculosis. Bacille Calmette Guérin has been used successfully for anti-*Leishmania* immunotherapy in South American patients without side effects. Bacille Calmette Guérin vectors carrying gp63 have also been used successfully to induce protection in the *L. major* system (Abdelhak et al., 1995; Aebischer et al., 2000). Convict and colleagues, some of the early pioneers of killed vaccines, used a combination of killed *L. mexicana* or *L. braziliensis* promastigotes and *Mycobacterium bovis* BCG both prophylactically and therapeutically against South American leishmaniasis (Castes et al., 1989). When used in the therapeutic mode, vaccination appeared to induce a high cure rate even in patients with severe cases. Cure was accompanied by the development of Th1-type immune responses in the recipients, with the production of IFN-γ and the absence of IL-4 (Castes et al., 1989; Cabrera et al., 2000).
Vaccination with BCG plus killed Leishmania promastigotes reduced acute infection by *T. cruzi* in mice, increasing survival time and decreasing parasitaemia and mortality (Araujo et al., 1999). Sohrabi et al. (2005) found out that liposomes containing autoclaved *L. major* antigens mixed with BCG could be used to induce a Th1 response in resistance C57 BL/6 mice. A recent vaccine study evaluated the individual safety and immunogenicity of alum and BCG with results associating alum with humoral antibody responses and BCG with both humoral and cellular immune responses.

There has been considerable effort to improve vaccine adjuvants in order to improve immunogenicity. Aluminium hydroxide gel (Jankovic et al., 1997) or water in oil emulsions (Ahlers et al., 1997) in combination with murine rIL-12 was found to enhance the protective immune response to HIV antigens in mice (Jankovic et al., 1997). This was characterized by both increased antibody production and Th1 cytokine response. In a primate model of cutaneous leishmaniasis, killed promastigotes gave effective protection when used in conjunction with alum and rhIL-12 (Kenney et al., 1999). These animals showed a distinct Th1 response. Alum has been conventionally used as an adjuvant for a wide range of antibody-inducing vaccines. However, it retains antigen at the site of injection and the slow release of antigen may have beneficial effects on diseases needing a TH1 boost. Alum-precipitated *L. Major* vaccine has been evaluated in conjunction with BCG in a single dose schedule against *L. donovani* in the langur model and was found to induce promising immune responses (Misra et al., 2001). This vaccine formulation was further evaluated in human volunteers and was found to induce a strong delayed type hypersensitivity reaction (Kamil et al., 2003).
Montanide ISA 720 has been shown to induce potent Th 1 immune response (Oliveira et al., 2005). This adjuvant has been used in malaria, HIV and cancer vaccine trials (Kenney and Edelman, 2003) and has shown impressive immune responses (Myriam et al., 2005; Oliveira et al., 2005; Collins et al., 2006). In *Leishmania* infection, the use of Montanide avoids problems that have been encountered with other adjuvants (for example, Freund’s) including adverse reactions (Sharples et al., 1994; Smrkovski and Larson, 1977). Vervet monkeys immunized with a combination of recombinant glutathione-S-transferase-Histone-1 (*Leishmania* antigen) and MISA720 adjuvant were able to generate durable cellular responses that were sufficient to control infection in the majority of the monkeys (Masina et al., 2003). This adjuvant has been recently shown to be superior to alum or BCG in vaccinated mice.

Monophosphoryl lipid A has also been found to be safe in a vaccine study in healthy human toddlers (Vernacchio et al., 2002). Additional safety evaluation of MPL as an adjuvant for clinical trials had been done earlier in dogs, rabbits and rats (Baldrick et al., 2002). This adjuvant has been associated with both humoral and cell mediated immune responses to DNA vaccination against HIV-1 (Sasaki et al., 1997). In leishmaniasis studies, MPL has been indicated to be safe and effective (Reed et al., 2003). Monophosphoryl lipid A combined with *Leishmania*-derived recombinant polyprotein Leish-111f in a vaccine (Leish-111f+MPL-SE) against *Leishmania infantum* in mice and hamsters demonstrated strong humoral and T-cell responses (Coler et al., 2007). This vaccination induced a significant increase in CD4+ T cells producing gamma interferon, interleukin 2, and tumor necrosis factor cytokines, indicating Th-1 immune response. In another study, monophosphoryl lipid A (MPL) in a stable emulsion (MPL-SE) formulated with rLeish-111f elicited protective immunity against *L. major* infection in mice (Skeiky et al., 2002).
2.5.4.3 The future of *Leishmania* vaccines

New vaccines will require adjuvants to elicit, as well as enhance, the appropriate immune response; therefore, the development of adjuvants is pivotal to the development of vaccines (Marciani, 2003). Furthermore, successful vaccine development requires knowing which adjuvants to use and knowing how to formulate adjuvants and antigens to achieve stable, safe and immunogenic vaccines (Reed *et al.*, 2009). The development of a *Leishmania* vaccine has been hampered by significant antigenic diversity and the fact that the parasites have digenetic life cycle in at least two hosts, sandfly vector and human, but there is also an animal reservoir (Garg and Dube, 2006).

The availability of many potential adjuvants will require research experiments designed to identify rational standards for the selection of adjuvant formulations based on safety and sound immunological principles for human vaccines. It is certain that, the use of an appropriate Th1 inducing adjuvant in combination with an antigen that includes all important *Leishmania* epitopes in a relevant animal model, will define an effective vaccine for end-point use in humans. Three adjuvants that have shown promising results in *Leishmania* vaccine studies have been identified in a recent review (Mutiso *et al.*, 2010). Despite a lot of vaccination research using these adjuvants, it is not readily clear which of them is more superior in terms of safety and immunogenicity and hence which of them is most appropriate for use in the development of *Leishmania* vaccines. It is of scientific merit to evaluate these promising adjuvants in a relevant animal model such as the vervet monkey with a view to defining an adjuvant with desirable features for quality human *Leishmania* vaccine.
CHAPTER 3: MATERIALS AND METHODS

3.1 Research facility

The research work was carried out in the leishmaniasis Laboratory, Department of Tropical and Infectious Diseases (TID) at the Institute of Primate Research (IPR), a Biomedical and Primatology Research Center collaborating with the World Health Organization (WHO). The Institute of Primate Research is located in Oloolua forest in Karen, Nairobi, Kenya. The Animal resource, Reproductive biology and TID departments are fully equipped with modern facilities and equipments for biomedical research. Animal housing and maintenance was carried out in the department of animal resources.

3.2 Leishmania parasites and preparation of vaccine antigen

3.2.1 Leishmania parasites

*Leishmania donovani* strain NLB-065 originated from the spleen of an infected patient in the Baringo district of Kenya and was maintained by intracardiac hamster-to-hamster passage at the Institute of Primate Research. A hamster splenic biopsy was cultured in Schneider’s Drosophila insect medium supplemented with 20% fetal bovine serum and 100 μg/ml of gentamycin at 25°C until stationary phase. Stationary phase promastigotes were harvested by centrifugation at 2500 rpm (Servoll 6000D) for 15 min at 4°C as described elsewhere (Gicheru et al., 2001). The pellets were washed three times in sterile phosphate buffered saline (PBS) by centrifugation. These parasites were used for animal challenge and to prepare antigens as detailed below.
3.2.2 Preparation of soluble *Leishmania* antigen

*Leishmania donovani* stationary phase promastigotes were harvested by centrifugation as described above. Harvested promastigotes were washed and rapidly frozen and thawed three times in liquid nitrogen before sonication at 18 kHz for five periods of 45 seconds each on ice, separated by intervals of 1 minute as described (Gicheru *et al.*, 1997). The parasite suspension was centrifuged at 10,000 g for 30 minutes at 4°C. Protein concentration of the supernatant was determined using Bio Rad protein assay kit (Bio Rad) and stored at -70°C until use. This antigen was used for coating ELISA plates for antibody assay.

3.2.3 Preparation of formalin-fixed *Leishmania* antigens

For *in vitro* lymphocyte proliferation and cytokine secretion assays, *L. donovani* promastigotes were harvested at stationary phase and washed three times in sterile PBS as described before. Parasites were fixed in 1% formal saline for 1 hr and then washed three times in PBS as above. Parasites were counted using haemocytometer counting chamber and resuspended in a concentration of $5 \times 10^8$ ml in sterile PBS and stored at -70°C until required.

3.2.4 Adjuvants and vaccine preparation

Monophosphoryl lipid A (CAYLA-InvivoGen) (1 mg/ml stock solution), Montanide ISA 720 V (Seppic, France) (Water-in-oil emulsion), alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ) and BCG (Serum Institute of India, Hadapsar, India) (supplied as lyophilized colony forming unit, CFU and reconstituted in 2 ml injection water to produce 20 doses) were used as adjuvants in this study. The vaccination antigen was prepared from *Leishmania donovani* promastigotes. Stationary phase promastigotes were harvested as described before, counted and resuspended at a concentration of $8 \times 10^8$ promastigotes in 3 ml PBS representing 80 vaccine doses. The resuspended promastigotes were freeze – thawed three times in liquid
nitrogen and sonicated at 18 kHz for five periods of 45 seconds each on ice, separated by intervals of 1 minute. The sonicated antigen (Ag) was formulated in the various adjuvants as follows: The Ag was added to each adjuvant at an equivalent of $1 \times 10^7$ promastigotes. In the case of alum-BCG, Ag was added to 1 mg of alum and incubated at room temperature for two hours to allow the Ag to adsorb to the alum. Fifty microliters of BCG was added to the alum-Ag admixture prior to vaccination. For formulation of the Ag in MPL, Ag was added to 40 μL of the adjuvant and mixed at room temperature by shaking the tube containing the mixture. Formulation of the Ag in MISA 720 involved the addition of sonicate Ag to the adjuvant to achieve an adjuvant: antigen ratio of 7:3. The final volume for the antigen-adjuvant mixture was 120 μL for single vaccine dose. The formulations were done in accordance with the manufacturers instructions, thus quality was controlled and assured.

3.3 Experimental research animals and related activities

3.3.1 Research animals

Both young and adult vervet monkeys (Figure 3.1) of both sexes were caught in the wild and quarantined for 120 days at the Institute of Primate Research animal quarantine area in Karen, Nairobi, Kenya. During the quarantine period, the monkeys were monitored for *Mycobacterium tuberculosis* infection and gastrointestinal and parasitic infections. The animals that passed the quarantine were further tested for antileishmanial antibodies against both *Leishmania donovani* and *L. major* antigen by ELISA and monkeys with no antibody titre were selected for the study. These animals of average body weight of 3.4 Kg were housed individually in squeeze-back cages and maintained on commercial non-human primate meal, supplemented weekly with fruits and vegetables and provided with water *ad libitum*. Institutional Animal Care and Use and Institutional Review Committee’s guidelines were strictly followed.
3.3.2 Study design and animal inoculations

The selected animals were divided into five groups of three monkeys each and treated as follows: Group 1 was administered with alum precipitated antigen plus BCG (AlBCG+Ag); Group 2 received antigen formulated in monophosphoryl lipid A (MPLA+Ag); Group 3 received antigen formulated in montanide ISA 720 (MISA+Ag); Group 4 were inoculated with antigen (Ag) alone while Group 5 served as non-vaccinated controls (naïve control). The animals were inoculated as follows: Briefly, each animal was inoculated intradermally (Appendix III (c)) with the respective antigen-adjuvant preparations and repeat doses were administered on days 28 and 42. The animals were subjected to post-vaccination monitoring for safety and immunogenicity of the vaccine prior to challenge with virulent *L. donovani* parasites. Challenge infections involved intravenous (femoral vein) (Appendix III (e))...
administration of 100 μl normal saline containing $2 \times 10^6$ viable parasites and were done on day 21 after the second vaccine booster.

### 3.4 Evaluation of vaccine safety

The animals were observed for skin induration and erythema at the sites of vaccine injection as previously described (Misra et al., 2001). Regional lymph nodes were visually examined for signs of swelling. Body temperature and weight (Appendices III (a) and (b)) were also measured to assess for any abnormal changes in the parameters.

