DETERMINATION OF THE ADJUVANT POTENTIAL OF BACILLE CALMETTE GUÉRIN WITH CULTURE DERIVED *LEISHMANIA MAJOR* SOLUBLE EXO-ANTIGENS

BY

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A thesis submitted to the University of Nairobi, School of Biological Sciences, in partial fulfilment of the requirements for the award of degree of Master of Science in Applied Parasitology of University of Nairobi

2009

DECLARATION

I Stella Kepha, hereby declare that this my original work and has not been presented for a degree in any other University.

Kepha
Signature

30th July 2009
Date

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This thesis has been submitted with our approval as supervisors:

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Prof Horace Ochanda (Ph.D.)
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Signature

July 29, 2009
Date

Dr. Willy Kiprotich Tonui (Ph.D.)
Senior Research Officer,
Centre for Biotechnology Research & Development (CBRD),
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Dedication

This thesis is dedicated to my father Kepha, my mother Grace, and my siblings Claire, Allan and Moraa. It has been a tough journey, thank you for your support morally, spiritually and materially. God bless you.

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I am indebted to God who has been so gracious, given me life, strength and wisdom. To you Father I give all the glory and honor, thank you for making this thesis become a reality.

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Acronyms and abbreviations

ALM	Autoclaved *Leishmania major*
APC	Antigen presenting cell
BCG	Bacille Calmette Guérin
CBA	Cytometric bead array
CBRD	Centre for Biotechnology Research and Development
CCIEP	Counter current immunoelectrophoresis
CD	Cluster of differentiation
CL	Cutaneous leishmaniasis
Con A	Concanavalin A
CP	Cysteine proteinase
CpG	Cytosine phosphate guanosine
DALY	Disability adjusted year
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
DDT	Dichloro diphenyl trichloroethane
Dhfr-ts	Dihydrofolate reductase-thymidylate synthetase
DCL	Diffuse cutaneous leishmaniasis
FACS	Fluorescence activated cell sorter
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GM-CSF	Granulocyte macrophage- colony stimulating factor

gp 63	63 kilo Dalton Glycoprotein
ID	Immunodiffusion
IFN-γ	Interferon-gamma
IHA	Indirect haemaglutination
IgG	Immunoglobulin G
IL	Interleukin
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-10	Interleukin-10
IL-12	Interleukin-12
ISCOMS	Immunostimulating complexes
KEMRI	Kenya Medical Research Institute
LACK	*Leishmania* Homologue of the mammalian Receptor for Activated C Kinase
LeIF	Leishmanial eukaryotic ribosomal protein
LCL	Localized cutaneous leishmaniasis
LPG	Lipophosphoglycan
***Lm*SEAgs**	*Leishmania major* soluble exo-antigens
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MTT	3-(4, 5-dimethylthiazol-2yl)-2, 5-Diphenyl tetrazolium bromide
nM	Nanomolar

NLB	Nairobi *Leishmania* bank
ODN	Oligodeoxynucleotides
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pg/ml	Pico grams per millilitre
PKDL	Post Kala-azar dermal leishmaniasis
PHA	Phytohaemagglutinin
PSA	Parasite surface antigen
rIL-12	Recombinant interleukin-12
Th1	T Helper 1
Th2	T Helper 2
TNF-α	Tumour necrosis factor-alpha
µL	Microlitre
µg	Microgram
µM	Micromolar
Mµ	Micrometers
VL	Visceral leishmaniasis
WHO	World Health Organization

Abstract

Leishmaniasis is one of the neglected diseases that afflicts residents of developing countries. To date, there are no proven vaccines against this disease. Previous studies have shown that *Leishmania major* soluble exo-antigens (*Lm*SEAgs) alone have the potential to confer protection to mice infected with *L. major*. There was need to investigate whether adjuvants could enhance the protective effects of the *Lm*SEAgs the subject of this thesis.

In this study, the immunoprophylatic and immunotherapeutic potential of Bacille Calmette Guérin (BCG) as an adjuvant was investigated. For immunoprophylaxis, susceptible BALB/c mice were vaccinated with *Lm*SEAgs with or without BCG on day 0 and boosted on day 13, then challenged with *L. major* metacyclic promastigotes a week later. The control group was unvaccinated. While for immunotherapy, mice were vaccinated with *Lm*SEAgs with or without BCG at day 21 and boosted on day 35. Disease progression was determined by measuring the size of lesions and quantifying parasite burdens in *L. major* infected footpads using a limiting dilution assay. While cytokine production from splenocytes was determined by flow cytometry.

For both immunoprophylaxis and immunotherapy, mice produced significantly high levels of IFN-γ ($P< 0.05$) and low levels of interleukin IL-4, ($P< 0.05$) compared to the unvaccinated mice. This was corroborated with reduction in lesion sizes and parasite burden compared to the controls ($P< 0.05$). In comparison to the group of mice treated with *Lm*SEAgs alone or BCG alone there was no significant difference in the levels of IFN-γ produced, lesion size reduction or parasite burdens ($P>0.05$) in both immunoprophylaxis and immunotherapy.

In conclusion, the results show that BCG does not enhance the protective effect of *Lm*SEAgs. Further studies should be done to search for molecules with potential of enhancing the immunotherapeutic and immunoprophylactic effects of *Lm*SEAgs.

CHAPTER 1- INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Background information

Leishmaniases are a group of diseases that are caused by obligate intracellular and kinetoplastid protozoa that belong to the genus *Leishmania* (family Trypanosomatidae) (Roberts, 2006). The parasite is transmitted by the bite of an infected sand fly of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world (WHO, 2007).

The diseases are geographically and ecologically widespread, occurring in tropical and subtropical regions in all continents except Australia. Leishmaniasis is endemic in 88 countries (WHO, 2007). The incidence of leishmaniasis is increasing, with many endemic areas reporting a 500 fold increase over the past seven years (Salam, 2004). It is estimated that there are 2 million new cases each year with about 350 million people being at risk and 14 million people infected worldwide (Roberts, 2006; WHO, 2007).

The disease exhibits a spectrum of disease from cutaneous, mucocutaneous to visceral leishmaniasis (Roberts, 2006). Cutaneous forms of the disease normally produce skin ulcers on the exposed parts of the body such as the face, arms and legs (Desjeux, 2004; WHO, 2006). In mucocutaneous forms of leishmaniasis, lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues (WHO, 2006). Visceral leishmaniasis (VL) also known as kala-azar is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia: if left untreated, it is fatal (Hailu *et al.*, 2005; WHO, 2006).

Currently, control strategies of leishmaniasis rely on chemotherapy to alleviate disease and on vector control to reduce transmission (Handman, 2001; Khamesipour *et al.*, 2006; Tonui and Titus, 2006). The drugs currently recommended for the treatments of leishmaniasis include the pentavalent antimonials; sodium stibogluconate (Pentostam), meglumine antimoniate (Glucantime) and amphotericin B (Guerin *et al.*, 2002). However these drugs have drawbacks such as serious side-effects, long courses of treatment, parenteral route of administration, high cost of treatment and rampant drug resistance especially of the antimonials (Guerin *et al.*, 2002; Croft *et al.*, 2006; WHO, 2006; WHO, 2007).

Leishmaniasis contributes significantly to the propagation of poverty, because treatment is expensive and hence either unaffordable or it imposes a substantial economic burden, including loss of wages (WHO, 2007). Neglected by researchers and funding agencies, leishmaniasis control strategies have varied little for decades although recently impressive advances have been made including vaccine development (Davies *et al.*, 2003).

1.1.2 Global distribution of leishmaniasis

Leishmaniasis is endemic in more than 88 countries found in the Indian sub continent, south western Asia, southern Europe, Africa, and central and south America (WHO, 2007) (Figure 1). There is a remarkable increase in risk factors for leishmaniases worldwide and the disease burden is increasing (Reithinger *et al.*, 2007). This is attributed to different phenomena such as economic hardship, natural disasters, armed conflict, and tourism that cause susceptible populations to migrate to areas endemic for leishmaniasis, where exposure to infection results in noticeable epidemics (Guthmann *et al.*, 1997; Blum *et al.*, 2004; Wenia *et al.*, 2004).

Globally, there are an estimated 350 million people at risk of infection and disease, 14 million people are infected and 2 million new cases are reported each year (Reithinger *et al.*, 2007; WHO, 2007). The global burden of leishmaniasis has remained stable for some years, causing a morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Davies *et al.*, 2003; Reithinger *et al.*, 2007).

The global estimate for new cases of VL is 500 000 cases per year out of which 90% of the cases occur in just five countries namely Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004; WHO, 2007).

Cutaneous leishmaniasis (CL) occurs in more than 70 countries worldwide with 90% of the cases reported in Afghanistan, Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Syria (Ghalib and Modabber, 2007). In addition each year, there are 1.5 million new cases (WHO, 2007).

Globally, muco-cutaneous leishmaniasis (MCL) cases are dominant in south America, especially in Brazil, Paraguay, Ecaudor, Bolivia, Peru, Colombia and Venezuela. Ninety of all cases of MCL cases occur in Bolivia, Brazil and Peru (Desjeux, 2004).

In Africa, both CL and VL are endemic to countries in the north, central, east, the Horn of Africa and West Africa (Boakye *et al.*, 2005). Sudan bears the heaviest burden of the disease, as it is one of the five countries that constitute 90% of all global cases of VL (Guerin *et al.*, 2002). The highest incidence of post kala-azar dermal leishmaniasis (PDKL) a complication of VL in the world is also found in Sudan (Ghalib and Modabber, 2007). Algeria is the only African country among the eight countries that contribute 90% of worldwide cases of CL (Murray *et al.*, 2005).

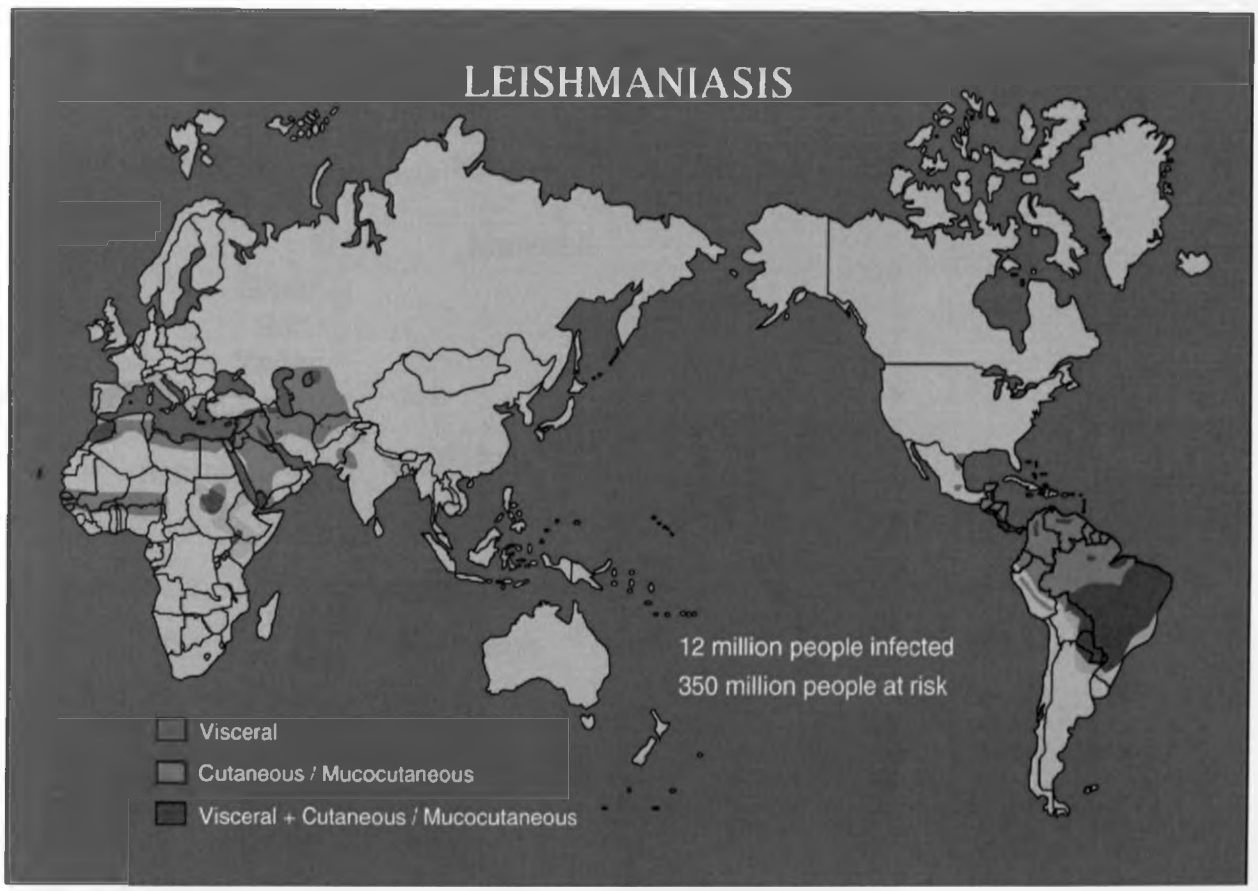

Figure 1: World map highlighting areas where cutaneous, visceral and mucocutaneous leishmaniasis is endemic (Adapted from Handman, 2001).

1.1.3 Epidemiology of leishmaniasis in Kenya.

In Kenya two forms of leishmaniasis are prevalent namely CL and VL (Tonui, 2006) (Figure 2). Leishmaniasis has been known to be endemic in parts of Kenya from as far back as early in the 20th century (Fendall, 1961). An outbreak of VL was first reported among King's African Rifles troops encamped north of Lake Turkana in the 1940s (Cole *et al.*, 1942). Turkana, Baringo, Kitui, West Pokot, Machakos, Meru, Keiyo and Marakwet districts have since been considered to be endemic for VL with Baringo and West Pokot being considered endemic foci (Tonui, 2006).

Figure 2: Map of Kenya showing areas where leishmaniasis is endemic Key: VL- Visceral leishmaniasis; CL- Cutaneous leishmaniasis;
DCL- Diffuse cutaneous leishmaniasis

Visceral leishmaniasis in Kenya is caused by *L. donovani* transmitted by *Phlebotomus martini* and is endemic in Baringo District, Kenya. The disease occurring in Baringo has a focal distribution in the dry, hot areas below 1500 metres. In the early 1990s, three Maasai children were diagnosed with VL acquired locally in Kajiado district in the Rift Valley of Kenya. In 2001, an outbreak of VL was reported in the previously non-endemic Wajir and Mandera districts of North Eastern province Kenya where between May 2000 to August 2001, 904 patients were diagnosed with VL, with patients coming from as far as southern Somalia and southeast Ethiopia (Marlet *et al.*, 2003). Unusual rainfall patterns malnutrition and migration of a *Leishmania* infected population, seeking food security are thought to have contributed to this outbreak (Marlet *et al.*, 2003).

In Kenya CL is caused by *Leishmania major, L. aethiopica and L. tropica* (Mebrahtu *et al.*, 1992). *Leishmania major* transmitted by *P. duboscqi* was first reported in Kitui district in the 1990s (Mutinga *et al.*, 1994). In this country CL due to *L. major* which is transmitted by *P. duboscqi* is rare in humans, but underreporting is likely. *Phlebotomous duboscqi* is mainly found in animal burrows where it feeds on rodents which are frequently infected (Schaefer *et al.*, 1994).

Diffuse cutaneous leishmaniasis (DCL) was first described in Kenya in 1969 with three patients from the indigenous communities in Bungoma district, around the Mt. Elgon area diagnosed (Kungu *et al.*, 1972). *Leishmania aethiopica* has since been identified as the aetiological agent of DCL in Mt Elgon and rodents as the animal reservoirs (Mutinga, 1975; Sang and Chance, 1993).

Although quite a number of studies on the transmission and control of leishmaniases have been done in Kenya; the burden of the disease is still enormous. Many cases of leishmaniases still go unreported or undiagnosed hence official statistics are currently not available for determining the actual number of cases in Kenya (Tonui, 2006).

1.1.4 Aetiology and clinical presentation of leishmaniasis

Leishmaniasis is characterized by both diversity and complexity: it is caused by more than twenty Leishmanial species and is transmitted to humans by approximately thirty different species of phlebotomine sandflies (Chappuis *et al.*, 2007). The leishmaniases cause considerable morbidity and mortality. In man, the disease occurs in at least four major forms: namely cutaneous, diffuse cutaneous, mucocutaneous and visceral (Desjeux, 2004).

1.1.4.1 Visceral Leishmaniasis

Visceral leishmaniasis is also known as kala-azar from the Hindu vernacular (Hailu *et al.*, 2005). It is a systemic disease and the most severe form of leishmaniasis that is fatal if left untreated (Desjeux, 2004; Chappuis *et al.*, 2007) (Figure 3a and 3b). *Leishmania donovani* complex is the main aetiological agent of VL (Chappuis *et al.*, 2007). In east Africa and the Indian subcontinent VL is caused by *L. donovani sensu stricto* while in Europe, north Africa, Mediterranean region and Latin America it is caused by *Leishmania infantum* (Chappuis *et al.*, 2007). On the other hand in the New World VL is caused by *L. chagasi* (Murray, 2001; Saha *et al.*, 2005). *Leishmania tropica* has been reported to produce visceral disease in immunocompromised persons (Herwaldt, 1999). *Leishmania amazonensis* is known to cause American tegumentary leishmaniasis, however there are reports of visceralization (Magill *et al.*, 1993; Herwaldt, 1999).

The classic kala-azar syndrome is exemplified by patients such as those in Sudan who are heavily infected throughout the mononuclear phagocyte system. They develop life-threatening disease after an incubation period of weeks to months; and have fever, severe cachexia, hepatosplenomegaly (splenomegaly usually predominates), pancytopenia anaemia, thrombocytopenia and leucopenia, with neutropenia, marked eosinopenia, a relative lymphocytosis and monocytosis, and hypergammaglobulinaemia (mainly IgG from polyclonal B-cell activation) with hypoalbuminaemia (Herwaldt, 1999; Desjeux, 2004; Saha *et al.*, 2005)

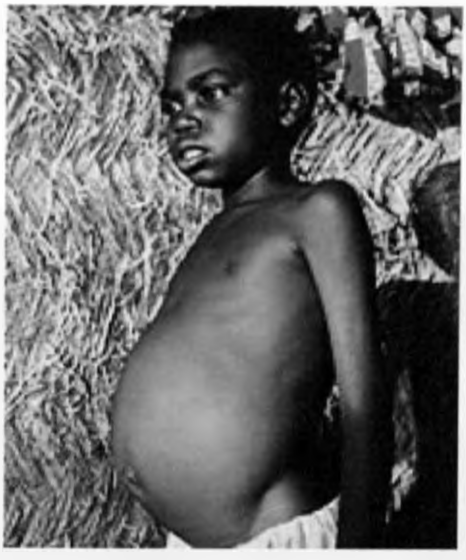

Figure 3a: Profile view of a teenage boy suffering from visceral leishmaniasis. The boy exhibits splenomegaly, distended abdomen and severe muscle wasting. (Adapted from WHO/TDR/Kuzoe)

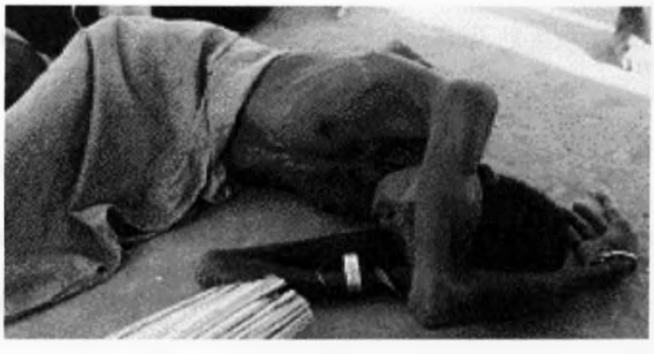

Figure 3b: Cachectic Sudanese woman with visceral Leishmaniasis. (Adapted from Herwaldt, 1999).

