Host and Parasite Factors that Regulate Secondary Immunity to Experimental Cutaneous Leishmaniasis

By

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ABSTRACT

Despite many years of intensive research, cutaneous leishmaniasis, one of the neglected tropical diseases, remains a global public health problem with no effective licensed vaccine for use in humans. Therefore, there is need to better understand the factors that regulate effective immunity to the disease, which will aid the development of an affective vaccine, vaccination and treatment strategies. The overall aim of this thesis is to decipher host factors that regulate the development and maintenance of immunity to cutaneous leishmaniasis.

Previous studies showed that $CD8^+$ T cells play a role in optimal primary immunity to low dose *L. major* infection. However, whether the initial parasite dose used during primary infection affects the overall T cell response as well as whether $CD8^+$ T cells contribute to secondary immunity following recovery from low dose infection is unknown. In the first part of this thesis, I show that low and high dose infections induced significantly more $CD8^+$ and $CD4^+$ T cells responses, respectively. Interestingly, although $CD8^+$ T cells were important for the development of optimal primary immunity to low dose infection, they where completely dispensable during secondary immunity suggesting that once protective primary immunity is developed, $CD8^+$ T cells are no longer necessary for its maintenance and/or for protection against subsequent virulent challenges.

We previously showed that LIGHT, (*lymphotoxin like, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes*) is important for initiation of IL-12-dependent optimal primary immunity to *L. major* in mice. However, the role of LIGHT in dendritic cell (DC) activation and IL-12 production as well as its role in secondary immune response to *L. major* infection is not known. I hypothesized that LIGHT plays a critical role in DC maturation and hence

optimal secondary immunity to *L. major*. In the second part of this thesis, I characterized the contribution of LIGHT in DC maturation, initiation and maintenance of primary immunity and secondary immunity to *L. major*. I showed that blockade of LIGHT interaction with its receptors significantly impaired DC maturation, expression of co-stimulatory molecules, and early cytokine production (IL-12 and IFN-γ) following *L. major* infection. However, I found that contrary to my hypothesis, LIGHT was completely dispensable during secondary immunity in WT mice. Interestingly, my studies revealed a critical redundant role for LIGHT for maintenance of IL-12 production and secondary immunity in the absence of CD40 signalling.

Although CD40-CD40L interaction is critical for IL-12 production and development of Th1 immunity, its role in the development of protective anti-*Leishmania* immunity is still controversial. The third part of this thesis I compared disease progression and immune response in CD40 and CD40L deficient mice infected with *L. major* under identical experimental conditions. Opposite to my hypothesis, I found significant differences in disease progression and immune response in rIL-12 treated CD40 KO and CD40L KO mice infected with *L. major*. While IL-12 treatment leads to lesion resolution and sustained resistance in CD40 KO, disease reactivation occurred in CD40L KO following cessation of IL-12 treatment. I discovered that in the absence of CD40, IL-12 production was maintained via the interaction CD40L and Mac-1, thereby revealing a crucial but redundant role for Mac-CD40L interaction in sustained IL-12 production and maintenance of immunity against *L. major*.

Collectively, the results from this thesis provide novel insights into the mechanisms involved in the development and maintenance of protective immunity against cutaneous leishmaniasis, which could aid the development of a more efficient and effective immunotherapeutic and/or vaccination strategies against the disease.

DEDICATION

This thesis is dedicated to the strongest, intelligent and compassionate woman I know: my one and only sister Obioma as well as the most open minded, loving and supportive husband any woman could ever ask for: my lovely husband Ashu-Oben.

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ABBREVIATIONS

Acronym	Definition
AIDS	Acquired Immune Deficiency Syndrome
AmphB	Amphotericin B
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guerin
BMDC	Bone Marrow Derived Dendritic Cells
BMDM	Bone Marrow Derived Macrophages
BSA	Bovine Serum Albumin
BTLA	B and T Lymphocyte Attenuator
CCR7	Chemokine Receptor seven
CD103	Cluster Differentiation one hundred and three
CD11b	Cluster Differentiation eleven b
CD11c	Cluster Differentiation eleven c
CD3	Cluster Differentiation three
CD4	Cluster Differentiation four
CD40	Cluster Differentiation forty
CD40L	Cluster Differentiation forty Ligand
CD45	Cluster Differentiation forty-five
CD62L	Cluster Differentiation sixty-two Ligand
CD8	Cluster Differentiation 8
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CL	Cutaneous Leishmaniasis
CPB	Cysteine proteinase B
CpG	Cytidine-phosphateguanosine
CR3	Complement Receptor three
DC	Dendritic Cell
DCL	Diffuse Cutaneous Leishmaniasis
DHFR-TS	Dihydrofolate Reductase-Thymidylate Synthetase
dLNs	Draining Lymphnodes
DNA	Deoxyribonucleic Acid
DNT	Double Negative T Cells
DTH	Delayed Type Hypersensitivity
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GMCSF	Granulocyte Monocyte Colony Stimulating Factor
GP63	Glycoprotein sixty-three
HEV	High Endothelial Venules
HIV	Human Immunodeficiency Syndrome
HVEM	Herpes Virus Entry Mediation
HVEM-Ig	Herpes Virus Entry Mediator –Immunoglobulin
IFN-γ	Interferon gamma

IL-10	Interleukin ten
IL-10 R	Interleukin ten Receptor
IL-12	Interleukin twelve
IL-13	Interleukin thirteen
IL-4	Interleukin four
iNOS	Inducible Nitric Oxide Synthase
KMBA	Killed But Metabolically Active
KMP11	Kinetoplastid Membrane Protein eleven
KO	Knock Out
L-NIL	L-N- (1-iminoethyl)- Lysine
LACK	*Leishmania* Homolog of Receptors for Activated C-kinase
LCL	Local Cutaneous Leishmaniasis
LIGHT	Lymphotoxin like Exhibits Inducible Expression Competes with HSV Glycoprotein D for HVEM, a Receptor Expressed by T Cells
LmST11	*Leishmania major* Stress Inducible Protein 1
LPG	Lipophosphoglycan
LPG2	Lipophosphoglycan two
LPS	Lipopolysaccharide
LST	Leishmanin Skin Test
LTß	Lymphotoxin Beta
LTßR-Ig	Lymphotoxin Beta Receptor –Immunoglobulin
mAbs	Monoclonal Antibodies
Mac-1	Macrophage 1 antigen
MBCL	Methylbenzethonium Chloride
MCL	Mucocutaneous Leishmaniasis
MFI	Mean Flourescence Intensity
mg	Milligram
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
ml	Millilitre
mM	Millimolar
MPL-SE	Monophosphoryl lipid A
mRNA	Messenger Ribonucleic Acid
ng	Nanogram
NHP	Non-Human Primate
NK	Natural Killer
NKT	Natural Killer T
NO	Nitric Oxide
ODN	OligoDeoxynucleotide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PKDL	Post Kalazar Dermal Leishmaniasis
PLGA	poly (D,L-lactide-co-glycolide)
PM	Paromomycin
PMSF	Phenylmethylsulfonyl Fluoride
rGP63	Recombinant Glycoprotein sixty-three

rIL-12	Recombinant Interleukin twelve
sCD40L	Soluble Cluster Différentiation forty Ligand
SCID	Severely Compromised Immuno Deficient
SD	Standard Deviation
SLA	Soluble Leishmania Antigen
SMT	Sterol 24-c-Methyltransferase
SSG	Sodium Stibogluconate
Tcm	Central Memory T Cells
Tem	Effector Memory T Cells
TGFß	Transforming Growth Factor Beta
Th1	T Helper one
Th2	T Helper two
TNFR 1	Tumour Necrosis Factor Receptor one
TNFR 2	Tumour Necrosis Factor Receptor two
TNFSF	Tumour Necrosis Factor Superfamily
TNFSFR	Tumour Necrosis Factor Superfamily Receptor
Tregs	Regulatory T Cells
T_{RM}	Resident Memory T Cell
TSA	Thiol-Specific Antioxidant
VL	Visceral Leishmaniasis
WHO	World Health Organization
WT	Wild Type
αβ TCR	Alpha Beta T Cell Receptor
γδ TCR	Gamma Delta T Cell Receptor
µg	Microgram

1. INTRODUCTION

1.1 Epidemiology of Leishmaniasis

The spectrum of disease known as leishmaniasis continues to pose a major public health problem worldwide. It is one of the neglected tropical diseases caused by different species of the protozoan parasite *Leishmania* [1]. Current estimates show that leishmaniasis affects 12 million people with 310 million people at risk of infection with an annual global incidence of 0.2-0.4 million cases of visceral leishmaniasis (VL) and 0.7 to 1.3 million cases of cutaneous leishmaniasis (CL) [2]. Leishmaniasis is endemic in 98 countries and greater than 90% of visceral leishmaniasis (VL) occur in poor rural and sub urban areas in six countries: Bangladesh, Ethiopia, Brazil, India, Sudan, and South Sudan. Unlike VL, CL is more wide spread and occurs in the Americas, the Mediterranean and Western Asia and 70 to 75% of global CL occur in ten countries: Afghanistan, Algeria, Brazil, Iran, Peru, Ethiopia, Northern Sudan, Costa Rica, Colombia and Syria [2]. The fact that leishmaniasis occurs as syndromes [1] coupled with lack of surveillance and vital records reporting in the countries most affected by the disease [2] limits the determination of actual burden of the disease. As such the current estimates of disease burden may be under reported and does not reflect the true impact of the diseases [1, 2].

High morbidity and low mortality of infectious diseases are well recognized determinants of poverty [3]. Leishmaniasis is ranked as one of the most neglected tropical diseases and has strong links with poverty [4] and together they create a mutual vicious cycle [3]. Compared to other diseases, treatment for leishmaniasis is very expensive ranging from 30 to 1500 USD for the drugs alone and this is considered a major contributor to poverty of the affected individuals [5]. Visceral leishmaniasis is a problem in immune-compromised people and in the early 1990s

Leishmania/HIV co-infection was recognised as a major health problem. The incidence of HIV/*Leishmania* co-infection has been on the increase since the worldwide epidemic of HIV infection especially in the south-western parts of Europe [6] where leishmaniasis is now recognized as one of AIDS defining diseases in HIV-infected people [7, 8]. Since 1994 the incidence of *Leishmania*/HIV co-infection has been monitored by a surveillance network consisting of 16 institutions [9] in four countries including France, Italy, Portugal and Spain [10] and cases of HIV/*Leishmania* co infection have been reported from 35 countries around the world [11].

1.2 Vector, Parasite and Transmission Cycle

The phlebotomine Sandflies are small insects found mostly in the tropics and subtropics. Out of the 500 known *Phlebotomine* species only about 30 have been positively identified as vectors of human leishmaniasis [12]. Females of the two Sandfly sub genera; *Phlebotomus spp.* and *Lutzomyia spp* are the main vectors of leishmaniasis in the Old and New World respectively [13]. Female sand flies are hematophagous and need blood meal for egg development every 4-5 days [14]. They get infected as well as transmit the parasite during feeding. In the sandfly vector, *Leishmania Spp* exist as extracellular flagellated spindle shaped organism known as promastigote, which develop in close approximation to the endothelial cells of the insect midgut [15] and during feeding, infected flies inject saliva and parasites into the host [14]. Once inside a host, the parasites are taken up by antigen presenting cells (APCs) such as epidermal macrophages or dendritic cells (DCs) and transforms into non-flagellated, non-motile and round amastigote form in the phagolysosome [16]. Inside the cell parasites replicate and rupture the cell to release daughter parasites. The released parasites are in turn taken up by other uninfected APCs and/or the vector during feeding on an infected person. If taken up by the Sandfly vector,

the amastigotes transforms into the, flagellated, motile and spindle shaped promastigotes in the insect mid gut thus completing the life cycle [17, 18].

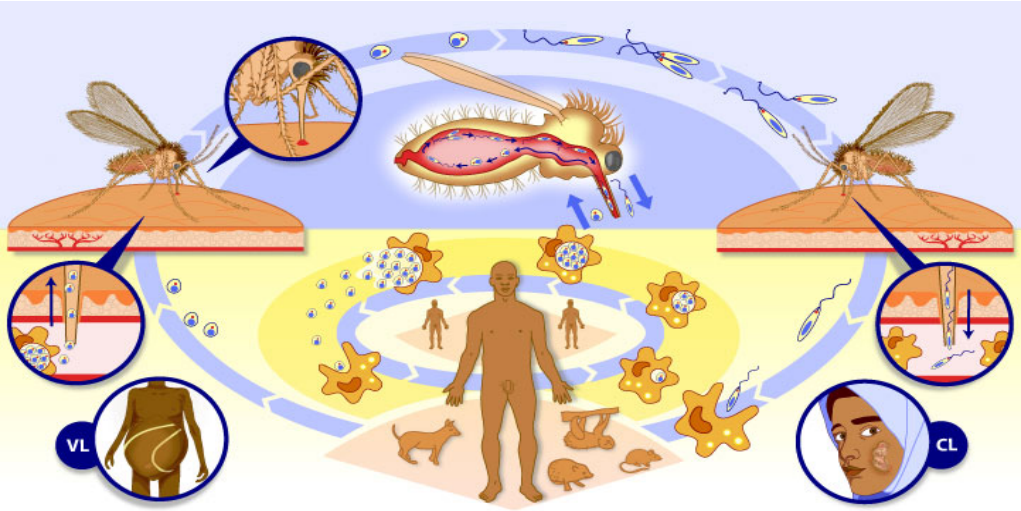

Figure 1. *Life Cycle of Leishmania parasites.* Sandfly vector takes a blood meal from an infected person or dog and the parasites transforms into promastigotes within the Sand flies. Inside the host, promastigotes are then taken up my antigen presenting cells where they multiply and rupture the cells and can be taken up by other antigen presenting cells and /or Sandflies. *Public Domain Material from: www.liquidjigsaw.com/science/illustration/index.html*

1.3 Disease Classification

There are about thirty species of *Leishmania* parasites that cause disease in both humans and animals [1]. Clinical leishmaniasis occurs as a spectrum of diseases depending on the infecting parasite specie, host and environmental factors. Host factors such as polymorphism in genes that regulate immune response has been shown to influence the outcome of human cutaneous leishmaniasis (CL) [19] and parasite factors such as expression of certain molecules have been shown to affect the tissue tropism of *Leishmania* parasites. For example, the expression of A2 protein in *L. major* increases its ability to colonize and infect visceral organs [20].

There are four main forms of the diseases.

1.3.1 Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis is caused by *Leishmania* parasites in the *L. mexicana* complex such as *L. major*, *L. tropica*, *L. aethiopica* in the old world and *L. venezuelensis*, *L. mexicana*, *L. amazonensis* in the new world [19]. CL is the most common form of leishmaniasis representing 50-75 % of all new cases. An estimated 0.7 million to 1.3 million cases occur annually with 95% of CL occurring in the Americas, the Mediterranean Basin, the Middle East and Central Asia [21]. Most importantly, the incidence of CL has increased in war veterans serving in endemic areas . In line with this CL have been diagnosed in American [22], Dutch [23] and Canadian [24] soldiers returning from active duty in Iraq and Afghanistan.

Following the bite of an infected Sandfly, a small erythema develops at the bite site. This later 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 cutaneous leishmaniasis [25]. These skin lesions are usually self-limiting/self-healing within a few months of infection [26] with lifelong

protection to re-infection. However, infected individuals develop of lifelong cutaneous scar, which, depending on its size and location, may cause substantial psychological trauma in affected individuals [27].

1.3.2 Diffuse Cutaneous Leishmaniasis (DCL)

DCL lesion is the parasite-laden, non-ulcerative nodule that disseminates from the initial site of infection and may cover a patient's entire body [25]. In comparison to the CL, DCL is difficult to treat and patients do not self-cure. This form of the disease is seen in individuals with defective cell mediated immune response and it is subject to relapse after treatment [28].