### 3.5 Evaluation of vaccine immunogenicity

#### 3.5.1 Enzyme linked immunosorbent assays for antibodies (ELISA)

#### 3.5.1.1 Quantification of total IgG

Parasite specific total IgG antibodies were quantified by ELISA as described (Gicheru et al., 1995). Briefly, polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 100 μl of soluble *L. donovani* antigen (10 μg/ml) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hr at 37°C. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred microlitres of diluted serum (1/125 in 1% BSA in PBS-Tween) samples were dispensed into the wells and incubated for 1 hr at 37°C. Unbound serum was washed off six times as above and 100 μl of 1/2000 horse radish peroxidase conjugated goat antimonkey IgG was added followed by incubation for 1 hr at 37°C. Unbound conjugate was washed off as above before adding 100 μl of Orthophenyldiamine substrate (OPD, Sigma, UK, final concentration 0.4 μg/ml) in citrate buffer. The plates were incubated at 37°C in the dark for 30 min and optical density was read at 630 nm in a microplate reader (Dynatech Laboratories).
3.5.1.2 Quantification of IgG subclasses (IgG1-4)

Polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 100 µl of soluble *L. donovani* antigen (10 µg/ml) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hr at 37°C. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred microlitres of diluted serum (1/125 in 1% BSA in PBS-Tween) samples were dispensed into the wells and incubated for 1 hr at 37°C. Unbound serum was washed off six times as above and 100 µl of sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Binding Site, UK) antibodies were added to individual well plates at a concentration of 10 µg/well followed by incubation for 1 hr at 37°C. Unbound primary antibodies were washed off as above before adding Donkey anti-sheep horse raddish peroxidase conjugate diluted to 1/5000. The plates were incubated for 1 hr at 37°C before washing to remove unbound secondary antibody. Tetramethylbenzidene (TMB) substrate was added and plates incubated at 37°C in the dark for 30 min and optical density read at 630 nm in a microplate reader (Dynatech Laboratories).

3.5.2 Cellular immune responses

3.5.2.1 Assessment of skin delayed-type hypersensitivity (DTH)

Three weeks after the third vaccination, animals were assessed for DTH responses by intradermal inoculation with 100 µg *L. donovani* antigen, equivalent to 1 x 10⁷ sonicated *Leishmania donovani* promastigotes in 100 µl PBS. Seventy two hours later, skin indurations at the inoculation sites were measured using a metric caliper (Appendix III (d)) as described (Gicheru et al., 2001). A mean induration diameter >5 mm was considered to be positive.
3.5.2.2 Lymphocyte recall proliferation assay

Peripheral blood mononuclear cells (PBMCs) were prepared from venous whole blood by
density centrifugation as described (Olobo and Reid, 1990). The cells were adjusted to $3 \times 10^6$ ml in complete RPMI 1640 medium (GIBCO), which consisted of 10% fetal bovine
serum (Flow Laboratories), 2 mM L-glutamine (Sigma Laboratories), 100 µg of gentamicin
(GIBCO) per ml, and 0.05 mM 2-mercaptoethanol (Sigma Laboratories). One hundred
microlitres of cell suspension were distributed to each well of 96-well round bottomed
microtitre plates (Nunc, Roskilde, Denmark). A 100 µl volume of $5 \times 10^6$ formalin-fixed *L.
donovani* promastigotes/ml or 10 µg of Concanavalin A (Sigma)/ml (Appendix I (h)) was
added to the wells. Control wells received 100 µl of complete RPMI 1640 medium. Cultures
were prepared in duplicate and incubated at 37°C in humidified atmosphere containing 5%
CO₂ for 5 days for *Leishmania* antigen cultures and 3 days for concanavalin A cultures. The
cells were pulsed with 0.5 µCi of [methyl-³H] over the last 18 hr and then harvested on fibre
filter (Whatman International Ltd., Maidstone, UK). Incorporation of radionuclide into DNA
was determined by liquid scintillation spectrometry. Proliferation was expressed as
stimulation index (SI).

\[
SI = \frac{\text{mean counts per minute in stimulated culture}}{\text{mean counts per minute in unstimulated culture}}
\]

The SI values of experimental groups were compared with control monkeys. SI value of > 2.5
was considered a positive response.
3.5.2.3 Quantification of cytokines

Cytokines (IFN-γ, TNF-α, IL-4 and IL-10) were measured in cell-culture supernatants. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation as described (Olobo and Reid, 1990). Purified cells were adjusted to 3 x 10⁶/ml in complete RPMI 1640 medium and stimulated with Leishmania donovani antigen as described previously (Gicheru et al., 1995). Cultures supernatants were collected from triplicate wells after 72 h of stimulation and the concentration of TNF-α, IFN-γ, IL-4 and IL-10 in the supernatant was determined by enzyme linked immunosorbent assay (ELISA). Briefly, polystyrene flat bottomed micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 50 µl of 2 µg/ml of capture monoclonal antibodies (Becton Dickinson and MabTech, Sweden) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was removed, and nonspecific binding sites blocked using 3% bovine serum albumin (Sigma) in PBS for 1 h at 37°C. The plates were washed four times with 0.005% Tween 20 in PBS, and 50 µl of culture supernatant was dispensed to appropriate wells. Human TNF-α (Becton Dickinson) and IFN-γ, IL-4 and IL-10 (MabTech) diluted (1 to 600 U/ml) in 1% bovine serum albumin in PBS-Tween were used as standards. The plates were incubated at 37°C for 1 h and washed four times before addition of biotinylated secondary monoclonal antibodies (50 µl of 1/2000 dilution) followed by incubation at 37°C for 1 h. The plates were washed four times as before, 50 µl of 1/300-diluted alkaline phosphate-conjugated streptavidin was added, and the mixtures were incubated for 1 h as described above. The plates were washed 10 times in PBS-Tween, and 50 µl of nitrophenyl phosphate substrate (1 mg/ml) in diethanolamine buffer was added. The plates were incubated at 37°C in the dark for 30 min, and absorbance was read at 405 nm.
3.5.2.4 Cell surface staining and measurements

Peripheral blood mononuclear cells (PBMCs) were isolated as described above. After counting, 3 million PBMCs/ml were cryopreserved using 1 ml of 10% Dimethyl Sulfoxide (Sigma, USA) and 90% Fetal Bovine Serum (Sigma, USA) cryopreservation solution. Samples were rate-controlled frozen to -70°C, stored overnight suspended over liquid nitrogen and then transferred into liquid nitrogen until analysis.

Before analysis, PBMC vials were thawed in a 37°C water bath, washed in complete RPMI medium, counted and suspended in PBS at a concentration of 0.5 – 1.0 x 10^6 cells/ml. The viability of cells recovered following storage in liquid nitrogen was estimated to be over 98%. Cells were transferred to individual tubes in 50 µl aliquots and 500 µl PBS wash added to each tube followed by centrifuge at 450 x g for 5 minutes at 10°C. Supernatants were aspirated and pellet disrupted resuspended in 200 µl complete RPMI before addition of 2 µl soluble Leishmania donovani antigen (10 µg/ml) to each desired tube. The tubes were placed at 37°C in a humidified CO₂ incubator for 3 hours and Brefelding A (10 µg/ml final) added to the desired tubes incubated for 20 hours. Positive control tubes with PHA stimulated samples were included. The tubes were centrifuged at 450 X g for 5 minutes at 10°C and supernatant aspirated and the cell pellets were resuspended in 50 µl PBS wash containing optimally titrated amount (10 µL of each antibody) of CD3 PerCP, CD8 FITC, and CD4 PE antibodies (BD, Biosciences, Belgium). Negative control tubes were included with PBS wash. Single colour controls were also included at this stage. Tubes were incubated for 20 minutes on ice and 500 µl PBS wash added to each tube before centrifuge at 450 X g for 5 minutes at 10°C. Supernatants were aspirated and tubes agitated to disrupt cell pellets.
To each sample tube, 200 μl 4% paraformaldehyde was added and tubes vortexed and incubated for 20 minutes on ice. To each sample tube 200 μl permeabilization buffer was added and tubes centrifuged at 450 X g for 5 minutes at 10°C. Supernatants were aspirated and cell pellet disrupted before addition of 100 μl permeabilization buffer to the sample tubes that were to be stained with anti-cytokine antibody. The remaining tubes had 100 μl PBS wash added. Tubes were incubated for 5 minutes at room temperature and 10 μl of APC-conjugated anti-IFN-γ cytokine antibody (BD, Biosciences, Belgium) added to the desired sample tubes and contents mixed. Tubes were incubated for 5 minutes at room temperature and 200 μl permeabilization buffer added to each tube and centrifuged at 450 x g for 5 minutes at 10°C. Supernatants were aspirated and tubes agitated to disrupt the cell pellets. Cells were then resuspended in 300 μl fix solution and the tubes vortexed before data acquisition on a four colour fluorescent-activated cell sorter (Becton Dickinson). Data were analyzed by CellQuest Software (Becton Dickinson).

3.6 Clinical monitoring of animals following challenge infection

Following challenge infection, all animals were monitored for body temperature and body weight (Appendices III (a and b)). Body temperature was assessed by measuring anal temperature using a clinical thermometer while body weights were measured using a weighing balance. At the time of termination of the experiment (day 103 post-challenge), animals were sacrificed and spleen and liver visually inspected for any signs of splenomegally and hepatomegally.
3.7 Quantification of parasitic load

For calculating parasitic burden, dab smears of spleens from individuals in various experimental and control animal groups were prepared on day 103 post-challenge. These smears were fixed with absolute methanol for 5 minutes and stained with 10% Giemsa stain for 20 minutes before counting the number of amastigotes/1000 cell nuclei using a microscope. Parasite loads in experimental groups were assessed against the control group.

3.8 Statistical analysis

GraphPad InStat software, version 3.05, 32 bit for Win 95/NT was used for data analyses. One-way analysis of variance (ANOVA) was used to compare mean parameter values between all study animal groups. Tukey-Kramer test was used for inter-group statistical analysis. Differences were considered significant where $P < 0.05$. Where applicable, Spearman rank correlation was used for correlation analysis while Wilcoxon matched-paired signed-rank test was used for analysis of clinical parameters in challenged animals.
4.1 Vaccine safety assessment

4.1.1 Local reaction to *Leishmania donovani* sonicate antigen-adjuvant vaccine

All animals in the AIBCG+Ag group showed indurations at the sites of vaccination. These indurations, measured seven days following immunizations, lasted between 34 and 41 days before complete resolution. These indurations were ulcerative and associated with erythema. Lymph nodes swelling were also observed in the AIBCG+Ag group and these resolved with disappearance of indurations. Animals in other groups did not show any lymphadenopathy or local skin reaction to the immunizations.

4.1.2 Body temperature (°C) measurements

Throughout the vaccination period, days 0 – 63, all measured body temperature remained comparable to the baseline values. There was no indication of fever in any of the vaccinated groups (Table 4.1). Wilcoxon matched-pairs test did not indicate any significant difference between body temperature values taken before and during vaccination period ([r] = 0.8208; \( P = 0.5000 \)).
Table 4.1 Animals body temperature measurements in vaccinated vervets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days during vaccination period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>AlBCG+Ag</td>
<td>38.2 ± 1.079</td>
</tr>
<tr>
<td>MPL+Ag</td>
<td>37.25 ± 0.59</td>
</tr>
<tr>
<td>MISA+Ag</td>
<td>37.70 ± 1.20</td>
</tr>
<tr>
<td>Ag</td>
<td>37.40 ± 0.53</td>
</tr>
<tr>
<td>Control</td>
<td>37.60 ± 0.32</td>
</tr>
</tbody>
</table>

AlBCG: alum +BCG; MPL: monophosphoryl lipid A; MISA: montanide ISA 720; Ag: antigen. "Temperatures were measured in degrees celcius (°C)

4.1.3 Body weight (Kg) measurements

There was no notable change in body weight in any of the vaccinated animal groups or in any individual animal during the vaccination period (Table 4.2). Statistical analysis using Wilcoxon matched-pairs test failed to conclude any significant difference in body weight values taken before and during the vaccination period ((r) = -0.9177; P = 0.0623).
Table 4.2 Animals body weight measurements during vaccination period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days during vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>AIBCG+Ag</td>
<td>3.37 ± 0.82</td>
</tr>
<tr>
<td>MPL+Ag</td>
<td>3.37 ± 0.15</td>
</tr>
<tr>
<td>MISA+Ag</td>
<td>3.36 ± 0.61</td>
</tr>
<tr>
<td>Ag</td>
<td>3.40 ± 0.26</td>
</tr>
<tr>
<td>Control</td>
<td>3.37 ± 0.71</td>
</tr>
</tbody>
</table>

AIBCG: alum + BCG; MPL: monophosphoryl lipid A; MISA: montanide ISA 720; Ag: antigen. *Body weights were measured in kilograms (Kg).