1.1.4.2 Post-kala-azar dermal leishmaniasis (PKDL)

Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL; it is characterized by a macular, maculopapular, and nodular rash in a patient who has recovered from VL and who is otherwise well (Zijlstra *et al.*, 2003) (Figure 4). The interval between treated VL and PKDL is 0–6 months in Sudan and 6 months to 3 years in India. People presenting with PKDL cases are highly infectious because the nodular lesions contain many parasites (Piscopo and Mallia, 2006) and such cases are the putative reservoir for anthroponotic VL between epidemic cycles (Zijlstra *et al.*, 2003). Most cases occur on the Indian subcontinent (India, Nepal, Bangladesh) and east Africa (Sudan, Ethiopia, Kenya), where *Leishmania donovani* is the causative parasite (Zijlstra *et al.*, 2003).

Figure 4: PKDL papular lesions (Adapted from Ziljstra *et al.*, 2003)

1.1.4.3 Cutaneous leishmaniasis

Cutaneous leishmaniasis occurs both in the Old World and New World (WHO, 2007). It encompasses a broad range of severity and manifestations of infection (Herwaldt, 1999). Many species of the *Leishmania* genus can serve as etiologic agents of cutaneous disease.

including *L. major*, *L. tropica*, *L. aethiopica*, *L. infantum* and *L. archibaldi* in Eurasia and Africa (Old world), and *L. mexicana*, *L. venezuelensis*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. chagasi L. guyanensis* and *L. peruviana* in South America (New World). Localized cutaneous leishmaniasis (LCL) is the most common and the least drastic form of CL. The lesions are caused by *L. major*, *L. tropica*, *L. aethiopica*, and subspecies of *L. mexicana* (Murray *et al.*, 2005). The erythema develops into a papule, then a nodule that progressively ulcerates over a period of 2 weeks to 6 months to become the lesion that is characteristic of LCL (Murray *et al.*, 2005) (Figure 5).

The recidivans form of CL is a complication of *L. tropica* infection (Akilov *et al.*, 2007). This form is rare (incidence about 3–10%) and resembles lupus vulgaris; therefore, it is also called "lupoid leishmaniasis" or "late leishmaniasis" (Akilov *et al.*, 2007). Diffuse CL begins as a single non ulcerative nodule that grows and becomes surrounded by other similar lesions. The nodules are shiny and often slightly red; their edges are usually distinct, with normal intervening skin. This process is repeated over many years the skin over the face, limbs, and buttocks becomes covered with symmetric non-ulcerating nodules (Reithinger *et al.*, 2007). To date, all isolates from Old World cases of DCL have been strains of *L. aethiopica* (Schonian *et al.*, 2000: Akilov *et al.*, 2007). The body's immune system apparently fails to battle the protozoa, which are free to spread throughout the body (Handman, 2001).

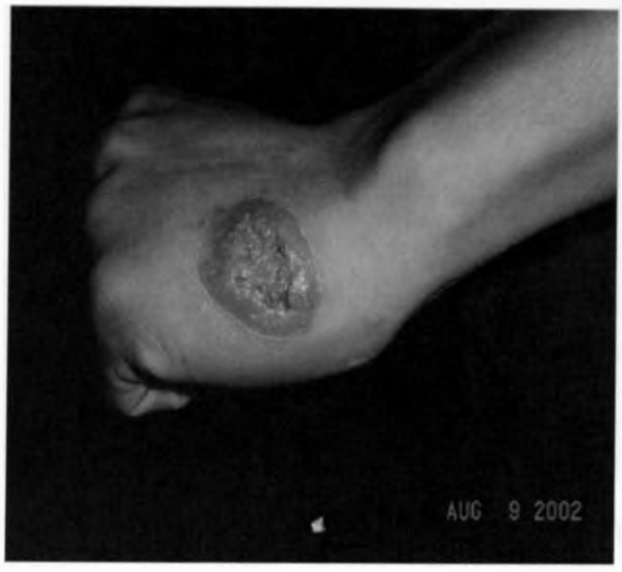

Figure 5: Cutaneous leishmaniasis lesion (Adapted from www.pdhealth.mil)

1.1.4.4 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis also known as 'espundia' causes extensive destruction of oral-nasal and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face and great suffering for life (WHO, 2007). Although mostly related to *Leishmania* species of the New World (America) such as *L. braziliensis L. guyanensis* and *L. peruviana* it has been also reported in the Old World (Africa, Asia and Europe) due to *L. donovani, L. major* in immmuno-suppressed patients (Desjeux, 2004) (Figure 6).

Figure 6: A patient from Bolivia with mucosal leishmaniasis (Adapted from Chappuis *et al.*, 2007)

1.1.5 The biology of *Leishmania* parasites

Leishmaniasis is a parasitic infection caused by the obligate, intracellular protozoan of the genus *Leishmania* (family Trypanosomatidae) under the kingdom Prostista and phylum Euglenoza (Singh, 2006). The parasite has a digenetic life cycle with an extra cellular developmental stage in the insect vector, a female phlebotomine sandfly, and a developmental stage in mammals, which is mostly intracellular (Roberts, 2006).

The invertebrate hosts or vectors are small insects of the order Diptera, belonging to the subfamily Phlebotominae and are commonly called phlebotomine sand flies (Banuls *et al.*, 2007). The female needs a blood meal for egg development and only the female is haematophagous (Roberts, 2006; Banuls *et al.*, 2007). When a sandfly feeds on an infected host during a blood meal the fly ingests parasitized macrophages which are then released into the gut (Roberts, 2006).

The first developmental event in the sandfly is the transformation of amastigotes to procyclic promastigotes. This transformation of amastigotes to promastigotes starts within hours of ingestion of the amastigotes (either free or intracellular) and occurs exclusively in the gut (Singh, 2006). The main difference of promastigotes from amastigotes is that the cell body is elongated, in the range of 8-15 μm, the flagellum emerges from the cell body, and is functional, making these cells motile (Roberts, 2006). By the time the sandfly takes a new blood meal, the metacyclics will have affected the feeding mechanism of the sandfly in such a way that they cause regurgitation of midgut content containing the parasites, into the feeding wound this is a survival mechanism that ensures efficient transmission to the vertebrate host (Rogers *et al.*, 2002).

The developmental cycle in the vertebrate host is initiated by the interaction of metacyclic promastigotes with skin macrophages. After uptake and internalization of metacyclic promastigotes in a phagolysosome, fusion with lysosomes proceeds as normal and the parasite inhabits a secondary lysosome or phagolysosome (Bates, 1993). During this process the metacyclic promastigote transforms into an amastigote within 12-24 h and continues to grow and divide within the phagolysosomal compartment. The amastigotes have to overcome two environmental challenges: the battery of lysosomal enzymes and low pH (4.5-5.5) (Zilberstein and Shapira, 1994). Low pH is not a problem as amastigotes seem to be acidophiles: they are metabolically more active at low pH (Singh, 2006). With this adaptation the parasite is able to survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow. The complete life cycle of the *Leishmania* parasite is summarized in the Figure 7 below.

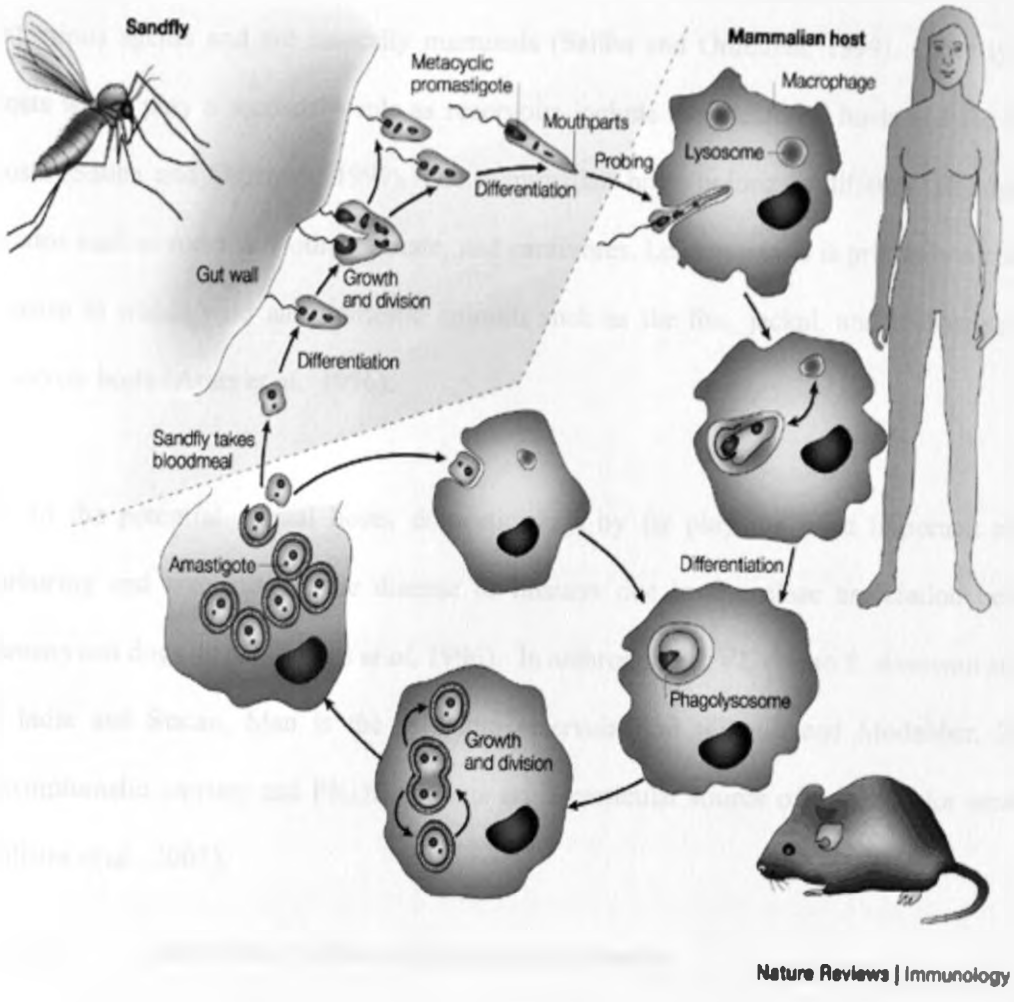

Figure 7: Life cycle of *Leishmania spp* (Adapted from Sacks and Noben-Trauth., 2003)

1.1.6 Transmission of leishmaniases.

1.1.6.1 Reservoir hosts of *Leishmania spp*

Primary reservoir hosts of leishmaniasis are responsible for the long-term maintenance of the infectious agents and are basically mammals (Saliba and Omenish, 1999). Other types of hosts which play a secondary role as reservoirs include the incidental hosts and the liaison hosts (Saliba and Omenish, 1999). The mammalian hosts belong to different phylogenetic groups such as rodents, sloths, primate, and carnivores. Leishmaniasis is primarily a zoonotic disease in which wild and domestic animals such as the fox, jackal, and wolves serve as reservoir hosts (Arias *et al.*, 1996).

Of all the potential animal hosts, domestic dogs by far play the most important role in harboring and transmitting the disease to humans due to the close association between humans and dogs as pets (Arias *et al*, 1996). In anthroponotic VL due to *L. donovani* such as in India and Sudan, Man is the principal reservoir host (Ghalib and Modabber, 2007). Asymptomatic carriers and PKDL patients are a particular source of infection for sandflies (Ziljstra *et al.*, 2003).

1.1.6.2 Sand flies: vectors of *Leishmania* parasite

The only proven vector of the *Leishmania* parasite is the blood-sucking female phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Murray *et al.*, 2005; Kato *et al.*, 2007). The insects are 2-3 mm long and are found throughout the tropical and temperate parts of the world. Only 30 or so of the over 500 species of Phlebotomine sandflies are known to transmit *Leishmania* parasites, these include *P. argentipes* on the Indian sub-continent, *P. duboscqi*, *P. martini* and *P.orientalis* in Africa and the Mediterranean basin, *P. chinensis* and *P. alexandri* in china. In the new world *L. longipalpis* is the only known vector of *L. chagasi* (Murray *et al.*, 2005; WHO, 2007).

1.1.7 Diagnosis of leishmaniases

The clinical signs and symptoms and epidemiological manifestations are not pathognomic of VL or CL (Singh, 2006). They can mimic several other conditions like; in the case of kala-azar it may be confused with other similar conditions such as malaria, tropical splenomegaly, schistosomiasis cirrhosis lymphoma or leukemia hence a laboratory diagnosis is necessary to confirm the clinical suspicions (Singh, 2006).

The gold standard and confirmatory diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes in the relevant tissues aspirates or biopsies such as bone marrow, spleen, lymph nodes, liver or skin slit smears or biopsies or in the peripheral blood buffy coat (Liarte *et al.*, 2001; Singh and Sivakumar, 2003; Singh, 2006). However there are various diagnostic tools used for each leishmanial syndrome.

Leishmania DNA can also be detected in tissue aspirates and peripheral blood using polymerase chain reaction (PCR) (Singh, 2006). Polymerase chain reaction has been used for clinical diagnosis albeit limited to tertiary care hospitals and research laboratories (Conjivaram, 2002; Singh, 2006). Amongst the molecular methods used for clinical diagnosis, PCR has been proven to be the most sensitive and specific technique. Bone marrow, lymph node aspirates, skin biopsy, skin scrape/exudates and blood samples have been used for PCR in several studies (Liarte *et al.*, 2001).

Exploiting the host-parasite interaction, for the diagnosis of visceral leishmaniasis a number of antibody detection methods have been developed from time to time. Some of these tests include indirect haemagglutination (IHA), counter current immunoelectrophoresis (CCIEP),

immunodiffusion (ID) and several others (Boelaert *et al.*, 2004). These tests are cumbersome and lack sensitivity and specificity. Antibody detection is poorly sensitive owing to the lack of significant antibody production in cutaneous leishmaniasis (Singh, 2006).

In other places such as Africa and Iran, serum antileishmanial immunoglobulin G (IgG) in high titre is the diagnostic standard, primarily with direct agglutination test (DAT) (Zijlstra and El-Hassan, 2001). The direct agglutination test, in which serum antibodies agglutinate stained parasite is popular in Iran and Africa (Conjivaram, 2002).

Delayed hypersensitivity is an important feature of cutaneous forms of human leishmaniasis and can be measured by the leishmanin test reaction (Singh, 2006). Enzyme linked immunosorbent assay (ELISA) is a valuable tool and one of the most sensitive tests for the serodiagnosis of visceral leishmaniasis. The test is useful for laboratory analysis or field applications and to screen a large number of samples at a rapid pace (Ryan *et al.*, 2002; Singh, 2006).

1.1.8 Control of leishmaniases

Leishmaniasis is one of the most neglected tropical diseases, in terms of the few tools available for control and the lack of clear criteria for methods of control (WHO, 2006). The diversity of epidemiological situations of leishmaniasis poses a challenge for any single diagnosis, treatment or control measure to be suitable for all (Guerin *et al.*, 2002). Current control measures rely on chemotherapy to alleviate disease and on vector control to reduce transmission (Handman, 2001; Guerin *et al.*, 2002).

1.1.8.1 Vector and reservoir control

Vector control may be feasible for domestic and para domestic however in sylvatic leishmaniasis vector control has not been successful (Feliciangeli *et al.*, 2003; Maroli and Khoury, 2006; Chappuis *et al.*, 2007). Where sandflies are endophagic (mainly feed indoors) and most active when people are asleep, bed nets provide considerable protection (Davies *et al.*, 2003). Protection provided by wide mesh nets is enhanced by treating them with pyrethroids reducing sandfly biting. This is a potent strategy where leishmaniasis is anthroponotic as is the case of *L. tropica* where the main aim is to block transmission (Davis *et al.*, 2003). However where transmission is anthroponotic the best control strategy is chemical and environmental so as to interrupt man-vector contact (Kishore *et al.*, 2006).

Spraying houses with insecticide is the most widely used intervention for controlling sandflies that are endophilic (Davies *et al.*, 2003). Indoor residual spraying is a simple and cost-effective method of controlling vector. The insecticide of choice still remains dichloro-diphenyl-trichloroethane (DDT) because of its low cost, high efficacy, long residual action and relative safety when used for indoor residual spray (Kishore *et al.*, 2006). Vector control using insecticides is possible because sandflies are still very sensitive to insecticides (Desjeux, 2004).

Environmental control is based on making the environment inhabitable to the vector, with an ultimate aim of breaking the parasite cycle. Sandflies breed in dark corners as well as in the crevices of the walls having rich humus and moisture. In the former Union of Soviet Socialist Republic (USSR) leishmaniasis was successfully controlled by destroying rodent burrows (Kishore *et al.*, 2006). In another study, it was demonstrated that filling all the cracks and

crevices of walls on which are sandfly rest using mud and lime. The breeding of sandflies could be stopped successfully (Kumar *et al.*, 1995). Lime has a powerful water absorbing capacity which makes it unsuitable for the sandfly breeding (Kishore *et al.*, 2006).

Personal protection such as the use of chemical repellents applied to skin and wearing of protective clothing is the most important preventive measure for human protection against haematophagus arthropods (Davies *et al.*, 2003). In areas where the transmission of leishmaniasis is extra-domiciliary, use of insect repellents or protective clothing may be the only preventative measure available (Maroli and Khoury, 2006).

In Latin America, the Mediterranean basin, central and south western Asia where VL is primarily zoonotic, transmission to human beings is reduced by targeting the animal reservoir as a feasible strategy (Davies *et al.*, 2003). In China, this strategy has been effective however not so in Brazil, where it has not been effective because of incomplete coverage; delays between taking blood samples, diagnosis and culling; and the high dog population turnover rate (Courtenay *et al.*, 2002). Therefore to circumvent these shortcomings alternative control strategies such as dipping dogs in insecticide, applying topical insecticide lotions and the use of deltamethrin treated collars are novel strategies that can substantially reduce sandfly bites on dogs and subsequent human infection (Davies *et al.*, 2003).

Studies suggest that there is a decrease in human prevalence of disease when seropositive dogs are removed, however such control should be combined with vector control and human case treatment (Saliba and Omenish, 1999).

1.1.8.2 Chemotherapy of leishmaniases

Pentavalent antimonials are the first line of drugs for the treatment of visceral and cutaneous leishmaniasis except in Bihar India where visceral leishmaniasis is highest, because of resistance (Guerin *et al.*, 2002). Current treatments require long courses and parenteral administration and most are expensive (Croft *et al.*, 2006; WHO, 2007).

1.1.8.2.1 Treatment of visceral leishmaniasis

The therapy against VL is limited; the available agents with established efficacy are all injectable (Guerin *et al.*, 2002). In addition to specific anti-leishmanial treatment of VL relies on the aggressive management of any concomitant bacterial or parasitic infections, anaemia, hypovolemia (decreased blood volume) and malnutrition (Chappius *et al.*, 2007).

For many decades, the treatment of VL has been based on pentavalent antimonials which are the first line of treatment, such as sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), given intramuscularly or intravenously for one month (Hailu *et al.*, 2005; Croft *et al.*, 2006). In addition cheaper generic forms of these drugs are available and have been shown to be equally effective as the branded products (Chappuis *et al.*, 2007).