1.3.3 Mucocutaneous Leishmaniasis (MCL)

Almost 90% of MCL occurs in Bolivia, Brazil and Peru [21] and is mostly caused by parasites from the *Leishmania Viannaia* subgenus such as *L. guyanensis, L. panamensis* and *L. braziliensis [29]*. Host factors such as ability of the infected individual to mount an inflammatory response influences whether an infected individual will develop CL or MCL: patients that are unable to express inflammatory genes were more likely to progress to MCL after infection with *L. braziliensis* [30]. Parasite factors such as its ability to metastasize has also been linked to the development of MCL which has been linked to the parasite's ability to survive oxidative stress [31] as well as high *Leishmania* RNA virus burden [32]. Mucocutaneous leishmaniasis typically begins with nasal inflammation and stuffiness (i.e. mild mucosal leishmaniasis), followed by ulceration of the nasal mucosa and perforation of the septum. In some cases, the lips, cheeks, soft palate, pharynx, or larynx are also involved (i.e.severe mucosal

leishmaniasis). There are extensive and disfiguring lesions of the mucous membranes of the nose, mouth and throat cavities due to the ability of the parasites to metastasize to mucous tissues by lymphatic or haematogenous dissemination [28, 33, 34]. Mucosal leishmaniasis never heals spontaneously is very difficult to treat and because secondary bacterial infections common, is potentially fatal [35]. In most endemic areas, 1–10% of CL infections result in mucosal leishmaniasis 1–5 years after LCL has healed, [35, 36] but reports do exist for which MCL presented at the same time as LCL [37].

1.3.4 Visceral Leishmaniasis (VL)

Visceral leishmaniasis is caused by *L. donovani*, *L. chagasi*, and *L. infantum* (*L. donovani* complex) and infection with these parasites is almost always associated with visceral disease with no cutaneous lesion in immunocompetent people [38]. The parasites in the *L. donovani* complex can invade internal organs and cause VL which is dependent on thermotolerance [39] due to the expression of A2 family of proteins that has been shown to protect against heat stress in the in the internal organs [20]. Visceral leishmaniasis has an annual incidence of 200, 000 - 400,000 world wide and is endemic in the Mediterranean, Central Asia, China and also in Latin America. 90% of new cases occur in Bangladesh, Brazil, India, Ethiopia, South Sudan and Sudan [21]. VL is the most dangerous form of leishmaniasis and is almost always fatal if left untreated. Following an incubation period that generally lasts between 2 and 6 months, VL patients present symptoms and signs of persistent systemic infection (like fever, fatigue, weakness, loss of appetite and weight loss) as well as parasitic invasion of the blood & reticulo-endothelial system; lymph nodes, spleen and liver. Patient develops fever rigor, chills, fatigue which is caused by

the persistent inflammatory state. After recovery some patients develop chronic skin disease known as the post kalazar dermal leishmaniasis (PKDL) [40, 41]. PKDL is a complication of VL that is frequently observed after treatment in Sudan and more rarely in other East African countries and in the Indian subcontinent [41]. The interval between treated VL and PKDL is 0–6 months in Sudan and 6 months to 3 years in India. PKDL cases are highly infectious because the nodular lesions contain many parasites and such cases could be the putative reservoir for anthroponotic VL between epidemic cycles [42].

1.4 Diagnosis

A clinical diagnosis of leishmaniasis may be possible in endemic areas or where there is an obvious travel history in non-endemic areas. Definitive diagnosis is almost always done by laboratory confirmation due to the broad differential diagnosis as well as the potential for the use of very toxic drugs [43].

1.4.1 Microscopy

Microscopy is used to look for the presence of *Leishmania* amastigotes in fluids, scrappings or biopsies from patients. *Leishmania* amastigotes can be visualized under the microscope in skin and bone marrow biopsy samples from patients with cutaneous and visceral leishmaniasis respectively [44]. The sensitivity of microscopy as a diagnostic tool range from 60-85% and it may produce non-specific results. However, it may be helpful in differentiating CL lesions from other similar clinical presentations [45] such as impetigo, dermatophyte and cutaneous mycobacterial infections [46]

1.4.2 Molecular Diagnosis

1.4.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) tests can be performed on aspirates, scrapings, biopsy smears, and fresh or preserved skin biopsies [44]. Detection of parasite DNA in lesion material by PCR is usually very sensitive in the diagnosis of both cutaneous and mucosal leishmaniasis [47, 48]. The latest DNA extraction methods and real-time PCR tests are increasingly automated and can generate results within a few hours [48]. This assay has revolutionized the diagnosis of CL with a high sensitivity, the detection of parasite ribonucleic acid (RNA) or DNA, with great specificity and may enable a species-specific diagnosis to be made [45].

1.4.2.2 *Leishmania* Antigen Enzyme Linked Immunosorbent Assay (ELISA).

In order to develop non-invasive and relatively easy methods for the diagnosis of leishmaniasis, ELISA for the detection of secreted *Leishmania* antigens in the urine of infected individuals have been developed with very high specificity and varying degrees of sensitivities ranging from 47% to 95% [49-51]. A Recent study by Abeijon et al [52] showed that the sensitivity of the assay can be improved by combining three *Leishmania* antigens with detection limit of 4-10 pg per ml of urine. The ELISA assay presents a simple, easy, non-invasive diagnostic methods that can be used in endemic regions.

1.4.3 Culture of *Leishmania* Parasites

The culture of *Leishmania* parasites can be performed from aspirates, scrapings, and fresh skin biopsies. This remains an important method because it is the only way to obtain enough parasite material to perform subspecies identification by either zymodeme (isoenzyme) analysis or DNA sequencing [45].

1.5 Treatment

It has been recognized that development of drugs is the first step in the control of neglected tropical diseases including leishmaniasis [5]. Current treatment regimens for leishmaniasis have many problems such as high cost, high toxicity, development of resistance, relapse and poor compliance due to painful injections for long durations and sometimes scarcity of drugs [53]. There has been at least one reported instance where hundreds of patients were treated with fake drugs in Bangladesh [54]. As such there is need for the development of new, effective and cost effective drugs as well as development and implementation of policies to ensure availability of good quality drugs.

1.5.1 Pentavalent Antimonials:

Meglumine antimoniate and sodium stilbogluconate are pentavalent antimonials commonly used for the treatment of leishmaniasis [55]. Although the mechanism of action remains unclear, it is believed that the pentavalent form of the drug is reduced to the active trivalent form inside the macrophage by using both host and parasite thiols [56]. In addition to its leishmanicidal activity, antimonials have the ability to modulate host immune response by enhancing conditions that favour killing of intracellular parasites by macrophages such as proinflammatory cytokine, superoxide and nitric oxide production as well as phagocytosis [57-59].

For treatment of CL intralesional injections of antimonials are typically administered at 1-5 ml/session every 3-7 days [60]. The disadvantage of this treatment is that it is long, painful and problematic in children with facial lesions [61]. For VL and severe CL, antimonials can be

injected systemically at a dose of 20mg/kg for 30 days. The systemic administration of these drugs is often associated with side effects such as renal insufficiency and electrolyte abnormalities in patients receiving these treatments [61]. A study in a cohort of children receiving antimonials in Brazil reported up to 15% rates for cardiac, hepatic and pancreatic toxicity [62]. Also strains of *Leishmania* that are resistant to pentavalent antimonials have been reported. Factors such as decreased drug intake, increased efflux mechanism, inhibition of drug activation have been associated with development of resistance [55].

1.5.2 Amphotericin B (Amph B)

Amphotericin B deoxyclolate is most commonly used for the treatment of VL. Amph B exerts its action by first binding to cholesterol and creating holes on host cells leading to leakage of cellular materials and eventually cell death [55]. However, immunomodulatory properties like enhancement of T cell proliferation and proinflammatory cytokine production have been reported [63, 64]. AmphB has excellent leishmanicidal activity with cure rates between 90-95%. The major disadvantage of this drug, is that it must be given in a hospital setting as well as the high nephrotoxicity rates [65]. The development of the lipid formulations of Amph B increased the safety of the medication [66]. To date, there are three formulations of lipid AmphB: amphotericin B lipid complex (Abelect^{R}), Amphhotericin colloidal dispersion (Amphocil^{R}) and liposomal amphotericin B (AmBisome^{R}). Data suggests that liposomal amphotericin B is the least toxic and is also the most studied of all three formulations for the treatment of VL [66, 67]. Dosing regimen range from a single dose of 10mg/kg [68] to 18-21mg/kg/day for 5-7 days [69]. The limiting factor to the wide spread use of this medication is cost [70] and differences in the response and curates depending on the region and predominant parasite specie endemic in the

region. For example, AmBisome^{R} is most effective in the treatment of VL caused by *L. donovani* in India and Ethiopia but was not very effective against VL caused by *L. infantum* in South America [71].

1.5.3 Miltefosine and Paromomycin

Miltefosine is the first oral drug for the treatment of VL. The mechanisms of action is not clear however, it is thought to disrupt the akyl-lipid metabolism leading to apoptotic cell death [55]. It has great leishmanicidal activity with 90 - 94% cure rates in Indian VL and PKDL patients [72-74]. Miltefosine is the first line drug for the treatment of VL in India [55]. Interestingly, studies carried out in Guatemala and Colombia showed that in the treatment of CL, the cure rates varied from 53% in Guatemala to 66% - 95% in Colombia [75]. More recently a phase III clinical trial reported that the efficacy of miltefosine against CL was 58.6% at a dose of 150mg/day for 28 days.

Paromomycin (PM): is a broad spectrum antibiotic that acts by inhibiting parasite protein synthesis [55]. It has been reported to be effective in the treatment of new infection of VL as well as treatment of simple CL. [76-78]. In controlled trials, paromomycin given together with methylbenzethonium chloride (MBCL) resulted in cure rates of 20% to 93% [79]. Later studies of 15% paromomycin with 12% MBCL applied twice daily for 20 to 30 days have shown cure rates of 79% to 86%. More recently, two studies tested a combination of paromomycin and gentamicin for the treatment of CL: one study conducted in Tunis, showed comparable curates in the patients treated with paromomycin plus gentamicin and paromomycin alone [80]. Interestingly, the second study conducted in Panama showed that a combination of paromomycin and Gentamicin was better than paromomycin alone in patients with CL [81].

1.5.4 Physical Therapy

Non-pharmacological therapies such as cryotherapy or thermotherapy have been used for the treatment of simple CL lesions.[82-84]. Reithinger and colleagues [29] demonstrated that a single treatment with accurately measured localized heat is as effective as the administration of intra-lesional sodium stibogluconate (SSG) and more effective than the administration of intramuscular SSG for the treatment of CL due to *L. tropica*. The time to cure was shown to be shorter with thermotherapy than with SSG regimens. The use of heat delivered in form of radio frequency has been tested for the treatment of human CL [81, 85, 86] and canine leishmaniasis [87]. It was shown to elicit systemic cytokine response [86] and is effective in individuals co-infected with HIV [88]. A recent paper explored the use of tattooing technology as a drug delivery system for the treatment of CL by targeting drugs directly to the dermis. In this experimental study, several daily-tattooing regimens as a delivery vehicle for a test drug in liposomes led to complete lesion clearance compared to either topical or intraperitoneal methods of administration [89]. Although this is a proof of concept study, the use of tattoo technology for drug delivery is advantageous since it is relatively easy to use and has been used in the cosmetics industry for a long time. The limited availability of equipment and expertise plus concerns over altered pigmentation, secondary infection, dissemination of parasites, slower healing, larger scars, and poor compliance have limited their acceptance.

1.6 Animal Models for Cutaneous Leishmaniasis

Animal models have been and continue to be used for immunologic studies, drug testing and vaccine development. The main prerequisite for choosing any animal model to study any disease is physiology, availability, ease of handling and cost. Models ranging from rodents, dogs and non-human primates have been used for experimental leishmaniasis. However, hamsters and mice are the two extensively studied animal models for studying the outcome of infection and chemotherapy in leishmaniasis [90].

1.6.1 Inbred Mouse Model

In the past four decades, mice models have been used to extensively study CL in order to understand signal transduction, primary and secondary effector mechanism in the control of experimental leishmaniasis [91]. C57BL/6 and BALB/c mice are resistant and susceptible to *Leishmania major* infection, respectively. Susceptibility in the BALB/c mice has been associated with the development of progressive non-healing lesion with Th2 response characterised by extensive production of interleukin 4 (IL-4), interleukin 10 (IL-10) [92, 93] and transforming growth factor beta (TGF-β) [94, 95]. In contrast, the C57BL/6 mice resolve lesions through the production of Th1 cytokines like interferon-gamma (IFN-γ), interleukin 12 (IL-12) and tumour necrosis factor (TNF) [96-98]. The use of mice in biomedical research is advantageous because they are readily available, easy to house and manipulate. Also since they have identical genetic backgrounds, it allows for the collection and combination of data over time thus allowing for in depth characterization of phenotype [99]. Different mice models have contributed immensely in our understanding of the host pathogen interaction in CL. However, due to the inherent

differences between humans and mice, results from mice studies are hard to extrapolate into humans [99].

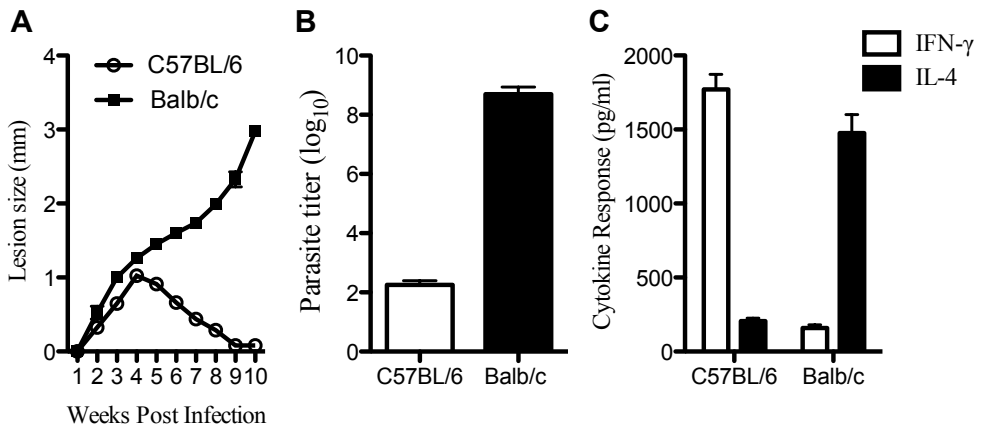

Figure 2. Typical disease development and immune response in resistant (C57BL/6) and susceptible (Balb/c) mouse strains infected with Leishmania major. Lesion development (A), parasite burden (B) and cytokine respnse (C) in C57BL/6 and Balb/c mice mice infected with *L. major*.

1.6.2 Non-Human Primates (NHP)

Non-Human primates are typically used for *Leishmania* vaccine efficacy studies [99]. Rhesus Macaques infected with *L. major* develop cutaneous lesions that ulcerate and spontaneously heal within three months just like in humans and develop varying levels of resistance following homologous reinfection [100]. This specie of Macaque seems to be a good model for the study of CL since they develop clinical disease and histopathological signs that are commonly observed in humans. However cost, availability and difficulty in handling hamper their wide spread use in biomedical and *Leishmania* research [99].

1.6.3 Wild Rodents

The use of wild rodents in the study of diseases is especially advantageous because unlike their inbred counterpart, they have genetic polymorphism because they are outbred like humans. Also some of the wild rodents represent natural reservoirs for diseases and understanding the host-pathogen interaction that occur in these natural reservoir is important in understanding the mechanism involved in maintaining a non pathogenic natural infections [99]. *Peromyscus yacatanicus* a primary reservoir for the causative agent of CL *L. mexicana* has been adapted to laboratory conditions and was infected with *L. mexicana.* Infected mice developed single ulcerated lesion with chronic inflammatory response similar to those observed in humans [101]. Interestingly and in contrast to result from inbred mice, nitric oxide (NO) produced in *L. mexicana* infected *Peromyscus yacantanicus* was not able to clear infection [102]. More studies are needed to further characterize this model of experimental leishmaniasis.

1.7 Immunity to Cutaneous Leishmaniasis

Over the years, a lot of studies (*in vitro* and *in vivo)* have been carried out in CL with the sole aim of understanding the correlates of immunity to the disease [103]. These studies show that both the innate and adaptive immune systems have roles to play in the control of CL. They also showed that cell mediated immunity is more critical for the control of CL since *Leishmania* is an obligate intracellular parasite and the induction and maintenance of Th1 response is required for the effective clearance of *Leishmania* parasites [104]. The early Th1 response is initiated by IL-12 produced by dendritic cells, which activates IFN-γ producing T cells. The IFN-γ produced by T cells further activates macrophages to produce NO that is responsible for killing the parasites within macrophages. On the other hand, the induction of a Th2 response provides an environment that is conducive for deactivation of macrophages leading to the survival of the *Leishmania* parasites located within the macrophages [93, 105].