4.2 Immune responses

4.2.1 Antileishmanial antibodies

4.2.1.1 Parasite-specific total IgG antibody responses

All vaccinated animals responded to *Leishmania donovani* antigen by producing antibodies in titres of between 0.48 ± 0.05 (Ag group) and 1.35 ± 0.08 (AIBCG+Ag) on day 21 following the second vaccine booster. The AIBCG±Ag and MISA±Ag groups produced comparable levels of IgG (*P* > 0.05) that were significantly higher than IgG levels induced by MPL+Ag.
vaccination ($P < 0.001$) as measured by ANOVA followed by Tukey-Kramer test as post hoc. The MPL±Ag vaccination produced significantly higher IgG antibody levels than the Ag group ($P < 0.01$). Significantly higher IgG antibody levels were produced in the animal group vaccinated with Ag alone as compared to the control group ($P < 0.001$; Figure 4.1).

Figure 4.1 Antiparasite total IgG antibody responses to *Leishmania donovani* in vaccinated animals on day 21 post last vaccination. Twelve vervets were vaccinated at days 0, 28 and 42 with sonicate antigen (Ag) alone; sonicate antigen plus alum plus BCG (AIBCG+Ag); sonicate antigen plus monophosphoryl lipid A (MPL+Ag) or sonicate plus montanide ISA 720 V (MISA+Ag) and antileishmanial antibody determined on day 21 following second vaccine booster vaccination. Data shown indicate mean antibody ± SD in each vaccination group (n = 3).

4.2.1.2 Parasite-specific IgG1-4 subclass antibody responses (IgG1-4)

Production of antigen specific IgG subclasses 1-4 was compared within and between groups. There was no significant difference in the production of IgG1 between all study groups ($F = 3.163; P = 0.0636$). The levels of IgG2 in the experimental groups were significantly higher
the levels obtained in the control group ($F = 34.283; P < 0.0001$; Figure 4.2). Vaccination with AlBCG+Ag induced the highest IgG2 levels as compared to other vaccinated groups ($P < 0.0001$). The MISA+Ag vaccinated group produced significantly higher ($P < 0.05$) levels of IgG2 than the MPL group. The IgG2 levels induced by the MPL group did not differ with levels induced by the Ag or control group ($P > 0.05$). Experimental groups produced higher Ig3 levels as compared to the control group ($F = 16.482; P = 0.0002$). Vaccination with AlBCG+Ag induced IgG3 levels comparable to those induced by the MISA group ($P > 0.05$) but significantly higher than in the MPL+Ag vaccinated group ($P < 0.01$). The IgG3 subclass levels induced by vaccination with MPL+Ag were not different from the levels observed in the Ag vaccinated or control group ($P > 0.05$). Although there was significant difference in the production of IgG4 subclass antibodies between experimental and control groups ($F = 4.715; P = 0.0213$), only the AlBCG+Ag and control intergroup analysis showed a significant difference ($P < 0.05$). Other intergroup computations did not indicate any significant differences.

Intragroup analysis indicated a significant difference in the production of IgG subclasses 1-4 following vaccination with AlBCG+Ag ($F = 31.614; P < 0.0001$). The absorbance values for subclasses were 0.105 (IgG1), 0.434 (IgG2), 0.142 (IgG3) and 0.144 (IgG4). Parasite specific IgG2 was significantly produced in large amounts as compared to all other subclasses ($P < 0.001$). The amounts of IgG1, 3 and 4 produced in this group were comparable ($P > 0.05$). As in the AlBCG group, there were significant differences in the production of IgG1-4 subclasses in the MISA group ($F = 12.945; P = 0.0019$). Absorbance values were 0.085 (IgG1), 0.26 (IgG2), 0.113 (IgG3) and 0.139 (IgG4). The subclass, IgG2 was significantly higher than any other subclass ($P < 0.01$) with no difference between levels IG1, IgG3 and IgG4 subclasses ($P > 0.05$).
There was no difference in the levels of IgG1-4 subclasses produced following vaccinations with MPL+Ag ($F = 0.8236; P = 0.5167$) with subclass absorbance values being 0.119 (IgG1), 0.116 (IgG2), 0.092 (IgG3) and 0.102 (IgG4). Absorbance values for subclasses in the Ag ranged from (0.072 to 0.089) while those in the control group were from 0.059 to 0.072. Analysis of levels of IgG1-4 subclasses in the Ag vaccinated ($F = 2.835; P = 0.1061$) and control ($F = 1.257; P = 0.3522$) groups did not indicate any statistical difference between values within each group.

![Graph](image-url)

**Figure 4.2** Antiparasite IgG subclass antibody responses to *Leishmania donovani* antigen in vaccinated animals. Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and IgG1-4 subclass antibody levels determined on day 21 after the third vaccination. Data shown indicate mean IgG subclass level ± SD in each vaccination group ($n = 3$).
4.2.1.3 Relationship between total IgG and IgG2 subclass responses

Parasite specific total IgG antibody responses were quantified and results correlated with the most prominent IgG subclass (IgG2). Spearman rank correlation analysis indicated a positive and significant correlation between total IgG and IgG2 antibody responses ($r = 1.000; P = 0.0167$; Figure 4.3).

![Figure 4.3](image)

Figure 4.3 Relationship between total IgG and IgG2 antibody responses to *Leishmania donovani* antigen in vaccinated animals. Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (A/BCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and total IgG and IgG2 antibody responses determined on day 21 after the third vaccination. Data shown indicate mean antibody level ± SD in each vaccination group (n = 3).
4.2.2 Cellular immune responses

4.2.2.1 Skin delayed-type hypersensitivity (DTH) response

All immunized monkeys developed positive DTH response to *Leishmania donovani* antigen as measured on day 63 following initial immunization (Figure 4.4). Post test analysis indicated a significantly higher DTH reaction in the MISA+Ag group as compared to each of the other groups \( (P < 0.001) \). The AlBCG+Ag group showed significantly higher DTH response to the sonicate antigen as compared to the MPL+Ag or Ag groups \( (P < 0.001) \). It was interesting to note that there was no difference in DTH responses between the MPL+Ag and Ag groups \( (P > 0.05) \). Control monkeys did not exhibit any DTH response. Delayed type hypersensitivity response significantly correlated positively with either IFN-\( \gamma \) or TNF-\( \alpha \) cytokine responses \( (r = 0.941; P < 0.0001) \).
Figure 4.4 Skin delayed-type hypersensitivity (DTH) responses in vervet monkeys following vaccinations. Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and DTH determined on day 63 following initial vaccination. Data shown indicate mean DTH ± SD in each vaccination group. DTH value < 5 mm was considered negative (n = 3).

4.2.2.2 Lymphoproliferative responses to Con A and antigens

All experimental and control groups responded positively to Con A in the range of between 237.54 ± 23.762 and 205 ± 74.55 stimulation indices (SI). The responses to Con A were comparable in all the groups (P > 0.05). Lymphoproliferative response to the *Leishmania donovani* antigen was highest in the MISA+Ag group but with no significant difference from the AIBCG+Ag group (P > 0.05). Response to the antigen in the MISA+Ag was significantly higher than in the MPL+Ag vaccinated group (P < 0.001). The AIBCG+Ag vaccinated animals showed significantly higher response to the sonicate antigen than the MPL+Ag
vaccinated animals \((P < 0.05)\). Although the Ag vaccinated group showed a slight positive response to the antigen, this response was no different from the control group (Table 4.3).

Table 4.3 Lymphoproliferative responses\(^{\dagger}\) (stimulation indices)* in vaccinated and control vervet monkey groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Con A</th>
<th>Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIBCG+Ag</td>
<td>218.29 ± 17.89</td>
<td>40.54 ± 3.2</td>
</tr>
<tr>
<td>MPL+Ag</td>
<td>205.33 ± 74.55</td>
<td>19.41 ± 3.65</td>
</tr>
<tr>
<td>MISA+Ag</td>
<td>225.99 ± 43.71</td>
<td>52.4 ± 13.58</td>
</tr>
<tr>
<td>Ag</td>
<td>210.48 ± 3.56</td>
<td>2.62 ± 0.64</td>
</tr>
<tr>
<td>Control</td>
<td>237.54 ± 0.97</td>
<td>1.44 ± 0.06</td>
</tr>
</tbody>
</table>

\(^{\dagger}\)responses were measured 21 days after the last vaccination

\(* > 2.5\) Stimulation index value to Con A/Ag was considered positive.

AIBCG: alum +BCG; MPL: monophosphoryl lipid A; MISA: montanide ISA 720; Ag: antigen.

4.2.2.3 T helper 2 (Th 2) cytokines (IL-4 and IL-10) responses

Type-2 cytokine responses to Con A were significantly higher than levels of the cytokines induced following lymphocyte stimulation with \textit{Leishmania} antigen. However, there were no significant inter-group differences \((P > 0.05)\) in the Con A stimulated cells. Antigen stimulated type 2 (Th2) cytokine levels or inhibitory cytokines, IL-4 and IL-10 were generally
low but prominent in the MPL+Ag vaccinated group. Significantly higher levels of IL-4 were observed in the vaccinated animal groups as compared to the control group ($F = 8.506; P = 0.0029$). Low levels of the cytokine were induced in all the vaccinated animal groups and these ranged from the highest level of 27.56 pg/ml in the MPL+Ag group to the lowest levels of 5.0006 pg/ml in the Ag group (Figure 4.5). The baseline mean level of IL-4 in the control group was 1.66 pg/ml. Levels of induced IL-4 did not differ amongst the antigen-adjuvant vaccinated groups ($P > 0.05$). Interleukin-4 levels induced in the AlBCG+Ag and MPL+Ag were significantly higher than those induced in the Ag group ($P < 0.05$). Among the experimental groups, the MPL+Ag group induced the highest IL-4 cytokine levels, followed by AlBCG+Ag, then MISA+Ag, while the Ag group recorded the least IL-4 amounts. Levels of IL-4 between MISA+Ag and the Ag groups were comparable ($P > 0.05$). Although the adjuvant+Ag vaccinated groups induced statistically comparable IL-4 levels, the IL-4 cytokine levels induced following vaccination with MISA+Ag were comparable to the baseline values induced by the control group while the IL-4 cytokine levels induced following immunization with AlBCG+Ag and MPL+Ag were significantly higher than the cytokine levels induced in the control group ($P < 0.05$ and $P < 0.01$ respectively).
Figure 4.5 Interleukin-4 cytokine responses to *Leishmania donovani* antigen in vaccinated animals. Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and IL-4 cytokine responses determined on day 21 after the third vaccination. Data shown indicate mean IL-4 level ± SD in each vaccination group (n = 3).