Antimonials are toxic drugs with sometimes life-threatening adverse side effects, including cardiac arrhythmia and acute pancreatitis. Patients under the age of two or aged forty five and above with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, VL complications or a combination of these factors (Chappuis *et al.*, 2007). Of major concern is the development of resistance to anitimonials. A case in point is in northeastern India, where the

incidence of visceral leishmaniasis is highest, due to resistance to antimonial drugs, the mainstay of treatment can no longer be used (Reithinger *et al.*, 2007).

Amphotericin B is the current alternative treatment of choice. This drug is highly efficacious but is associated with serious side effects and can only be given in a hospital setting (Hailu *et al.*, 2005). Its drawbacks are cost, limited availability in some areas and toxicity notably infusion-related side-effects (fever, chills, and bone pain) and hypocalcaemia, renal impairment, and anemia. These problems are generally tolerable at the doses used in Bihar, where conventional amphotericin B is now the first-line drug in kala-azar treatment centres (Guerin *et al.*, 2002).

Paromomycin (aminosidine) is an old aminoglycoside antibiotic with unique antileishmanial activity. It acts synergistically with antimony in vitro. The results from initial studies in India and Africa were promising (Chappuis *et al.*, 2007).The drug is effective, well tolerated, and as cheap as conventional amphotericin B, but it must be administered parenterally. Paromomycin was registered in India in August 2006 (Croft *et al.*, 2006). In east Africa, paromomycin is currently being evaluated in mono and combination (with sodium stibogluconate) therapy. Another advantage of paromomycin is the fact that it is active against a wide variety of pathogens, including bacteria (Chappuis *et al.*, 2007).

Miltefosine, an alkylphospholipid that was originally developed as an oral antineoplastic agent, is the first effective oral drug and is the most advanced drug in *Leishmania* drug development (Guerin *et al.*, 2002; Chappuis *et al.*, 2007).

1.1.8.2.2 Treatment of cutaneous leishmaniasis

Cutaneous Leishmaniasis often heals spontaneously within 1 week to 3 years, usually within 1 year, however this varies with the strain of *Leishmania* involved. Ninety percent of patients of Old World cutaneous leishmaniasis heal spontaneously within 3–18 months (Davies *et al.*, 2003). In New World CL, self-healing after 3 months is rapid in *L. mexicana* (75%) but slow in *L. braziliensis* (about 10%) and *L. panamensis* infections (about 35%) (Soto *et al.*, 2004). Although not fatal, CL is treated to accelerate cure thereby reducing scarring, especially in cosmetic sites and to prevent parasite dissemination (i.e. mucosal leishmaniasis) or relapse (Reithinger *et al.*, 2007).

Major emphasis has been placed on topical application of Paromycin. In the Old World Paromycin preparation has shown improvement in the protozoal clearing in patients with CL caused by *L. major* in Israel. However, Paromycin-ointment was shown to be ineffective against *L. tropica* in Turkey and *L. major* in several randomized, placebo-controlled trials in the Sudan, Tunisia, and Iran. Thus the effectiveness of paromomycin remains controversial. In the New World topical treatment of *L. braziliensis* is not advisable as dissemination to mucocutaneous disease can occur. Major concern about the use of local therapy is that it does not cure lymph node infection or protect against mucosal disease if metastasis has already started (Moskowitz and Kurban, 1999).

Reithinger in (2007) reviewed currently available data and concluded that for cutaneous leishmaniasis, pentavalent antimony, given parenterally or intralesionally, remains the first-line treatment approach. Apart from the immunotherapy policy in Venezuela Convit (1996) and the pentamidine treatment policies in French Guyana and Suriname, Roussel (2006) WHO recommends treating cutaneous leishmaniasis with pentavalent antimonial drugs (i.e.

sodium stibogluconate or meglumine antimonate) at 20 mg/kg per day for 20–28 consecutive days. Barring one exception, this regimen has been shown to be more efficient than a daily dose of 10 mg/kg, 13 mg/kg, or 15 mg/kg in treating LCL (Berman, 1997; Moskowitz and Kurban, 1999; Reithinger *et al.*, 2007). Alternative treatment regimens include amphotericin B, especially for mucosal leishmaniasis (Davies *et al.*, 2003).

1.2 Literature review

1.2.1 Immunity against *Leishmania major* in mice

The most frequently studied model for leishmaniasis by far is that of *L. major* (Noben – Trauth, 2000; Garg and Dube, 2006). Although not perfect, the spectrum of disease manifestations observed in human leishmaniasis can be mimicked in the laboratory by infection of inbred strains of mice with *L. major* (Handman, 2001). Studies to elucidate the immunologic pathways responsible for resistance or susceptibility to *L. major* in the murine model have given rise to the identification of the T-helper 1/T-helper 2 (Th1/Th2) dichotomy in the development of the immune response (Roberts, 2006). The subdivision of $CD4^+$ cells into Th1 and Th2 is based on the pattern of cytokines the cells produce (Handman, 2001).

Immunity to cutaneous leishmaniasis is a well studied subject, in which mice are the commonly used models. It has been established that *L. major* infection of inbred mice leads to a major dichotomous response death or survival that depends on the strain of mice (Campos-Neto, 2005). Inbred strains of mice experimentally infected with *L .major* have been classified as genetically resistant or susceptible on the basis of their ability to mount an effective immune response to the parasite (Nabors *et al.*, 1994). This mouse model has been subsequently used to demonstrate that susceptibility of BALB/c mice was caused by Th2 response to the parasite in contrast to the protective Th1 responses in the engineered resistant

inbred strains of mice such as C57BL/6, C3H and CBA (Fowell *et al.*, 1997). This view is supported by classical experiments which established that T-cell deficient mice rapidly succumb to inoculation with any one of several species of *Leishmania*, and that transfer of normal T cells confers resistance to the animals (Preston and Dumonde, 1976; Scott *et al.*, 1988; Campos-Neto, 2005).

Healing responses in the susceptible breeds such as BALB/c are known to be dependent on the expansion of IFN-γ producing CD4+ Th I helper T cells which contribute to the control of the primary infection (Noben-Trauth *et al.*, 2003). Interferon gamma can activate macrophages to kill a number of intracellular parasites including *L. major* and acts to perpetuate Th I responses by suppressing the development and proliferation of Th2 cells (Renier and Locksley, 1995). Susceptibility to *L. major* resulting in uncontrolled, non healing infections are associated with the proliferation of Th2 cells and the production of IL-4, IL-5, resulting in an ineffective anti-parasitic response (Nabors *et al.*, 1994; Mahmoodi *et al.*, 2005). There is new evidence that IL-10 is an additional factor controlling susceptibility to *L major* in BALB/c (Noben-Trauth *et al.*, 2003).

In experimental infection of inbred mice with *L. major*, activation of two distinct CD4+ T helper cell subsets, Th1 and Th2, have been described. Resistant mice such as C57BL/6 and C3H when infected with *L. major*, resolution of infection is associated with expansion of Th1 cells that produces IFN-γ, IL-2 and IL-12 (Tonui *et al.*, 2004). In contrast, other strains of mice, notably BALB/c, develop severe and uncontrolled lesions following infection with *L. major* (DeKrey *et al.*, 1998). In this strain of mice, disease progression is associated with expansion of Th2 subset that secretes IL-4, IL-5, IL-10 and IL-13 (Mahmoodi *et al.*, 2005).

Leishmania amastigotes reside in phagolysosomes inside macrophages. For the host to be able to control *Leishmania* parasites, infected macrophages need to be activated to induce parasite killing through the production of reactive oxygen intermediates and nitric oxide (Serarslan and Atik, 2005). Several Th1 type cytokines (IFN-γ, TNF-α) are strong inducers of the macrophage enzyme inducible nitric oxide synthase (iNOS) that produce nitric oxide (Serarslan and Atik, 2005). Inbred strains of mice such as C57BL/6, C3H and CBA/N produce Th1 cytokine profiles and consequently are resistant to infection by *L. major* (Noben-Trauth *et al.*, 2003). They develop small lesions which resolve in 10 to 12 weeks and are resistant to re-infection (Preston and Dumonde, 1976; Garg and Dube, 2006). On the other hand, BALB/c mice are highly susceptible; they mount a Th2 mediated immune response. Because macrophages are not activated to kill intracellular amastigotes, they develop severe and uncontrolled skin ulcers, which expand and metastasize; leading to death (De Krey *et al.*, 1998). Most other strains of mice are intermediate in susceptibility (Preston and Dumonde, 1976).

There are two other mechanisms of intracellular killing that have been proposed involving destruction of infected macrophages by cytotoxic T lymphocytes (CTL) (Muller *et al.*, 1991) and Fas Ligand (Fas-L)-mediated macrophage apoptosis (Huang *et al.*, 1998). In addition studies have suggested that susceptibility and resistance to *Leishmania* infection is associated with the emergence of a unique subset of T cells, namely T regulatory cells (T reg) (Belkaid *et al.*, 2002).

1.2.2 Immune responses to leishmaniasis in humans

The immune response of humans to infection with *Leishmania* is not as well characterized as the response of mice (Rogers and Titus, 2004). Much of the research with human leishmaniasis has involved the use of infected or recovered patients. Cutaneous leishmaniasis often heals spontaneously with one or a few skin lesions, which leave a depressive scar (Ajdary *et al.*, 2000). Resolution is characterized by induction of specific IFN-γ releasing CD4+ T cells (Kemp *et al.*, 1994). Failure to cure disease is associated with elevated IL-4 with low IFN-γ responses from *Leishmania* specific CD-4+ T cells. Patients with VL usually demonstrate anergy with negative skin test to *Leishmania* antigens (Roberts, 2006). 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). It is well known that IFN-γ induces microbicidal activity against both the promastigote and amastigote of *L. donovani* in monocyte derived human macrophages, in hydrogen peroxide dependent as well as in NO mediated pathway (Saha *et al.*, 2005). Therefore, the reason why high parasite burden is favored in active VL patients can be explained by the deficiency of IFN-γ during disease. On the potential source of immunoregulatory cytokines investigators have found a population of antigen specific T cells co-producing IL-10 and IFN-γ which expand response to *L. donovani* infection in humans (Kemp *et al.*, 1999).

1.2.3 Vaccine development against leishmaniases

There are no vaccines against leishmaniasis to date. However, there is consensus that vaccines ought to become a major tool in the control of leishmaniasis in the long term (Handman, 2001; Piscopo and Mallia, 2006). The rationale for leishmaniasis vaccine development lies in the simple nature of the parasite lifecycle and the fact that healing and

recovery protects individuals from reinfection indicating that it should be possible to develop a vaccine against leishmaniasis (Selvapandinyan *et al.*, 2006). The extensive knowledge that has been gathered from animal models has shown that protection against live challenge could be achieved using parasite-specific proteins or DNA (Chappuis *et al.*, 2007; Reithinger, 2007).

The completion of sequencing of *L. major* genome has added momentum to attempts to identify the genes that are responsible for resistance or susceptibility to leishmaniasis (Ivens *et al.*, 2005 Reithinger, 2007).

In general, the leishmaniasis vaccines in development can be divided into three categories: (*i*) Live *Leishmania*; including new genetically modified constructs; (*ii*) First generation vaccines (FGV) consisting fractions of the parasite or whole killed *Leishmania* with or without adjuvants (*iii*) Second generation vaccines (SGV) including all defined vaccines, *i.e.*, recombinant proteins, DNA vaccines and combinations thereof (Khamesipour *et al.*, 2006; Ghalib and Modabber, 2007).

Several vaccine preparations are in more or less advanced stages of testing they include, use of whole lysate, killed, avirulent or irradiated parasites (Handman, 2001; Requena *et al* 2004; Ghalib and Modabber, 2007). The newer vaccines under consideration comprise recombinant DNA-derived antigens and peptides. Some of the target antigens are species and life cycle stage specific, while others are shared by promastigotes and amastigotes. Most of these strategies have shown some degree of effectiveness in animal models but little or no protection in humans (Requena *et al.*, 2004).

1.2.3.1 Live *Leishmania* vaccines

1.2.3.1.1 Live *Leishmania* as a prophylactic vaccine

For prophylaxis, live virulent *L. major* is the best vaccine against human cutaneous leishmaniasis (Ghalib and Moddaber, 2007). It has been used for centuries, by inoculating material from exudates of an active lesion into a covered part of the body (Khamesipour *et al.*, 2005). Inoculation of live virulent *Leishmania* became known as leishmanization (LZ) has been used for over 60 years in several countries (Khamesipour *et al.*, 2005). Bedouin or some Kurdistani tribal societies traditionally expose their babies' bottoms to sand fly bites in order to protect them from facial lesions (Khamesipour *et al.*, 2006).

Another ancient technique practiced in the Middle East has been the use of a thorn to transfer infectious material from lesions to uninfected individuals. In more recent years in Iran, Israel and Uzbekistan, leishmanization has been performed by inoculating cultured *L. major* (Khamesipour *et al.*, 2005).

Leishmanization has many setbacks such as, the difficulty with cultured promastigotes is the loss of virulence of parasites after several *in vitro* passages, areas. In addition there is reported immunosuppression as seen by reduced responsiveness to diphtheria, pertussis, and tetanus (DPT) vaccine in children following LZ (Khamesipour *et al.*, 2005). In addition, *Leishmania* parasites are believed to persist for a long time, as a result, leishmanization cannot be used on a large scale or in HIV endemic areas (Ghalib and Modabber, 2007).

At present there is only one prophylactic live vaccine in use which is a mixture of live virulent *L. major* mixed with killed parasite registered in Uzbekistan (Khamesipour *et al.*, 2006; Noazin *et al.*, 2008).

1.2.3.2 First generation vaccines for treatment

The first generation vaccines have not been efficient for prophylaxis (Khamesipour *et al.*, 2006).Various killed parasites have been tested for decades with or without different adjuvants. The earliest trials with killed *Leishmania* as a vaccine were conducted in Brazil in the 1940s. Later, from 1970s onwards Mayrink and colleagues developed a killed vaccine composed of five isolates of *Leishmania* containing four different species which was later simplified to a single *L. amazonensis* vaccine and tested for prophylactic potential in Columbia and Ecuador and as an adjunct to chemotherapy in Brazil (Modabber, 1995; Genaro *et al.*, 1996).

In Venezuela, Convit *et al.* (1989) used a combination of autoclaved *L. mexicana or L. braziliensis* promastigotes and *M. bovis* BCG either prophylactically, immunotherapeutically and/or immunochemotherapeutically against South American leishmaniasis (Castes *et al.*, 1989). Cure was associated with the development of Th-1 type of immune responces in the recipients with production of IFN-γ (Cabera *et al.*, 2000). In the monkey model of CL, protective immunity was achieved using killed *L. amazonensis* co-administered with recombinant IL-12 as adjuvant while another study using *L. major* with recombinant human IL-12 resulted in a skewed Th1 immune response but did not protect the primates against challenge infection with the parasite (Kenney *et al.*, 1999; Gicheru *et al.*, 2001).

1.2.3.2.1 Live attenuated vaccines

Discovery of new knowledge of *Leishmania* genome and the ability to manipulate it by introducing or eliminating genes have the potential to make live-attenuated vaccines a reality. The objective of live-attenuated vaccine is to expose the recipient to complex antigens in the right context over time without producing pathology (Selvapandiyan *et al.*, 2006). Using gene-targeting tools, 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). In a mouse model, *L. major* parasites lacking *dhfr-ts* induced protection against *L. major* and *L. amazonensis* but no protection was conferred to Rhesus monkeys (Titus *et al.*, 1995; Veras *et al.*, 1999; Amaral *et al.*, 2002).

Other genetically engineered and tested mutant parasites include the null mutants for the glucose transporter gene family in *L. mexicana* that exhibited reduced infectivity to BALB/c mouse macrophages, demonstrating that a single gene deletion can render a parasite virulent (Burchmore *et al.*, 2003). Additionally, although *L. major* mutants deficient for leishmanolysin genes showed normal development in macrophages *in vitro*, they showed delayed lesion development in susceptible BALB/c mice (Joshi *et al.*, 1998, 2002). *Leishmania major* mutants that lack LPG1 (the gene encoding a galactofuranosyl transferase) showed attenuated virulence in mice whereas the parasites that lacked LPG2 (the gene encoding a golgi GDP-mannose transporter) persisted indefinitely at low level in mice without displaying disease and provided protection from virulent *L. major* challenge (Spath *et al.*, 2003; Uzonna *et al.*, 2004). Such protection from virulent challenges in mice has also been achieved after gene knockout for other genes: cysteine protease in *L. mexicana* and biopterin transporter in *L. donovani* (Alexander *et al.*, 1998; Papadopoulou *et al.*, 2002).

Attempts to develop avirulent parasites as vaccine candidates against leishmaniasis by irradiation, temperature sensitive mutations or random mutations induced by chemical agents has been hampered by reversion to virulence (Selvapandiyan *et al.*, 2006). These challenges can be met with the use of live attenuated strains which posses genetically defined mutations, can persist in the host without being virulent, have less chance of reversion to the virulent phenotype and can be produced in large quantities.

1.2.3.3 Second generation vaccines

1.2.3.3.1 Recombinant and synthetic vaccines

Newer vaccines under consideration for leishmaniasis comprise recombinant DNA-derived antigens and peptides (Handman, 2001; Khamesipour *et al.*, 2006). Some of the target antigens are species and life cycle stage specific while others are shared by promastigotes and amastigotes. Some are conserved among *Leishmania* species, while others are not. Since T cells usually recognize peptides derived from cytosolic proteins bound in the major histocompatibility complex (MHC) class I groove or peptides derived from the lysosomal compartment bound in the MHC class II groove on the antigen-presenting cell (APC) surface, all parasite proteins can function as antigens regardless of their location in the parasite (Handman, 2001).

Recombinant antigens can be delivered as purified proteins, as the naked DNA encoding them, or as bacteria manufacturing proteins *in situ*. Manipulations now allow targeting of the antigen to specific locations or to particular APCs, 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 adjuvant effect, which

may "activate" or "licence" these APCs (Matzinger, 1998; Handman, 2001).

1.2.3.3.2 Synthetic peptides

In the early eighties there was much enthusiasm on the use of synthetic peptides and especially those with T-cell epitopes as vaccine candidates against leishmaniasis. In recent times however, this seems to have waned and focus has shifted to the use of recombinant DNA-produced polypeptides and to naked DNA (Handman, 2001; Khamesipour *et al.*, 2006).

Data obtained from experimental studies has provided evidence that a single subcutaneous injection of a single synthetic T cell epitope is sufficient to provide long-lasting protection against highly virulent strains of *L. major* in BALB/c mice (Spitzer *et al.*, 1999). In another study the results showed that a single immunization with the peptide/adjuvant preparation was highly effective in protection of mice against both strains of *L. major* for at least ten months (Spitzer *et al.*, 1999).

Several considerations make the peptide antigens less attractive. These include the magnitude of the T-cell memory induction, the inability of all individuals in the population to respond to the peptide and the economics of production. Some polypeptides such as PSA-2 need to be in their native conformation for antigen processing to take place, a requirement that may not be fulfilled by *Eschericia coli*-derived recombinant proteins (Handman, 2001). In addition, most synthetic peptides require delivery systems such as adjuvants but most effective adjuvants generally cause strong inflammation which although necessary for adjuvanticity, may preclude their use in humans owing to unacceptable side effects (Howard, 1993; Handman, 2001).