1.7. 1 Role of T Cells

1.7.1.1 CD4 Helper T cells and Resistance to Cutaneous Leishmaniasis

Following the identification of distinct mouse $CD4^+$ T helper cell subsets by Mossman and Coffman [106], it was demonstrated that IFN-γ production by $CD4^+$ T cells was associated with healing of *L. major*-infected C57BL/6 mice, while IL-4 production was associated with susceptibility in the BALB/c mice [105]. Scott *et al.* [107] demonstrated that adoptive transfer of polarized T cell clones can change the outcome of *L. major* infection: Th1 clones were "protective" while Th2 clones were "non-protective" [107]. Holaday and colleagues [108] further confirmed this finding by transferring Th1-like or Th2-like cell lines into SCID mice,

which resulted in the recipient mice becoming resistant or susceptible, respectively. Thus, the balance of Th1/Th2 cytokines determines disease outcome in mice model of cutaneous *leishmaniasis*: healing in resistant mice is associated with the development of $CD4^+$ Th1 cells that produce IFN-γ [109] whereas early production of IL-4 by $CD4^+$ T cells promotes the development and expansion of Th2 cells in the susceptible mice [15, 110-112]. A recent study by Lazarski et. al [113] showed that in addition to orchestrating the development of early Th2 response, IL-4 also affects the migration of immune cells to infection sites thus limiting host ability to clear parasites. Using MHC II tetramer technology, the trafficking of $CD4^+$ T cells after *L. major* infection in the resistant mouse strain was recently studied. The main finding from this study was that the peak expansion of antigen specific $CD4^+$ T cells was at 2 weeks post infection and they differentiated mostly in to Th1 cells due to their expression of the transcription factor T-bet as well as their ability to produce IFN-γ [114, 115].

Studies in humans with CL showed that $CD4^+$ T cells producing IFN-γ is critical for the activation of macrophages and killing of intracellular parasites [116]. In VL, $CD4^+$ T cells was shown to be critical for the development and maintenance of immunity in the liver of *L. donovani* infected mice as exemplified by enhanced disease in mice that were depleted of $CD4^+$ T cells. Even though it is known that $CD4^+$ T cells play an important in role in experimental leishmaniasis, there is little information regarding its role in human disease. A recent study investigated the role of $CD4^+$ T cells, in human CL; the depletion of $CD4^+$ T cells in the PBMC of VL patients led to significant reduction in level of IFN-γ suggesting that $CD4^+$ T cells were the main producers of soluble *Leishmania* induced IFN-γ in these patients. The neutralization of IFN-γ in *ex vivo* splenic aspirates led to increase in parasite load in 61% of the patients [117].

Furthermore, $CD4^+ CD25^- FoxP3^- CD127^{low}$ T cells were shown to be the main producers of IL-10 in patients with CL [118].

1.7.1.2 CD8 Helper T cells and Resistance to Cutaneous Leishmaniasis

The fact that activated $CD8^+$ T cells also produce IFN-γ (a critical macrophage activating cytokine for intracellular parasite killing), it was speculated that they would also contribute to optimal immunity to *Leishmania major*. Interestingly, older studies demonstrated that mice lacking $CD8^+$ T cells or MHC-class I expression were not impaired in their ability to control primary *L. major* infections [119, 120]. However, studies utilizing low dose intradermal infection that closely mimic natural infections show that $CD8^+$ T cells are important for anti-*Leishmania* immunity. Inoculation of low dose metacyclic promastigotes into the ear dermis of CD8 deficient C57BL/6 mice led to uncontrolled parasite proliferation [121, 122]. Also, low dose infection induced a transient Th2 response in naive WT mice which was sustained in the absence of $CD8^+$ T cells suggesting that the major role of $CD8^+$ T cells is to produce IFN-γ that down-modulates the early $CD4^+$ Th2 cell development [121]. Protection in CL caused by *L. major* was associated with the expansion of IFN-γ producing $CD8^+$ T cells [123]. Recently, cytotoxic $CD8^+$T cells were shown to be important in the efficacy of amphotericin B PLGA nanospheres against experimental VL [124]. Despite the protective role of $CD8^+$ T cell in murine CL studies, there is evidence suggesting that $CD8^+$ T cells also play a role in immunopathology in humans with CL. Studies show that the frequency of $CD8^+$ T cells was higher in ulcerated lesions and this higher frequency also correlated with higher intensity of inflammation in the lesions, indicating that $CD8^+$ cells that produce granzyme B may contribute to immunopathology in patients with CL [116, 125, 126]. Transcriptome analysis of patients with CL caused by *L. brazilensis* showed that

genes associated with cytolytic pathway was highly expressed and $CD8^+$ T cells present in the lesions exhibited increased cytolytic phenotype [30]. In murine model of *L. brazilensis*, it was shown that cytotoxic $CD8^+$ T cells were associated with the lesion metastasis, suggesting that cytolytic $CD8^+$ cells could mediate immunopathology and drive the development of the metastatic lesions in CL [127]. Taken together, the role of $CD8^+$ T cells in CL leishmaniasis remains unclear. Also it seems like two types of $CD8^+$ T cells can be induced during CL; IFN-γ producing Th1 cells and a cytolytic granzyme B^+ that mediate protection and immunopathology, respectively [128].

1.7.1.3 Double Negative (DN) T cells

Double negative T cells refer to a subpopulation of T cells that express CD3 but are negative for both CD4 and CD8. In both humans and mice, DN T cells represent 1-3% of total T cells [129, 130] but there is growing evidence to show that DN T cells are important players in different immune-mediated and infectious diseases [131]. Several studies have documented the expansion of $CD3^+CD4^-CD8^-$ (double negative, DN) cells in the PBMC of *Leishmania* patients [132] and dog [133] and in spleens of *L. major*-infected mice [134], where they were proposed to contribute to primary and vaccine-induced immunity [134, 135]. Studies showed that in human leishmaniasis, DN T cells were the predominant cell type producing large amount of IFN-γ following short-term stimulation of PBMCs with *Leishmania* antigens [132]. In humans with CL, two subpopulations of DN T cells have been identified in the PBMC; αβ TCR which represented 75% of the total DN T cell population and gamma/delta TCR representing 25% of total DN T cell population [136]. These αβ TCR DN T cells exhibited high level activation profile and produced large amounts of proinflammatory cytokines like IFN-γ and TNF [136, 137], while the

γδ DN cells produced more regulatory cytokine like IL-10. This suggests that DN T cells could play a role in parasite killing and protection as well as posses immunoregulatory functions [136]. The problem with the study of DN T cells is their small numbers (1-3% of total T cells) and lack of specific markers for their identification as such there is a possibility that double negative T cells are antigen activated T cells CD4 and/or CD8 T cells that have simply down regulated their expression of these molecules. Interestingly, a recent study by Mou et.al [137] showed that DN T cells do not express mRNA for CD4 and CD8 suggesting that DN cells are not T cells that down regulated their CD4 or CD8 expression. In addition, they demonstrated that DN T cells with innate-like gene signatures, restricted by MHC II are specific to *Leishmania* and play an important role in both primary and secondary anti-*Leishmania* immunity.

1.8 Role of Cytokines

1.8.1 Interleukin 12 (IL-12)

IL-12 was initially identified for its ability to stimulate natural killer cells. It is a heterodimeric cytokine made up of two subunits: a 40kDa and 35kDa chains. The physiological relevance of IL-12 in CL came from mouse studies that showed that resistant mice infected with *L. major* produced high levels of IL-12, activated NK cells and produced large amount of IFN-γ [138]. Treatment of susceptible Balb/c mice with rIL-12 at time of infection led to robust Th1 response and healing [139, 140] and the blockade of IL-12 or deletion of IL-12 in resistant mice strain led to susceptibility to *Leishmania* infection [141]. Further, the difference in the susceptible and resistant mice strains following infection with *Leishmania majo*r may be attributed to the differences in their ability to regulate IL-12 receptor expression and signaling. The resistant mice strain treated with anti-IL-12 mAbs maintained a population of $CD4^+$ T cells

that expressed the IL-12 receptor beta 1 and 2 and were able to produce IFN-γ, which was in contrast to the susceptible strain [142]. The ability of IL-12 to induce a robust Th1 response prompted studies to test whether IL-12 can be used as a vaccine adjuvant in experimental leishmaniasis. Thus Balb/c mice where vaccinated with *Leishmania* antigen with or without rIL-12. Mice vaccinated with rIL-12 were protected against virulent challenge which was associated with the early induction of IFN-γ producing NK cells (0-2 weeks post infection) followed by the induction of IFN-γ producing $CD4^+$ T cells later on in the infection (>2 weeks) [143]. IL-12 deficient mice treated with rIL-12 heal their lesion but will spontaneously reactivate disease when treatment is stopped, suggesting that IL-12 many be required for the maintenance of Th1 cell mediated immunity to *L. major [141]*. It was later confirmed that IL-12 is indeed required for the maintenance of established cell mediated immunity [144]. Recently, vaccination of Balb/c mice with *Lactococcus lactis* co-expressing LACK and IL-12 protected mice against virulent challenge exemplified by delayed lesion development and reduced parasite burden [145, 146]. The role of IL-12 in humans with leishmaniasis is not very clear. However, PBMCs from humans with active CL produced more IL-12 compared to individuals that have healed their lesions [147]. This could represent an attempt by the host immune system to fight the infection. Even though immunotherapy with IL-12 in different diseases showed very promising results in preclinical studies, treatment with recombinant IL-12 in different forms of human cancers not only demonstrated poor efficacy but also severe adverse effects [148]. Following, intravenous injection of recombinant human IL-12, toxicities such as fever, chills, nausea, fatigue were observed at 3ng/kg however, more severe side effects such as anemia, lymphopenia, neutropenia, thrombocytopenia, and elevated liver enzymes were observed at 1000ng/kg cancer patients [149]. In a subsequent study, 12 out of 17 patients developed severe side effects that required

hospitalization. The Food and Drug Administration subsequently stopped this study due to the death of two patients [150]. Based on subsequent studies the local administration of recombinant IL-12 in accessible tumors seems to be a more successful approach [151].

1.8.2 Interferon gamma (IFN-γ)

Interferon gamma is a critical cytokine for resistance to *Leishmania major* infection in mice because it plays a crucial role in macrophage activation and the production of leishmanicidal molecules. In addition, IFN-γ is also necessary for the suppression of Th2 cell development. A single injection of anti-IFN-γ antibodies to resistant mice 2 days prior to *L. major* infection resulted in enhanced Th2 response and increased susceptibility [152, 153]. In contrast, administration of recombinant IFN-γ at the time of infection of susceptible mice dramatically reduced lesion sizes and parasite burden [152]. Furthermore, IFN-γ or IFN-γ receptor deficient mice on the usually resistant C57BL/6 background develop progressive lesion associated with uncontrolled parasite proliferation after *L. major* infection [154]. The induction of IFN-γ producing T cells was associated with protection in mice vaccinated with live parasites or multiple doses of killed *L. major* parasites [155, 156]. In a recent study, Balb/c mice that received Influenza-LACK vaccine were protected against virulent challenge which was associated with increased numbers of IFN-γ producing $CD4^+$ T cells [157]. It is important to note the robust IFN-γ response in not always required to achieve protection. Vaccination with mutant *Leishmania* parasites (*lpg2)* induced protection that was not associated with enhanced IFN-γ response [158]. In line with this, it was reported that mice with a knock-in mutation in the P110 delta isoform of the P13 kinase were resistant to *L. major* infection with minimal IFN-ϒ response [159]. Interferon gamma producing CD4 T cells were detected in PBMCs of individuals that healed their cutaneous lesions [160]. The pattern of cytokine response in patients with local

cutaneous leishmaniasis revealed that early on in the infection, there is a mixed Th1 and Th2 response however this response switches to a predominant Th1 response characterised by high level of IFN-γ in older lesion [161]. It was not clear from this study if this increase in IFN-γ correlated with lesion resolution, however other studies reported high level of IFN-γ response in individuals that healed their disease compared to those with active disease [162, 163].

1.8.3 Interleukin 4 (IL-4)/ Interleukin 13 (IL-13)

In contrast to elevated IFN-γ level in *L. major*-infected resistant mice, high levels of IL-4 are found in infected susceptible BALB/c mice and is associated with disease progression. Administration of IL-4 neutralizing antibodies renders susceptible BALB/c mice resistant to *L. major* [164]. Surprisingly, IL-4 deficient BALB/c mice remain susceptible to *L. major* (LV39 sub-strain) infection [165], suggesting that another cytokine contributes to the susceptibility in BALB/c mice. Signaling through IL-4Rα subunit is shared between IL-4 and IL-13, and IL-13 is a major Th2 cytokine [166]. Studies show that IL-13 promotes disease in *L. major*-infected mice [167-170] by down-regulating macrophage function such as IL-12 [171], iNOS [172, 173], and TNF [174, 175] production. Interestingly, there is evidence that the role of IL-4 in CL depends on cell type, time and stage of immune response. Injection of IL-4 at 0-8hrs of infection with *L. major* led to increased IL-12 mRNA production in dendritic cells, enhanced Th1 response and protection BALB/c mice. On the other hand injection of IL-4 at 12-16 hours after infection (during T cell priming) lead to susceptibility in the BALB/c mice [176]. The ability of IL-4 signaling in DCs to promote Th1 response was tested using a CD11cCre /IL-4Rα$^{-/lox}$ mice. Deletion of IL-4Rα only in DCs led to severe disease and exaggerated Th2 response compared to

the WT BALB/c indicating that IL-4 directs DCs to induce Th1 response [177]. Overall, the definitive role of IL-4 and IL-13 in resistance and susceptibility in CL leishmaniasis remains unclear since robust Th2 response can be induced in the absence of IL-4 and/or IL-13 and both IL-4 and IL-13 can mediate their action independent of each other and can either enhance disease or promote healing [178].

1.8.4 Interleukin 10 (IL-10)

The finding that IL-4Rα deficient BALB/c mice remain highly susceptible to *L. major* infection and this susceptibility could be abolished by treatment with anti-IL-10R antibody suggests that the susceptibility of BALB/c mice to *L. major* is IL-10 dependent [179]. In addition, IL-10 deficient BALB/c mice are resistant to *L. major* infection despite intact IL-4 signaling [180]. IL-10 mediates its effect by blocking macrophage activation by IFN-γ thereby preventing the production of parasiticidal NO [181]. IL-10 also directly inhibits the development of Th1 cells and their production of IFN-γ [182, 183]. Both macrophages [184] and $CD4^+$ Th2 cells [185] are important sources of IL-10 in *Leishmania*-infected mice. Interestingly, $IL10^{-/-}$ mice on C57BL/6 background do not show any enhanced resistance to *L. amazonensis*, despite mounting a stronger Th1-type response [186]. Moreover, another study reported that $IL10^{-/-}$ BALB/c mice infected with *L. mexicana* and *L. amazonesis* fail to control disease progression but the lesions were less severe than their WT controls, suggesting that the genetic background and parasite species may influence the requirement for IL-10 in resistance [187]. Healing of primary *L. major* infection is typically accompanied by parasite persistence [188] which seems to be controlled by regulatory T cells (Tregs) [189]. Increased number of regulatory T cells and

elevated levels of IL-10 led to loss of immunity in healed and resistant C57BL/6 mice treated with killed *Leishmania* promastigotes [190]. Since different cells types can produce IL-10, Schwartz et.al. [191] used the Cre/Lox system to address the question of the cellular source of pathogenic IL-10. They showed that T cell derived IL-10 was more important in driving the immune response and as well as disease progression in murine model of leishmaniasis. C57BL/6 mice with complete IL-10 or T cell specific deficiency in IL-10 was susceptible to infection while deficiency of IL-10 in macrophages or neutrophils had no effect on disease progression [191]. Also, elevated levels of IL-10 and Tregs were observed in skin biopsies of patients with CL caused by *L. brazilensis* [192]. Taken together, these data clearly indicate a central role for IL-10 in susceptibility, immunopathology and parasite persistence in *L. major*-infected mice and humans.