Results on IL-10 cytokine production indicated a significant difference between the experimental and the control groups (F = 38.67; P < 0.0001). Slightly higher amounts of IL-10 were produced in the corresponding experimental animal groups as compared to IL-4 levels. However, as in the case for IL-4, the IL-10 values were low and they ranged from a low of 10.79 pg/ml in the Ag vaccinated group to a high of 41.208 pg/ml in the MPL+Ag vaccinated animals. Baseline mean value was 1.802 pg/ml in the control group. Adjuvant-Ag vaccinated groups produced significantly higher IL-10 than the Ag group (P < 0.01; Figure 4.6). As in the case for IL-4, there was no statistical difference in production of IL-10 amongst the adjuvant-Ag vaccinated groups (P > 0.05). Levels of IL-10 induced by Ag vaccination were no different from those in the control group (P > 0.05). There was a
positive and significant correlation between IL-4 and IL-10 levels in the corresponding experimental and control groups \( (r = 1.000; P = 0.0167) \).

![Graph showing IL-10 cytokine responses to Leishmania donovani antigen in vaccinated animals.](image)

**Figure 4.6 Interleukin-10 cytokine responses to Leishmania donovani antigen in vaccinated animals.** Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and IL-10 cytokine responses determined on day 21 after the third vaccination. A control (naive) group was also included. Data shown indicate mean IL-10 level ± SD in each group \( (n = 3) \).

### 4.2.2.4 T helper 1 (Th1) cytokine responses

#### 4.2.2.4.1 Gamma-interferon response to Con A and *L. donovani* sonicate antigen

Concanavalin A (Con A) induced proliferation and production of IFN-γ in both vaccinated and control groups (Figure 4.7). The IFN-γ production in response to Con A showed wide variations within and between the groups but with no significant inter-group differences \( (P > 0.05) \). When *Leishmania donovani* antigen was used to induce secretion of IFN-γ, only the antigen plus adjuvant groups had detectable levels of the cytokine. Animal groups vaccinated with either AIBCG+Ag and MISA+Ag induced the highest IFN-γ cytokine levels which were
comparable ($P > 0.05$). These two groups induced significantly higher IFN-γ cytokine levels than the MPL+Ag vaccinated group ($P < 0.001$). The MPL+Ag group induced significantly higher IFN-γ levels when compared to the Ag or control group ($P < 0.01$; Figure 4.7).

**Figure 4.7 Level of γ-interferon in response to Con A and Leishmania donovani antigen.** Twelve vervets were vaccinated at three different time points with sonicate antigen (Ag) alone or in combination with plus alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and 21 days following the third vaccination PBMCs were stimulated in vitro with Leishmania donovani antigen and IFN-γ production determined. Data shown indicate mean IFN-γ ± SD in each group (n = 3).

### 4.2.2.4.2 Tumour necrosis factor alpha (TNF-α)

As in the case for IFN-γ responses, Con A induced proliferation and production of TNF-α in both vaccinated and control groups. The TNF-α production in response to Con A did not show any significant inter-group differences ($P > 0.05$). Compared with the control group, the vaccinated animal groups induced significantly higher TNF-α cytokine levels in response to *L. donovani* antigen ($F = 17.413; P = 0.0002$; Figure 4.8). The MISA+Ag group induced the highest TNF-α cytokine levels which were significantly higher than the MPL+Ag group ($P <$
0.05) but comparable to the AIBCG+Ag group \( (P > 0.05) \). The amount of TNF-\( \alpha \) secretion induced in the MPL+Ag group did not differ significantly from levels induced by the Ag group \( (P > 0.05) \). The Ag vaccinated and control groups induced comparable amounts of TN-\( \alpha \) cytokine \( (P > 0.05) \).

![Graph showing TNF-\( \alpha \) levels](image)

**Figure 4.8 Level of Tumour necrosis factor (TNF) in response to Leishmania donovani antigen.** Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and 21 days following the third vaccination PBMCs were stimulated *in vitro* for 72 hours with *Leishmania donovani* antigen and TNF-\( \alpha \) production determined. Data shown indicate mean TNF \( \pm \) SD in each group \( (n = 3) \).

**4.2.2.5 Interferon gamma-producing CD4\(^+\) and CD8\(^+\) T cell populations**

Parasite specific CD4\(^+\) T cells expressing IFN-\( \gamma \) were significantly higher in the experimental group compared to the control group \( (P = 0.0001) \). All groups vaccinated with adjuvant plus antigen produced significantly higher IFN-\( \gamma \) inducing CD4\(^+\) T cells as compared to either the
Ag or the control groups (Figure 4.9 (a) – (e)). Vaccinations with MISA+Ag induced the highest numbers of CD4+ T cells involved in IFN-γ production (Figure 4.9 (c)). These cells were significantly more than those induced by AlBCG+Ag vaccinations (P < 0.01). However, the AlBCG+Ag group (Figure 4.9 (a)) induced significantly higher IFN-γ inducing CD4+ T cells than the MPL+Ag group (Figure 4.9 (b)) (P < 0.001). The IFN-γ inducing CD4+ T cells stimulated in MPL+Ag vaccinated group were significantly more than those induced following vaccination by Ag alone (Figure 4.9 (d)) (P < 0.01). The IFN-γ positive CD4+ T cells did not differ between the Ag vaccinated and the control (Figure 4.9 (e)) groups (P > 0.05).

As for the IFN-γ+ CD4+ T cell population, parasite specific IFN-γ inducing CD8+ T cells also differed significantly in numbers between the experimental-adjuvant and the control groups (P = 0.0001). These cells were the highest but comparable in AlBCG+Ag and MISA+Ag vaccinated groups. A narrow gap of difference in numbers of the IFN-γ positive CD8+ T cells was observed between the MPL+Ag and the Ag vaccinated groups (P < 0.05). The IFN-γ positive CD8+ T cells did not differ in numbers between the Ag vaccinated and the control groups (P > 0.05). Figure 4.10 shows a summary of IFN-γ producing CD4+ and CD8+ T cells in all the study groups.
Figure 4.9 (a) Scatter graph indicating levels of interferon gamma producing CD4⁺ and CD8⁺ T cells induced in vervet monkeys following vaccination with sonicate antigen formulated in alum-BCG (AlBCG+Ag). Animals were vaccinated intradermally at three time points and PBMCs harvested on day 63 following initial vaccination. Harvested cells were stained intracellularly and levels of T cells involved in IFN-γ production quantified in flow cytometer.
Figure 4.9 (b) Scatter graph indicating levels of interferon gamma producing CD4\(^+\) and CD8\(^+\) T cells induced in vervet monkeys following vaccination with sonicate antigen formulated in monophosphoryl lipid A (MPL+Ag). Animals were vaccinated intradermally at three time points and PBMCs harvested on day 63 following initial vaccination. Harvested cells were stained intracellularly and levels of T cells involved in IFN-\(\gamma\) production quantified in flow cytometer.
Figure 4.9 (c) Scatter graph indicating levels of interferon gamma producing CD4\(^+\) and CD8\(^+\) T cells induced in vervet monkeys following vaccination with sonicate antigen formulated in montanide ISA 720 (MISA+Ag). Animals were vaccinated intradermally at three time points and PBMCs harvested on day 63 following initial vaccination. Harvested cells were stained intracellularly and levels of T cells involved in IFN-\(\gamma\) production quantified in flow cytometer.
Figure 4.9 (d) Scatter graph indicating levels of interferon gamma producing CD4$^+$ and CD8$^+$ T cells induced in vervet monkeys following vaccination with sonicate antigen (Ag). Animals were vaccinated intradermally at three time points and PBMCs harvested on day 63 following initial vaccination. Harvested cells were stained intracellularly and levels of T cells involved in IFN-γ production quantified in flow cytometer.
Figure 4.9 (e) Scatter graph indicating levels of interferon gamma producing CD4⁺ and CD8⁺ T cells induced in non-vaccinated (naïve) vervet monkeys. Harvested PBMC cells were stained intracellularly and levels of T cells involved in IFN-γ production quantified in flow cytometer.
Figure 4.10 Interferon gamma-producing CD4$^+$ and CD8$^+$ T cell populations. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBC+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third vaccination were stimulated \textit{in vitro} with \textit{Leishmania donovani} antigen and cells stained for measurement of interferon gamma (IFN-$\gamma$) producing CD4$^+$ and CD8$^+$ T cell populations in flow cytometer. Data shown indicate mean CD4 or CD8 ± SD in each group (n = 3).

4.2.3 Relationships between immunological parameters

4.2.3.1 Correlation between IFN-$\gamma$ producing CD4$^+$ and CD8$^+$ T cell populations

Spearman rank correlation analysis between IFN-$\gamma$ positive CD4+ and CD8+ T cells indicated a very strong positive correlation between the two T cell populations (r = 1.00; \(P = 0.0167\)). An increase in parasite specific CD4+ T cells involved in IFN-$\gamma$ induction was accompanied by a corresponding increase in CD8+ T cells involved in IFN-$\gamma$ production (Figure 4.11).
Figure 4.11 Association between IFN-γ producing CD4+ and CD8+ T cell populations. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third vaccination were stimulated \textit{in vitro} with \textit{Leishmania donovani} antigen and cells stained for measurement of interferon gamma positive (IFN-γ+) producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate correlation of mean CD4 or CD8 ± SD in each group (n = 3).

4.2.3.2 Association between IFN-γ producing CD4+ and CD8+ T cell populations with Th1 cytokines

Tumour necrosis factor alpha, TNF-α and IFN-γ cytokines were measured and levels were correlated with CD4+ and CD8+ T cell populations involved in the production of IFN-γ. Both CD4+ and CD8+ cells positively and significantly correlated with either TNF-α or IFN-γ levels in antigen stimulated lymphocyte supernatants (r = 1.000; \( P = 0.0167 \)). Figure 4.12 shows the relationship between IFN-γ+ CD4+ T cells and IFN-γ levels in corresponding experimental and control groups.
Figure 4.12 Association between IFN-γ levels and IFN-γ+ CD4+ cell population. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third vaccination were stimulated in vitro with \textit{Leishmania donovani} antigen and IFN-γ levels determined and cells stained for measurement of interferon gamma positive (IFN-γ+) producing CD4+ and CD8+ T cell populations in flow cytometer. Data shown indicate relationship between mean IFN-γ and CD4 ± SD in each group. A similar relationship was observed for IFN-γ levels with CD8+ T cells and also between TNF-α and CD4+ or CD8+ T cells (n = 3).

4.2.3.3 Associations between IFN-γ+ CD4+ and CD8+ T cell populations with DTH responses

Skin - delayed type hypersensitivity (DTH) responses in vaccinated and control animals were determined and values correlated with cytotoxic and Th1 cell populations. There were significant correlations between IFN-γ+ CD4+ and CD8+ T cells with DTH responses (r = 1.000; P = 0.0167; Figure 4.13).
Figure 4.13 Association between DTH and IFN-γ producing CD4⁺ cell population. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBC+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag). DTH responses were determined and peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third vaccination were stimulated in vitro with *Leishmania donovani* antigen and cells stained for measurement of interferon gamma positive (IFN-γ⁺) producing CD4⁺ and CD8⁺ T cell populations in flow cytometer. Data shown indicate relationship between of mean DTH and CD4⁺ ± SD in each group. A similar relationship was observed for DTH levels with CD8⁺ T cells (n = 3).

### 4.3 Clinical parameters in *Leishmania donovani* challenged animals

#### 4.3.1 Body temperature (°C) changes

Challenged animals were monitored for clinical parameters including body temperature and weight. Measured body temperatures remained within the normal range of between 37°C and 40°C documented for vervet monkeys. Wilcoxon matched-pairs signed-ranks test did not indicate any significant difference between body temperature values measured before challenge and those taken at termination ((r) = -0.1622; P > 0.8750; Table 4.4).
Table 4.4 The body temperature* measurements in challenged vervets

<table>
<thead>
<tr>
<th>Days post challenge</th>
<th>0</th>
<th>63</th>
<th>103</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlBCG+Ag</td>
<td>37.7 ± 0.25</td>
<td>37.3 ± 0.61</td>
<td>37.2 ± 0.55</td>
</tr>
<tr>
<td>MPL+Ag</td>
<td>37.3 ± 0.29</td>
<td>37.3 ± 0.40</td>
<td>37.2 ± 0.61</td>
</tr>
<tr>
<td>MISA+Ag</td>
<td>37.4 ± 0.52</td>
<td>37.4 ± 0.65</td>
<td>37.6 ± 0.42</td>
</tr>
<tr>
<td>Ag</td>
<td>37.3 ± 0.46</td>
<td>37.5 ± 0.49</td>
<td>37.5 ± 0.35</td>
</tr>
<tr>
<td>Control</td>
<td>37.6 ± 0.40</td>
<td>37.5 ± 0.46</td>
<td>37.5 ± 0.35</td>
</tr>
</tbody>
</table>

AlBCG: alum +BCG; MPL: monophosphoryl lipid A; MISA: montanide ISA 720; Ag: antigen. *Temperature measurements were in degrees celsius (°C).