1.2.3.3.3 Non-protein antigens

Early studies on vaccine development demonstrated that glycolipids such as the *Leishmania* lipophosphoglycan (LPG) provided excellent protection. However, protection was shown to rely on the use of adjuvants such as liposomes or *C. parvum* and on the integrity of the molecule (Russel and Alexander, 1988). In other studies, the water-soluble LPG lacking the glycophosphatidylinositol (GPI) anchor not only proved non protective, but it also exacerbated disease (Mitchell and Handman, 1986; Handman, 2001). 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 (Sjölander *et al.*, 1998).

1.2.3.3.4 Expression of immunogens in bacteria and viruses

The encoding for the protein portion of the *Leishmania* surface glycoprotein (gp63) or leishmaniolysin was the first *Leishmania* vaccine delivered as a plasmid (Xu and Liew, 1994). Leishmaniolysin is a membrane protease present in promastigotes of all species and is one of the parasite receptors for host macrophages such that parasite mutants lacking the protein are avirulent, belongs to a multigene family, with different members being expressed in promastigotes and amastigotes (Chang *et al.*, 1990; Handman, 2001).

Overall, gp63 is still considered a promising vaccine candidate the gene has been engineered in a number of delivery systems including BCG, vaccinia virus and *Salmonella typhimurium* with an aim of inducing the appropriate Th1 immune response (Handman, 2001). The protective efficacy of purified gp63 has been tested in several experimental models using different strains and adjuvants, giving rise to conflicting results (Khamesipour *et al.*, 2006).

Interestingly, both the recombinant and native proteins seem to protect better against infection with *L. amazonensis* than against infection with *L. major*, suggesting species-specific epitopes, in animal models (Russsell and Alexander, 1988; Olobo *et al.*, 1995).

Parasite surface antigen 2 (PSA-2) or gp46/M2, a membrane antigen of unknown function is another vaccine candidate that has been tested in animal models. This antigen belongs to a family of glycoinositol phospholipids anchored glycoproteins expressed in both promastigotes and amastigotes of all *Leishmania* species except *L. braziliensis* (Handman *et al.*, 1995; Handman, 2001).

It has been demonstrated that while vaccination with native PSA-2 with *C. parvum* as adjuvant protects mice against *Leishmania* through a Th1 mediated response, the recombinant PSA-2 purified from *Eschericia coli* and administered in immunostimulating complexes (ISCOMs) or mixed with *C. parvum* as adjuvant, does not induce protective immunity despite induction of Th1 responses (Sjölander *et al.*, 1998; Khamesipour *et al.*, 2006).

The *Leishmania* homologue of the mammalian receptor for activated c kinase (LACK) is a known antigen of *Leishmania* spp. that might be a candidate for vaccination (Mougneau *et al.*, 1995). The antigen is expressed by both promastigotes and amastigotes and has been shown to protect mice from infection, especially when administered with IL-12 (Gurunathan *et al.*, 1997). Very rapid IL-4 production in response to LACK has been documented in susceptible mice infected with *L. major*, which induced the typical Th2 cell response (Launois *et al.*, 1997). In addition, immunization with LACK protein and IL-12 along with deoxyribonucleic acid (DNA) plasmids containing LACK gene and 'altered' LACK peptides

made susceptible-mice resistant to infection with *L. major* (Mougneau *et al.*, 1995; Guranathan *et al.*, 1997). Thus, the role of LACK-specific cells in resistance and susceptibility to *Leishmania* infection (cutaneous leishmaniasis) is far from being established (Bourreau *et al.*, 2003).

The leishmanial eukaryotic ribosomal protein (LeIF), a homologue of the ribosomal of the ribosomal protein eIF4A, 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 (Handman, 2001).

Several other vaccine candidates that are amastigote specific include the A2, P4 and P8 of *L. mexicana pifanoi* (Soong *et al.*, 1995). The A2 genes form part of a multigene family of at least 11 genes and are considered to be virulence factors that are required for the survival of *Leishmania* parasites in the mammalian host (Ghosh and Bandyopadhyay, 2003). Other antigens expressed in amastigotes are the cathepsin L-like cysteine proteinases (CPs), enzymes that belong to the papain superfamily (Mottram *et al.*, 2004). These enzymes have been used in immunization experiments in the mouse model and it has been shown that the recombinant cysteine proteinases class B (CPB), in combination with adjuvant, induces long lasting immunity against *L. major* infection in BALB/c mice, while DNA vaccination is more efficient when a cocktail of plasmid DNAs encoding cysteine proteases class A (CPA) and CPB indicating the usefulness of a combination of these antigens (Rafati *et al.*, 2000). It has been shown that *Leishmania* histones are relevant immunogens for the host immune system during both *Leishmania* infection and disease.

1.2.3.3.5 Naked DNA vaccines

A new tool, 'naked DNA', has become available in vaccine development. It shows great promise for both the improvement of existing vaccines and the development of vaccines against disease targets for which there are so far no effective vaccines (Khamesipour *et al.*, 2006). DNA vaccines induce a complete immune response against the encoded antigen. The gene encoding the vaccine candidate is cloned in a mammalian expression vector, and the DNA is injected directly into muscle or skin (Sjölander *et al.*, 1998).

DNA vaccines encoding gp63, LACK and PSA-2 have all been shown to protect both genetically resistant and susceptible mice from with *L. major* by inducing Th1 immune responses (Guranathan *et al.*, 1997; Sjölander *et al.*, 1998; Handman, 2001).

The nucleoside hydrolase (NH36) DNA vaccine of *Leishmania donovani* has been shown to be cross-protective against murine VL caused by *L. chagasi* while vaccination with a multi-component DNA vaccine against VL in dogs produced Th1 cytokines and was not only shown to reduce incidences of canine VL, but also indirectly reduced the incidence of human VL (Gamboa-León *et al.*, 2006).

Further improvements of the DNA vaccination approach are still needed to design a fully protective vaccine against leishmaniasis (Ahmed *et al.*, 2004).

1.2.3.3.6 Transmission blocking vaccines

Transmission blocking vaccine studies in leishmaniasis have also been undertaken. These studies have shown that LPG is an excellent candidate as a transmission blocking vaccine against *L. major* infections (Tonui, 1999; Tonui *et al.*, 2001). In these studies, sand flies,

which fed on BALB/c mice, immunized with *L. major*-derived LPG or monoclonal antibodies raised against LPG showed that parasite development was inhibited at the log phase (procyclic) of the parasite (Tonui *et al.*, 2003). There was also a marked reduction in the numbers of metacyclic promastigotes developing, leading to reduced transmission of *L. major* to naïve BALB/c mice (Tonui *et al.*, 2001). It has also been shown that *P. duboscqi* gut lysates and proteins present in *L. major*-derived LPG share two common proteins of molecular weights 105 kDa and 106 kDa (Tonui *et al.*, 2003). The main focus of these studies has been to develop a vaccine that can be utilized both to reduce transmissible infections within the sand fly and disease severity within the host. They have also contributed to some of the important findings relevant to vaccine research (Tonui, 2006).

1.2.4 *Leishmania* excreted/secreted exo-antigens

Excretory and secretory molecules are released from parasite in all stages of the parasite life cycle and may have immunomodulatory and protective effects. *Leishmania* species produce and release a variety of proteins during their growth *in vitro*. The secreted proteins have distinct functions during *Leishmania* infection. First, they play a role in the establishment of the infection in conjunction with important elements existent in the saliva of the sandfly vector (Kamhawi, 2000; Rittig and Bogdan, 2000; Almeida *et al.*, 2003). They are thought to contribute to the maintenance of the infection by interfering with the macrophagic microbicidal functions, cytokine production, antigen presentation, and effector cells activation. This is achieved by repression of gene expression, post-translation protein modification or degradation, and by activation of suppressive pathways (Santarem *et al.*, 2007).

Like many other pathogenic protozoa, *Leishmania* parasites are purine auxotrophs that must salvage preformed nucleosides from their hosts (Marr *et al.*, 1978; Miller *et al.*, 2007). Due to lack of the pathways for *de novo* purine biosynthesis and reliance on preformed purine nucleosides or bases to meet their growth demands. The parasite may have evolved unique pathways to salvage purine nucleosides or bases from their environment (LaFon *et al.*, 1982). These unique pathways present targets against which safe and effective antileishmanial drugs and vaccines can be designed.

There are several enzymes used by the parasite in the salvage pathways one of them is nucleoside hydrolase (NuH). The *Leishmania* non-specific NuH is an essential enzyme in the purine–pyrimidine salvage pathway utilized for DNA and RNA synthesis (Miller *et al.*, 2007). Nucleoside hydrolase is implicated as an important catalytic activity of purine salvage in several trypanosomatid and other parasites. The absence of this enzyme in the mammalian hosts makes it an ideal target for chemotherapy (Cui *et al.*, 2001).

The bulk of the knowledge on surface and secreted molecules of *Leishmania* is focused on lypophosphoglycan (LPG). This is a surface molecule in *Leishmania*, the LPG is composed mainly of repetitive units of a disaccharide and a phosphate. LPG is linked to the membrane by a glycosylphosphatidylinositol anchor (Santarem *et al.*, 2007).

Several attempts to use LPG to confer protection were unproductive (Tonui *et al.*, 2003; Tonui, 2003). Constitutively shed by several Leishmania species. the LPG is the paradigm molecule referred to as evasive and invasive (Santarem *et al.*, 2007).

Soluble *L. major* exo-antigens (*Lm*SEAgs) were shown to be immunogenic and protective (both with respect to lesion size and parasite burden within lesions) in susceptible adult

BALB/c mice in the absence of any adjuvant (Tonui *et al.*, 2004). Although this suggests that *Lm*SEAgs might have vaccine potential for humans, such a claim was reinforced by evidence that a *Lm*SEAgs-based vaccine might be useful in neonates (a common approach towards vaccinating humans) and that *Lm*SEAgs actually stimulate human cells in a protective manner (Tonui and Titus, 2006). Results demonstrated that *Lm*SEAgs were able to confer resistance to challenge in vaccinated neonatal susceptible BALB/c mice (Tonui and Titus, 2006). This ability to vaccinate neonates suggests that a vaccine based upon *Lm*SEAgs might be effective in children vaccinated against leishmaniasis (Tonui and Titus, 2006).

Studies have shown that both the lipophosphogycan and M2/gp46 are present in the *Lm*SEAgs. In addition *Lm*SEAgs have also been used to develop serological assays that demonstrate very high degree of sensitivity and specificity (Martin *et al.*, 1998; Ryan *et al.*, 2002).

In a follow-up study recombinant nucleoside hydrolase (rNuH), a single component of *Lm*SEAgs induced marked protection in susceptible BALB/c mice. In addition the protective potential of NuH was not enhanced by co-injection with IL-12 (Al-Wabel *et al.*, 2007). Finally, protection after vaccination with rNuH correlated with increased production of IFN-γ and decreased production of IL-10 (Al- Wabel *et al.*, 2007). These results suggest that NuH should be considered as a component of subunit vaccines against leishmaniasis.

1.2.5 Adjuvants in *Leishmania* vaccine development

An adjuvant is a substance that helps and enhances the pharmacological effect of a drug or increases the ability of an antigen to stimulate the immune system. An adjuvant when administered with an antigen influences the quantity and quality of ensuing response (Tonui and Titus, 2006). There are Recombinant granulocyte-macrophage colony-stimulating factor

(GM-CSF) as an adjuvant has been shown to be well tolerated when used in patients with VL and has been successful used with a defined antigen to treat MCL refractory to antimony (Badaro *et al.*, 2001).

Immunostimulatory DNA (Oligodeoxynucleotides (ODN) which contain immunostimulatory cytosine-phosphate-guanosine (CpG) motifs (CpG ODN) have been used as adjuvant to soluble *Leishmania* antigen and Montanide ISA 720 used with histone 1 (H1) recombinant vaccine have been tested in BALB/c mice and proved to be partially protective (Stacey and Blackwell, 1999; Masina *et al.*, 2003). However when montanide 720 was tried with *Lm*SEAgs it found that it did not enhance the activity of the antigens (Tonui *et al.*, 2004).

Soluble cytokines known to promote Th1 immune responses have been used to induce and maintain immune responses by *Leishmania* vaccines. Interleukin-12 has been shown to induce the development of CD4+ Th1 cytokines (Aebischer *et al.*, 2000; Tonui *et al.*, 2004). Other types of particulate adjuvants and delivery systems that have been tested in animal models include liposomes, microparticles, immunostimulating complexes (ISCOMS) (Handman, 2001). In general, these substances induced partially protective immunity.

1.2.5.1 Bacille Calmette Guérin (B.C.G)

One of the well known vaccine that also acts as an adjuvant is BCG. It is a vaccine against tuberculosis that is prepared from a strain of the attenuated (weakened) live bovine tuberculosis bacillus, *Mycobacterium bovis* that has lost its virulence in humans by being specially cultured in an artificial medium for years. The bacilli have retained enough antigenicity to become a somewhat effective vaccine for the prevention of human

tuberculosis (Fine *et al.*, 1999). It was discovered by Albert Calmette, and his colleague, Camille Guérin, in 1908 from whom the vaccine derives its name. The BCG vaccine was first used in humans in 1921 (Fine *et al.*, 1999).

Bacille Calmette Guérin has also been used in many other diseases successfully. It has been shown to have a small protective effect against leprosy (Setia *et al.*, 2006). It is possible that BCG may protect against or delay the onset of Buruli ulcer (Tanghe *et al.*, 2001). Bacille Calmette Guérin is useful in the treatment of superficial forms of bladder cancer (Lamm *et al.*, 1991). Since the late 1980s evidence has become available that instillation of BCG into the bladder is an effective form of immunotherapy in this disease (Lamm *et al.*, 1991). Immunotherapy with BCG prevents recurrence in up to two thirds of cases of superficial bladder cancer BCG (Mosolits *et al.*, 2005).

Bacille Calmette Guérin as an adjuvant has been used in vaccination and immunotherapy trials against leishmaniasis. As adjuvant, BCG has been used successfully in many trials in both clinical and pre clinical trials (Misra *et al.*, 2001). Previous studies have shown that the combination of live BCG with killed *Leishmania* promastigotes was efficient in the cure of Venezuelan patients suffering American cutaneous leishmaniasis (Castes *et al.*, 1989; Convit *et al.*, 1989). In addition the application of this combination in the immunoprophylaxis of cutaneous leishmaniasis is encouraging, since the vaccine resulted in a long-term stimulation of the immune response to *Leishmania* antigens in a high percentage of healthy volunteers (Castes *et al.*, 1989).

The vaccination with BCG plus killed *Leishmania* promastigotes against *Trypanosoma cruzi* in mice reduced acute infection increasing survival time and decreased parasitaemia and

mortality (Araujo *et al.*, 1999). Successful vaccination with autoclaved *Leishmania major* (ALM) along with BCG has been evaluated in single and triple dose schedules against *L. donovani* in Indian langurs for its vaccine potential against cutaneous leishmaniasis (Misra *et al.*, 2001). The findings from this study suggest ALM+BCG as a potential vaccine against visceral leishmaniasis (Misra *et al.*, 2001). In a vaccine trial in Bam, Iran a single dose of ALM plus BCG was found to be safe and more immunogenic than BCG alone, showing that BCG influences the prophylactic effect of ALM (Sharifi *et al.*, 1998). In a randomized trial in Sudan using BCG plus ALM it was found that BCG alone was found to be more protective than BCG plus ALM (Khalil *et al.*, 2000).

In experimental studies treatment of leishmaniasis, BCG has been fairly successful. Early studies suggested that intraperitoneal or subcutaneous inoculation of C3H mice with BCG did not alter lesion size in *Leishmania mexicana* infected animals (Grimaldi *et al.*, 1980). Intravenous pretreatment of BALB/c mice with BCG was protective against systemic *L. major* disease but did little to protect these animals from cutaneous infection (Weintraub and Weinbaum, 1977). However, later studies to examine the protective effects of BCG on *Leishmania major* infections of BALB/c and P/J mice showed that BCG induced protection against chronic cutaneous disease, as well as lethal systemic infections (Fortier *et al.*, 1987).

1.2.6 Problem statement and justification

Leishmaniasis is of public health importance and is highly endemic in developing countries affecting predominantly the poor in the society (WHO, 2007). First-line treatment is expensive, toxic and needs to be administered by injection in hospital (WHO, 2007). On the other hand *Leishmania* coinfection with human immunodeficiency virus (HIV) is an emerging condition that demands urgent attention (WHO, 2007). Even when co-infected patients receive proper treatments, they relapse repeatedly and the outcome is fatal frequently. Currently, there are reports of drug resistance against pentavalent antimonials (Hadighi *et al.*, 2006; WHO, 2006).

Leishmaniasis in general, but particularly cutaneous leishmaniasis is probably one of a few parasitic diseases that is most likely to be controlled by vaccines. The relatively uncomplicated leishmanial life cycle and the fact that recovery from a primary infection renders the host resistant to subsequent infections indicate that a successful vaccine is feasible (Handman, 2001; Kedzierski *et al.*, 2006). With regard, to the severity of human kala-azar, the spreading of the epidemic and the limitations of chemotherapy (toxicity and need for hospitalization), the development of a vaccine against the human and canine diseases is strongly encouraged by the World Health Organization. In view of this, there is an urgent need for a vaccine against leishmaniasis that would be both therapeutic and prophylactic (Kedzierski *et al.*, 2006).

Protection against *L. major* infection depends on the ability to generate macrophage-activating Th1 responses resulting in the production of IFN-γ and adjuvants are tools to help achieve this (Tonui *et al.*, 2004). Frequently used adjuvants like Freund's incomplete adjuvant and aluminium hydroxide (alum) are known to induce mainly antibody and biased

Th2 responses thus limiting their application in vaccines against *Leishmania* (Conacher *et al.*, 2000). Coinjecting *Lm*SEAgs together with alum, rmIL-12, or Montanide ISA 720 did not increase protection. The only adjuvant system that equaled the protection seen with *Lm*SEAgs alone was IL-12 plus alum (Tonui *et al.*, 2004).

Therefore, there is need to look for an adjuvant that would enhance the activity of *Lm*SEAgs. In the other hand, BCG has proved to be a non-specific stimulant of immune system, it is also a powerful adjuvant, enhancing immune reaction. Such enhancement is likely to produce long-term immunity. Experimental studies on clinical leishmaniasis, have shown that BCG elicits partial protective Th1 responses (Mahmoodi *et al.*, 2005; Khamesipour *et al.*, 2006). In Iran, a study showed that in the long term, the combination of BCG and ALM provided better efficacy than BCG alone (Sharifi *et al.*, 1998).

Leishmania Soluble exo-antigens are potential vaccine candidates against leishmaniasis. The exo antigen is able to elicit Th-1 type response that has been shown to confer protection against leishmaniasis (Tonui *et al.*, 2004; Tonui and Titus, 2006; Al-Wabel *et al.*, 2007). In a study by Tonui *et al.*, (2004) *L. major* soluble exo-antigens were found to be immunogenic eliciting significant protection against the challenge with *L. major* in susceptible BALB/c mice without an adjuvant. There is no documented study on the use of BCG as an adjuvant to *Lm*SEAgs. Considering the fact that both BCG and *Lm*SEAgs are immunoprophylatic, the purpose of this study was to determine their combined potential in vaccination against leishmaniasis.

1.2.7 Null hypothesis

BCG does not enhance protective immune responses in *Lm*SEAgs vaccinated BALB/c mice.

1.2.8 Objectives

1.2.8.1 General objective

To determine the adjuvant potential of BCG in *Lm*SEAgs vaccinated BALB/c mice.