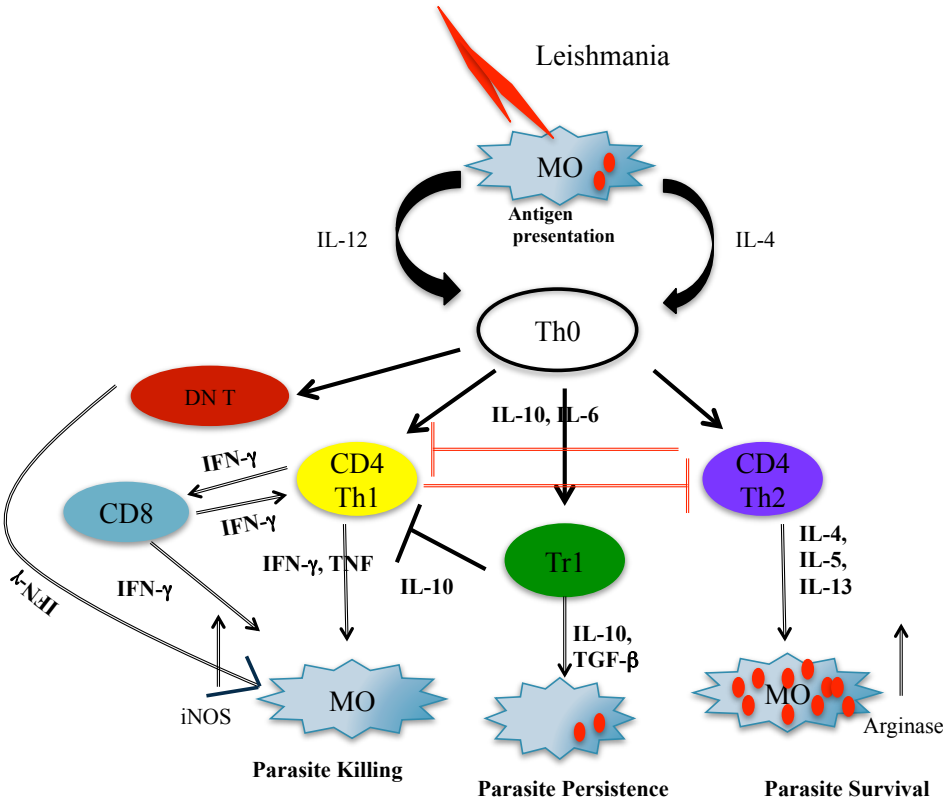

Figure 3: Correlates of protection, susceptibility and parasite persistence in cutaneous leishmaniasis. After entering the *Leishmania* is taken by antigen presenting cells with three possible outcomes. 1. The APCs can prodduce IL-12 which activates Th1 CD4, CD8 and double negative T cells to produce IFN-γ which further activate macrophages to upregulate inducible nitric oxide synthetase (iNOS) and the production of nitric oxide (NO) leading to parasite killing inside macrophages. 2. They can also produce IL-10 and IL-6 which activate naive T cells to differentiate into inducible Regulatory T cells. These Tregs further produce IL-10 and transforming growth factor beta (TGF-β) which leads to parasite persistence. 3. *Leishmania* infected APCs, produce IL-14 leading to the activation and expansion of Th2 CD4 T cells. These Th2 CD4 T cells further produce, IL-14 and other Th2 cytokines which deactivate macrophages and upregulate of arginase resulting in parasite survival.

1.9 Tumour Necrosis Factor Superfamily (TNSF) and Tumour Necrosis Factor Superfamily Receptors (TNSFR).

The tumour necrosis superfamily (TNFSF) and their receptors (TNFSFR) are found in all mammals and are highly conserved. There are over 50 members that share the canonical tumour necrosis factor (TNF) homology domain and are mostly active in the trimeric form occurring as cell surface or soluble molecules following extracellular cleavage [193]. Members of the TNFSF of cytokines and their cognate receptors play significant roles in modulating disease outcome in experimental cutaneous leishmaniasis.

1.9.1 Tumour Necrosis Factor (TNF)

TNF is the canonical and most studied member of the TNFSF. It has been shown to play a protective role in leishmaniasis by synergizing with IFN-γ in mediating parasite killing [194, 195]. Peritoneal macrophages from TNFR1 deficient mice are grossly defective in NO production and their ability to kill parasites *in vitro*. In contrast, macrophages from TNFR2 deficient mice are normal; suggesting that TNF-dependent macrophage activation for *in vitro* parasite killing was mediated via signaling through TNFR1 [196]. Treatment of infected mice with recombinant TNF resulted in reduced lesion size and lower parasite burden, while the administration of anti-TNF antibodies results in larger lesions and higher parasite burden [197]. Disruption of the TNF gene in the resistant mice leads to visceralization of *L. major* infection

and death within a few weeks [198]. Different drugs targeting TNF is currently used clinically for the treatment of different inflammatory disorders and both VL and CL has been diagnosed in patients that received anti-TNF antibody medication [199]. However, it was not clear from this case report if these VL and CL infections were new infections or reactivation of latent infection but the fact these patients lived in endemic area suggests that the infections could be due to reactivation of latent infection in the absence of TNF- signaling.

1.9.2 *Homologous to Lymphotoxin, exhibits inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed on T cells* (LIGHT) Lymphotoxin Beta (LTβ) and Herpes Virus Entry Mediator (HVEM).

LIGHT is expressed on activated T cells, monocytes, granulocytes and immature dendritic cells [200-202] and binds to three receptors: LTβR, HVEM and in humans, the decoy receptor, DcR3/TR6 [200, 203, 204]. HVEM is expressed on resting T cells, NK cells, monocytes, immature DCs and endothelial cells [203, 205, 206], whereas LTβR is expressed mostly on stromal cells, DCs and endothelial cells but absent on lymphocytes [207, 208]. In leishmaniasis, the lymphotoxin beta deficient mice develop fatal disseminating disease that resulted in death with 8-14 weeks [209]. In addition, lymphotoxin beta knock out (KO) develop chronic non healing disease associated with reduced IL-12 production [210]. Interestingly, HVEM can also bind another ligand B and T lymphocyte attenuator (BTLA). Binding of HVEM to BTLA induces inhibitory signals by recruiting tyrosine phosphatases [211, 212].

LIGHT expression enhances cytolytic T lymphocyte-mediated tumour immunity and allograft rejection [202, 213] and its overexpression on T cells results in extensive T cell proliferative disorders that is characterized by massive polyclonal expansion of $CD4^+$ and $CD8^+$ T cells [214,

215]. Blockade of LIGHT with fusion proteins in the resistant C57BL/6 mice led to susceptibility exemplified by increased lesion size and visceralization following infection with *L. major*. This susceptibility to *L. major* infection in normally resistant mice was due to impaired ability of dendritic cells to produce IL-12, an important cytokine required for the initiation of protective Th1 response to *L. major* [216]. This finding was corroborated by a recent study by Stanley and colleagues [217] which showed that the level of expression of LIGHT mRNA increased in mice infected with *L. donovani* and blocking the interaction of LIGHT with its receptors using fusion protein led to enhanced susceptibility. Together, these findings suggest an important role for LIGHT in the control of primary infection to leishmaniasis but more studies are required to clearly define the role LIGHT in cutaneous leishmaniasis. It is not clear whether LIGHT plays a role in *L. major* mediated dendritic cell maturation, the maintenance of protective immunity in *L. major* infected mice as well as secondary immunity to *L. major*.

1.9.3 CD40-CD40L Interaction

CD40 is a type I transmembrane protein that plays a role in many aspects of the immune system. It was initially described in B cells where it was shown to induce B cell proliferation and isotype switching [218, 219]. It is constitutively expressed on basophils, dendritic cells, B cells and epithelial cells but can be induced on macrophages, endothelial cells, smooth muscle cells and fibroblasts. CD40L is a type II transmembrane protein that was discovered as a ligand for CD40 [220]. A soluble form of CD40L (sCD40L) was shown to have biological activity similar to the transmembrane CD40L [221]. CD40L is inducible on $CD4^+$ and $CD8^+$ T cells, B cells, epithelial cells, eosinophils, monocytes, macrophages, NK cells and mast cells [193]. CD40

/CD40L pathway, is a well-characterised co-stimulatory pathway. This pathway is essential for T and B cell responses and is critical for development of humoral and cellular immunity [222]. The interaction of CD40 and CD40L is known to be critical in the production of IL-12p70 [223] and in leishmaniasis, IL-12 is important in protection. Mice deficient in CD40L or CD40 were shown to be susceptible to *L. major* [224, 225] and *L. amazonensis* [226] infections. Treatment of susceptible Balb/c mice with CD40 agonist antibody led to healing following infection with *L. major* [227] while injection of anti-CD40L antagonists led to susceptibility associated with reduced IL-12 production [228]. Also blocking CD40-CD40L interaction in human PBMCs stimulated with or without *Leishmania* led to reduced IFN-γ response [229]. Interestingly, there are studies that suggest that the interaction of CD40 and its ligand is not required for protection against *L. major* [230, 231]. Also in patients with CL due to *L. brazilensis*, blocking the interaction of CD40 and CD40L did not make any difference in the production of IFN-γ. Together, the role of CD40-CD40L interaction in leishmaniasis remains controversial, which could be attributed to differences in experimental models and parasite species, studied.

1.10 Macrophage Antigen 1 (Mac-1) /(CD11b/118)/Complement Receptor 3 (CR3)

Macrophage antigen 1 is a β2 integrin that is present on leukocytes and plays a role in immunity, adhesion and phagocytic migration [232]. It is composed of two chains CD11b and CD18. CD11b is a type 1 transmembrane receptor composed of extracellular, transmembrane and cytoplasmic domains [233]. Mac -1 is an adhesion molecule that is important for adhesion to tissues and migration of phagocytic cells to sites of infection [234]. Mac-1 is expressed on monocytes, neutrophils and macrophages [235]. The C- domain is believed to be important in

the recognition of pathogen surface molecules like *Mycobacterium tuberculosis* oligosaccharides and *Leishmania* lipophosphoglycan (LPG) [236] while the I domain near the N terminal is responsible for binding to C3bi, fibrinogen and other bacterial antigen. The combination of the C and I domain may explain the wide range of ligands bound by Mac-1 [237, 238]. Uptake of *Leishmania* parasite is a receptor-mediated process. Using a Mac-1 blocking antibody Mosser et. al [239] showed that the uptake of C3bi opsonized or non opsonized *L. major* parasites was through the binding of *Leishmania* parasites to the Mac-1 and there was a 63% reduction in parasite uptake in the presence of Mac-1 blocking antibody. Also *Leishmania* LPG can directly bind to Mac-1 [240] and it is thought that *Leishmania* utilizes Mac-1 to gain entry into the host without activating anti-*Leishmania* defensive response and leading to establishment of infection [241]. The role of Mac-1 in experimental CL was studied using Mac-1 deficient mice, data from this study showed that susceptible Balb/c mice lacking Mac-1 displayed intermediate disease phenotype characterised by smaller lesion and slower progression to ulceration with equivalent parasite burden compared to the their WT counter part [241].

1.11 Parasite Dose and Cutaneous Leishmaniasis

Antigen dose is one of the factors that has been show to influence the differentiation of T cells to either Th1 or Th2 subsets. Early studies utilizing model soluble antigens report that low antigen dose favours the differentiation of cells to a Th2 phenotype characterised by high IL-4 production while high antigen dose favours the IFN-γ producing Th1 cells [242-244]. The role of parasite dose in the development of Th1 or Th2 phenotype in cutaneous leishmaniasis is well documented in the literature with conflicting results. Overall, *in vitro* studies show that high and

low antigen doses promote Th1 and Th2 responses, respectively. However, early studies in BALB/c mice infected with *L. major* suggest that low and high infection doses promote Th1 and Th2 responses, respectively [245]. Cells from the resistant C57BL/6 mice infected with high dose *L. major* exhibited a strong IFN-γ response, whereas cells from mice given a low dose of parasites produced substantially more IL-4 [121]. This early low dose-induced IL-4 (Th2) response was transient because these mice eventually control parasite replication and develop a strong IFN-γ response. Interestingly, in the absence of $CD8^+$ T cells, this early and transient IL-4 response in C57BL/6 mice given low dose infection is sustained leading to uncontrolled parasite replication and the development of progressive disease. The dependence on $CD8^+$ T cells for optimal Th1 response was only observed at low parasite doses, indicating that their role might be to modulate the early Th2 response. Further studies showed that $CD8^+$ T cells mediate their modulatory effect on $CD4^+$ Th2 cells by producing IFN-γ, a key cytokine that enhances Th1 development [121]. In a mouse model of Influenza, low antigen dose was shown to induce a population of cytolytic $CD4^+$ T cells through an IL-2 dependent process [246]. Even though antigen dose has also been shown to have an effect on the type and quality of memory T cells generated following infection [247], its definitive role in immunity to *Leishmania* parasite remains unclear.

1.12 Immunologic Memory

Immunologic memory is the hallmark of the adaptive immune system and is the ability of the host immune system to recognize and responds fast to a previously encountered antigen. It is the main idea behind any successful vaccine and vaccination strategy [248, 249]. In order to provide efficient and effective immunity to a secondary challenge, memory T cells must rapidly

home to the lymph node draining the challenge site, proliferate and then migrate to the site of antigenic challenge in the peripheral tissues to mediate their effector function. This proliferation in the lymph node is critical because the frequency of antigen-specific T cells in the memory pool, although higher than naïve cells, is too low to mediate sufficient response to provide rapid immunity [248, 250].

1.12.1 Classification of Memory Cells

Earlier studies classified memory cells based on their location, functional capabilities and expression of certain markers. As such two major types memory T cells were described in humans [251-254] and mice [255-257]. Central memory T cells (Tcm) express high levels of CCR7 and CD62L molecules that are important for extravasation of T cells through the high endothelial venules (HEV) and homing to the secondary lymphoid organs [252, 258]. In contrast, effector memory T cells (Tem) do not express significant levels of these molecules and home preferentially to non lymphoid tissue where they exert effector functions [252, 258]. Upon antigenic recall stimulation, Tcm cells produce only IL-2 and do not make effector cytokines such as IFN-γ. In contrast, tissue homing Tem cells produce copious amounts of effector cytokines (IL-4 and IFN-γ) upon antigenic challenge and may constitutively express other cytotoxic effector molecules such as perforins and granzymes. It is important to note that other markers have been used to identify memory T cells, for example, the leucocyte common antigen, CD45 (T200, Ly-5); while naïve T cells are $CD45RA^{hi}$ memory T cells are $CD45^{lo}$ [259, 260]. More recently a new group of memory T cells known as tissue resident memory T cell (T_{RM}) has been described. These cells can reside for long periods of time in organs such as brain and mucosal tissues (lung, gut and skin) [261-263] and have limited ability to egress and recirculate

throughout the body. T_{RM} have mostly been studied within the $CD8^+$ T cell population [264, 265]. They are known to be $CD103^{hi}$, $CD69^{hi}$ and $CD27^{lo}$ and sometimes can express high levels of granzyme B [264, 265]. It will be interesting to investigate whether T_{RM} develops and play any role in immune response to *Leishmania* parasites.

1.12. 2 Immunologic Memory and Cutaneous Leishmaniasis

Since resistance to cutaneous leishmaniasis is primarily due to cell-mediated immunity (T cells), it is therefore reasonable to assume that memory T cells mediate secondary anti-*Leishmania* immunity. It has been shown that in healed C57BL/6 mice, *Leishmania*-specific $CD4^+$ memory cells proliferating in response to secondary challenge differentially express CD62L depending on their location [256]. In the draining lymph node, most of the proliferating cells expressed high levels of CD62L, whereas in the footpad, 98% of the proliferating cells were $CD62L^{lo}$ [256]. This dichotomy in phenotype was also reflected in their effector function such that $CD62L^{hi}$ cells produced predominantly IL-2 while $CD62L^{lo}$ cells in the periphery (footpad) produced high levels of IL-12 and IFN-γ [255, 256]. Functional studies revealed that although both subsets of anti-*Leishmania* memory cells are protective, their efficiency of protection is dramatically different. The $CD62L^{lo}$ cells (Tem) mediate rapid protection whereas the protection mediated by $CD62L^{hi}$ cells (Tcm) is delayed. Thus, as seen in other systems, both Tcm and Tem cells develop in *Leishmania*-infected mice [256]. A recent study by Pereira-Carvalho and colleagues [266] showed that effector memory $CD4^+$ and $CD8^+$ T cells contract following healing from CL caused by *L. braziliensis*. The number of effector memory cells correlated negatively with time elapsed after healing. Interestingly, these effector memory cells retained the

ability to expand and exhibit recall responses following exposure to *Leishmania* antigen *in vitro.* Furthermore, the number of IFN-γ–producing $CD8^+$ $CD45RO^+$ $CD45RA^+$ memory T cells were found to be higher in individuals that recovered from CL compared to the healthy controls, suggesting that protection in these individuals could be due to rapid response from *Leishmania* specific memory $CD8^+$ T cells. Unfortunately, the phenotype of the memory T cells in the $CD4^+$ T cell compartment was not characterised in this study. It will be interesting to see if studies in humans will corroborate findings in mice.