4.3.2 Body weight (Kg) changes

Body weight did not differ significantly in challenged animals at day 0 and 103 post challenge (Table 4.5). It was however clear that the body weight was gradually reducing in the control group as indicated by a reduction from 3.65 ± 0.7 (day 0 p.c.) to 3.43 ± 0.9 Kg (day 103 p.c.). This difference was however not significant ((r) = 0.2895; P = 0.3125).
Table 4.5 The body weight\textsuperscript{+} measurements in challenged vervets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>AlBCG+Ag</td>
<td>3.64 ± 1.06</td>
</tr>
<tr>
<td>MPL+Ag</td>
<td>3.64 ± 1.07</td>
</tr>
<tr>
<td>MISA+Ag</td>
<td>3.66 ± 0.75</td>
</tr>
<tr>
<td>Ag</td>
<td>3.6 ± 0.26</td>
</tr>
<tr>
<td>Control</td>
<td>3.65 ± 0.7</td>
</tr>
</tbody>
</table>

AlBCG: alum + BCG; MPL: monophosphoryl lipid A; MISA: montanide ISA 720; Ag: antigen. \textsuperscript{+}MBody weights were measured in kilograms (Kg).

4.4 Efficacy evaluation

4.4.1 Assessment of parasite burden

A highly significant reduction in parasite loads was associated with the antigen-adjuvant vaccinated animal groups as compared to the Ag or control groups ($P < 0.0001$). The levels of parasite reductions associated with the AlBCG±Ag and MISA±Ag were comparable ($P > 0.05$). These two groups significantly reduced parasite numbers as compared to the MPL±Ag vaccinated group ($P < 0.05$). Parasite loads were comparable between the Ag and control groups ($P > 0.05$; Figure 4.14).
Figure 4.14 Parasite burden in vaccinated and unvaccinated vervet monkeys. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AlBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and challenged with virulent *Leishmania donovani* promastigotes 21 days after the third vaccination. Parasite numbers were determined on Giemsa stained splenic impression smears on day 103 post challenge. Data shown indicate mean parasite load ± SD in each group (n = 3).

4.4.2 Association between DTH and protection

There was a highly significant inverse association between DTH reaction and parasite numbers as indicated by non-parametric spearman-rank correlation analysis ($r = -0.9290; P < 0.0001$). An increase in DTH reaction was associated by a corresponding decrease in parasite load (Figure 4.15).
Figure 4.15 Relationship between DTH and parasitic load in vervet monkeys following vaccinated and challenge. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in combination with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and DTH reaction determined 21 days after the third vaccination. Vaccinated animals were challenged with *Leishmania donovani* parasites and parasitic loads determined on day 103 post challenge. Data were analyzed with spearman rank correlation test. The figure presents means of DTH and parasitic loads ± SD in each group (n = 3).

4.4.3 Association between antileishmanial antibodies and disease outcome

Spearman rank correlation analysis did not conclude significant association between antibody responses (either total IgG or IgG2 subclasses) and disease outcome ($r = -0.9000; P = 0.0833$). However, high levels of total IgG or IgG2 subclass antibody responses were associated with reduced parasite numbers in both AIBCG+Ag and MISA+Ag vaccinated groups while low IgG2 antibodies in the Ag or control groups were associated with high parasite numbers (Data not shown).
4.4.4 Association between interferon gamma levels and protection

Spearman rank correlation analysis indicated a highly significant inverse association ($r = -0.9435; P < 0.0001$) between IFN-γ responses and parasite loads. The results showed that, an increase in interferon gamma level was accompanied by a decrease in parasite numbers (Figure 4.16).

![Graph showing relationship between interferon gamma (IFN-γ) and parasitic load in vervet monkeys following vaccination and challenge](image)

Figure 4.16 Relationship between IFN-γ and parasitic load in vervet monkeys following vaccination and challenge. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag) and IFN-γ levels determined on 21 days after the third vaccination. Vaccinated animals were challenged with *Leishmania donovani* parasites and parasitic loads determined on day 103 p.c. Data were analyzed with spearman rank correlation. The figure presents means of IFN-γ and parasitic loads ± SD in each group (n = 3).
4.4.5 Associations between IFN-γ producing CD4⁺ and CD8⁺ T cell populations with protection

There were significant negative correlations between IFN-γ⁺ CD4⁺ or CD8⁺ T cell populations with parasite loads in both experimental and control animal groups ($r = -1.000; P = 0.0167$; Figure 4.17).

**Figure 4.17** Association between IFN-γ producing CD4⁺ cell population and parasite numbers. Twelve vervets were vaccinated at three time points with sonicate antigen (Ag) alone or in conjunction with alum plus BCG (AIBCG+Ag), monophosphoryl lipid A (MPL+Ag) or montanide ISA 720 V (MISA+Ag). Peripheral blood mononuclear cells (PBMCs) harvested 21 days after the third vaccination were stimulated *in vitro* with *Leishmania donovani* antigen and cells stained for measurement of interferon gamma positive (IFN-γ⁺) producing CD4⁺ and CD8⁺ T cell populations in flow cytometer. Animals were challenged with $2 \times 10^6$ virulent *Leishmania donovani* parasites intravenously through the femoral vein and parasitic burden was assessed on day 103 post challenge in splenic impression smears by counting the number of infected macrophages per 1000 cell nuclei using a microscope. Data shown indicate relationship between mean parasite loads and CD4 ± SD in each group. A similar relationship was observed for parasite loads with CD8 + T cells ($n = 3$).
CHAPTER 5: DISCUSSION

5.1 Introduction

Visceral leishmaniasis is a major health problem in East Africa where it is associated with frequent epidemics that claim hundreds of lives especially in the remote areas where medical care facilities are limited. Available drugs are very expensive and highly toxic (WHO, 2007). Development of a safe vaccine may provide an immunological control strategy. In humans, recovery from *Leishmania* infection usually results in long-lasting immunity, thus indicating that vaccines against leishmaniasis are achievable. However, to date, there is no available vaccine for control of leishmaniasis (Garg and Dube, 2006). It is apparent that an effective *Leishmania* vaccine will only be achieved by careful selection of both an appropriate adjuvant as well as an antigen (Mutiso *et al.*, 2010).

Hundreds of different adjuvants exist, and these need to be selected on basis of safety and sound immunological principles. Promising adjuvants include Montanide ISA 720 (Masina *et al.*, 2003), alum/BCG (Misra *et al.*, 2001; Kamil *et al.*, 2003) and Monophosphoryl lipid A (Coler *et al.*, 2007). These adjuvants have been associated with Th1 as well as humoral immune responses. However, it is not yet clear, which of these adjuvants would be superior in inducing cellular immune response required for effective control of leishmaniasis. In this study, we used a nonhuman primate model to assess the safety, immunogenicity and protective capacity of a vaccine that combines *Leishmania donovani* sonicate antigen delivered with either alum-BCG, monophosphoryl lipid A or montanide ISA 720 as adjuvants. This kind of study was the first of its kind in this animal model.
5.2 Vaccine safety

The results of the present study show that based on two clinical parameters including fever and changes in body weight, the immunization protocols are safe. Following initial immunization, daily observation of immunized animals was carried out and there was no any local or systemic side effects in any of the animals immunized with MPL+Ag or MISA+Ag. The safety of MISA has been reported elsewhere including vaccination studies carried out in the *Leishmania* Research Laboratory, Institute of Primate Research (Toledo *et al.*, 2001; Masina *et al.*, 2003; Oliveira *et al.*, 2005). The present safety report on MISA as an adjuvant for *Leishmania* vaccine differs with other studies that have associated this adjuvant with adverse reactions including swelling and erythema at the injection site observed within a few days following vaccination (Pierce *et al.*, 2010). Other studies have reported occasional unacceptable local reactogenicity in vaccines containing montanides (Wu *et al.*, 2008).

It is likely that adverse reactions observed following immunizations with vaccines containing MISA may be a result of the immunizing antigen. Monophosphoryl lipid A has also been found to be safe in a vaccine study in healthy human infants (Vernacchio *et al.*, 2002). Earlier studies had also indicated the safety and effectiveness of MPL in *Leishmania* vaccine studies (Reed *et al.*, 2003). Additional safety evaluation of MPL as an adjuvant for clinical trials had been done earlier in dogs, rabbits and rats (Baldrick *et al.*, 2002). However, as a matter of serious concern, the study has established that, based on adverse local skin reaction, an immunization containing BCG is not safe as it is associated with erythematous ulcerative skin nodules or indurations. This confirms results observed in earlier studies on *Leishmania* vaccines in humans (Khamil *et al.*, 2003) where vaccination with alum-BCG was associated with adverse reaction. Other studies involving the use of the monkey system have also...
associated BCG with severe reactions (Misra et al., 2001). Lymphadenopathy was also an undesirable feature in the use of BCG as an adjuvant in this study.

5.3 Immune responses following vaccinations with *L. donovani* antigen mixed with different adjuvants

The association of AIBC+Ag or MISA+Ag vaccinations with high antibody levels was expected considering that these adjuvants are known to induce both cellular and humoral antibodies (Toledo et al., 2001; Masina et al., 2003; Oliveira et al., 2005; Nateghi et al., 2010). The lack of high antibody responses in MPL+Ag vaccinated group may be due to formulation of this adjuvant in water instead of stable emulsion as used elsewhere (Coler et al., 2007). In any case, this study expected to get comparable antibody levels in the three adjuvant groups since MPL is also an adjuvant associated with both cellular and high antibody responses (Sasaki et al., 1997; Macleod et al., 2011). However, similarly to the present results, a different previous study failed to get higher antibody levels in MPL toddler vaccination as compared to the control group (Vernacchio et al., 2002). Antibody levels were not predictive of disease outcome since antibody responses do not have any protective value in leishmaniasis. Furthermore high antibody levels are associated with active visceral leishmaniasis infection (Olobo et al., 1980; Gicheru et al., 1995; Saha et al., 2006; Nylen and Guatam, 2010).

The three adjuvants evaluated in this study have been associated with both cellular and humoral immune responses and it would be expected that these adjuvants induce high antibody responses following vaccinations. The predominant production of IgG2 and IgG4 following vaccination with AIBC+Ag may indicate this adjuvant as a potent inducer of mixed type 1 (IgG2) and type 2 (IgG4) immune response associated antibodies. The high
production of IgG2 and low production of all other subclasses upon vaccination with MISA+Ag clearly indicates that this adjuvant is a superior Th1 antibody inducer. Vaccination against murine malaria using MISA 720 related adjuvant, MISA 51, was predominantly associated with type 1 (IgG2a and IgG2b) antibody responses and low IgG1 and IgG3 subclass responses. In a murine malaria vaccine study, MISA 720 was associated with high antibody responses including high IgG2a antibody responses (Mata et al., 2007). In the present study, it was interesting to note that only the two adjuvant groups, AlBCG and MISA, inducing the highest IgG2 subclass antibodies were associated with protection of challenged monkeys against visceral disease.