1.2.8.2 Specific objectives

1. To determine cytokine immune responses (IL-4. IL-5 and IFN-γ) in *Lm*SEAgs vaccinated BALB/c mice with or without BCG as an adjuvant.
2. To determine disease progression in *Lm*SEAgs vaccinated BALB/c mice with or without BCG as an adjuvant.
3. To determine the parasite burden in *Lm*SEAgs vaccinated BALB/c mice with or without BCG as an adjuvant.

1.2.9 Significance of the study

The results of this study will be useful in providing new insights on the adjuvant potential of BCG on *L. major* soluble exo-antigen against *L. major* infection in BALB/c mice.

CHAPTER 2-MATERIALS AND METHODS

2.1 Study site

The study was carried out in the Leishmaniasis laboratory at the Center for Biotechnology Research and Development at the Kenya Medical Research Institute (KEMRI).

2.2 *Leishmania major* parasites and culture

Metacyclic promastigotes of *L. major* strain (Strain IDC/KE/ 83=NLB-144) were used. Parasites were maintained in Schneider's Drosophila insect medium described by Titus *et al.*, (1995). Stationary phase promastigotes were obtained from 5 to 7 day old cultures. The metacyclics were isolated from stationary-phase cultures by negative selection using peanut agglutination described in Sack and Perkins (1984) and Tonui *et al.*, (2004).

2.3 Preparation of *Leishmania major* soluble excreted/secreted exo-antigens

Leishmania major soluble exo-antigens were produced as previously described by Ryan *et al.*, 2002. Briefly *L. major* parasites were initially grown in supplemented medium to the late log phase. The parasites were spun for 9000 rounds per minute and then washed in serum-free medium (XOM) described by Martin *et al.*, (1998). Washed promastigotes were inoculated into XOM to give a final density of 10^8cells/ml and incubated at 26^0C for 72h in roller bottles. Thereafter the spent medium was harvested by centrifugation twice at 11000rpm for 30 minutes and the relative protein concentration of *Lm*SEAgs was estimated by measuring the optical density (OD) at 280nm (Tonui *et al.*, 2004).

2.4 Study design

The study was carried out using a completely randomized block design in two phases. Ninety six female BALB/c mice aged between 6-8 weeks were used for the study. The mice that

were used in the experiments were bred and maintained at KEMRI's animal house facility following the laid down institutional guidelines. The mice were randomly divided into two groups: immunotherapy and immunoprophylaxis. In each of the two experimental set-ups the mice were randomly assigned to four groups of twelve mice each. In the immunoprophylaxis experiments the mice were vaccinated before infection while in the immunotherapy experiments the mice were challenged before vaccination.

2.4.1 Immunoprophylaxis of a combination of *Lm*SEAgs and BCG.

The first four groups were vaccinated before challenge with *L. major* metacyclic promastigotes. The first group in this experimental set-up was treated with 50µg *Lm*SEAgs subcutaneous injection alone; the second group received soluble 50µg of *Lm*SEAgs in combination with 0.1ml of *Mycobacterium bovis* BCG suspension that contained 10^5 colony forming units per milliliter by subcutaneous injection. The third group received BCG alone that contained 10^5 colony forming units per milliliter by subcutaneous injection alone, while the control group received subcutaneous injection of phosphate buffered saline (PBS). These treatments were carried out at, day 0 and 13 before challenge with *L. major*. Seven days after the second immunization, all the mice were challenged (Figure 8) by subcutaneous injection with 1 x 10^5 metacyclic *L. major* in 50 µL of saline solution in their hind right footpad as described in Tonui *et al*., (2004).

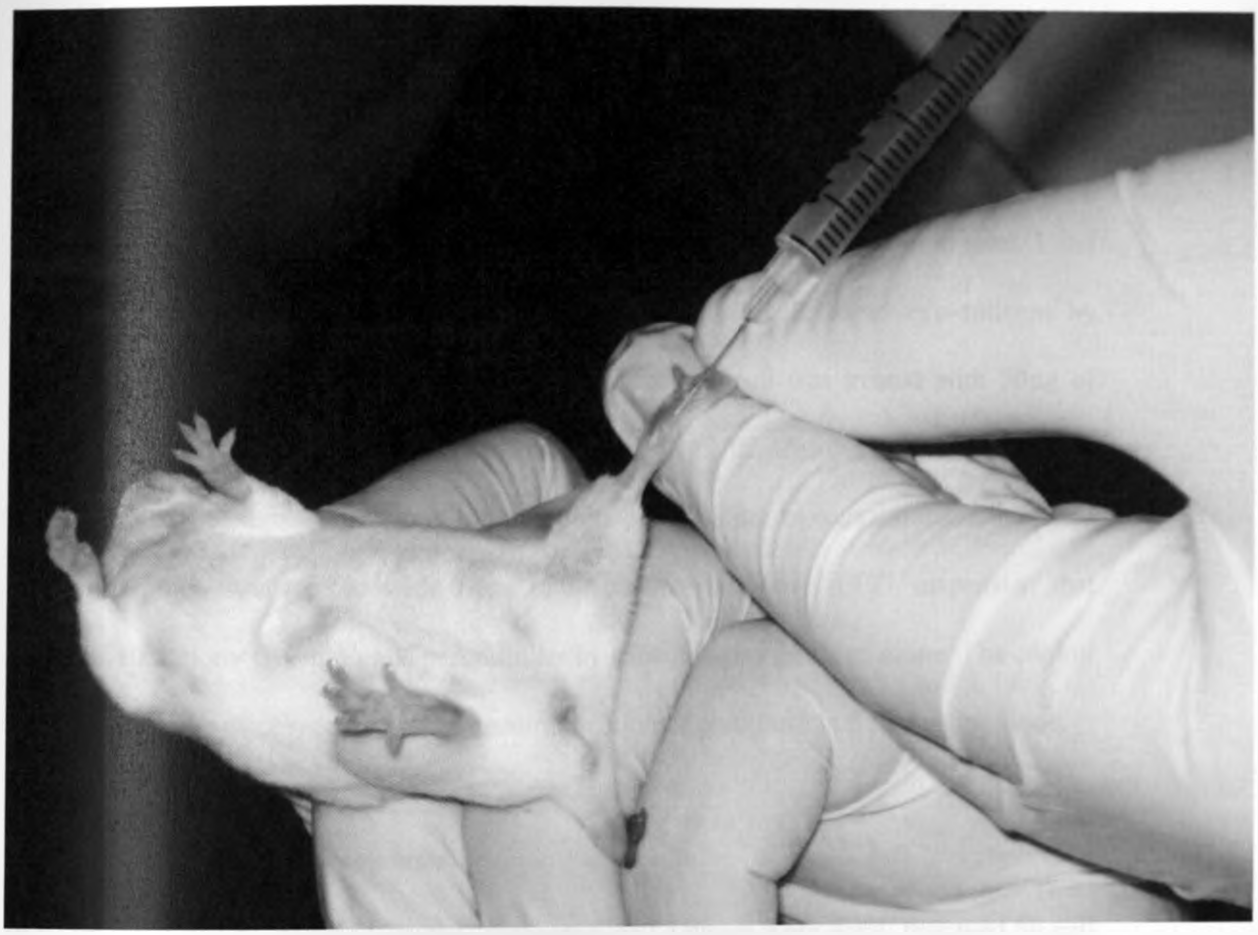

Figure 8: Subcutaneous infection of a BALB/c mouse on the left footpad mice with 10^5 *L. major* metacyclic promastigotes.

2.4.2 Immunotherapy of a combination of *Lm*SEAgs and BCG

All the mice in the other four groups were challenged at day zero with 1 x 10^5 metacyclic *L. major* in 50 µL of saline solution in by subcutaneous injection on their hind right footpad (Tonui *et al.*, 2004; Al-Wabel *et al.*, 2007). The vaccinations were carried out at week 3 and 5 post infection. One group was treated with 50µg of soluble *L. major* exo-antigens by subcutaneous injection (Tonui *et al.*, 2004). The second group was treated with 50µg of soluble *L. major* exo-antigens in combination with 0.1ml of *Mycobacterium bovis* BCG suspension that contains 10^5 colony forming units per milliliter by subcutaneous injection. The third group was treated with 0.1ml of *Mycobacterium bovis* BCG suspension that contains 10^5 colony forming units per milliliter by subcutaneous injection alone. The control group of BALB/c mice received subcutaneous injection of phosphate buffered saline alone.

2.5 Cytokine immune responses.

In order to determine acquired immune responses, cell cultures were done, followed by cell proliferation. From the cultures supernatants were harvested and used for measurement of cytokines.

2.5.1 Cell cultures

Spleens from mice infected with *L. major* were harvested from duplicate mice in each experimental group just prior to infection (day 0) and at day 35 post-infection (Immunoprophylaxis) and at day 7 and 28 post vaccination (immunotherapy). The mononuclear cells were purified with Ficoll-Paque ingredients (Bøyum, 1968). The viability of mononuclear cells was examined by trypan blue exclusion test. Splenocytes were then adjusted to 10^6/ml in complete RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units of penicillin per ml, and 100 µg of streptomycin

per ml. The splenocytes were then dispensed at the rate of 100 µL per well in 96 well flat bottomed tissue culture plate in triplicate for each of the experimental groups and either cultured alone or stimulated with Con A, 100 µg/ml of *Lm*SEAgs. The cells were then incubated in a humidified atmosphere at 37 ^{0}C and 5% CO2 for 48 h. Colorimetric microassay was used to determine cell proliferation (Hay and Westwood, 2002).

2.5.2 Colorimetric assay for cell proliferation

Colorimetric microassay for cell proliferation was performed as described in (Hay and Westwood, 2002). Forty eight hours after culture, the plates were centrifuged at 90 g for 10 minutes to remove medium. After which 100 µL of thiazolyl blue tetrazolium bromide (MTT) was added to all wells of the plates then cells were incubated at 37 ^{0}C for 3-4 h. One hundred microlitres of culture supernatant was removed after centrifugation and 100 µL of dimethyl sulfoxide (DMSO) was added to all the wells followed by vigorous pipetting to dissolve the formazan crystals formed by metabolization of MTT by live cells. The plates were then read at 570 nm in an ELISA reader.

2.5.3 Measurement of cytokines

The cytometric bead array (CBA) kit was used to measure levels of IL-4, IL-5 and IFN-γ as previously described by Hodge *et al.*, 2004. Briefly, the Th1/Th2 cytokine standards were reconstituted and serially diluted using assay diluent. Ten microlitre of each mouse cytokine capture bead suspension was mixed and 50µl of mixed beads transferred to each assay tube. After the addition, 50 µl each of the standard dilutions and test samples to the appropriate sample tubes, 50 µl of phycoerythrin (PE) detection reagent was added and the mixture incubated for 2 h in the dark at room temperature. The samples will be washed and centrifuged at 500g for 5 min and the pellet resuspended in 300 µl of wash buffer. The FACS

Calibur flow cytometer (BD Pharmingen) was calibrated with setup beads and the sample acquired. Individual cytokine concentration was indicated by its fluorescent intensities and then computed using the standard reference curve of CELLQUEST and CBA software (BD Pharmingen).

2.6 Limiting dilution assay for quantification of *L. major* parasite numbers

The number of living *L. major* parasites in infected footpads was quantified at 21, 42 and 63 days post infection in the immunoprophylatic experiments while in the immunotherapeutic experiments the parasite load was quantified at 21, 35, 49 and 63. In both experiments the quantification was done using the parasite-limiting dilution assay (Lima *et al.*, 1997). Mice were sacrificed and the feet removed between the ankle joint and toes. Skin-peeled tissue from the footpad lesion was cut into several pieces then homogenized in a glass tissue homogenizer in complete Schneider's insect medium. Pooled samples were diluted in 5 ml complete Schneider's medium per infected footpad. Serial dilutions of 200 µl of the footpad homogenate were then distributed in replicate wells of a 24-well plate. The plates were sealed using a parafilm and incubated in the dark at 26°C. After 7 to 14 days, the assay was read microscopically, and the number of viable parasites in the tissue was determined as described by Titus *et al.*, (1995). Plates were scored and the results evaluated using the ELIDA program (ELIDA software 1985–2005; Carl Taswell) for statistical analysis (Taswell, 1987).

2.7 Determination of disease progression by measuring lesion sizes

To determine the disease progression, development of lesions (Figure 9) for both experimental setups was followed by measuring the thickness (Figure 9) of the infected

footpad as compared to the thickness of the contra lateral footpad prior to infection using a vernier caliper (Titus *et al.*, 1989; Tonui *et al.*, 2004). The feet of infected mice were monitored weekly.

Figure 9: An ulcerating lesion of an infected footpad (above) and measuring lesion sizes of *L. major* challenged-mice (below).

2.8 Ethical and biosafety considerations

Approval for the study was sought from KEMRI ethical review committee and the Board of Postgraduate Studies of the University of Nairobi. The experiments were done in compliance with KEMRI's Animal Care and Use Committee (ACUC). Standard operating procedures (SOPs) available at the *Leishmania* laboratory at the CBRD include immunizing of animals using standard 21gauge needle, anesthetizing them using 6% sodium Pentobarbitone (Sagatal ®) and killing them by using CO_2 asphyxiation. Biosafety issues were addressed by sterilizing dead animals through dipping them into 70% ethanol, disposing into appropriate biohazard bags before transfer to the incinerator at KEMRI. Further precautionary measures involved putting on protective gear and carrying out the experiments in a laminar flow hood (Nuair Biological Cabinets).

2.9 Statistical analyses

Statistical analysis was performed using Microsoft Excel. Cytokine concentrations were expressed as means ± the standard error. The Student *t* test was used to examine the differences in cytokine concentrations in culture supernatants as well as proliferation of stimulated and unstimulated splenocyte cultures. Data on lesion progression was analyzed for statistical significance using the analysis of variance (ANOVA) and T test. *P* value of ≤ 0.05 was considered significant. Results from the LDA were evaluated using the ELIDA program (ELIDA software 1985–2005; Carl Taswell, 1987).

CHAPTER THREE-RESULTS

3.1 Immunoprophylaxis of combination of BCG and *Lm*SEAgs

3.1.1 Lymphoproliferative responses of splenocyte cultures before infection with *Leishmania major*

The *in vitro* lymphocyte proliferative response to *Lm*SEAgs was conducted at 7 days after the booster vaccination to determine the effect of vaccination alone in the absence of challenge with *L. major*. The results for the *Lm*SEAgs stimulated splenocytes of mice that had received BCG + *Lm*SEAgs, *Lm*SEAgs alone, BCG alone and the control unvaccinated groups before infection with *L. major* are summarized in Table 1. As a control for cell viability, splenocytes were also stimulated with a mitogen, con A. The cells from all the groups stimulated with con A showed high degrees of proliferative responses with no statistical significance among the groups ($P > 0.05$).

The proliferative responses of splenocyte cultures to *Lm*SEAgs obtained from mice that had been vaccinated with *Lm*SEAgs + BCG was significantly higher ($P<0.01$; O.D± S.E 0.633± 0.016) compared to the unvaccinated control (O.D± S.E 0.466 ± 0.015).

For the group of mice vaccinated with BCG alone stimulated with *Lm*SEAgs, the proliferative response was significantly higher ($P< 0.01$; O.D ±S.E 0.689 ± 0.015) compared to the unvaccinated controls. While for the group of mice vaccinated with *Lm*SEAgs alone, there was a significant difference in the proliferation compared to the controls ($P=0.001$; O.D ± S.E 0.689 ± 0.015).

There was no significant difference ($P>0.05$) in the proliferative responses of splenocytes of from group of mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone.

Table 1: Mean optical density (OD) readings (540nm) of *In vitro* proliferative responses of splenocytes before and after infection with *L. major*.

Treatment groups	Cell Proliferation before infection with *L. major*			Cell proliferation 30 days after infection with *L. major*		
	Mean OD readings (540 nm) ± S.E			Mean OD readings (540 nm) ± S.E		
	*Lm*SEAgs	Medium	*P* value (compared to the unvaccinated control)	*Lm*SEAgs	Medium	*P* value(compared to the unvaccinated control)
*Lm*SEAgs	0.633± 0.016	0.362 ± 0.007	0.001	0.861 ± 0.006	0.416 ± 0.01	< 0.001
*Lm*SEAgs+ BCG	0.668 ± 0.025	0.346 ± 0.039	0.001	0.749 ± 0.016	0.374 ± 0.008	< 0.01
BCG	0.689 ± 0.015	0.354 ± 0.021	0.002	0.881 ± 0.009	0.388± 0.006	< 0.001
PBS	0.466 ± 0.015	0.327 ± 0.029	N/A	0.508 ± 0.037	0.301 ± 0.026	N/A

3.1.2 Lymphoproliferative responses of splenocyte cultures after infection with *Leishmania major.*

The second cell proliferation assay was carried out on the 30th day after infection of mice with *L. major.* There was marked increase in the proliferative response to *Lm*SEAgs for the groups vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone compared to the proliferation before infection ($P<0.05$). This is an indication of recall proliferative response.

The proliferative response after infection was significantly higher ($P<0.001$) in the vaccinated groups compared to the unvaccinated controls (Table 1).

In the group of mice vaccinated with *Lm*SEAgs + BCG, the proliferative response of splenocyte cultures to *Lm*SEAgs was not significantly different compared to the proliferation before infection (0.668 ± 0.025 *vs* 0.749 ± 0.016; $P=0.08$).

While in the group of mice vaccinated with *Lm*SEAgs the proliferative response of splenocyte culture was higher than it was before infection (O.D ± S.E 0.633 ± 0.016 *vs* 0.861 ± 0.006; $P=0.007$).

Finally in the group of mice vaccinated with BCG the proliferative response of splenocyte culture was high after infection compared to before infection (O.D ± S.E 0.881 ± 0.009 *vs* 0.689 ± 0.015; $P=0.02$).

3.1.3 Cytokine production

3.1.3.1 Interferon gamma production of splenocyte cultures before infection with *Leishmania major*

The mean production of IFN-γ by splenocytes of mice vaccinated with *Lm*SEAgs co-administered with BCG in response to *in vitro* stimulation with *Lm*SEAgs before challenge was significantly higher compared to the control unvaccinated group ($P<0.05$). The *Lm*SEAgs group had the highest production of IFN-γ (mean ± S.E., 640.447 ± 20.734 pg/ml), followed by the group of mice vaccinated with BCG alone (Table 2). However, there was no significant difference in the *in vitro* production of IFN-γ in mice vaccinated with *Lm*SEAgs alone, BCG alone and *Lm*SEAgs + BCG ($P=0.173$).

Table 2: *In vitro* IFN-γ production (mean ± SE) before and after infection with *L. major*.

Treatment groups	Mean IFN-γ production before infection		Mean IFN-γ production 30 days after infection	
	Mean production of IFN-γ (pg/ml) induced by *Lm*SEAgs	Mean IFN-γ production (pg/ml) by cells in medium	Mean production of IFN-γ (pg/ml) induced by *Lm*SEAgs	Mean IFN-γ production (pg/ml) by cells in medium
*Lm*SEAgs	640.447 ± 20.734	15.143 ± 0.282	803.368 ± 17.697	15.742 ± 2.015
*Lm*SEAgs + BCG	581.106 ± 24.384	12.225 ± 2.831	730.490 ± 7.384	10.861 ± 2.831
BCG	604.640 ± 9.964	10.339 ± 1.897	1117.882 ± 84.903	12.565 ± 2.39
PBS	177.743 ± 10.430	0.000 ± 0.000	292.715 ± 10.430	0.000 ± 0.000

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate Buffered Saline

3.1.3.2 Interferon gamma production of splenocyte cultures after infection with *Leishmania major*

The levels of IFN-γ were determined at 30 days post-infection with *L. major* parasites. There was no significant increase in the level of IFN-γ produced by the group of mice vaccinated with *Lm*SEAgs + BCG as compared to the amount produced before infection (P= 0.255). However, there was significant increase in the level of IFN-γ produced from the *Lm*SEAgs group compared to the levels produced before infection with *L. major* (P= 0.003). Finally in the group vaccinated with BCG there was significant increase of IFN-γ production (mean ± S.E., 803.249 ± 17.697; P=0.01) compared to before infection (mean ± S.E., 604.447 ± 20.734).