1.12.3 Infection induced Immunity in Cutaneous Leishmaniasis

Recovery from natural or experimental *L. major* infection in humans and mice is associated with the development of strong and durable immunity to rechallenge infection. This so-called infection-induced resistance is the fundamental principle underlying leishmanization, a practice in which individuals are deliberately injected with live organisms to protect against more serious ulcers after natural infection [267]. Understanding the factors that regulate and mediate infection-induced resistance is critically important for designing an effective vaccine and vaccination strategies against leishmaniasis. Infection-induced resistance in mice is mediated by IFN-γ-producing $CD4^+$ T cells [153] and its maintenance is dependent on IL-12 produced by antigen presenting cells because the highly susceptible IL-12 deficient mice treated with rIL-12 develop Th1 response and resolve their lesion. However, in contrast to WT mice, these rIL-12-treated mice develop progressive disease and uncontrolled parasite replication upon re-challenge infection [268]. In fact, lesion/disease reactivation occurs at the primary infection site in healed IL-12-deficient mice upon cessation of IL-12 treatment [268], suggesting that exogenous administration of rIL-12 was able to only promote short-term resistance. It is conceivable that

IL-12 may be required for optimal proliferation and differentiation of memory $CD4^+$ T into IFN-γ producing effector cells. Alternatively, IL-12 could be acting to enhance the development and survival of *Leishmania*-specific effector memory cells that are important for mediating rapid secondary anti-*Leishmania* immunity [256, 269].

Under certain conditions, infection-induced resistance can be lost and previously immune animals become highly susceptible to re-challenge infections [189, 270]. This loss of resistance has been linked to complete parasite clearance, suggesting that persistent parasites are important for the maintenance of anti-*Leishmania* immunity. Recent studies from our group showed that infection-induced resistance could also be lost in the presence of persistent parasites [190]; injection of killed parasites into mice that have healed their primary *L. major* infection results in rapid expansion of IL-10-producing Tregs, a concomitant loss of infection-induced resistance and susceptibility to virulent *L. major* challenge [190].

1.12. 4 Parasite Persistence and Immunity to Leishmaniasis

One of the hallmarks of natural or experimental leishmaniasis is the persistence of a small number of viable parasites at the primary site of infection and draining lymph node of the host [188, 271]. This phenomenon led to the common belief that *Leishmania* parasites are never completely eliminated from infected host and immunity in leishmaniasis is dependent on these persisting parasites. The disadvantage of parasite persistence is that under certain conditions including malnutrition and immunosuppression, recrudescence (reactivation leishmaniasis) can occur. For example, leishmaniasis is a major complication of AIDS in the sub-Saharan Africa and India [272-275]. However, whether disease in immune suppressed individuals is due to new

infection or actual reactivation from persistent parasites remains unclear. Interestingly, the same parasite strain that caused initial disease was isolated from 50% of individuals with recurrent cutaneous leishmaniasis due to *L. braziliensis* [276]. The greatest evidence that support the theory that persistent parasites lead to reactivation disease comes from murine studies. Treatment with L-NIL, the competitive inhibitor of nitric oxide synthase (important for nitric oxide production), [277] adoptive transfer with $CD4^+CD25^+$ cells isolated from infected mice [278, 279] or injection of killed parasites [190] into healed led to recrudescence and progressive disease at the primary site of infection. Collectively, these murine studies suggest that some cases of reactivation disease in humans could arise from persistent parasites. The factors and conditions that favour parasite persistence are not clearly understood but there is evidence that the nature of immune response generated can play a role. For example, ultra low dose infection of Balb/c mice led to the induction of an exclusive Th1 immune response (with no detectable Th2 component) and resistance that is associated with complete parasite clearance [280]. In contrast, infection with an intermediate dose led to parasite persistence due to the development of the development of a weak Th1 response with a substantial Th2 component. Also extent of IL-10 production during infection influences parasite persistence. For instance, IL-10 receptor deficient mice or healed WT mice treated with anti-IL-10R mAb mount strong Th1 response and completely clear parasite [189]. Other studies have implicated natural $CD4^+CD25^+Foxp3^+$ regulatory T cells as the major source of IL-10 and the key cells that mediate parasite persistence in mice [278, 279]. Unlike murine studies, the factors responsible for parasite persistence and development of subclinical disease in humans are not known and have not been properly investigated. However, given that $CD4^+CD25^+$ T cells isolated from lesions of infected patients mediate suppressive activities *in vitro* [281], coupled with the presence of high levels of IL-10 in

the plasma of human patients [282, 283], one can argue that as in mice, both $CD4^+CD25^+$ T cells and IL-10 may also play important roles in the persistence of parasites in humans.

The observation that immunity in leishmaniasis is lost following complete clearance of live parasites is suggestive that true memory cells do not develop following *Leishmania* infection. It has been suggested that in chronic infections like leishmaniasis, continued antigenic stimulation inhibits memory T cell development [250, 284]. Consistent with this, *L. major* infected mice presenting non-healing lesion in one foot or ear have been shown to resist virulent contralateral challenges [285]. However, there is evidence that true memory cells and not concomitant immunity is responsible for secondary anti-*Leishmania* immunity. Both effector and central memory T cells are induced following *L. major* infection in mice [256, 257, 286]. Adoptive transfer study show that these cells and are able to mediate protection against secondary *L. major* challenge [256]. Interestingly, even though *Leishmania*-specific effector memory cells decline in the absence of parasites, the maintenance of central memory cells appears to be independent of live parasites [256]. Mutant *Leishmania* parasites have been used to investigate the influence of persistent parasite on the maintenance of anti-*Leishmania* memory cells. *Leishmania major* parasites that are non pathogenic and are completely cleared by the host in 8 weeks [287] induced immune response that is comparable in quality to those infected with wild type parasites, and are resistant to early virulent challenge (Uzonna et al unpublished data). Even though the mice infected with the mutant parasite were not protected against virulent challenge at later time points compared to mice infected with WT parasites, they showed delayed but significant protection over their naïve age-matched controls [256]. These results suggest that anti-*Leishmania* memory cells develop after infection and are maintained in the absence of live parasites. In line with this, repeated inoculation of C57BL/6 mice with killed *Leishmania*

parasites provides a relatively long-term protection against virulent challenge. Together, the data suggest that the nature and quality of memory cells maintained in the presence and absence of live parasites may be different. These findings, if shown to be true in humans, must be considered in vaccine designs and strategies against leishmaniasis [288].

1.13 Vaccines and Vaccination Strategies in Cutaneous Leishmaniasis

There is no universally acceptable effective vaccine against leishmanias, thus suggesting that we still do not fully understand the requirements for maintenance of anti-*Leishmania* immunity [289]. When developed a good anti-*Leishmania* vaccine should protect against different species of *Leishmania* in the absence of persistent parasites [288] and induce long term protective immunity [290]. An effective vaccine work by generating long-lived memory cells in vaccinated individuals that are able to recognize and eliminate the invading organism upon subsequent encounter. Many successful anti-*Leishmania* vaccination strategies have been studied in mice, however vaccination trials in non human primates and humans yielded very disappointing results [291].

1.13.1 Leishmanization

This vaccination strategy is the injection of live virulent parasites or tissue extracts from infected lesions into hidden parts of the body of non-immune individual with a view to preventing the formation of visible lesions following natural infection [292]. It is the oldest and most effective vaccination strategy against cutaneous leishmaniasis and was used successfully for a long time to contain epidemics of cutaneous leishmaniasis in the republics of the former

Soviet Union, Israel and Iran. However, the development of non-healing lesions that require medical treatment in some vaccinated individuals [292] has led to the abandonment of this practice. Despite the associated morbidities, leishmanization remains the only effective vaccine with proven efficacy in humans to date. As such, several strategies have been employed to make it safer such as inclusion of killed parasites [293], the use of adjuvants like CpG that promote rapid onset of anti-*Leishmania* immunity and swift healing of lesions [294] and vaccination with genetically attenuated parasites [291]. Attenuated parasites such as *lpg2- L. major* persist at the local site of injection and draining lymph node, does not cause pathology and protect mice against virulent *L. major* challenge [295, 296].

1.13.2 Live-Attenuated Parasite Vaccine

Vaccination with live attenuated parasites is a more practical approach over traditional leishmanization since it will provide durable immunity without the potential safety concerns of disease development in vaccinated individuals [297, 298]. To date different strategies are currently used to attenuate live virulent parasites such as long-term *in vitro* culture with or without antibiotic pressure [299], irradiation [300], chemical mutagenesis [301] and more recently targeted deletion of essential virulence genes [287]. Among these, targeted gene deletion has shown much promise because of the reduced risk of reversal to virulence. Immunization with phosphomannomutase-deficient *L. major* protected the highly susceptible BALB/c mice against virulent challenge. The protection was associated with suppression of early IL-10 and IL-13 production as well as expansion of $CD44^{hi}$ $CD4^{+}$ and $CD8^{+}$ T cells [302]. In a similar study, *Leishmania* parasite that lacks the gene that encodes for dihydrofolate reductase-thymidylate synthetase (*dhfr-ts*), which is essential for long-term parasite survival, was tested as potential

vaccines [287, 303]. This mutant parasite showed limited protection in mice against *L. major* and *L. amazonensis* infection but failed to protect non-human primates against virulent challenge [304]. This lack of protection may be attributed to the rapid elimination of the parasites from the host because parasite persistence is associated with maintenance of anti-*Leishmania* immunity [188, 270]. *lpg2* deficient parasites lacks the gene that encodes an enzyme involved in the transport of GDP-mannose to the Golgi apparatus and are able to persist indefinitely in infected mice without causing obvious pathology [295]. Vaccination of mice with these mutant parasites induced very strong protection against virulent *L. major* challenge [305]. Interestingly, the protection induced by *lpg2*-parasites was not associated with the development of delayed-type hypersensitivity (DTH) and enhanced IFN-γ responses, suggesting that the induction of Th1-like responses might not always be essential or correlate with protective immunity [158]. Whether *lpg2*- could mediate protection in non-human primates has not yet been investigated. Thus, caution must be exercised in using this mutant parasite as a potential live-attenuated vaccine. Further, the gene encoding cysteine proteinase in *L. mexicana* has also been targeted to create attenuated parasite for vaccination studies [306]. Cysteine proteinase deficient *L. mexicana* are highly attenuated *in vitro* and induced protection against a homologous challenge in hamsters [307] and mice [306]. Another live-attenuated vaccination approach involves the use of *Leishmania* strains that are not pathogenic to humans. Vaccination with *L. tarentolae*, a lizard parasite, was shown to induce DC maturation, Th1 response and protection against virulent *L. donovani* challenge [308]. In addition, vaccination with *L. tarentolae* expressing A2 (amastigote-specific) antigen of *L. donovani* induced strong Th1 response leading to protection against *L. donovani* challenge [309]. The use of non-pathogenic *Leishmania* parasite would most likely eliminate the fear of disease development following vaccination. However, many questions

remain to be answered, such as how long these parasites would persist in the vaccinated host, what is the quality and durability of the primary and memory immune responses? Can these non- virulent parasites cross-protect against other *Leishmania* species? [289].

1.13.3 Killed whole parasite vaccines

Vaccination with killed *Leishmania* parasites was the first bold step to tackle epidemics of cutaneous leishmaniasis by vaccination in endemic countries and dates back to the 1940's [310]. Killed vaccines are attractive because they are easy and cheap to make, do not require sophisticated technology, there is no worry about lesion development, reversion to virulence and can be used in immunocompromised individuals [289]. However, standardization of cultured parasite-derived vaccines from one culture to another is a major drawback that could impede the registration and marketing of killed vaccines [310]. A vaccine containing promastigotes of five killed *Leishmania* strains was shown to be safe and immunogenic as measured by the leishmanin skin test (LST) reactivity, but conferred only a small degree of protection (50%). Phase III clinical trials in Ecuador and Colombia showed that heat-killed *L. amazonensis* vaccine was safe, induced strong IFN-γ response but did not prevent clinical disease [311, 312]. The apparent lack of protection despite strong Th1 response is consistent with similar observations in mice and primates, and suggests that the induction of Th1 immune response may be necessary but not sufficient for protection against cutaneous leishmaniasis. In contrast to the reported benefits of heat-killed vaccines in South America, studies utilizing heat-killed *L. major* with or without BCG in Iran and East African countries yielded disappointing results [313, 314]. Studies in Vervet monkeys show that killed *Leishmania* vaccine induced robust Th1 response but could not

protect against virulent challenge [315, 316]. Thus suggesting that the failure of killed parasites to induce long-term protection may be related to their inability to maintain memory cells [288]. This scenario maybe likely since repeated injection of killed parasites led to robust expansion of effector-like memory T cells resulting in durable protection against virulent challenge [155]. This supports the notion that killed parasites have the ability to induce and maintain memory cells provided enough effector memory-like cells are generated and continuously re-stimulated by booster immunizations there may be no obligatory requirement for live parasites for maintaining anti-*Leishmania* immunity. Treatment of *Leishmania* parasites with a compound amotososalen (S-59) produced killed but metabolically active (KMBA) parasites. Vaccination of mice with these KMBA parasites protected them against virulent challenge [317]. Together, these data provide strong rationale for continued evaluation of mechanisms of secondary protective immunity against *L. major* [289]. In line with this recent studies show that killed parasite administered with adjuvants are able to protect mice against virulent *L. donovani* challenge [318, 319].

1.13.4 Subunit Vaccines

Subunit vaccines are made of single or multiple proteins /antigens from a microbe that has been shown to simulate the immune system. They are particularly attractive because they lack the ability to cause disease and are relatively cheap to produce and standardize. Several *Leishmania* protein antigens have been used as subunit vaccine candidates against leishmaniasis. Vaccination with Leish-111f, a recombinant polyprotein vaccine that contains thiol-specific antioxidant (TSA), *Leishmania major* stress inducible protein 1 (LmST11) and *L. major* elongation initiation factor (LeIF) was shown to protect against both visceral and cutaneous

leishmaniasis [320]. Phases 1 and II clinical trials for Leish-111f vaccine have been completed and show that the vaccine is safe and immunogenic in healthy adult patients with mucocutaneous and cutaneous leishmaniasis [321]. This vaccine has also been used therapeutically in combination with sodium stilbogluconate for treatment of mucosal leishmaniasis [321] and in combination with meglumine for the treatment of human cutaneous leishmaniasis [322]. Interestingly and although a recent study suggested that the Leish-111f vaccine could also partially protect dogs against visceral leishmaniasis [320], it failed to induce any significant protection in vaccinated dogs in a well-controlled Phase III trial [323]. More clinical studies are needed to determine the potential of using this vaccine to control human leishmaniasis. A more recent study showed that another polyprotein comprising of kinetoplastid membrane protein 11 (KMP11), Sterol 24-c-methyltransferase (SMT), A2, and cysteine proteinase B (CPB) given with monophosphoryl lipid A (MPL-SE) as adjuvant was able to protect mice against visceral and cutaneous leishmaniasis caused by *L. infantum* and *L. major*, respectively [324]. It will be interesting to determine the clinical efficacy of this vaccine as a prophylactic or therapeutic vaccine in human or canine leishmaniasis [289]. One study showed that vaccination with recombinant gp63 expressed in *E. coli* failed to protect mice against virulent *L. major* challenge [325]. In another report, BALB/c mice vaccinated with recombinant gp63 (rgp63) encapsulated in cationic liposome with CpG ODN as an adjuvant had significant reduction in parasite burden, lower Th2 response and higher Th1 response compared to mice that received rgp63 alone or rgp63 plus CpG ODN following virulent *L. major* challenge [326]. Recently, a third polyprotein vaccine made up of LACK, TSA, LbSTI, or LeIF was shown to protect mice against cutaneous leishmaniasis caused by *L. major* but failed to protect disease caused by *L. braziliensis* [327]. This result is rather puzzling given that the vaccine consisted of components derived from

antigens that are conserved across all *Leishmania* and hence should cross protect against different *Leishmania* species [289].