It has been shown that murine IgG2a and IgG2b equivalent to human IgG2 have been implicated in conferring protection against various forms of infection (Takayama et al., 1991). The failure to induce type 1 antibody responses by MPL in the present study may be a matter of the formulation of the adjuvant used (aqueous). In a murine *L. infantum* study, immunization with MPL in stable emulsion with Leish-111f antigen produced a robust IgG1 and IgG2a antibody responses (Coler et al., 2007). The selective stimulation for IgG2 production coupled with depressed secretion of IgG1, IgG3 and IgG4 in both the AlBCG and MISA adjuvant groups may add new knowledge on immunogenicity of these adjuvants in vaccine studies. Furthermore this was the first study to try to characterize IgG subclass responses in *Leishmania* vaccine studies. It may therefore be right to suggest that, although both adjuvants are associated with cellular as well as humoral immune responses, the antibody responses is selective for type 1 immune response associated antibody (IgG2) response. It may be right to believe that, high IgG2 antibody response is associated with protection against leishmaniasis since this was the outcome in this study where high IgG2 responses induced by AlBCG or MISA vaccination significantly reduced parasite numbers. In
any case, high levels of IgG1 and IgG3 production have been associated with IL-10 activity and blunting of IFN-γ activity (IgG2) in visceral leishmaniasis (Garraud et al., 2003; Caldas et al., 2005). The role of IgG4 in parasitic infections is not clear but it has been suggested to play a blocking role in parasite killing and clearance (Jassim et al., 1987; Dafa’alla and Ghalib, 1992). This IgG subclass (IgG4) as well as IgG1 and IgG3 have been shown to increase in patients with active VL disease (Shiddo et al., 1996). In the present study, comparable IgG1-4 subclasses responses observed both between and within the MPL+Ag and Ag vaccinated groups, may indicate a failure of the MPL used in this study to immunopotentiate the sonicate antigen.

Leishmania donovani sonicate antigen specific IL-10 production, which is reported to be important for the cellular immune depression that accompanies active VL disease, as well as that of IL-4 promoting susceptibility to Leishmania infections, was low and did not cause vaccine failure. It is likely that the high Th1 responses in both AlBCG+Ag and MISA+Ag vaccinated groups may have depressed the polarization of Th2 immune responses, thereby driving the response to predominantly Th1 with high IFN-γ production. Furthermore, on presentation of Leishmania antigens to CD4+ T cells, the concomitant secretion of IL-12 drives the proliferation of (IFN-γ secreting) Th1 cells and NK cells, which activate macrophages and inhibit Th2 responses (Ruiz and Becker, 2007). There is hardly any data on evaluation of type 2 immune responses in Leishmania vaccine studies using alum-BCG and this is the first study to measure type 2 cytokine responses in vaccine trials in animal models of leishmaniasis using MISA 720 as an adjuvant. However, in a murine L. Infantum vaccine study, monophosphoryl lipid A delivered with Leish-11f antigen induced only marginal Th2 cytokines and strong Th1 immune responses (Coler et al., 2007). In the present study, MPL
induced relatively higher Th2 cytokine responses than other adjuvant-Ag groups leading to failure of Th1/Th2 cytokine responses to correlate within the study animal groups.

The relatively higher Th2 cytokine responses in the Ag and MPL+Ag vaccinated groups as opposed to other experimental groups may have depressed the development of type 1 responses in the two animal groups. Studies have indicated that, the secretion of IL-4 during antigen presentation to CD4+ T cells drives Th2 cell development that inhibits Th1 responses and promotes B lymphocyte growth and development (Von Stebut and Udey, 2004). This may clearly point out that although AIBCG and MISA adjuvants are believed to induce both type 2 and cell mediated immune responses (Sasaki et al., 1997; Toledo et al., 2001), the response is biased towards cellular which consequently may inhibit the development of strong type 2 responses. With regard to specific T-helper-type 1 or 2 (Th1 or Th2)-derived cell-mediated immunity, studies have shown clearly that MPL has greater Th1 than Th2 stimulating potential (Baldrick et al., 2002).

The associations of significant positive DTH with AIBCG and MISA adjuvants in vaccinated animals indicate the importance of these adjuvants in the induction of strong Th1 immune responses as compared to vaccinations with the Ag alone or when combined with MPL. Positive DTH response is a marker of type 1 immune response and has been used to assess the immunogenicity of candidate vaccine antigens against leishmaniasis (Misra et al., 2001; Gicheru et al., 2001; Masina et al., 2003). Earlier studies had shown that, in an endemic area, the prevalence of DTH skin test positivity increases and the incidence of disease decreases with age, indicating the acquisition of immunity in the population over time (Melby and Anstead, 2001). Indeed, the strong correlation between DTH and Th1 cytokines as well as IFN-γ producing CD4+ and CD8+ T cell responses in the present study indicates the
importance of DTH response as an early marker of outcome of a vaccination study and may as well predict the outcome of disease. Delayed type hypersensitivity to leishmanial antigens has been widely used in leishmaniasis to indicate exposure to leishmanial infections and to assess the level of host protection to the disease (Gicheru et al., 1995; Gicheru et al., 2001). In the present study, DTH response was found to be a good predictor of late immune responses in immunized subjects and a good marker of disease outcome.

Parasite specific lymphocyte proliferation was demonstrated in all vaccinated animals with marked Con A stimulation demonstrated in all animals was an indication of viability of the cells used in this assay. All vaccinated animals responded positively to *Leishmania donovani* antigen signifying the importance of this antigen in priming of lymphocytes in vaccinated animals which may translate into strong T cell memory for long lasting immunity. In earlier reports on leishmaniasis vaccine studies, vaccinations with BCG (Misra et al., 2001) and MISA 720 (Masina et al., 2003) were associated with high recall lymphocyte proliferative responses. In the present report, the higher recall lymphoproliferative responses induced in PBMCs harvested in animals vaccinated with AlBCG+Ag and MISA+Ag as compared to vaccination with MPL+Ag suggest that both AlBCG and MISA have a strong ability to amplify the priming of T cell responses to the sonicate antigen. Furthermore lymphoproliferative responses of PBMCs to leishmanial antigens have been used as a measure of exposure to the parasite, as well as a measure of protection (Gicheru et al., 2001). The ability of MISA 720 to induce high recall proliferative responses has also been reported in HIV-1 negative human volunteers receiving a multi-polypeptide TAB9 antigen in HIV-1 vaccine studies (Toledo et al., 2001) where vaccinated individuals were associated with intense lymphoproliferative responses.
The production of higher levels of IFN-γ in AlBCG+Ag and MISA+Ag vaccinated groups than the MPLA+Ag group may indicate the superiority in potency of the former two adjuvants as compared to the latter. High IFN-γ level has been considered one of the correlates of resistance (Gicheru et al., 2001; Roberts, 2006) and the cytokine is elevated in *L. major* self cured animals (Ho et al., 1982; Gicheru et al., 1997). The strong association with IFN-γ and resistance would be expected, since the parasites are killed when macrophages are activated by IFN-γ.

Remarkable levels of TNF-α and IFN-γ induced by groups vaccinated with antigen containing Alum-BCG and MISA may also point to the superiority of these adjuvants in the selective induction of Th1 immune responses. Alum-BCG has been indicated to induce strong Th1 immune responses in visceral *Leishmania* vaccine studies (Misra et al., 2001; Kamil et al., 2003). In malaria, HIV and cancer vaccine studies, MISA 720 has been indicated to induce strong Th1 immune responses important for control of these diseases (Kenney and Eldelman, 2003). This adjuvant has also been shown to induce strong Th1 cytokines in *Leishmania* vaccination studies (Masina, et al., 2003). Immunity against leishmaniasis is dependent on high IFN-γ levels. The cytokine activates macrophages to kill the intracellular parasites through induction of nitric oxide (NO) which is lethal to the parasites (Roberts, 2006). Tumour necrosis factor is a co-factor with NO (Roberts, 2006) and it contributes to protective immunity by synergizing with IFN-γ to activate macrophages (Melby and Anstead, 2001).

Acquired immunity in murine *L. major* cutaneous leishmaniasis is mediated by parasite-induced production of IFN-γ by CD4 T cells (Th1 subset), and can develop in the absence of CD8 T cells (Reiner and Locksley, 1995). However, as both CD4 and CD8 T cells are required for an effective defense against visceral *L. donovani* infection (Melby and Anstead,
Furthermore, cure for all forms of leishmaniasis is affected through cellular immune response capable of activating host macrophages to eliminate the parasite (Tripathi et al., 2007). Considering the central role played by both CD4+ T cell (Th1) and CD8+ T cells in the effective control of leishmaniasis, it is therefore important that an appropriate vaccine-adjuvant against visceral leishmaniasis induces high levels of these lymphocytes. The present study evaluated the expression of IFN-γ inducing CD4 and CD8 cells in vervet monkeys following vaccinations with *Leishmania* sonicate antigen delivered with AlBCG, MPL or MISA as adjuvants.

The expression of the highest numbers of IFN-γ inducing CD4 T cells in animals vaccinated with MISA+Ag is a confirmation that montanide ISA 720 is a superior adjuvant associated with induction of Th1 immune responses. This is also confirmed by the expression of the highest numbers of IFN-γ producing CD8 T cells in the same adjuvant vaccinated animals as compared to other adjuvant+Ag vaccinated groups. Reports on montanide trials done with HIV- and malaria-derived antigens as well as in a cancer vaccine have shown that this adjuvant is highly immunogenic inducing both Th1 type cellular and humoral responses (Kenney and Edelman, 2003; Myriam et al., 2005; Oliveira et al., 2005; Collins et al., 2006). The present results indicate alum-BCG as a lesser adjuvant than MISA 720 in the production of IFN-γ- inducing CD4 T cells as well as in the expression of IFN-γ inducing CD8 T cells despite the response being comparable in the latter lymphocyte expression. However, AlBCG has been evaluated in *Leishmania* vaccine studies and has been associated with strong Th1 immune responses including production of IFN-γ (Misra et al., 2001; Kamil et al., 2003). Both AlBCG and MISA can equally be used to induce effective Th1 immune responses against visceral *L. donovani* infection.
The present results failed to establish MPL as a competitive adjuvant in the expression of IFN-\(\gamma\)-inducing CD4 and CD8 T cells as compared to AIBCG and MISA adjuvants. Monophosphoryl lipid A used with Leishmania-derived recombinant polyprotein Leish-111f antigen was shown to be highly immunogenic in a vaccine against murine \(L. \text{ infantum}\) leishmaniasis (Coler \textit{et al.}, 2007). Failure of the MPL+Ag used in this study to induce high levels of FN-\(\gamma\)-producing CD4 and CD8 T cell levels comparable to other antigen-adjuvant groups may be attributed to the formulation of this adjuvant. In the study carried out by Coler \textit{et al.} (2007), the monophosphoryl lipid A was in a stable emulsion while our present study used monophosphoryl lipid A formulated in water. It may appear that, the aqueous formulation of monophosphoryl lipid A may be considered less effective than the emulsion-based formulation. However, in a different study using pneumococcal-CRM\(_{197}\) conjugate vaccine in health infants, MPL in aqueous formulation was associated with high cellular immune responses (Vernacchio \textit{et al.}, 2002). The difference in the toddler studies with our results may be attributable to batch to batch disparities or due to the antigens used. The almost baseline IFN-\(\gamma\)-inducing CD4 or CD8 T cell numbers associated with vaccinations with sonicate antigen alone indicates the importance of the adjuvants used in this study as inducers of Th1 immune responses. The positive and significant correlation between IFN-\(\gamma\)-producing CD4+ and CD8+ T cells clearly indicate that both types of T lymphocytes are important in the production of IFN-\(\gamma\) for effective control against visceral leishmaniasis. Furthermore previous studies on the immunology of leishmaniasis have clearly shown that both CD4+ and CD8+ T lymphocytes are induced and are required to control the disease (Garcia-Miss, 1995). The positive and significant correlation of these lymphocytes with type 1 cytokines and DTH was a confirmation of how strong these adjuvants are in inducing type 1 immune responses. It further indicates that early positive DTH response could be used to
predict the importance of a vaccination protocol and therefore the need to continue or discontinue the immunization experiment.