Overall, there was an increase (P>0.05)in the amount of IFN-γ detected in splenocyte culture supernatants of all the groups after infection as compared to before infection (Table 2). Very low levels of IFN-γ was observed from wells with unstimulated cultures.

3.1.3.3 Interleukin-4 production before infection with *Leishmania major*

Splenocytes from mice vaccinated with *Lm*SEAgs + BCG produced the highest level of IL-4 when stimulated *in vitro* with *Lm*SEAgs (mean ± S.E., 31.651 ± 1.667). This cytokine production was significantly higher than that produced by the unvaccinated controls (P= 0.01). The level of IL-4 observed from splenocyte cultures of mice vaccinated with *Lm*SEAgs (mean ± S.E., 26.992 ± 2.284), was not significantly different from that produced by the control unvaccinated mice (P=0.07) (Table 3). The lowest production of IL-4 in response to stimulation with *Lm*SEAgs *in vitro* was observed in the group of mice vaccinated with BCG (mean ± S.E., 15.452 ± 2.45). This cytokine level did not reach any statistical significance compared to that of the control unvaccinated group (P= 0.715).

The IL-4 production from splenocyte cultures of mice vaccinated with *Lm*SEAgs alone, BCG alone after *in vitro* stimulation with *Lm*SEAgs did not differ significantly from the controls ($P< 0.05$). Very low levels of IL-4 was observed from wells with unstimulated cultures.

Table 3: *In vitro* IL-4 production (mean ± SE) before and after infection with *L. major*.

Treatment groups	Mean IL-4 production before infection		Mean IL-4 production 30 days after infection	
	Mean production of IL-4 (pg/ml) induced by *Lm*SEAgs	Mean production of IL-4 (pg/ml) by cells in medium	Mean production of IL-4 (pg/ml) induced by *Lm*SEAgs	Mean production of IL-4 (pg/ml) by cells in medium
*Lm*SEAgs	26.622 ± 2.284	7.315 ± 1.122	11.404 ± 1.421	1.73 ± 0.898
*Lm*SEAgs + BCG	35.651 ± 1.669	3.485 ± 0.297	13.476 ± 4.966	4.388 ± 0.832
BCG	15.452 ± 2.45	5.965 ± 1.065	9.673 ± 3.551	0.000 ± 0.000
PBS	17.119 ± 3.476	1.151 ± 0.665	35.339 ± 5.31	6.948 ± 0.43

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate Buffered Saline

3.1.3.4 Interleukin-4 production after infection with *Leishmania major*

Data on IL-4 production 30 days after vaccination is summarized in Table 3. There was a significant decrease in IL-4 production for all the vaccinated groups on the 30th day post infection with *L. major* ($P < 0.05$). However, in the control unvaccinated group there was a 2.1 fold increase in IL-4 produced compared to before infection. In the *Lm*SEAgs + BCG group there was a significant decrease in comparison to before infection (mean ± S.E., 32.651 ±1.669 pg/ml). This represented a 2.4 fold reduction compared to before infection ($P= 0.002$).

The splenocytes from mice vaccinated with BCG had a significant reduction in IL-4 production compared to before infection (mean ± S.E., 9.673 ± 3.551 pg/ml; $P =0.02$). This represented a 2.3-fold decrease and was significantly lower compared to that of the unvaccinated controls. Finally, there was a significant decrease in the IL-4 production in the splenocytes from mice vaccinated with *Lm*SEAgs after infection compared to before (mean ± S.E., 11.404 ± 1.421 vs 26.622 ± 2.284 pg/ml; $P<0.05$).

On the overall, the controls unvaccinated but *L. major* challenged mice produced the highest amount of IL-4 compared to the treatment groups (mean ± S.E., 35.339 ± 5.31 pg/ml; $P= 0.007$). There was no significant difference in the production of IL-4 in the vaccinated groups ($P= 0.767$). Very low levels of IL-4 was observed from wells with unstimulated cultures.

3.1.3.5 Interleukin-5 production before infection with *Leishmania major*

Data on IL-5 production by splenocytes of mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone, BCG alone and control unvaccinated in response to *in vitro* stimulation with *Lm*SEAgs, before challenge with *L. major* is presented in Table 4.

The mean production of IL-5 by splenocytes of mice vaccinated with *Lm*SEAgs + BCG did not differ significantly from that of the control unvaccinated group (mean ± S.E., 13.514 ± 1.958 *vs* 15.216 ± 3.321 pg/ml; P=0.6). Similarly, the mean production of IL-5 of splenocytes from mice vaccinated with *Lm*SEAgs did not reach any statistical significance compared to the unvaccinated control (P = 0.1). However levels of IL-5 produced by splenocytes from mice vaccinated with BCG reached statistical significance (P = 0.003) compared to the unvaccinated controls.

There was significant difference in the production of IL-5 by splenocytes of mice vaccinated with *Lm*SEAgs + BCG, BCG alone and *Lm*SEAgs in response to *Lm*SEAgs (P=0.04). The highest production of IL-5 was realized from the unvaccinated group of mice (mean ± S.E., 19.514 ± 1.251pg/ml). Very low levels of IL-5 were observed from wells with unstimulated cultures.

Table 4: *In vitro* IL-5 production (mean ± SE) before and after infection with *L. major*.

Treatment groups	Mean IL-5production before infection		Mean IL-5 production 30 days after infection	
	Mean production of IL-5 (pg/ml) induced by *Lm*SEAgs	Mean production of IL-5 (pg/ml) by cells in medium	Mean production of IL-5 (pg/ml) induced by *Lm*SEAgs	Mean production of IL-5 (pg/ml) by cells in medium
*Lm*SEAgs	8.547 ± 0.891	4.330 ± 0.787	5.653 ± 2.204	0.000 ± 0.000
BCG	7.996 ± 0.377	5.114 ± 0.407	0.000 ± 0.000	0.000 ± 0.000
PBS	15.216 ± 3.312	4.442 ± 0.336	38.320 ± 4.778	3.861 ± 0.376

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate Buffered Saline

3.1.3.6 Interleukin-5 production after infection with *Leishmania major*

Data on IL-5 production by cell cultures of splenocytes from mice 30 days after infection with *L. major* in response to stimulation with *Lm*SEAgs are presented in Table 4. The mean production of IL-5 by cells of mice vaccinated with *Lm*SEAgs + BCG, in response to *Lm*SEAgs was significantly lower compared to before infection with *L. major* (mean ± S.E., 15.962 ± 1.585 pg/ml; P=0.02). In addition, this cytokine production was significantly lower compared to the unvaccinated controls (P= 0.01). This was also 2.4 fold lower than the controls. On the other hand, there was no significant difference in the mean production of IL-5 by splenocytes of mice vaccinated with *Lm*SEAgs compared to before infection (mean ± S.E., 5.653 ± 2.204 pg/ml; P =0.2). However this production was significantly different from the control mice (P= 0.003) and was 6.7 times lower than the amount produced by the control mice.

There was no production of IL-5 from splenocyte cultures from mice vaccinated with BCG stimulated *in vitro* with *Lm*SEAgs. The highest production of IL-5 from splenocyte culture supernatants stimulated *in vitro* with *Lm*SEAgs was observed in the control group of mice (mean ± S.E., 38.320 ± 4.778 pg/ml). In contrast, very low levels of IL-5 were observed from wells with unstimulated cultures.

3.1.4 Lesion size measurements for BALB/c mice vaccinated before infection with *L. major*.

In order to evaluate the disease progression in mice vaccinated with *Lm*SEAgs alone, *Lm*SEAgs + BCG, BCG alone and PBS as the control; lesion size was followed by measuring the increase in the thickness of the infected foot compared with the contralateral uninfected footpad on a week-to-week basis. As shown in Figure 10 lesion sizes had not developed in all the groups of mice between days 0 and 7.

To evaluate the efficacy of *Lm*SEAgs + BCG, lesion size was compared with that in unvaccinated controls. The lesion development started at the end of week 1. At week 2, 3, 4 and 5 the lesion sizes were not significantly different from the controls ($P > 0.05$). From week 6 to week 10 there was a steady reduction in lesion sizes that was significantly different from that of the unvaccinated controls ($P < 0.01$).

In the group of mice vaccinated with *Lm*SEAgs alone, lesion development started at the end of the first week. At week 2 and week 3 lesion sizes were not significantly different compared to the controls ($P > 0.05$). From week 4 to week 10 the lesion sizes were smaller than the control ($P < 0.01$).

In mice vaccinated with BCG, lesion development started at the end of the first week. From week 2 to week 5 lesion sizes were significantly smaller compared to the controls ($P<0.01$) although they were increasing in size (Figure 10). From week 6 to week 10

there was a steady reduction in the lesion sizes that was statistically significant from the controls ($P< 0.01$).

On the overall, there was no significant difference in lesion sizes among the groups of mice vaccinated with *Lm*SEAgs + BCG, BCG alone and *Lm*SEAgs alone ($P> 0.05$).

Mice in the control unvaccinated but *L. major* challenged group developed large progressive footpad lesions reaching a peak at week 10 (mean ± S.E., 1.2 ± 0.071 mm Figure 10). Unvaccinated control groups were euthanatized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain.

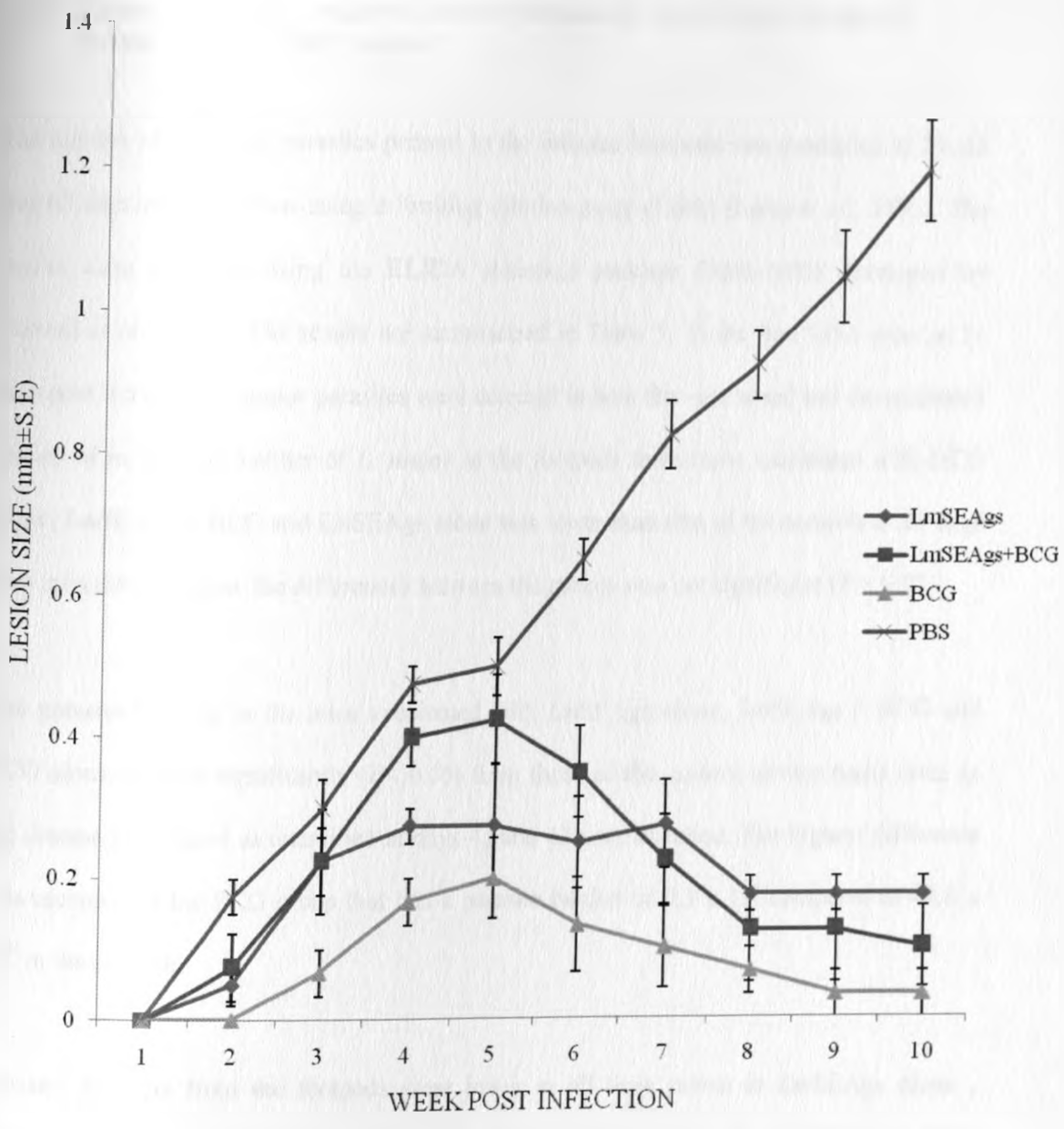

Figure 10: Progression of *L. major* infection as represented with footpad swelling in BALB/c mice of mice vaccinated twice subcutaneously with various regiments before challenge with *L. major*.

Data represent the mean ± S.E. of the difference between the infected and contralateral uninfected footpad from the four experiments with 4 mice per group.

3.1.5 Quantification of *L. major* in infected footpads of BALB/c mice vaccinated before infection with *L. major*.

The number of *L. major* parasites present in the infected footpads was quantified at 21, 42 and 63 days post infection using a limiting dilution assay (LDA) (Lima *et al.*, 1997). The results were analyzed using the ELIDA statistical package (2001-2005) developed by Taswell *et al.* (1981). The results are summarized in Table 5. In the first LDA done at 21 days post infection, *L. major* parasites were detected in both the vaccinated and unvaccinated groups of mice. The number of *L. major* in the footpads from mice vaccinated with BCG alone, *Lm*SEAgs + BCG and *Lm*SEAgs alone was lower than that of the controls at 21 days post infection, however the differences between the groups was not significant ($P > 0.05$).

The parasite burdens in the mice vaccinated with *Lm*SEAgs alone, *Lm*SEAgs + BCG and BCG alone differed significantly ($P< 0.05$) from those of the control unvaccinated mice as the disease progressed as quantified at days 42 and 63 post infection. The highest difference was recorded in the BCG group that had a parasite burden of 0.3×10^4 compared to 48.8×10^6 in the controls.

Parasite numbers from the footpads were lower at all time points in *Lm*SEAgs alone , *Lm*SEAgs+BCG and BCG alone (Table 5). The parasite loads in the *Lm*SEAgs + BCG vaccinated BALB/c mice footpads were 75 fold less, those in *Lm*SEAgs was 232.3 fold less than their unvaccinated counterparts. Lastly, those of the BCG vaccinated mice ranged 1626 fold less than the control unvaccinated mice at day 63. The parasite loads for mice vaccinated with *Lm*SEAgs, *Lm*SEAgs+BCG and BCG were significantly lower than those of the unvaccinated mice ($P < 0.05$). On the other hand, parasite burdens in the unvaccinated control increased persistently as the disease progressed.

Overall, the parasite burdens in the footpads of mice vaccinated with *Lm*SEAgs, *Lm*SEAgs + BCG alone did not differ significantly from each other ($P>0.05$) but were significantly different ($P< 0.05$) from the unvaccinated control mice at day 42 and day 35.

Table 5: Parasite burden in the *L. major* infected footpads after vaccination with various regimes before challenge during the course of infection.

Days post-infection	Experimental group (No. of *L. major* (10^6)/footpad (95% confidence limits)			
	Control	*Lm*SEAgs	*Lm*SEAgs + BCG	BCG
21	10.2(29.4-1.23)	1.5(1.7-1.3)	4.13 (12.1-3.8)	0.94(1.19-0.68)
42	19.3(28.75 -13.8)	0.85(1.21-0.55)*	1.88 (3.38-0.41)*	0.52 (0.13-1.21)*
63	48.8(87.2-19.8)	0.21(0.15-0.09)*	0.65(1.54-0.54)*	0.03(0.01-0.06)*

(*) $P < 0.05$ parasite burden is significantly different from mice that received PBS (control unvaccinated group).

3.2 Determination of the Immunotherapy of a combination of *Lm*SEAgs + BCG:

3.2.1 Lymphoproliferative responses of splenocytes cultures a week post vaccination

The first experiment, conducted at 7 days after the booster vaccinations sought to determine the effect of vaccination five weeks after challenge with *L. major* on the potential of the immune system to respond to stimulation *in vitro* with soluble *Lm*SEAgs. The *in vitro* lymphocyte proliferative responses of unstimulated and *Lm*SEAgs stimulated splenocytes of mice that had received BCG + *Lm*SEAgs, *Lm*SEAgs alone, BCG alone and the control unvaccinated groups after infection with *L. major* are summarized in Table 6.

As a control for cell viability, splenocytes were stimulated with a mitogen, con A. The cells from all the groups stimulated with con A showed high degrees of proliferative responses over the ones stimulated with *Lm*SEAgs and medium ($P<0.05$). In addition, the results of these cultures demonstrated that the degree of proliferation of cells from all the groups stimulated with con A was almost equivalent with no statistical significance among the groups.

The proliferative responses of splenocyte cultures to *Lm*SEAgs obtained from mice that had been vaccinated with *Lm*SEAgs + BCG was significantly higher compared to the control unvaccinated group ($P=0.008$; mean ± SE, 0.536 ± 0.04 *vs* Mean ± S.E, 0.312 ± 0.017).

For the group of mice vaccinated with *Lm*SEAgs alone, the proliferative response was significantly higher ($P=0.002$; mean ± S.E., 0.558± 0.006) compared to the control unvaccinated mice.

While for the group of mice vaccinated with BCG, there was a significant high proliferative response compared to the controls ($P< 0.01$; mean ± S.E., 0.617 ± 0.001).

However, there was no significant difference in the proliferative responses of splenocytes of groups of mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone ($P> 0.05$).

Table 6: Mean optical density (OD) readings (540 nm) of *in vitro* proliferative responses of splenocytes one week and four weeks post vaccination. of mice vaccinated after challenge with *L. major*.

Treatment groups	Proliferative responses one week after vaccination			Proliferative responses four weeks after vaccination		
	Mean OD readings (540 nm) ± S.E			Stimulation index (540 nm) ± S.E		
	*Lm*SEAgs	Medium	*P* value compared to the unvaccinated control	*Lm*SEAgs	Medium	*P* value compared to the unvaccinated control
*Lm*SEAgs	0.558± 0.006	0.369± 0.012	0.002	0.845± 0.042	0.407± 0.026	0.006
*Lm*SEAgs+ BCG	0.536 ± 0.04	0.337 ± 0.024	0.008	0.781 ± 0.027	0.317 ± 0.033	< 0.01
BCG	0.617 ± 0.001	0.380 ± 0.008	< 0.01	1.021 ± 0.049	0.408 ± 0.021	< 0.01
PBS	0.312 ± 0.017	0.303± 0.028	N/A	0.313 ± 0.035	0.307± 0.002	N/A

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate

3.2.2 Lymphoproliferative responses of splenocyte cultures four weeks post vaccination

The second cell proliferation assay carried out on the 28th day after booster vaccinations. The *in vitro* lymphocyte proliferative responses of unstimulated and *Lm*SEAgs stimulated splenocytes of mice who had received *Lm*SEAgs + BCG, *Lm*SEAgs alone, BCG alone and the control unvaccinated groups are summarized in Table 6.