1.13.5 DNA vaccines

In the recent past, deoxyribonucleic acid (DNA) has been used as vaccine delivery method. To make a DNA vaccine, genes encoding the target proteins are cloned into an expression vector, which is then delivered to the host by intramuscular or intradermal route of injection [328]. DNA vaccines are capable of eliciting strong immune responses similar to those induced by protein antigens and this could be further enhanced and modulated by the inclusion of adjuvants or cytokines [329, 330]. Since protection against leishmaniasis requires the induction of early Th1 response, DNA vaccination is a very attractive strategy because of the propensity of DNA vaccines to elicit strong cell-mediated immunity [331, 332]. In addition, DNA vaccines mimic the protective effects of live vaccines without the potential danger of disease development, they are relatively easy and inexpensive to produce and unlike protein or live-attenuated vaccines, does not require the maintenance of "cold chain" sequence [333]. Several experimental studies have shown that DNA vaccines confer strong protection against cutaneous [334, 335] and visceral [336-340] leishmaniasis. However, although DNA vaccination is considered a promising technology, it still remains an experimental practice because no development of such vaccines for use in humans has been reported so far. In addition, the conflicting reports on the protective efficacy of this vaccination strategy add to the confusion in the field [158]. There are also genuine concerns about ethics, safety and delivery systems, which collectively have hampered the application of this technology in humans. At present, DNA

vaccination remains a very attractive experimental research area with possible benefits in human medicine [289].

2. RATIONALE AND OBJECTIVES

Despite years of intense research in the immunology of cutaneous leishmaniasis, there is no licensed effective anti-*Leishmania* vaccine for use in humans. However, the fact that natural and experimental infection with *L. major* leads to life long immunity to reinfection suggests that the development of an effective anti-*Leishmania* vaccine is feasible. However, more research is needed to completely understand the factors that regulate secondary immunity to cutaneous leishmaniasis.

The type and quality of memory T cells developed following primary antigen exposure has been shown to play a major role in shaping the nature of secondary/memory response [341]. Using CD8 deficient mice it was previously shown that CD8 T cells are critical in optimal primary immunity to low dose but not high dose *L. major* infection [121]. The finding that the role of $CD8^+$ T cells was dependent on infection doses raises the question whether parasite dose affects the induction and expansion of T cells following primary *L. major* infection and subsequently affects the nature of memory response following virulent challenge. In the first part of this thesis, I investigated whether the initial parasite dose affects the quality (subset) and magnitude of the initial (primary) T cell expansion and the impact of this on infection-induced (secondary) anti-*Leishmania* immunity.

The tumor necrosis factor super family (TNFSF) of cytokines and their receptors including CD40, CD40L, LIGHT *(lymphotoxin like, exhibits inducible expression and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes),* tumour necrosis factor (TNF) and Herpes Virus Entry Mediator (HVEM) play critical roles in the development of the immune system, immune regulation, inflammation and autoimmunity by regulating cell

death, survival and differentiation [342, 343]. It was previously shown that LIGHT and its receptors (HVEM and LTßR) are important for initiation of $CD4^+$ T helper (Th1) cell response and development of primary immunity to *Leishmania major* infection in mice [210, 216]. However, whether LIGHT is important for dendritic cell maturation, initiation and maintenance of primary immunity and also contributes to secondary immunity to *Leishmania major* is unknown. In the second part of the thesis I further characterized the contribution of LIGHT in the maturation of dendritic cells (DCs) as well as primary and secondary anti-*Leishmania* immunity.

The interaction between CD40 and CD40L is important in the production of IL-12 [344], a key cytokine for protection against *L. major* infection [141]. Interestingly, there are conflicting reports regarding the role of CD40-CD40L interaction in cutaneous leishmaniasis. While some reports show that mice deficient in CD40L are resistant to cutaneous leishmaniasis [231] others show that CD40L deficient mice are highly susceptible [226]. This discrepancy could be due to differences in experimental design and methodologies used in the studies. In the third part of the thesis I further investigated the role of CD40 and CD40L interaction in cutaneous leishmaniasis by characterizing and comparing disease outcome and immune response to *L. major* in CD40 and CD40L KO mice.

2.1 Hypotheses

1. Based on the observation that primary immunity to low dose *L. major* is dependent in $CD8^+$ but not $CD4^+$ T cells, I hypothesized that primary infection with low and high dose *L. major* will induce different cell mediated immune response leading to differences in memory response to *L. major*.

2. Since LIGHT is critical during primary immune response to *L. major*, I hypothesized that LIGHT will also play a critical role in secondary immunity to *L. major* as such blockade of LIGHT will lead to loss of infection–induced resistance following virulent challenge in normally resistant healed C57BL/6 mice.

3. Since the interaction of CD40 and CD40L is important for IL-12 production, I hypothesized that there will be comparable immune response and disease outcome in CD40 and CD40L deficient mice infected with *L.* major and treated with recombinant interleukin 12.

2.2 Overall Objectives

There are three main objectives in this thesis

1. To determine the effect of antigen dose on primary and secondary immunity to *Leishmania major*.
    a. Compare the magnitude and quality of primary and secondary immune response to low and high dose *L. major* infection.
    b. Investigate if there are differences in the types of memory cells induced following low and high dose infection with *L. major*.
    c. Investigate the role of $CD8^+$ T cells in secondary immune response following low dose infection.

2. To determine the role of LIGHT in secondary anti-*Leishmania* immunity.

    a. Determine if LIGHT is critical for maturation of dendritic cells following *L. major* infection *in vivo*.

    b. Determine if continuous LIGHT interaction with its ligand(s) is important for maintenance of primary *L. major* immunity.

    c. Investigate if LIGHT is critical for maintenance secondary immunity to *L. major*.

3. To determine the role CD40-CD40L interaction in anti-*Leishmania* immunity

    a. To fully characterise and compare the disease progression and immune response to *Leishmania major* in CD40 and CD40L deficient mice.

    b. Use *in vitro* system to test if Mac-1 binds to CD40L in the absence of CD40 (CD40KO mice).

    c. To investigate the role of Mac-1 binding to CD40L in reactivation of *L. major* infection in CD40 deficient mice.

    d. To determine the role of Mac-1 -CD40L interaction in secondary immune response to *L. major* in CD40 deficient mice.

3. MATERIALS AND METHODS

3.1 Mice

Six to eight weeks old female naive C57BL/6 (Thy1.2) mice were purchased either from Charles River Laboratory, St. Constante, Quebec or from the University of Manitoba Central Animal Care Services (CACS) breeding facility. C57BL/6 (Thy1.1), CD40 knock out (KO), CD40L KO and IL-12 KO mice were purchased from The Jackson Laboratory (Bar Harbour ME). All mice were maintained in the specific pathogen free environment at the University of Manitoba CACS and were used according to the guidelines stipulated by the Canadian Council for Animal Care.

3.2 Parasites

Leishmania major friedlin (MHOM/IL/80/Friedlin) were grown in 25 cm tissue culture flask (Corning, VWR, Missisuaga, ON) containing 10 or 20 ml complete parasite medium (Graces insect medium) (Invitrogen, Life Technologies, Burlington, Ontario) supplemented with 20% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 25 mM HEPES (Invitrogen) for 7 days in a 27^{o}C parasite incubator (Thermo Fisher, Ottawa, ON). The 7-day stationary phase promastigotes were harvested and used for infection. Briefly, parasites were transferred to a 50 ml tube (BD Falcon VWR) and washed two times in a centrifuge (Eppendorf 5810R, Mississuaga, ON) at 3000 rpm for 15 minutes. The supernatant was decanted and the pellet was re-suspended in 10 ml phosphate buffered saline (PBS) (Invitrogen). A 1: 100 dilution was made by adding 10 µl of the parasites suspension to 990 µl of PBS in a 1.5 ml eppendorf tube (VWR) and vortexed (VWR) for 30 seconds. The parasites were counted by adding 10 µl of the suspension to a haemocytometer (Fisher Scientific,

Whitby, ON), covered with a cover slip (Fisher) and counted under a microscope (Fisher) at x40 magnification. The parasites were re-suspended in appropriate volume of PBS for injection.

3.3 Soluble *Leishmania* Antigen (SLA):

Seven day *L. major* promastigotes frozen at a concentration of $1x10^{10}$/ml was thawed and diluted to $2.5x10^{9}$/ml in a solution containing 100 mM Tris,1 mM EDTA, 1.6 mM PMSF and 50 µg/ml leupeptin (Sigma, Oakville, ON) pH 8.0 and incubated on ice for ten minutes. Using a sonicator, (Fisher) parasite membranes were disrupted (20 secs/burst) and repeated 3-4 times at the highest setting. The sonicated parasites solution was transferred into 30 ml autoclaved oakridge tubes, placed in a Ti60 rotor (Beckman Coulter, Mississuaga,ON) and spun using an ultracentrifuge (Beckman Coulter) at 15,000 rpm at 4^{0}C for 30 minutes. Supernatant was transferred to ultracentrifuge tubes (Beckman) placed in Ti60 rotor and spun again at 35,000 rpm at 4 ^{0}C for 3 hours. Carefully collected supernatant was transferred into a dialysis tube and properly clamped to avoid leakage. The filled dialysis tube was placed in a 5L container (Fisher) containing 1x PBS in 4 ^{0}C walk-in cold room and allowed to dialyse over night with the 1x PBS (Invitrogen) changed 4 times. The dialysed solution was transferred into 30ml autoclaved oakridge tubes placed in Ti60 rotor (Beckman) and spun using an ultracentrifuge (Beckman Coulter) at 10,000 rpm at 4 ^{0}C for 10 minutes. The supernatant was filtered using a low protein binding 0.2µm syringe filter (VWR). A Bradford protein assay was run to determine protein concentration (See below). The SLA was stored at -80 ^{0}C in aliquots.

3.4 Bradford Protein Assay

The protein concentration in the prepared SLA was measured using the Bradford protein assay kit (BIO-RAD, Hercules, USA) according to the manufacturers suggested protocols. Briefly**,** four milligram per millilitre (mg/ml) of bovine serum albumin (BSA) (Sigma) was made and used as standard solution. First, six serial dilutions of the 4 mg/ml BSA standard solution were made in PBS. Ten microliter of each concentration of the standard solution and sample was transferred to properly labelled 1ml eppendorf tubes (VWR). A working solution was prepared by adding 1ml of Solution A and 20 µl of solution S. Then 50 µl of working solution was added to each tube containing 10 µl of the either standard solutions or samples. The next step was the addition of 400 µl of solution B to make a final volume of 460 µl. The solutions were then properly mixed by vortexing (Fisher) and allowed to develop at room temperature for 15 minutes. Finally, 100 µl of sample and the different concentrations of the standards were transferred to a flat bottom 96 well plate (VWR) in triplicates and then read on an ELISA reader (Spectra Max 190 Molecular Devices, Sunnyvale, CA) at 400 nm.

3.5 Infections and Challenge:

For primary infection in Aim 1, naive mice were injected subcutaneously in the right footpad with $5x10^6$ (high dose) or $1x10^3$ (low dose) *L. major* parasites. For aims 2 and 3, naïve CD40, CD40L, IL-12 and WT mice were infected with 1 million *L. major* parasites in the right footpad. Healed WT mice were generated by infecting naïve C57BL/6 mice with 1 million virulent *L. major* parasites on the right foot pad and allowing them to heal their primary lesion for over 12- 14 weeks. For challenge healed mice were infected in the left footpads with 5

million live virulent, *L. major* parasites. All parasites where injected in 50 µl PBS using a 1 ml syringe and 30G needles (VWR).

3.6 Isolation of Cells from Spleen:

At indicated times, infected or uninfected mice were sacrificed by cervical dislocation. The spleens were excised and collected on ice in 15 ml centrifuge tubes (BD, WR) containing 5ml complete culture medium i.e. DMEM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and 5 x 10^{-5} 2-mercaptoethanol (Invitrogen). To isolate cells from the spleen, whole spleens were homogenized using a sterile 15 ml tissue grinder (Fisher) with 10 ml complete culture medium. The homogenate was passed through a sterile 70µm cell strainer (VWR). The single cell suspension was centrifuged (Eppendorf) at 4 ^{o}C for 5 minutes at 1200 rpm. Supernatant was discarded and the pellet re-suspended in 5 ml red blood cell lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM NaEDTA (Sigma) per litre of deionised water pH 7.4) and incubated for 5 minutes at room temperature with intermittent shaking. Cells were washed two times with 10 ml complete culture medium centrifuged for 5 minutes at 1200 rpm. Supernatant was discarded and the pellet re-suspended in 10 ml complete culture medium. A 1:10 dilution was made in complete culture medium. Cells were further diluted in trypan blue (Invitrogen) to make a final dilution of 1:20. Cells were counted using a haemocytometer.

3.7 Isolation of Cells from Lymph-nodes:

At indicated times, infected or uninfected mice were sacrificed by cervical dislocation and the popliteal lymph-nodes excised and collected in 5 ml centrifuge tubes (BD VWR) containing 2 ml complete culture medium. The draining lymphnodes were placed in a 70 µm cell strainer and homogenized using a sterile 3 ml syringe plunger. The single cells were centrifuged (Eppendorf) at 4 ^{o}C for 5 minutes at 1200 rpm. Supernatant was discarded and the pellet re-suspended in 2 ml complete culture medium, diluted as described for spleen cells and counted using a haemocytometer.

3.8 Cell Culture with Soluble *Leishmania* Antigen:

Single cell suspension was diluted in complete culture medium to a final concentration of 4 million cells/ml. One mililitre of cells was plated in 24 well plates (BD Falcon), stimulated with or without 50 µg/ml soluble *Leishmania* antigen (SLA) and incubated for 72 hours at 37 ^{0}C with 5% CO_2 (Thermo Fisher Scientific, Ottawa, ON).

3.9 Cytokine Enzyme Linked Immunosorbent Assay (ELISA):

The levels of IL-10, IFN-γ, IL-4, IL-12 and TNF in the supernatants collected from cell cultures were determined by sandwich ELISA technique. Briefly, high binding ELISA plates (Immulon® VWR, Mississuaga, ON) were coated with primary antibodies (Biolegend, San Diego, CA) at a concentration of 1-1.2 µg/ml in (50 µl/well) and incubated overnight (O/N) at 4^{0}C. Antibodies were diluted in carbonate-bicarbonate coating buffer with a final pH of 9.6. After incubation, plates were washed 5 times with wash buffer (1x PBS, 0.05 % Tween 20 (Sigma) pH 7.4) using the automated ELISA washing machine BIOTEK ELX405 plate washer,

Biotek Instrument, Winooski, VT). A blocking buffer solution (5% new calf serum in PBS, pH 7.4) was added to all wells (200µl) and incubated for 2 hours at 37°C, to block non specific binding. Plates were then washed 5 times with wash buffer using the automated washer (Biotek). Recombinant cytokine at 2 ng/ml (Preprotech) was applied to the plate and titrated 2 fold in dilution buffer for 7 wells. Samples were appropriately diluted in dilution buffer and then titrated 2 fold. Samples were incubated O/N at 4°C. Plates were then washed 5 times with wash buffer using the ELISA washer (Biotek). Fifty microlitre of biotinylated detection antibody at 2 µg/ml (Biolegend) in dilution buffer was added to all wells. Plates were incubated for 1-2 hours at 37°C. After washing 5 times, streptavidin horseradishperoxidase at 1:3000 dilution (BD Pharmagen, San Jose, CA) in dilution buffer was added to all wells and incubated for 30 minutes at 37°C. Plates were washed 10 times and two component ABTS substrate (Mandel Scientific, Guelph, ON) was then added to plates. Plates were read at 405 nm (Spectra Max) after the appropriate color development.

Table 1 List of cytokines analyzed by ELISA

Cytokine	**Standard (pg/ml)**	**Sample Dilution**	**Sensitivity (pg/ml)**
Interferon gamma	2000	1:4	30.25
Tumour necrosis factor	2000	1:2	30.25
Interleukin 4	2000	1:2	30.25
Interleukin 12	2000	1:2	30.25

3.10 Intracellular Cytokine Detection by Flow Cytometry:

After collecting 72 hours cell culture supernatant, cells were stimulated with 50ng/ml phorbol myristate acetate (PMA) (Sigma) and 500 ng/ml ionomycin (Sigma) for 4 hours. Ten microgram per millilitre (µg/ml) brefeldin A (Sigma) was added in the last 2 hours for a total of 6 hours. After which cells were harvested and transferred into flow cytometry tubes (BD Falcon), washed in 1 ml FACS buffer (PBS containing 0.1% new calf serum and 0.1% sodium azide (NaN_3) by spinning in a centrifuge (Eppendorf) for 5 minutes at 1200 rpm at 4^0c. Thereafter supernatant was discarded and the excess fluid was blotted out.