5.4 Protection against visceral leishmaniasis following vaccination with *L. donovani* antigen mixed with different adjuvants

The failure of the present study to observe any clinical symptoms in challenged animals may only be attributed to the limited duration of the study. Animals were only kept for a total of 103 days following challenge and during this period there were no observable body temperature changes (chills or fever) or body weight changes such as wasting which is a common feature in advanced visceral leishmaniasis. It would probably require a longer duration to be able to establish the clinical symptoms. Furthermore other clinical features such as splenomegally or hepatomegally were also absent at the time of termination of the study. However, the slight decrease in body weights in the control animal group observed towards the termination of the experiment may have been an early indication of severe disease outcome.

The ability to induce a protective immune response is the principal test of a new vaccine and adjuvant combination (Masina *et al*., 2003). It was demonstrated that it is possible to generate a cellular immune response that was sufficient to control parasite multiplication in the animal groups vaccinated with AIBCG+Ag or MISA+Ag. The high antigen recall lymphoproliferative responses, high IFN-γ and TNF-α cytokines and robust IFN-γ producing CD4+ and CD8+ T lymphocytes induced in the animal groups vaccinated with AIBCG+Ag or MISA+Ag were all negatively and significantly correlated with parasitic burden indicating significant protection by these vaccines. With high numbers of Th1 CD4+ and cytotoxic CD8+ T lymphocytes, protection is achievable since these cells are involved in the production
of type 1 cytokines including IFN-γ which is important for effective control of intracellular parasite.

It was also interesting to notice that the positive DTH response was associated with significant protection against disease indicating the importance of DTH response as a predictive marker of protection and as a strong Th1 immune response. The study has also established the importance of INF-γ associated IgG2 antibody response in the control of visceral disease and that both AIBCG and MISA adjuvants may be important in vaccination protocols aimed at induction of high levels of protective IgG2 antibody responses. Failure of the MPL+Ag to control disease to levels comparable to the other two antigen-adjuvant groups may be attributed to the formulation of this adjuvant. In a previous study using Leishmania-derived recombinant polyprotein Leish-111f antigen plus monophosphoryl lipid A, protection against visceral leishmaniasis caused by Leishmania infantum was reported to be 99.6% (Coler et al., 2007). However, the monophosphoryl lipid A used in the study was formulated in stable oil emulsion while the monophosphoryl lipid A used in the present study was formulated in water. However, batch to batch disparities have a play as well as antigen type and dosage.

When considering the production of a Leishmania vaccine for clinical use, it would be desirable to produce a vaccine that is safe and able to control disease. The present study reports that, of the two most immunogenic and protective antigen-adjuvant combinations: AIBCG+Ag and MISA+Ag, only MISA+Ag passed the criteria for a promising Leishmania vaccine in terms of safety, immunogenicity and efficacy. Reports of adverse effects of BCG in Leishmania vaccine studies have been indicated in the India langur that used the intradermal route (Misra et al., 2001) for a vaccine against visceral leishmaniasis and in the
rhesus monkeys inoculated by subcutaneous route (Kenney et al., 1999) for a vaccine targeting cutaneous disease. Human Leishmania vaccine studies have also associated use of BCG adjuvant with ulcerative indurations (Kamil et al., 2003). Large ulcerating nodules as produced following vaccination with BCG may not be acceptable for field use of a vaccine.

On the basis of the results presented in the present study, we report the suitability for use of montanide ISA 720 as a safe and effective adjuvant in the delivery of vaccines against visceral leishmaniasis in the vervet monkey model. The vervet monkey model has been well documented (Gicheru et al., 1995; Gicheru et al., 1997; Gicheru et al., 2001; Olobo et al., 2001; Masina et al., 2003) and these results may be evaluated in humans. It has the additional advantage that montanide ISA 720 has been used in human vaccine trials (Toledo et al., 2001; Oliveira et al., 2005; Pierce et al., 2010; Herrera et al., 2011) and is strongly recommended by the manufacturer for clinical trials in humans (Gomez et al., 1999). Based on this study, it can further be recommended that montanide ISA 720 replaces BCG as an adjuvant for Leishmania vaccines. However, considering that, the monophosphoryl lipid A formulated in stable emulsion was shown to be highly effective in the control of L. infantum visceral leishmaniasis (Coler et al., 2007) it is important that a comparative study be carried out on the immunogenicity and efficacy between monophosphoryl lipid A in stable emulsion and montanide ISA 720 in non-human primate model of visceral leishmaniasis.
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

i. The study concludes that a *Leishmania* vaccine containing montanide ISA 720 or monophosphoryl lipid A is safe and is not associated with either local or systemic adverse reactions. However, as a matter of concern, a vaccine containing BCG is associated with both local and systemic side effects as indicated by ulcerative indurations and swollen lymph nodes.

ii. This study has clearly established that AIBCG or MISA 720 as adjuvants combined with *Leishmania donovani* sonicate antigen induces high quantities of parasite specific total IgG antibodies as well as robust levels of IgG2 subclass antibodies as compared to vaccination with MPL adjuvant.

iii. On the other hand, data from this study suggests that Montanide ISA 720 can be safely used in vaccination protocols that require induction of strong cellular immunity, including high levels of IFN-γ inducing CD4+ and CD8+ T cells, IFN-γ, TNF-α and strong DTH responses as well as recall lymphoproliferative responses as compared to vaccination with MPL.

iv. Vaccinations with sonicate antigen containing either AIBCG or MISA 720 was associated with low IL-4 and IL-10 cytokine responses as compared to MPL which induced higher Th2 cytokine levels.
v. Based on the efficacy evaluation data, it can be concluded that the best adjuvants for use with *Leishmania* donovani whole cell sonicate antigen are AlBCG and Montanide ISA 720 as indicated by the significantly reduced parasite numbers in challenged animals following vaccinations as compared to MPL adjuvant. However, the significantly lower parasitic loads associated with vaccination with MPL as compared to the sonicate antigen alone may point to the potential efficacy of this adjuvant if reformulated.

vi. Vaccinations with AlBCG or MISA 720 concluded equal protective capacity and it may be worthwhile to emphasise that the induction of strong cellular immune responses and high levels of IgG2 antibodies associated with these two adjuvants were responsible for their high efficacy.

6.2 RECOMMENDATIONS

6.2.1 Application of the research findings

i. The safety of BCG as an adjuvant in human vaccines needs to be thoroughly investigated

ii. Montanide ISA 720 adjuvant should be considered as an alternative immunostimulator in human *Leishmania* vaccines due to its safety and high efficacy.

iii. The use of DTH response as an early predictor of expected immune responses and protection of a vaccination protocol is validated by this study.
iv. The safety, immunogenicity and protective capacity of MISA 720 can be evaluated in human subjects. It is important though, to note that, the monkey model may not have exactly the same immune responses as human subjects and one may therefore not get exactly the same outcome of this study in humans as observed in the vervet monkey system.

6.2.2 Suggestions for future research work

i. Investigation on the most efficacious *Leishmania* antigen- MISA 720 combination against *Leishmania donovani* disease.

ii. Recommendation is made for a study to establish the most effective dosage of MISA 720 - containing *Leishmania* vaccine for clinical application.

iii. Investigation on the comparative safety, immunogenicity and efficacy of monophosphoryl lipid A in stable emulsion (MPL-SE) and montanide ISA 720 (MISA 720) in in non-human primates model of *L. donovani* visceral disease.

iv. Recommendations are made for human clinical trial of a *Leishmania* vaccine containing montanide ISA 720 V as an adjuvant.

v. Determination of immune responses induced by antigen-adjuvant combinations at different time points should be carried out.
REFERENCES


when administered as an adjuvant with pneumococcal-CRM$_{197}$ conjugate vaccine in healthy toddlers. *Vaccine* 20: 3658-3667.


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APPENDICES

APPENDIX I: PREPARATION OF BUFFERS AND REAGENTS USED IN VARIOUS ASSAYS

(a): Preparation of Alsever’s solution

To make 1000 ml of Alsever’s solution;

Mix and dissolve the following salts

Dextrose (Glucose) 20.5g
Sodium chloride 4.2g
Trisodium citrate 8.0g
Double distilled water 1000 ml

Adjust pH to 7.2, sterilize by membrane filtration and store at 4°C.

(b): Preparation of Carbonate-Bicarbonate buffer (pH 9.6)

NaCl 8.0g
KH₂PO₄ 0.2g
Na₂HPO₄.12H₂O 2.9g
KCl 0.2g
NaN₃ 0.2g

Make up to 1 liter with distilled water, store at 4°C.

(c): Preparation of Phosphate buffered saline (PBS, 10X)

NaH₂PO₄ H₂O 20.5g
Na₂HPO₄.7H₂O 179.9g
H₂O 4 litres
NaCl 701.3g
Dissolve and adjust the volume to 8 liters, adjust pH to 7.2-7.4

Store the stock solution at room temperature

Dilute to 1 X for use.

(d): Preparation of physiological saline (Sodium chloride, NaCl)

Sodium chloride 8.5g
Double distilled water 1 litre

Dissolve and filter sterilize.

(e): Giemsa stain

Giemsa stain 4ml
Methanol 4ml
Double distilled water 92ml

Store at room temperature.

(f): RPMI 1640 supplemented with 10% fetal bovine serum (FBS)

To 445 ml of RPMI -1640, add 50 ml of fetal bovine serum (heat inactivated for 1 hr at 56°C), L-glutamine (final concentration of 200 mM/ml), 1 ml of gentamycin (50 mg/ml) and 2-mercapto-ethanol (to make a final concentration of 5x10^{-5} millimole). Adjust the pH to 7.2 and filter sterilize with 0.22 micron Millipore filter. Store at 4°C.

(g): Schneider’s Drosophilla Insect media supplemented with 20% fetal bovine serum (FBS)

To 80ml of of Schneider’s medium add 20 ml of fetal serum (heat in-activated for 1 hr at 56°C), 0.2 ml gentamycin (50 mg/ml), filter sterilize with 0.22 micron milipore filter and store at 4°C.
(h) Concanavalin A (Con A)

To make 1 mg/ml of Concanavalin A (Con A):

1. Weigh 1 mg of Con A powder (Sigma) and transfer to sterile 15 ml centrifuge tube.
2. Add 1 ml of incomplete RPMI-1640 medium (Sigma) to the centrifuge containing the Con A powder and shake the tube mix the contents.
3. Aliquot in 100 μL/vial and store at -20°C. This should be used within 90 days.
4. For cell culture use 10 μg/ml of Con A prepared by reconstituting 100 μL/ml Con A in 10 ml of complete RPMI medium.

APPENDIX II: PROTEIN ESTIMATION ASSAY

Reagents

1. Complex-forming reagent. Prepare immediately before use by mixing the following 3 stock solutions A, B, and C in the proportion 100: 1: 1, respectively.

2. Solution A: 2% (w/v) NaCO₃ in distilled water

3. Solution B: 1% (w/v) CuSO₄ .5H₂O in distilled water.

4. Solution C: 2% (w/v) Sodium potassium tartrate in distilled water.

5. 2N NaOH.

6. Folin reagent (Commercially available; Sigma, immunochemical, UK): Use at 1N concentration

7. Standards: Use stock solution of standard (bovine serum albumin fraction V; Sigma, immunochemical, UK) containing 4mg/ml protein in distilled water stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

<table>
<thead>
<tr>
<th>Sample tubes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution μl</td>
<td>0</td>
<td>1.25</td>
<td>2.50</td>
<td>6.25</td>
<td>12.5</td>
<td>25.0</td>
<td>62.5</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>Water μl</td>
<td>500</td>
<td>499</td>
<td>498</td>
<td>494</td>
<td>488</td>
<td>475</td>
<td>438</td>
<td>375</td>
<td>250</td>
</tr>
<tr>
<td>Protein concentration μg/ml</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>500</td>
<td>1000</td>
<td>2000</td>
</tr>
</tbody>
</table>
Method

1. To 0.1ml of sample or standard, add 0.1ml of 2N NaOH. Hydrolyse at 100° C for 10 minutes in a heating block or boiling water bath.