There was a significant increase in the proliferative responses to *Lm*SEAgs at 28 days post vaccination as compared to 7 days post vaccination in all the vaccinated groups ($P< 0.05$) an indication of recall proliferative responses.

In the group of mice vaccinated with *Lm*SEAgs + BCG the proliferative response of splenocyte cultures to *Lm*SEAgs was significantly higher compared to 7 days post vaccination ($P=0.04$; mean ± S.E., 0.781 ± 0.027 *vs* 0.536 ± 0.04).

While for the group of mice vaccinated with *Lm*SEAgs there was an increase in the proliferative response of splenocyte culture compared to 7 days post infection ($P=0.02$; mean ± S.E., 0.845± 0.042 *vs* 0.558± 0.006).

Finally for the group vaccinated with BCG alone there was a marked increase in the proliferative response compared to 7 days post vaccination ($P< 0.01$; mean ± S.E., 1.021 ± 0.049 *vs* 0.617 ± 0.001).

The proliferative response of splenocytes from mice immunized with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone differed significantly among the groups ($P= 0.01$).

3.2.3 Cytokine production

3.2.3.1 Interferon gamma production of splenocyte cultures a week post vaccination.

The mean production of IFN-γ production by splenocytes of mice vaccinated with *Lm*SEAgs + BCG in response to *in vitro* stimulation with *Lm*SEAgs was significantly higher ($P < 0.01$) compared to the control unvaccinated group (Table 7). The BCG group produced high levels of IFN-γ ($P< 0.01$; mean ± S.E; 481.106 ± 25.847 pg/ml) compared to the unvaccinated control group. Finally mice vaccinated with *Lm*SEAgs had significantly high production of IFN-γ ($P< 0.01$; mean ± S.E., 473.78 ± 14.676 pg/ml) compared to the controls.

However there was no significant difference in the *in vitro* production of IFN-γ in mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone ($P = 0.08$).

Table 7: *In vitro* IFN-γ production (mean ± SE) one week and four weeks after vaccination of mice challenged before vaccination.

Treatment groups	Mean IFN-γ production one week after vaccination		Mean IFN-γ production four weeks after infection	
	Mean production of IFN-γ (pg/ml) induced by *Lm*SEAgs	Mean IFN-γ production (pg/ml) by cells in medium	Mean production of IFN-γ (pg/ml) induced by *Lm*SEAgs	Mean IFN-γ production (pg/ml) by cells in medium
*Lm*SEAgs	473.78 ± 14.676	15.143 ± 0.282	1103.249 ± 40.850	9.143 ± 3.742
*Lm*SEAgs + BCG	384.973 ± 36.336	12.225 ± 2.831	1165.548 ± 60.073	8.558 ± 0.502
BCG	481.106 ± 25.847	10.339 ± 1.897	967.156 ± 50.769	10.339 ± 1.897
PBS	82.077 ± 2.646	11.645 ± 5.378	389.382 ± 25.399	0.000 ± 0.000

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate

3.2.3.2 Interferon gamma production of splenocyte cultures four weeks post vaccination

The levels of IFN-γ were determined at 28 days in the groups of mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone, BCG alone and control (Table 7). There was a significant increase in the level of IFN-γ produced by the group of mice vaccinated with *Lm*SEAgs + BCG as compared to the amount produced one week post vaccination (P= 0.01). In addition there was a significant increase in the level of IFN-γ produced by spleen cells stimulated with *Lm*SEAgs *in vitro* from the *Lm*SEAgs group compared to one week after infection with *L. major* (P= 0.002). This was a highly significant difference. A significant increase of IFN-γ production was also observed in the spleen cells stimulated splenocytes from the BCG group stimulated by *Lm*SEAgs (P=0.01) compared to the earlier production of interferon gamma.

Overall, there was an increase in the amount of IFN-γ detected in splenocyte culture in the group of mice vaccinated with *Lm*SEAgs + BCG, BCG alone and *Lm*SEAgs alone compared to the controls ($P< 0.05$).

3.2.3.3 *In vitro* interleukin-4 production of splenocyte cultures a week after post vaccination

The mean production of IL-4 seven days post vaccination by splenocytes of mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone, BCG alone and control unvaccinated in response to *in vitro* stimulation with *Lm*SEAgs are summarized in Table 8. There was relatively low production of IL-4 by splenocytes from mice vaccinated with *Lm*SEAgs + BCG, *Lm*SEAgs alone and BCG alone. That did not differ significantly from each other ($P> 0.05$). However, this production was significantly lower than the controls ($P < 0.05$).

Splenocytes from mice vaccinated with *Lm*SEAgs + BCG produced the high level of IL-4 among the vaccinated groups when stimulated *in vitro* with *Lm*SEAgs ($P< 0.01$; mean ± S.E., 78.318 ± 10.380).

Table 8: *In vitro* Interleukin-4 production (mean ± SE) one week and four weeks after vaccination of mice that were challenged before vaccination

Treatment groups	Mean IL-4 production one week post vaccination		Mean IL-4 production four weeks post vaccination	
	Mean production of IL-4 (pg/ml) induced by *Lm*SEAgs	Mean IL-4 production (pg/ml) by cells in medium	Mean production of IL-4 (pg/ml) induced by *Lm*SEAgs	Mean IL-4 production (pg/ml) by cells in medium
*Lm*SEAgs	66.922 ± 2.341	11.449 ± 0.819	22.441 ± 3.440	0.000 ± 0.000
*Lm*SEAgs + BCG	79.318 ± 10.380	12.845 ± 0.393	32.143 ± 2.869	1.069 ± 3.253
BCG	65.522 ± 11.618	15.219 ± 0.456	13.006 ± 1.993	0.000 ± 0.000
PBS	121.079 ± 6.634	13.618 ± 1.971	176.123 ± 8.945	6.796 ± 6.100

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate

3.2.3.4 *In vitro* Interleukin-4 production of splenocyte cultures four weeks post vaccination

There was a significant in decrease IL-4 production in all the vaccinated groups on the 28th day post vaccination with *L. major* ($P > 0.05$). However in the control unvaccinated group there was a 2.8 fold increase in IL-4 produced compared to 7 days post vaccination. Data on IL-4 production 28 days post vaccination is summarized in Table 8.

In the group of mice vaccinated with *Lm*SEAgs + BCG there was a significant decrease in IL-4 production ($P= 0.01$; mean ± S.E., 79.318 ± 10.380 *vs* 32.651 ±1.669 pg/ml) compared to the IL-4 production 7 days post vaccination.

The splenocytes from mice vaccinated with BCG also had a significant reduction in IL-4 production compared to 7 days post vaccination ($P =0.01$; mean ± S.E.. 13.006±1.993pg/ml) that represented a 2.9 fold decrease.

In the group of mice vaccinated with *Lm*SEAgs there was a significant decrease in the IL-4 production (mean ± S.E., 22.441 ± 3.440 *vs* 66.922 ± 2.241 pg/ml $P < 0.01$) compared to 7 days post vaccination.

On the overall, the controls unvaccinated but *L. major* challenged mice produced the highest amount of IL-4 compared to the treatment groups (mean ± S.E., 176.123 ± 8.945pg/ml) and differed significantly from the vaccinated groups ($P<0.01$).

3.2.4 Results of lesion size measurements for BALB/c mice vaccinated 21 days post infection with 10^5 *L. major* metacyclic promastigotes.

In order to evaluate the effect of vaccination of *Lm*SEAgs alone, *Lm*SEAgs + BCG, BCG alone and PBS as the control on disease progression in *L .major* infected mice; lesion size was followed by measuring the increase in the thickness of the infected foot compared with the contralateral uninfected footpad on a week-to-week basis. As shown (Figure 11) lesions had not developed in all the groups of mice between days 0 and 7.

To evaluate the efficacy of BCG plus *Lm*SEAgs, lesion size was compared with that in unvaccinated controls. The lesion development started at the end of week 1. At week 2, 3 and 4 the lesions were not significantly different from the controls ($P> 0.05$). From week 5 to week 10 there was a steady reduction in lesion sizes that was significantly different from the unvaccinated controls ($P< 0.01$).

In the group of mice vaccinated with *Lm*SEAgs alone, lesion development started at the end of the first week. From week 2 to week 10 lesion sizes were significantly smaller compared to the controls ($P<0.01$).

In mice vaccinated with BCG lesion development started at the end of the first week. From week 2 to week 8 lesion sizes were significantly smaller compared to the controls ($P<0.01$). By the end of week 9 the mice had no lesions.

Mice in the control unvaccinated but *L. major* challenged group developed large progressive footpad lesions reaching a peak at week 10 (mean ± S.E., 1.2 ± 0.071 mm, Figure 10).

It was also demonstrated that lesion sizes of mice vaccinated with *Lm*SEAgs + BCG compared to those of BCG were not statistically significant ($P > 0.05$). For *Lm*SEAgs plus BCG compared with *Lm*SEAgs the lesion sizes did not differ significantly ($P=0.508$).While for *Lm*SEAgs compared to BCG was not significantly different ($P > 0.05$).

Unvaccinated control groups were euthanatized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain.

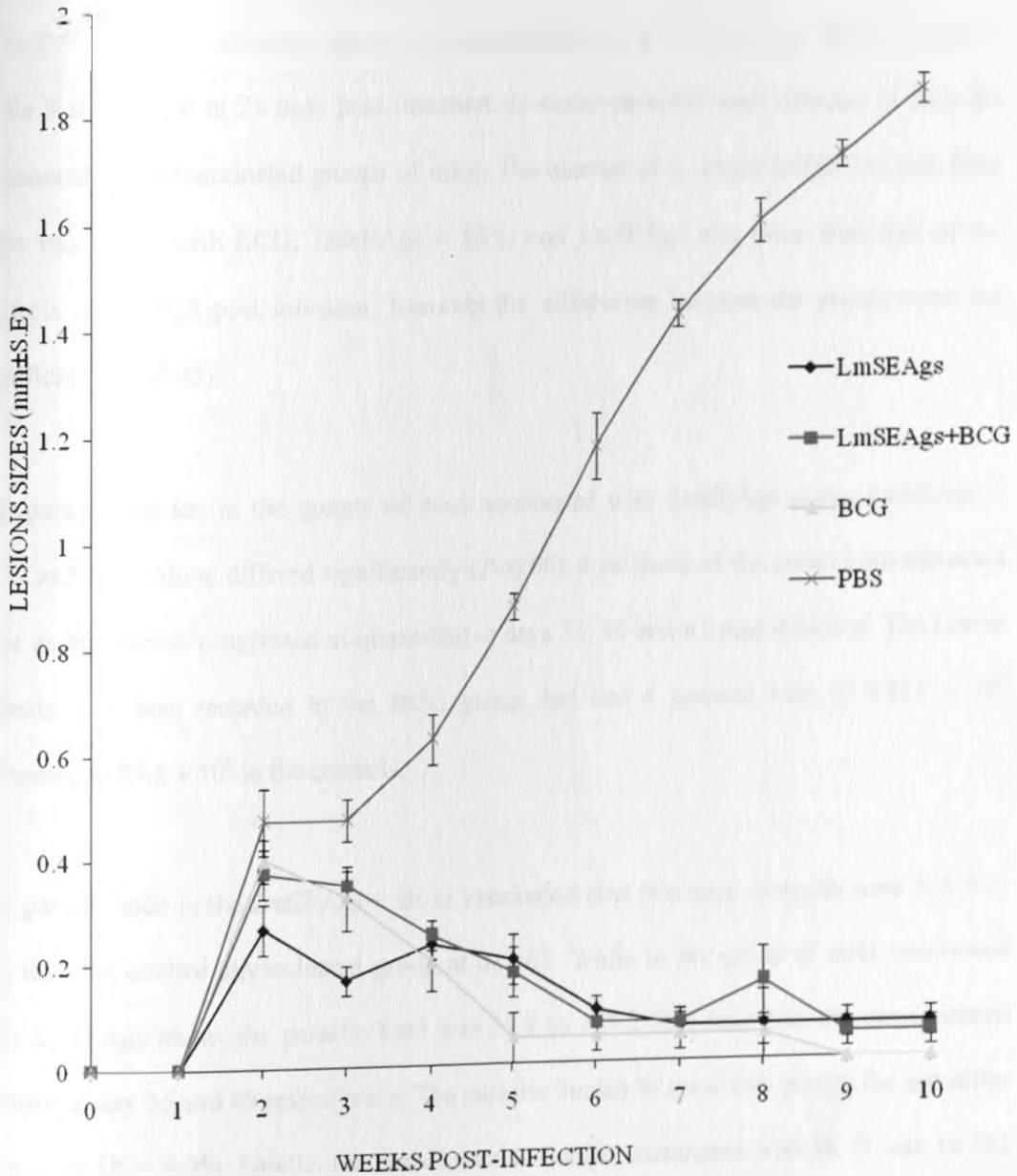

Figure 11: Lesion sizes measurements for BALB/c mice vaccinated twice subcutaneously with various regimes after challenge with *L .major*.

Key: BCG - Bacille Calmette Guerin
*Lm*SEAgs - *Leishmania major* soluble exo-antigens
PBS - Phosphate

3.2.5 Quantification of *L. major* in infected footpads of BALB/c mice vaccinated 21 days post infection.

The number of *L. major* parasites present in the infected footpads was quantified at 21, 35, 49 and 63 days post infection using a limiting dilution assay (Lima *et al.*, 1997). Results in Table 9 shows that at 21 days post infection, *L. major* parasites were detected in both the vaccinated and unvaccinated groups of mice. The number of *L. major* in the footpads from mice vaccinated with BCG, *Lm*SEAgs + BCG and *Lm*SEAgs was lower than that of the controls at 21 days post infection, however the differences between the groups were not significant ($P > 0.05$).

The parasite burden in the groups of mice vaccinated with *Lm*SEAgs alone, *Lm*SEAgs + BCG and BCG alone differed significantly ($P<0.05$) from those of the control unvaccinated mice as the disease progressed as quantified at days 35, 49 and 63 post infection. The lowest parasite load was recorded in the BCG group that had a parasite load of 0.525×10^3 compared to 77.5×10^6 in the controls.

The parasite load in the *Lm*SEAgs + BCG vaccinated BALB/c mice footpads were 155 fold less than the control unvaccinated group at day 63. While in the group of mice vaccinated with *Lm*SEAgs alone, the parasite load was 33.9 to 129.2 fold less than the unvaccinated controls at day 35 and 49 respectively. The parasite burden in these two groups did not differ from each ($P > 0.05$). Finally, the parasite load in mice vaccinated with BCG was 14,761 fold less than the control unvaccinated mice at day 63.

The figures obtained from *Lm*SEAgs, *Lm*SEAgs + BCG were statistically lower than those of the unvaccinated mice ($P < 0.05$). On the other hand, parasite burdens in the unvaccinated control increased steadily as the disease progressed.

Overall, the parasite burdens in the footpads of mice vaccinated with *Lm*SEAgs, *Lm*SEAgs + BCG alone did not differ significantly from each other but were significantly different from the unvaccinated control mice ($P < 0.05$).

Table 9: Parasite burden in mice vaccinated with various regimens after challenge with *L. major* during the course of infection.

	Experimental groups (No. of *L. major* (10^6)/footpad (95% confidence limits)			
Day	Control	*Lm*SEAgs	*Lm*SEAgs + BCG	BCG
21	15.45(19.4-11.5)	8.2(12.5-3.99)	9.35(11.9-6.75)	4.97 (2.63-6.75)*
35	32.5(53 -11.6)	3.44(0.595-6.09)	6.46(7.08-5.49)	0.1635(0.188-0.139) **
49	53.5(67.5-39.9)	1.58(1.83-1.32)*	1.54(1.94-1.15)*	0.0065(0.273-0.002) **
63	77.5(125.5-29.1)	0.6(0.9-0.3)*	0.5(0.95-0.15)*	0.005(0.008-0.003) **

(*) $P < 0.05$ parasite burden is significantly different from mice that received PBS (control unvaccinated group).

Chapter 4- DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Introduction

Several vaccines candidates against leishmaniasis have been identified although no vaccine has been discovered yet (Handman, 2001; Kedzierski *et al.*, 2006). In this study *Leishmania major* soluble exo-antigens (*Lm*SEAgs) which have been previously identified as immunogenic were investigated in combination with BCG. These *Lm*SEAgs have been shown to elicit a protective immune response against challenge with *L. major* in susceptible BALB/c mice, in absence of any adjuvant (Tonui *et al.*, 2004). In addition they have also been shown to protect neonatal BALB/c mice from challenge with *L. major* (Tonui *et al.*, 2006). On the other hand BCG has also been successfully used as an adjuvant in various animal and clinical *Leishmania* vaccine studies (Sharifi *et al.*, 1998; Misra *et al.*, 2001). This study sought to investigate the adjuvant potential of BCG with *Lm*SEAgs in vaccination against Leishmaniasis. The study was done in two experimental set-ups; immunoprophylaxis and immunotherapy.

4.2 Immunoprophylaxis of combination of BCG and *Lm*SEAgs

Animal models of cutaneous leishmaniasis indicate that Th1 responses are essential for protection by vaccination (Latifynia and Hazrati, 2008). These Th1 responses are predicated on the induction of high levels of IFN-γ and low levels of IL-4. The production of IFN-γ after stimulation of lymphocytes with leishmanial antigen has been considered one of the best correlates of resistance (Gicheru *et al.*, 2001). Interferon-gamma a Th1 cytokine is known to be a strong inducer of: the macrophage enzyme inducible nitric oxide synthase (iNOS) that produce nitric oxide (Serarslan and Atik, 2005). *Leishmania major* replicates intracellularly

in macrophages, and effective control requires macrophage activation and nitric oxide (NO)-mediated killing in response to the Th1-produced cytokine IFN-γ (Stacey and Blackwell, 1999). This was demonstrated in this study by the fact that the levels of Interferon gamma increased significantly compared to the control group while the levels of IL-4 was significantly low ($P<0.01$). In this study the immunoprophylactic potential of the combination of BCG and *Lm*SEAgs was investigated. The results show that combination of BCG + *Lm*SEAgs conferred protection in BALB/ c mice challenged with *L. major*.

In this study IL-4 production by mice injected with combination of BCG and *Lm*SEAgs was significantly lower than IFN-γ ($P<0.01$). In addition the IL-4 production after infection was 2.4 fold lower than it was before infection. Uncontrolled, non-healing lesions are associated with the proliferation of Th2 cells and the production of IL-4 resulting in an ineffective antiparasite response. This is accomplished by IL-4 promoting the differentiation of naive T cells into IL-4-secreting Th2 cells and suppressing IFN-γ responses (Seder *et al.*, 1992; Noben-Trauth, 2000). This results in a skewing toward Th2 responses that is thought to be involved in the susceptibility to infectious diseases such as leishmaniasis (Noben-Trauth, 2000). In addition IL-4 is known to suppress the production of IFN-γ resulting in exacerbation of the disease (Sacks and Noben-Trauth, 2002). In this study vaccination with a combination of BCG and *Lm*SEAgs resulted in low production of IL-4 and high IFN-γ thus resulting in protection. The high production of IFN-γ lead to activation of macrophages and induction of nitric oxide to kill the parasite. On the other hand the unvaccinated (control) group developed large uncontrolled lesions that were characterized by low levels of IFN-γ and high levels of IL-4.