Cells were incubated with Fc receptor blocker (50 µl 2.4G2 Hybridoma supernatant) for 5 minutes on ice, washed by centrifuging at 1200rpm for 5 minutes, fixed in 0.5 ml 2% paraformaldeyde (Sigma) and incubated on ice for 15 minutes and washed. One millilitre FACS buffer was added per tube to wash the cells. The cells were then incubated for another 10 minutes on ice in 0.1% saponin (Sigma) in FACS buffer to open up the membrane pores. A cocktail of flourochrome labelled antibodies (BD Bioscience, Mississauga, Ontario; 0.5 ug/tube/ antibody) was made in 0.1% saponinat a final volume of 20 µl/tube was added and the tubes were incubated for 25 minutes on ice and in the dark. Labelled cells were then washed first with 1 ml saponin (Sigma) in FACS buffer and then with FACS buffer to allow membrane closure. Cells were re-suspended in 300 µl/tube and were acquired using FACS Canto II (BD Bioscience) and analyzed using Flowjo (Flow Jo, Ashland, OR)

3.11 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Assay

In vitro cell proliferation was measured using CFSE dilution method. Single cell suspensions were made from whole spleen or draining lymphnodes and counted using a haemocytometer as described above. Cells were washed with 5 ml warm PBS, the supernatant was decanted and the pellet re-suspended in warm PBS to a final concentration of 8 million cells/ml. Five million cells were removed as unlabelled controls. Five milliliters of cell suspension was transferred into 15 ml centrifuge tube (BD Falcon), and 5 ml of 5 mM 1:2000 dilution CFSE was added to the tube and rocked gently by inversion for 5 minutes. Thereafter 5 ml warm FBS (Hyclone), was added to the tube and cells spun at 4 ^{0}C for 5 minutes at 1200 rpm. The pellet was re-suspended in 5 ml complete culture medium. Viable cells were counted as described above in section 3.6. Cells were re-suspended to final concentration of 2 million cells/ml and 200 µl aliquots were plated in 96 well round bottom plates (BD Falcon) and stimulated with or without SLA (50 µg/ml). Stimulated cells were incubated for 4 days at 37 ^{0}C in 5% CO_2. Thereafter, the supernatants were collected for ELISA and the cells stimulated and stained as described in 3.10 above.

3.12 Ex vivo Staining of Dendritic Cells

High and low dose *L. major* infected mice were sacrificed at days 7 and 14 weeks post-infection and the draining lymph nodes collected, minced into small pieces and digested with 2 ml RPMI-1640 medium containing 2% FBS and 1 mg/ml collagenase Type 1 A (Sigma, Oakville ON, Canada) and DNase I (Roche, Mississauga ON, Canada) by incubating for 25 minutes at 37 ^{0}C. The tissues were further disrupted with a 3 ml syringe plunger and the

suspension was filtered through a 70 μm cell strainer (VWR) to remove tissue debris, washed with 10 ml complete medium by centrifuging at 1200 rpm for 5 minutes. The cell pellets were re-suspended in 2 ml complete medium, counted, stained directly *ex vivo* with different flourochrome-conjugated mAbs against CD11c, CD40, CD86, CD103, CD8α and MHC II and analyzed by flow cytometry.

3.13 Assessment of Memory T Cell Subsets:

High and low dose *L. major* infected mice were sacrificed at 14 weeks post-infection and single cell suspensions of the draining lymph nodes and spleens were made. The cell were counted and adjusted to 4 x 10^6/ml and 100 μl aliquots were stained directly *ex vivo* with different flourochrome-conjugated mAbs against CD3, CD4, CD8, CD44 and CD62L and analyzed by Flow cytometry.

Table 2: List of flourochrome-conjugated antibodies used in flow cytometry

Antibody	**Clone**	**Location on cell**	**Fluorochrome**
CD4	RM4-5	Surface	FITC, PE, APC, APC-Cy7, Eflour
CD8	53-6.7	Surface	FITC, PE, APC, PE Cy5.5
IL-4	11B11	Intracellular	PE, APC
IL-10	JESS16E3	Intracellular	PE
CD25	PC61	Surface	FITC, PE, APC

IFN-γ	XMG1.2	Intracellular	Eflour PE, APC
FoxP3	FJK-16s	Intracellular	FITC, PE, APC
IL-12	C15.6	Intracellular	PE, APC
CD3	17A2	Surface	FITC, PE, APC, Eflour, APC-Cy7
CD40	IC10	Surface	PE, APC
MHC II	M5/114.15.2	Surface	PE, APC, FITC, Eflour
CD11c	HL3	Surface	PE, APC, FITC, Eflour
CD11b	M1/70	Surface	PE, APC, FITC, Eflour
CD 86	GL1	Surface	FITC
CD103	2E7	Surface	PE
CD40L	MR1	Surface	APC
CD44	1M7	Surface	APC,
CD62L	MEL-14	Surface	PE, APC

3.14 Flow Cytometry Gating Strategy

Cells were first gated of live lymphocytes and then gated on CD3 or CD11c or CD11b cells. I further gated on cells expressing other surface and intracellular markers depending on the particular study.

3.15 Delayed Type Hypersensitivity (DTH):

DTH was determined at 72 hours post challenge with live parasites by measuring foot pad swelling using digital calipers (Fisher).

3.16 Determination of Parasite Burden:

At the indicated times mice were sacrificed and the foot cut off just above the ankle and placed in a 15 ml centrifuge tube containing 2 ml 200 U/ml penicillin, 100 µg/ml streptomycin in PBS (Invitrogen) (2X P/S in PBS). Each foot was transferred sequentially every five minutes into 20 ml 70% ethanol, 20 ml chlorhexiderm, (DVM Pharmaceuticals, Maimi, FL), 70% ethanol and finally into 2X P/S in PBS. (Invitrogen). Toes and skin were removed from feet using stainless steel blade (Fisher). Feet were homogenized in a 15 ml autoclaved tissue grinder (Fisher) with 2x P/S. Tissue grinder was washed out with PBS and fluid was collected into a 15 ml centrifuge tube (BD Falcon) and was spun at 500 rpm for 5 minutes. The supernatant was transferred into another 15 ml centrifuge tube and was spun at 3000 rpm for 15 minutes. The supernatant was decanted and the pellet re-suspended in 2ml complete Schneider medium (Invitrogen) supplemented with 20% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 25 mM HEPES (Invitrogen). Twenty microliters per well of

parasite suspension was seeded into each flat bottom 96 well plates containing 180 µl/well of complete Schnieder medium. Ten fold serial dilutions were made and the last 20 µl discarded. Plates were wrapped in plastic wrap and incubated at 27 ^{0}C for 7 days (Fisher) and parasite growth was assessed by microscopy.

3.17 I*n vivo* Depletion of Cells

$CD4^{+}$ and $CD8^{+}$ T cells were depleted in healed mice 24 hr. before *L. major* challenge by injection of 500 µg of anti-CD4 (GK1.5) and anti-CD8 (TIB 210) monoclonal antibody intraperitoneally respectively. Titration studies from the lab show that this dose of antibody leads to complete depletion (> 98%) of $CD4^{+}$ and $CD8^{+}$ positive cells, respectively, for up to 7 days.

3.18 Injection of Fusion Proteins and Recombinant IL-12

Naïve or healed mice were treated with 100 ug of HVEM-Ig or LTßR-Ig to block the interaction of LIGHT and its receptors HVEM and LTβR respectively 24 hours before injection of parasites and once weekly for 2 more weeks. CD40, CD40L and IL-12 deficient mice were treated with 0.3 ug rIL-12 subcutaneously in the infection foot three times weekly for the first two weeks of infection.

3.19 Preparation and Infection of Bone marrow Derived Dendritic Cells (BMDC) and Bone Marrow Derived Macrophages (BMDM).

Bone marrow (BM) was flushed out from the tibia and femur of naïve mice with complete medium with 10ml syringe and 30-gauge needle. Clumps of BM were disrupted by aspirating and pushing out the cells 10 times. The BM suspension was then spun at 1200 rpm for 5 minutes. Red blood cells were lysed using 2 ml red blood cell lysing buffer for 3 minutes. Cells were then counted and re-suspended in complete medium at $2x10^6$/ml (macrophages) and $4x10^6$/ml for (dendritic cell) differentiation. The cells were differentiated into macrophages using complete medium supplemented with 30% L929 cell culture supernatant. BMDCs were differentiated in petri dishes in the presence of 20 ng/ml of recombinant granulocyte monocyte colony stimulating factor (rGM-CSF) (Peprotec). Immature dendritic cells and macrophages were harvested on day 7 and assessed for the expression of CD11c, CD40, CD80, CD86, and MHC class II by flow cytometry. The BMDCs were infected with 7 day *Leishmania* promatigotes at a cell-to-parasite ratio of 1:5 in propylene tube for 5 hours. After 6 hours of infection, free parasites were washed away by spinning at 500 rpm for 5 minutes in a centrifuge for a total of 5 times.

3.20 Cytospin of Infected BMDCs

To confirm that BMDCs were infected with *Leishmania* parasites, we performed cytospin. Briefly, 50 µl of infected cells were mixed with 50 µl of FBS (Hyclone) to make a final volume of 100 µl. The parasite and FBS mix was transferred to cytospin tubes and metal holder

containing a glass slide and spun at 1200 rpm for 5 minutes in a cytospin machine (Thermo Fisher).

3.21 Geimsa Staining

The glass slide (from the cytospin above) was fixed in alcohol (Sigma) for 30 seconds and excess fluid removed by dabbing on paper towel. The fixed slide was then transferred to a container with the red dye hematoxylin (Sigma) for 30 seconds excess fluid was removed by dabbing on a paper towel. Finally, the slide was immersed in eosin dye (Sigma) for an additional 30 seconds and excess fluid was removed by running slide under clod tap water for 30 seconds. The stained slide was allowed to air dry.

3.22 Infectivity of Bone Marrow Derived Dendritic Cells (BMDC)

Rate of infectivity was determined by viewing the Geimsa stained slides under the microscope at 100 times magnification (oil immersion).

3.23 Adoptive Transfer of Cells and *in vivo* Recall Response

Draining lymph-node (dLN) and spleen cells from healed (> 12 weeks post-infection) donor (Thy1.2) mice infected with low or high dose *Leishmania major* were labeled with CFSE dye as described in 3.12. CFSE labelled cells were then transferred into naïve Thy1.1 recipient mice by intravenous injection (30 million cells per recipient mouse). Twenty-four hours after adoptive transfer, recipient mice were infected with 5 million *L. major*. After 7 days, mice were sacrificed and dLN cells were assessed for proliferation and cytokine secretion directly *ex vivo* by intracellular cytokine staining described in section 3.10.

3.24 I*n vitro* Co-culture with Infected BMDC

In some experiments, lymph-nodes and spleen cells were co-cultured with infected BMDCs for 4 days (at a ratio of one dendritic cell to one hundred lymph-node cells). At the end of the culture period, the cells were stimulated with PMA (50 ng/mL), ionomycin (500 ng/mL) and brefeldin A (BFA; 10 mg/mL) for 4 hours and routinely stained intracellularly and analyzed by flow cytometry.

3.25 Isolation of Splenic $CD11c^+$, $CD11b^+$ and $CD90^+$ Cells

Splenic macrophages were isolated by positive selection using Stem Cell EasySep^R magnetic isolation kit according to manufacturer's instructions. Briefly, Single cell suspension was prepared from whole spleens at $1x10^8$ / cells in recommended medium (2% fetal bovine serum with 1mM EDTA in phosphate buffered saline). Cells were the placed in 5ml polystyrene tube. EasySep^R CD11c, CD11b or CD90 PE labelling antibody was added at 50 µl/ml of cell and then incubated at room temperature for 15 minutes. EasySep^R PE selection cocktail was added at 100 µl/ml of cells for another 15 minutes at room temperature. Next, magnetic nanoparticles were added at 50 µl/ml of cell and incubated for 10 minutes at room temperature. The cell suspension was brought to final volume of 2.5 ml by adding predetermined volume of recommended medium. The cell suspension was mixed thoroughly by pipetting up and down 3 times. Magnetically labelled cells were selected by placing the cells in the EasySep^R magnet for 5 minutes and the supernatant removed. The tube was then removed and cells were resuspended in 2.5 ml recommended medium, placed back in the magnet and set aside for another 5 minutes.

The last step was repeated 2 more times. The tube was removed from the magnet and the cells resuspended in 1-3 ml complete medium and counted.

3.26. Treatment of BMDC and BMDM and Splenic Macrophages with Soluble CD40L, anti-mac-1 antibody and Lipopolysaccharide

BMDM, BMDC and splenic $CD11b^+$, $CD11c^+$ cells were diluted in complete cell culture medium at a concentration of 2 million cells/ml. Cells were then cultured at 100 µl/ well in 96 well flat bottom cell culture plates (Falcon) at a final dilution concentration of 200,000 cell/well. Cells were stimulated with sCD40L at a final concentration of 5 µg/ml in 100 µl of complete medium. In some cases where anti-mac antibody was added, 5 µg/ml of sCD40L was added in 50 µl and 5 µg/ml of anti-mac-1 antibody was also added in 50 µl. The final volume for all culture condition was 200 µl. Lipopolysaccharide (LPS) from E. coli (Sigma) was added at a concentration of 1µg/ml in 100 µl of complete medium.

Table 3. List of Stimulants used for Cell Culture

Stimulant	**Concentration**
Lipopolysaccharide	1 µg/ml
Anti-Mac-1 antibody	5 µg/ml
Soluble CD40 ligand	5 µg/ml
Soluble *Leishmania* antigen	50 µg/ml
Granulocyte monocyte colony stimulating factor	20 ng/ml

3.27 Statistical Analysis

Two-tailed student T test was used to compare mean and standard error of mean (SEM) between two groups. In some other experiments, one-way analysis of variance (ANOVA) non-parametric was used to compare mean and standard deviation (SD) of more than two groups. Tukeys post-test was used where there was significant difference in ANOVA. Differences were considered significant when $p < 0.05$**.** Unless otherwise stated, there were at least four mice per group and each experiment was carried out at three times.

4. RESULTS

4.1 Determine the Effect of Antigen Dose in Primary Immune Response after Infection with Low and High Dose *L. major*

4.1.1 Introduction

Dendritic cells are important components of the immune system and are capable of sensing and recognizing different pathogens and damage signals. As the predominant antigen presenting cells, dendritic cells bridge the innate and adaptive immune responses by initiating and maintaining T dependent immune responses and are important considerations for the design of vaccines that are dependent on T cells [345, 346]. When dendritic cells receive activating signals, they migrate to the draining lymph-nodes where they undergo a maturation process characterized by increased expression of major histocompatibility complex (MHC) I and II which allows them present antigens to T cells in the context of MHC I and II to $CD8^+$ and $CD4^+$ T cells, respectively.

Even though a lot is known about the immune response to *Leishmania* parasites, there is currently no effective licensed vaccine for prevention of human leishmaniasis. This could in part be related to lack of proper understanding of the immunobiology of the disease, especially the factors that regulate the induction, maintenance and loss of protective immunity. Also because *Leishmania* are obligate intracellular parasites, a strong T cell-mediated immunity is critical for effective control of the infection. In line with this, T cell deficient mice are highly susceptible to *Leishmania* infection, and adoptive transfer of T cells restores resistance in these mice [347]. Mice deficient in MHC II molecule and as such do not develop $CD4^+$ T cells develop progressive non-healing lesion after *Leishmania major* infection [348]. It is commonly believed that $CD4^+$ T cells that produce Th1 cytokines like IFN-γ and TNF are the key lymphocyte subset that

regulates anti-*Leishmania* immunity [110]. However, there is evidence in the literature suggesting that both $CD4^+$ and $CD8^+$ T cells are involved in effector immune response to leishmaniasis. Studies utilizing low dose infections showed that $CD8^+$ T cells are also important for optimal primary immunity [121, 122]. Thus, while CD8 deficient mice are still resistant to high dose *L. major* infection, they were susceptible to low dose infection characterised by uncontrolled parasite proliferation and impaired IFN-γ response [121]. Data from this study suggests that parasite dose may influence the activation of dendritic cells and subsequently type of T cells that are induced following *L. major* infection. However, no study has addressed the impact of parasite dose on the magnitude of initial T cell (both $CD4^+$ and $CD8^+$) expansion, and its effects the development of secondary anti-*Leishmania* immunity. In addition, the contribution of $CD8^+$ T cells to low dose infection-induced (secondary) resistance is unknown.