2. Cool the hydrolyzate to room temperature and add 1ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 minutes.

3. Add 0.1ml of folin reagent, using vortex mixture, and let it stand at room temperature for 30-60 minutes (do not exceed 60 min).

4. Read the absorbance at 750nm if the protein concentration is below 500 μg/ml or at 550nm if the protein concentration is between 100 and 2000 μg/ml.

5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

APPENDIX III: EXPERIMENTATION ON THE VERVET MONKEY

a) Weight measurement
b) Temperature measurement

c) Intradermal vaccination of the vervet monkey
d) Measurement of DTH responses using metric caliper

e) Intravenous challenge of vervet monkey with *L. donovani* parasites
APPENDIX IV: MANUSCRIPTS PUBLISHED

(a)

Leishmania donovani whole cell antigen delivered with adjuvants protects against visceral leishmaniasis in vervet monkeys (Chlorocebus aethiops)<sup>2</sup>

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Abstract

In a previous immunogenicity and efficacy study in mice, montanide ISA 720 (MISA) was indicated to be a better adjuvant than bacillus calmette guerin vaccine (BCG) for a Leishmania vaccine. In the present study, we report the safety, immunogenicity and efficacy of Leishmania donovani (L. donovani) sonicated antigen delivered with alum-BCG (AIBCG), MISA or monophosphoryl lipid A (MPLA) in vervet monkeys following intradermal inoculums. Vaccinated and control animals were challenged with virulent L. donovani parasites and the parasitic burden was determined. Only animals vaccinated with alum-BCG adversely reacted to the inoculum by producing ulcerative erythematous edematous induration. Non-parametric ANOVA followed by a post test showed significantly higher IgG antibodies, and revealed the presence of lymphoproliferative and interferon gamma responses in both AIBCG+Ag and MISA+Ag as compared to the MPLA+Ag or other groups (P < 0.001). We conclude that L. donovani sonicated antigen containing MISA is safe and is associated with protective immune response against Leishmania donovani infection in the vervet monkey model.

Keywords: visceral leishmaniasis, Leishmania donovani, vervet monkey, sonicated antigen, adjuvants

INTRODUCTION

Leishmaniasis, a clinically heterogeneous group of diseases, caused by infection with protozoa of the genus Leishmania, is one of the world's most important infectious diseases<sup>1</sup>. Visceral leishmaniasis or kala-azar is the most dreaded and devastating amongst the various forms of leishmaniasis<sup>2</sup>. The disease is fatal in almost all cases if left untreated<sup>3</sup>. It may cause epidemic outbreaks with high mortality<sup>4</sup>. There is currently no vaccine against leishmaniasis in routine use anywhere in the world<sup>5</sup>. A vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on the genetics and biology of the parasite, as well as on the clinical and experimental immunology of leishmaniasis, and the availability of Leishmania vaccines that can protect experimental animals against challenge with different Leishmania species<sup>6</sup>. Clinical and experimental studies demonstrate that generation of an effective cellular immune response is required for protection against this disease<sup>7,8</sup>

The only successful intervention against leishmaniasis is inoculation using virulent parasites, a process...
SAFETY AND SKIN DELAYED-TYPE HYPERSENSITIVITY RESPONSE IN VERVET MONKEYS IMMUNIZED WITH Leishmania donovani SONICATE ANTIGEN DELIVERED WITH ADJUVANTS

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SUMMARY

In this study, we report on the safety and skin delayed-type hypersensitivity (DTH) responses of the Leishmania donovani whole cell sonicate antigen delivered in conjunction with alum-BCG (AlBGC), Montanide ISA 720 (MISA) or Monophosphoryl lipid A (MPLA) in groups of vervet monkeys. Following three intradermal injections of the inoculums on days 0, 28 and 42, safety and DTH responses were assessed. Preliminary tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) levels were also measured and these were compared with DTH. Only those animals immunized with alum-BCG reacted adversely to the inoculum by producing ulcerative erythematous skin inurations. Non-parametric analysis of variance followed by a post-test showed significantly higher DTH responses in the MISA+Ag group compared with other immunized groups (p < 0.001). The MPLA+Ag group indicated significantly lower DTH responses to the sonicate antigen compared with the AlBGC+Ag group. There was a significant correlation between the DTH and cytokine responses (p < 0.0001). Based on this study we conclude that Leishmania donovani sonicate antigen containing MISA 720 is safe and is associated with a strong DTH reaction following immunization.

KEYWORDS: Safety; Delayed-type hypersensitivity (DTH); Vervet monkeys; Leishmania donovani sonicate antigen; Adjuvants.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is the most dreaded and devastating amongst the various forms of leishmaniasis (GARG & DUBE, 2006). The disease is a major cause of morbidity and mortality in East Africa and the Indian subcontinent. Coinfection with HIV enhances the risk of the disease (NGURE et al., 2009). The only control measures currently available in East Africa are case detection and treatment with antimonials, drugs which are expensive, toxic, not always available and cannot be self-administered. Vector and reservoir control is difficult due to the elusive nature of the vector and the diversity of the animal reservoir. Cases of self-cure in cutaneous leishmaniasis, accompanied by solid immunity to reinfection, make vaccine development a feasible control method (MUTISO et al., 2010a). Celluar or Th1 immune response is important for the cure of leishmaniasis. This involves the activation of infected macrophages by sensitized T cells to produce parasite lethal nitric oxide. The use of adjuvants in effective Leishmania vaccine development cannot be avoided. The availability of many potential adjuvants has prompted the need for identifying rational standards for the selection of adjuvant formulations based on safety and sound immunological principles for human vaccines (ALVING, 2002). Three effective adjuvants that have shown promising results in Leishmania vaccine studies have been identified in a recent review (MUTISO et al., 2010b). These adjuvants include: Montanide ISA 720 (MUTISO et al., 2010b), alum formulated with BCG (MISRA et al., 2001; KAMIL et al., 2003) and Monophosphoryl lipid A (SKEIKY et al., 2002; COLER & REED, 2005; COLER et al., 2007). Despite a lot of vaccination research using these adjuvants, it is not readily clear which of them is more superior in terms of safety and immunogenicity and hence which of them is most appropriate for use in the development of Leishmania vaccines. Skin delayed-type hypersensitivity (DTH) response has been used as an indicator of the immunogenicity in an immunization antigen measured by the presence of a specific cellular type of immune reaction (GICHERU et al., 2001; RITTER & KORNER, 2002; MASINA et al., 2003). The present report describes a study undertaken to evaluate the safety and DTH response of Leishmania donovani sonicate antigen delivered together with each of the three selected adjuvants in the vervet monkey model with visceral leishmaniasis. The study also measured and compared preliminary TNF-α and IFN-γ levels with the DTH response.

MATERIALS AND METHODS

Leishmania parasites: The Leishmania donovani strain NLB-065 originated from the spleen of an infected patient in the Baringo district of Kenya and was maintained by intracardiac hamster-to-hamster passage at the Institute of Primate Research. A hamster spleenic biopsy was cultured in Schneider's drosophila insect medium and was supplemented

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Immunization with *Leishmania* Vaccine-Alum-BCG and Montanide ISA 720 Adjuvants Induces Low-Grade Type 2 Cytokines and High Levels of IgG2 Subclass Antibodies in the Vervet Monkey (*Chlorocebus aethiops*) Model

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Abstract

The availability of hundreds of adjuvants has prompted a need for identifying rational standards for the selection of adjuvant formulation based on sound immunological principles for human vaccines. As cytokines elaborated by activated T cells are required for the regulation of isotype switch during B-cell development, a study of Th2 cytokines and subclass distribution of the antibodies may shed new light on the processes involved in the polarization of the immune responses during vaccination studies. The aim of this study was to identify an appropriate *Leishmania* vaccine adjuvant based on low Th2 cytokine and high value IgG2 antibody responses. Groups of vervet monkeys were immunized with *Leishmania donovani* sorbite antigen (Ag) alone or in conjunction with alum-BCG (ABCG), monophosphoryl lipid A (MPL) or montanide ISA 720 (MISA) as adjuvants. Following three time point intradermal injections on days 0, 28 and 42, IL-4, IL-10 and IgG2 antibody subclasses were quantified by enzyme-linked immunosorbent assay (ELISA) and data analysed by one-way analysis of variance, Tukey–Kramer test and Spearman’s rank correlation analysis. Results indicated relatively higher IL-4 and IL-10 cytokine responses following MPL + Ag as compared to ABCG + Ag or MISA + Ag immunization. There was a positive significant correlation between IL-4 and IL-10 levels (r = 1.000; P = 0.0167). Significantly higher IgG2 antibody responses were associated with either ABCG + Ag or MISA + Ag as compared to MPL + Ag immunization (P < 0.05). The study concludes that both ABCG and MISA may be used in *Leishmania* vaccine studies that favour low Th2 cytokine and strong IgG2 antibody responses.

Introduction

Human visceral leishmaniasis (VL), kala-azar, is a tropical disease caused by the protozoan parasites of the *Leishmania donovani* complex. The parasites multiply in the macrophages of the spleen, liver, bone marrow and lymph nodes, resulting in a progressive disease which is invariably fatal if untreated. Infection by *L. donovani* in humans induces T cell energy as assessed by the depression of delayed-type hypersensitivity reaction and failure of peripheral blood T cells to proliferate [1, 2] and to produce gamma interferon (IFN-γ) and interleukin (IL)-2 in response to *Leishmania* antigens [3, 4]. Cytokine analysis reveals enhanced induction of IL-10 and/or IL-4 mRNA in tissues [5, 6], and the enhanced presence of IL-4 in circulation [7] of patients with kala-azar. The disease is also characterized by high levels of *Leishmania*-specific antibodies [8]. Resolution of VL and cure in humans is associated with predominant Th1 response, good cell-mediated immunity (CDM), production of interferon-gamma (IFN-γ) and macrophage activation [3]. VL disease (Kala-azar) is associated with a Th2 response, poor
Leishmania parasite specific CD4+ synergizes and correlates positively with CD8+ T cells in the production of gamma interferon following immunization of the vervet monkey (Chlorocebus aethiops) model

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Abstract. Although there is currently no vaccine against leishmaniasis in routine use anywhere in the world, cases of self cure in cutaneous leishmaniasis, accompanied by solid immunity to reinfection, make vaccine development a feasible control method. Immunity against visceral leishmaniasis is mediated by IFN-γ-inducing parasite specific CD4+ and CD8+ T cells. We assessed the capacity of Leishmania donovani sonicate antigen delivered with alum-BCG (AIBCG), monophosphoryl lipid A (MPL) or monranide ISA 720 (MISA) to induce parasite specific CD4+ and CD8+ T cells involved in IFN-γ production following immunization of groups of the vervet monkey model of visceral leishmaniasis. Groups of vervet monkeys were immunized intradermally at three time points on days 0, 28 and 42 and T cell populations involved in the production of IFN-γ measured 21 days after final immunization. Significantly higher CD4+ T cells were induced in the group immunized with MISA+Ag compared to the AIBCG+Ag immunized animals (P<0.01) with both groups inducing significantly more CD4+ T cells than other groups (P<0.0001). Levels of CD8+ T cells were comparable between AIBCG+Ag and MISA+Ag groups, being significantly higher compared to the MPL+Ag group (P<0.001). The CD4+ T cells significantly correlated positively with CD8+ T cells in the studied groups (r=1.00; P=0.0167). We conclude that, immunization with MISA+Ag induces robust CD4+ as well as CD8+ T cells involved in the production of IFN-γ indicating stronger ability of this adjuvant over AIBCG in directing cellular immune response in the vervet monkey model.

Keywords: Immunization; Adjuvants; CD4+ Th1 and CD8+ T cells; Th1 immune response; Visceral leishmaniasis; Vervet monkey model.