Tracking lesion size (as a measure of the pathologic response to infection) is one method of monitoring the development of infection with *L. major* (Al-Wabel *et al.*, 2007). The protective effects of BCG and *Lm*SEAgs are supported by the fact that the lesion sizes in the vaccinated mice reduced significantly compared to the unvaccinated controls. The mice vaccinated with a combination of *Lm*SEAgs and BCG initially had a steady increase in the lesion sizes, with a significant difference being reported at week six ($P<0.05$) post infection, at this point a decrease in lesion was observed until the 9th week.

Overall, the mice were protected from the disease as demonstrated by the reduction in lesion sizes and subsequent recovery. Tracking the parasite burden in unvaccinated (control) or vaccinated mice challenged with *L. major* is thought to add weight to the data from lesions sizes. In that control of infection should be a result of both reduction lesion sizes and parasite burden (Al-Wabel *et al.*, 2007). Lima *et al.*, (1997) suggest that interpreting experiments with *Leishmania* based on lesion size alone can be quite misleading. Therefore, it is imperative to monitor parasite burden when testing any vaccine for leishmaniasis. Congruent with this, in this study, the parasite numbers were determined using published techniques (Lima *et al.*, 1997). In the group of mice vaccinated with a combination of BCG and *Lm*SEAgs, there was a strong correlation between the parasite burdens and the lesions sizes. This was evidenced by a decrease in the lesion sizes as well as in the parasite numbers. On the 9th week the parasite burden was 75 fold lower than the control. The reduction in lesion sizes and parasite numbers during the course of infection is due to a Th1-dominated response, with high production of IFN-γ. This consequently led to induction of inducible nitric oxide synthase (iNOS) in the infected macrophages that resulted in the activation of macrophages thus killing intracellular parasites (Noben-Trauth *et al.*, 2003; Cruz *et al.*, 2006).

In vitro lymphocyte cell proliferation is one of the immunological parameters used to assess the development of Th-1 responses to leishmanial antigens (Gicheru *et al.*, 2001). The study found that *Lm*SEAgs stimulated splenocytes from BALB/c mice vaccinated with combination of BCG and *Lm*SEAgs. This proliferation was significantly higher than the controls $P< 0.05$ Indicating induction of a recall response.

In this study the protective potential of a combination of BCG and *Lm*SEAgs was compared with that of BCG alone. There was no significant difference in the protection conferred to mice vaccinated with a combination of BCG and *Lm*SEAgs in comparison to the BCG vaccinated group ($P>0.05$). This was observed in all the parameters measured. However vaccination with BCG alone compared to the unvaccinated (controls) was found to induce healing and protection from disease. Results from this group demonstrated high IFN- γ levels production compared to the controls ($P= 0.01$). This indicated a Th1-dominated response, with high production of IFN-γ and consequently induction of inducible nitric oxide synthase (iNOS) in the infected macrophages that resulted in the activation of macrophages to kill intracellular parasites (Noben-Trauth *et al.*, 2003; Cruz *et al.*, 2006). In addition the lesion sizes were small compared to the unvaccinated (controls) $P< 0.01$. These results were further corroborated with low parasite numbers in the lesion sizes compared to the control ($P< 0.01$) and with low IL-4 production from splenocytes from this group compared to the control ($P< 0.05$). Further to this there was 2 fold reduction in the IL-4 production after infection. The ability of BCG alone to confer protection against leishmaniasis is presumably due to antigenic cross-reactivity between *Mycobacteria* and *Leishmania* (Roberts, 2006). This antigenic cross-reactivity between *Mycobacterium* and *Leishmania* has been documented (Almahamoodi *et al.*, 2002). In experimental murine leishmaniasis, innate

immunity is controlled by Lsh gene that is thought to be identical to the BCG gene hence cross antigenic reaction between BCG and *Leishmania.*

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. It is used alone or in combination with other antigens is routinely in vaccination trials against leishmaniasis (Ghalib and Moddaber, 2007). The results from this study are in agreement with previous findings where BCG has been demonstrated to enhance the protective effects of various vaccine candidates. A study by Khalil *et al.* (2000) in Sudan found no evidence that two doses of ALM plus BCG offered significant protective immunity against leishmaniasis compared with BCG alone. While Misra *et al* (2001) demonstrated that a single dose of alum precipitated autoclaved *L. major* vaccine in combination with BCG could give successful protection against parasite challenge. While in Ecuador using the langur model for leishmaniasis, two doses of vaccine composed of *L. amazonensis* and *L. mexicana* mixed with BCG was shown to induce protection (Armijos *et al.*, 2004).

In addition to comparing the protective potential of a combination BCG and *Lm*SEAgs with that of BCG alone, a comparison was also drawn with *Lm*SEAgs alone. Similarly no significant protective potential in the group vaccinated with BCG and *Lm*SEAgs was observed compared to the group vaccinated with *Lm*SEAgs alone ($P>0.05$). However results obtained in this group concur with previous finding where *Lm*SEAgs have been demonstrated to the protective (Tonui *et al.*, 2004; Tonui and Titus, 2006). This was evidenced by a shift towards the Th-1 path where the highest production of IFN-γ was noted that was highly significant compared to the unvaccinated (control) ($P< 0.01$). Also there was a low IL-4 production compared to the control. This was further supported by small lesion

sizes ($P< 0.05$) from week 4 that was maintained to the end of the experiment compared to the controls suggesting protection.

Lastly the parasite burdens in the infected lesions also significantly reduced during the course of the study. In the last experiment the parasite load was 232 fold less than the controls. The results gave a clear indication of protection. These results are in agreement with previous studies that have demonstrated *Lm*SEAgs alone are highly immunogenic and are able to elicit significant protection against challenge with *L. major* in susceptible BALB/c mice (Tonui *et al.*, 2004). In addition the results from the group of mice vaccinated with *Lm*SEAgs alone concur with the findings of Tonui and Titus, (2006) that *Lm*SEAgs were able to confer resistance to vaccinated neonatal susceptible BALB/c mice when challenge with *L .major*. In an earlier study Webb *et al* (1998) demonstrated that leishmanial culture filtrate proteins plus *Corynebacterium parvum* induced a mixed type 1and type 2 response and resistance to infection with *L. major* when injected in mice. Al-wabel *et al* (2007) showed that nuclease hydrolase an isolate of the *Lm*SEAgs was able to confer resistance to mice against *L. major* challenge. The results from the group of mice vaccinated with *Lm*SEAgs alone are in agreement with the finding from the studies mentioned studies that *Lm*SEAgs alone are able to protect mice from infection with *L. major*.

4.3 Immunotherapeutic potential of combination of BCG and *LmSEAgs*

The results show that combination of BCG and *Lm*SEAgs cured BALB/ c mice challenged with *L. major*. This was demonstrated by the fact that the levels of IFN-γ increased significantly compared to the control group while the levels of IL-4 was significantly low. However in the second cytokine experiment there was a significant ($P< 0.05$) increase in the

IFN-γ production in this BCG and *Lm*SEAgs group. Interferon-gamma is known to be a strong inducer of: the macrophage enzyme inducible nitric oxide synthase (iNOS) (Serarslan and Atik, 2005). The high IFN-γ production observed in this group resulted in effective macrophage activation and nitric oxide (NO) mediated cytokine (Stacey and Blackwell, 1999). This consequently led to destruction *Leishmania major* that replicates intracellulary in macrophages.

In murine *L. major* infection, resistance is associated with the capacity of CD4 Th1 cells to generate IFN-γ (Cabera *et al.*, 2000). The down regulation of Th2 and up regulation of Th1 results in control of the infection observed in this study. This was demonstrated by low IL-4 production by mice injected with combination of BCG and *Lm*SEAgs than IFN-γ (P <0.01). In addition the IL-4 production after infection was significantly lower than before infection (P=0.01). Interferon gamma, secreted by Th1 cells, is the most potent macrophage-activating cytokine leading to host resistance to infection with *Leishmania* parasites whereas IL-4 secreted by Th2 cells, is associated with down-regulation of IFN-γ-mediated macrophage activation (Sacks and Noben-Trauth, 2002). The protection observed in this group concurs with a study by Cabrera *et al.*, (2000) that reported that immunotherapy with BCG enhances proliferative responses and IFN-γ production to the promastigotes antigens, as well as IFN-γ production to BCG. This was observed in the current study, splenocytes injected with a combination of BCG and *Lm*SEAgs proliferated in response to *in vitro* stimulation with *Lm*SEAgs compared to the control groups both one week post treatment and four weeks after treatment with the named substances ($P < 0.05$). It is a widely accepted fact that cellular immune responses play a vital role in the pathogenesis and healing of leishmaniasis see Garg *et al.*, (2005).

The protective effects of a combination of BCG and *Lm*SEAgs were supported by the fact that the lesion sizes in the treated mice reduced significantly compared to the unvaccinated controls. In the mice vaccinated with a combination of *Lm*SEAgs and BCG a significant difference was noted on day 35 post infection and day 14 post vaccination ($P<0.05$). Overall, the mice were protected from the disease as demonstrated by the reduction in lesion sizes and subsequent recovery. Congruent with this, in this study, the parasite numbers were determined using published techniques (Lima *et al.*, 1997). In the group of mice vaccinated with a combination of BCG and *Lm*SEAgs, there was a correlation between the parasite burdens and the lesions sizes. This was evidenced by a decrease in the lesion sizes as well as in the parasite numbers. On the 5^{th} week the parasite burden was 155 fold lower than the control. The reduction in lesion sizes and parasite numbers during the course of infection is due to a Th1-dominated response, with high production of IFN-γ. This consequently led to induction of inducible nitric oxide synthase (iNOS) in the infected macrophages that resulted in the activation of macrophages thus killing intracellular parasites (Noben-Trauth *et al.*, 2003).

The question that arises is how BCG mediates its immunotherapeutic effect. BCG is known to activate macrophages to produce TNFα and nitric oxide which could contribute to leishmanicidal activity (Marshall *et al.*, 1997). However, since parasitized macrophages in the lesion are at sites remote from administration of the BCG, this is not likely to directly contribute to the therapeutic effect. The nonspecific activation of macrophages will, however, act as a local adjuvant in promoting T cell responses to both BCG and leishmanial antigens. The ability of BCG to trigger IL-12 production by macrophages, which is enhanced in the presence of natural killer cells, will bias this towards a Th1 response (Matsumoto *et al.*, 1997).

In this study the immunotherapeutic potential, of BCG was investigated as a positive control compared with that of the unvaccinated (control). Results of this study show that immunotherapy is associated with enhanced antigen specific IFN-γ responses. There was significant high production of IFN-γ in the BCG group compared to the controls ($P<0.01$). This is consistent with a shift in balance of T cell response towards a Th1 response, and suggests that cure is mediated by IFN-γ. These finding are in agreement with results by Convit *et al.*, (1989) that reported immunotherapy using a combination of heat killed *Leishmania amazonensis* promastigotes and live Bacille Calmette Gue´rin (BCG) was effective at resolving CL. In addition IL-4 production was significantly lower than the IFN-γ and also as compared to the controls ($P< 0.01$). This shift towards Th-1 contributed to the control of the disease and cure.

Progression of disease in *L. major* infection has been correlated with the production of IL-4, IL-5, by CD4 T helper 2 (Th2) cells (Scott *et al.*, 1988, Sack and Noben-Trauth, 2002). This was not observed in this group as the IL-4 production was low with high IFN-γ. As earlier stated tracking the parasite burden in unvaccinated (control) or vaccinated mice challenged with *L. major* is thought to boost the data from lesions sizes (Al-Wabel *et al.*, 2007). The parasite burden was 15,500 fold less than the controls at the end of the study. In addition to this the lesion size was significantly lower as compared to the unvaccinated (controls) from week 4 post infection. The parasite burden and the lesion sizes correlated to each other as is expected. This reduction can be linked with the production of IFN-γ (Scott *et al.* 1988; Noben-Trauth, 2000), which activates the parasitized macrophage to kill the intracellular *Leishmania* (Mosmann and Coffman, 1989). On the overall the results from this group are in

agreement with those from (Frommel & Lagrange 1989) that concluded that in mice. BCG alone administered at a site remote to the lesions is capable of mediating protection or cure.

Finally there was also a group that was treated with *Lm*SEAgs. These antigens were found to be immunotherapeutic as demonstrated by significantly high IFN-γ production that was lower than the unvaccinated (controls) ($P< 0.05$). Further to this there was a correlation between the lesion sizes and the parasite burdens, whereby a reduction in both the lesion sizes and parasite burdens was observed as the disease progressed. However the parasite burden reduction was not as remarkable compared to the BCG group at day 63. The parasite numbers were 15,500 fold less than control while that of *Lm*SEAgs was 129 fold less compared than the control. Interestingly the lesion sizes in this group were not significantly different from those of vaccinated with BCG. Lima *et al.*, (1997) concluded that interpreting experiments with *Leishmania* based on lesion size alone can be quite misleading. This was the case in the group of mice vaccinated with *Lm*SEAgs. Therefore, the need to monitor parasite burden in addition lesion sizes leishmaniasis. This probably implies that *Lm*SEAgs is not a good immunotherapeutic agent as BCG. In that when it is administered in a site far-off from the lesion it is not capable of mediating cure. It is probable that *Lm*SEAgs lacks the natural stimulus of Thl immune responses, present in BCG.

Leishmania major exo-antigens are known to be highly immunogenic (Ryan *et al.*, 2002) and proved to be protect mice from subsequent challenge by *L .major* (Tonui *et al.*, 2004). The protection is associated with high IFN-γ, by low lesion sizes and low parasite burdens in comparison to the unvaccinated control. This trend was demonstrated in the immunotherapeutic set-up in the current study.

4.4 Conclusions

From this study the following conclusion can be drawn:

1. Bacille Calmette Gue´rin as an adjuvant did not enhance the immunoprophylactic or immunotherapeutic potential of *Lm*SEAgs.
2. Bacille Calmette Gue´rin had both immunoprophylactic and immunotherapeutic protective effect in murine leishmaniasis.
3. There was no significant difference in the immunotherapeutic and immunoprophylactic effects of BCG combined with *Lm*SEAgs.

4.4 Recommendations

This study focused on evaluating the immunoprophylactic and immunotherapeutic effects of BCG combined with *Lm*SEAgs in cutaneous leishmaniasis. Further studies should be done to search for molecules with potential of enhancing the immunotherapeutic and immunoprophylactic effects of *Lm*SEAgs in vaccination against other forms of leishmaniasis including visceral and mucocutaneous leishmaniasis.

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Appendix

KENYA MEDICAL RESEARCH INSTITUTE (KEMRI) LEISHMANIA LAB

STANDARD OPERATING PROCEDURE (S.O.P)\

PROJECT TITLE: Determination of the adjuvant potential of Bacille Calmette Guérin with culture derived *leishmania major* soluble exo-antigen.

PURPOSE: Preparation of *Leishmania major* soluble excreted/secreted (exo) antigens *Lm*SEAgs for immunization of mice.

PROTOCAL

1. Grow Leishmania promastigotes in normal supplemented media (Shneiders's Drosophila Insect medium supplement with 20% FBS- Heat inactivated) at 26%C to mid log at a density of 10^9cells/ml.
2. Pellet parasite and wash 6 times in XOM medium.
3. Resuspend in XOM to a final density of promastigotes/ml.
4. Incubate at 26°C in a roller tube with 0.01% Tween 80 (Sigma) for 72 h
5. Centrifuge at 9000 r. p. m for 30 min and collect supernatant.
6. Determine relative protein concentration of soluble antigens at OD 280nm Store at 4^0C

NB medium consist of RPIM 1640 consisting of D-xylose at 0.076mM, Hepes buffer at 25mM, L- glutamine and sodium bicarbonate at 30mM without phenol red.

Title: Cell cultures for proliferation and cytokine assays

Objective: To determine cell proliferation and cytokine production by proliferated cells

Materials and methods

Requirements:

-Immunized mice (Two each from groups immunized LmSEAgs. LmSEAgs+BCG, BCG alone and phosphate buffered saline (PBS).

-Sterile wire mesh

-Sterile RPMI wash media

-Sterile complete RPMI supplemented with 5% FBS

-Sterile Petri dishes

-Ficoll-paque

-Sterile syringe plunger

-Sterile 15 ml centrifuge tubes

-Microscope

-Haemocytometer

-Trypan blue

- 96 well plates

-Soluble *Leishmania* antigen 100 µg/ml

-Concanavalin A mitogen (10 µg/ml).

Procedure

a) Preparation of splenocytes

1. Sacrifice two BALB/c mice from each experimental group by CO_2 asphyxiation
2. Excise spleens aseptically through a fine wire mesh using a syringe plunger
3. Collect crushed spleen into sterile Petri dishes containing 5-7 m RPMI wash media

4. Allow cells to stand in ice for 5-10 min for sedimenting of clumps

5. Transfer the upper portion of the medium containing the splenocytes using 1ml pipette, to a 15 ml sterile centrifuge tube.

6. Wet walls of a 15ml centrifuge tube with RPMI wash containing 5% FBS.

7. Remove RPMI and add 3 ml of thoroughly well mixed Ficoll-Paque™ PLUS to a 15 ml tube (mix Ficoll-Paque™ PLUS (Ficoll) thoroughly before use by inverting the bottle several times).

8. Carefully layer cell suspension (max of 500 x 10^6 cells per gradient in 5ml of wash containing 5% FBS) over Ficoll being careful to minimize mixing with Ficoll.

9. Centrifuge the cell suspension at 400 X g for 30 min and wash cells once again in RPMI medium.

10. Remove and discard upper plasma layer without disturbing the Plasma-Ficoll interface

11. Remove and retain lymphocytes at the plasma-Ficoll interface without disturbing the erythrocyte/granulocyte pellet.

12. Wash mononuclear cells once with medium at between 1500-2500 rpm.

13. Count cells using a small aliquot of cell suspension in Neubeur chamber and Trypan blue exclusion.

b) Dispensing and culturing of lymphocytes

1. Make cell suspension containing 2 x 10^6 viable cells/ ml. in culture media containing 5% FBS

2. Dispense the cells at the rate of 100 µl per well in 96 well flat bottomed tissue culture plates in triplicate for each of the experimental groups

3. Culture cells from each experimental group with either 10 µg/ml of 100 µl of Con A, 100 µg/mL of *Lm*SEAgs and medium alone.

4. Incubate cells in a humidified CO_2 incubator (5%) at 37 ^{0}C for 48h.

5. Harvest supernatants from each of the triplicate wells of Con A, *Lm*SEAgs and medium alone for each of the experimental groups and pooled. They were then stored at --20 ^{0}C till assayed using a flow cytometer (FACSCalibur^{TM}).

c) Cell proliferation assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

1. Add 100 µl of 5 mg/ml of MTT solution to all wells of 48 h cultured lymphocytes and incubate for 4 h at 37 ^{0}C (formazan crystals will be formed at the bottom of each well).

2. Pipette out 100 µl of the spent medium along with suspension of cultured wells

3. Add 100 µl of dimethyl sulfoxide (DMSO) to all wells and pipette thoroughly to dissolve the dark blue crystals.

4. After a few minutes read the plates at room temperature using an ELISA reader at 540nm wavelength

5. Read plates normally within 1 hour of adding DMSO

6. Calculate cell proliferation as stimulation index

$$\text{Stimulation Index} = \frac{\text{A540 nm Stimulated lymphocytes}}{\text{A540 nm unstimulated lymphocytes}}$$

Where A540 = Absorbance at 540 nm