4.1.2 Hypothesis

I hypothesized that infection with low dose and high dose *L. major* will lead to differences in dendritic cell activation with subsequent differences in T cell response.

4.1.3 Objectives

a. To characterize the quality and quantity of primary and secondary immune response to low and high dose *Leishmania major*.

b. Determine the impact of *Leishmania* dose on the induction and maturation of dendritic cells.

c. Compare the ability of low and high antigen dose to protect mice against virulent challenge.

d. Investigate the role of CD8 and CD4 T cells in protection against virulent challenge in mice that received primary low and high dose infection.

4.1.4: Results

4.1.4.1 Kinetics of Lesion Development and Cell Recruitment Following Low and High Dose *L. Major* Infection.

To determine if dose of infecting parasites affects the pattern of lesion development we infected C57BL/6 mice with low dose (1000) and high dose (2 million) parasites and compared lesion size, total number of cells in the draining lymph-nodes (dLNs) and parasite burden between mice infected with low and high dose *L. major* weekly. The pattern of lesion development in the footpads was similar in both low and high dose-infected mice although the overall lesion sizes were significantly ($p < 0.05$ (wk 2), $p<0.01$ (wk 3) and $p < 0.001$ wk 7) higher in mice infected with high dose parasites from 2-7 weeks post-infection (Fig. 4A). In addition, high dose-infected mice had significantly ($p < 0.05$ and $p < 0.001$) more cells in their dLNs at 3 and 21 days post-infection than those infected with low dose parasites (Fig. 4B). Beyond 21 days, there was no significant difference in the number of cells in the dLNs, despite continued difference in lesion size that lasted up to 8 weeks post-infection (Fig. 4B). Furthermore, parasite burden in the high dose-infected mice was also significantly ($p < 0.05$ and $p < 0.001$) higher at 3, 5 and 7 weeks post-infection compared to the low dose-infected mice but this difference was abolished by 9 weeks post-infection (Fig. 4C). These results show that although the size of cutaneous lesion, inflammation and cell recruitment into the dLN are different following high and low dose *L. major* infections, the pattern and time to resolve cutaneous lesion and parasite clearance are comparable.

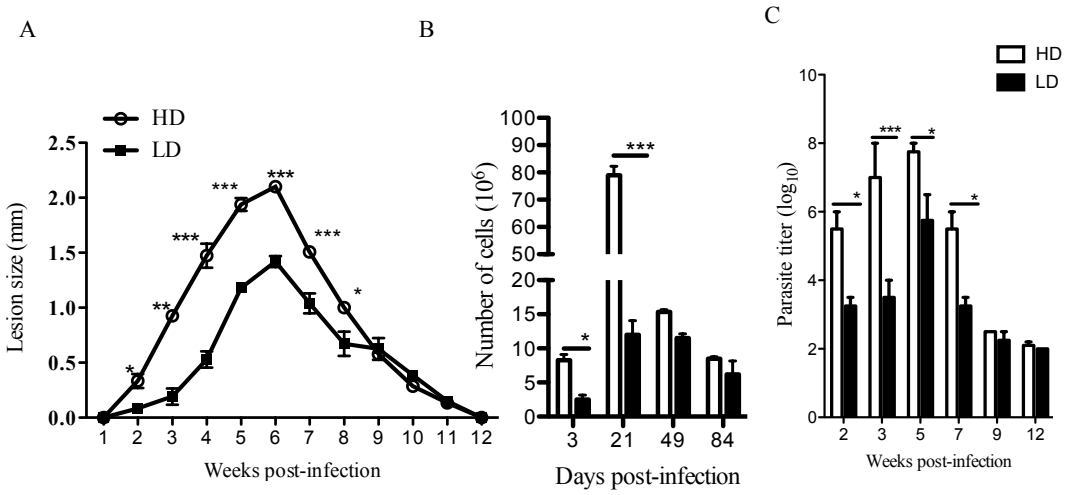

Figure 4. Kinetics of lesion development, cell recruitment in the draining lymph nodes and parasite burden following high and low dose L. major infections. C57BL/6 mice were infected with 10^3 low dose (LD) or 2 x 10^6 high dose (HD) *L. major* promastigotes in their right hind footpad and lesion size was measured weekly with Vernier callipers (A). At indicated times, some mice were sacrificed and the number of cells in the draining lymph nodes (dLNs) was determined by trypan blue exclusion test (B). Parasite burden in the infected footpads was determined by limiting dilution assay (C). Results presented are representative of 3 independent experiments (n = 4 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.4.1.2: Differential Expansion and Induction Of T Cell Subsets After Low and High Dose *L. Major* Infection

To determine the influence of parasite dose on the early expansion and activation of T cell subsets following *L. major* infection, we co-cultured CFSE-labeled dLN cells from mice infected with low and high dose *L. major* (one week post-infection) with *L. major*-infected BMDCs and assessed T cell proliferation and cytokine (IFN-γ and TNF) production by flow cytometry. There was a striking difference in proliferation of $CD4^+$ and $CD8^+$ T cells from dLNs of low and high dose-infected mice with high dose infection inducing significantly ($p < 0.05$-0.01) higher numbers of proliferating $CD4^+$ T cells compared to low dose infections (Figs. 5A and 5B). Conversely, low dose infection induced significantly ($p < 0.05$-0.01) higher numbers of proliferating $CD8^+$ T cells than high dose infection (Fig. 5A). Consistent with this, the percentage and mean fluorescence intensity (MFI) of IFN-γ and TNF-producing $CD4^+$ T cells in dLNs of high dose-infected mice were higher than those of low dose-infected mice (Figs. 5 C- 5D & 5G). In contrast, the percentage and MFI of IFN-γ and TNF-producing $CD8^+$ T cells were significantly higher in low dose-infected mice (Figs. 5 E-5F & 5H). Taken together, the data presented here show that the initial parasite dose affects the nature of T cell response in *L. major* infected mice.

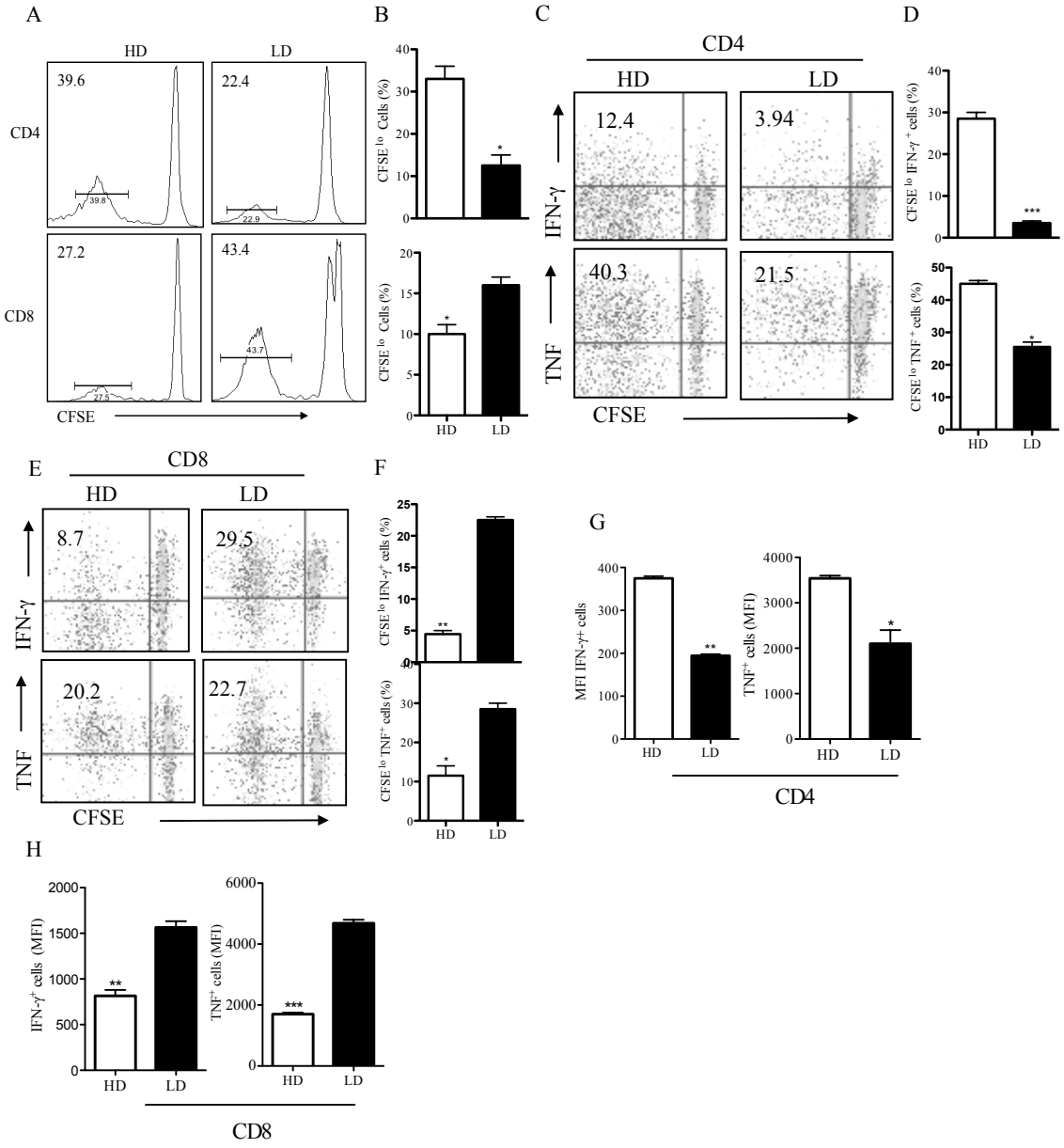

Figure 5. High and low dose infections preferentially expand CD4$^+$ and CD8$^+$ T cells, respectively. C57BL/6 mice were infected with 10^3 low dose (LD) or 2 x 10^6 high dose (HD) *L. major* promastigotes in their right hind footpad, sacrificed after 7 days and the draining lymph-node cells were labelled with CFSE dye and co-cultured with *L. major*-infected bone marrow-derived dendritic cells (BMDC) at a DC: lymph-node cell ratio of 1:100. After 4 days, the percentages of proliferating (CFSE^{lo}) CD4$^+$ and CD8$^+$ T cells (A-B), proliferating (CFSE^{lo}) IFN-γ (upper panel) and TNF (lower panel) -producing CD4$^+$ (C-D) and CD8$^+$ (E-F) T cells and mean flourescence intensity of IFN-γ and TNF in the CD4 (G) and CD8 (H) populations were determined by flow cytometry. Results are representative of 3 independent experiments (n = 6 mice/group) with similar results. * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$.

4.1.4.3 Differential Expansion of T Cells Following Low and High Dose Infection is Sustained Through out Infection

It is plausible that the early differential expansion of $CD4^+$ and $CD8^+$ T cells subsets by high and low dose *L. major* infection, respectively, is transient and related to differences in parasite number at the infection site (see Fig. 4C). We therefore wondered if differences in T cell expansion would be lost when both low and high dose infected mice have healed their lesion. To investigate this we assessed T cell responses (proliferation and cytokine production) after 12 weeks when lesion is fully resolved and parasite burden in the footpads (Fig. 4C) of mice infected with high and low dose *L. major* are comparable. Flow cytometric analyses show that just like the observations during early infection, the percentage of total proliferating $CD4^+$ and $CD8^+$ T cells was significantly ($p < 0.01$) higher in high dose and low dose infected mice respectively (Figs. 6A and B). Proliferating IFN-γ and TNF-secreting $CD4^+$ T cells was significantly ($p < 0.01$-$p < 0.001$) higher in mice that received high dose infection compared to those that healed low dose infection (Figs. 6 C and D). Also proliferating IFN-γ and TNF-secreting $CD8^+$ T cells in mice infected with low dose parasites were significantly ($p < 0.05$) higher than those from high dose infected mice (Figs. 6 E and F). Together, these results suggest that an early antigen encounter creates an immunological imprint in T cell subset responses that is maintained throughout the course of *L. major* infection.

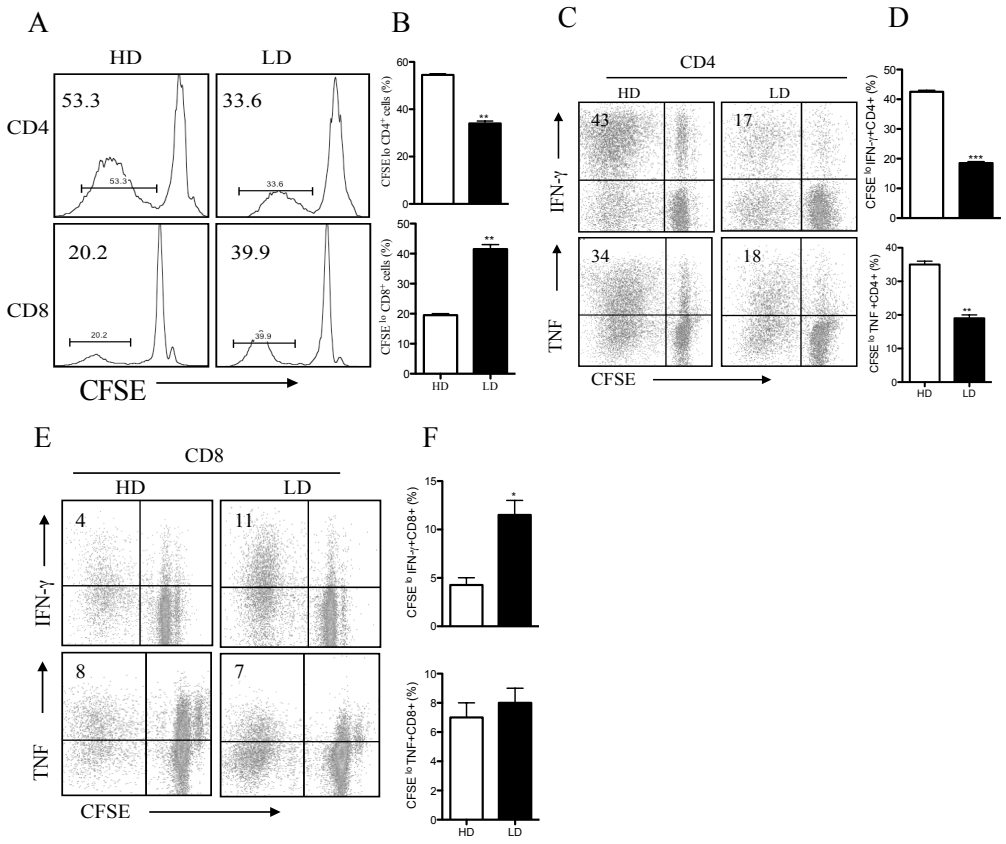

Figure 6. Sustained differential expansion of CD4$^+$ and CD8$^+$ T cells by high and low dose infections, respectively during recall response. C57BL/6 were mice infected with 10^3 low dose (LD) or 2 x 10^6 high dose (HD) *L. major* and allowed to completely resolve (heal) their lesion (> 12 wks.). Healed mice were sacrificed and dLN cells were labelled with CFSE dye and co-cultured with *L. major*-infected BMDCs at a DC:dLN cell ratio of 1:100. After 4 days, the percentages of proliferating (CFSE^{lo}) CD4$^+$ and CD8$^+$ T cells (A), proliferating (CFSE^{lo}) IFN-γ (upper panel) and TNF (lower panel) producing CD4$^+$ (C-D) and CD8$^+$ (E-F) T cells was measured by flow cytometry. Results presented are representative of 3 independent experiments (n = 3-4 mice/group) with similar results. * $P < 0.05$, ** $p < 0.01$, ***, $p < 0.001$.

4.1.4.4 Antigen Dose Affects the Induction and Maturation of Dendritic Cells.

Dendritic cells present antigen to $CD8^+$ and $CD4^+$ T cell on major histocompatibility complex (MHC) I and II respectively [349]. Since we observed a sustained expansion of $CD4^+$ and $CD8^+$ T cells following high dose and low dose infection respectively, we therefore investigated whether this differential expansion is dependent on the type of dendritic cell induced early on in the infection. We infected mice with low dose and high dose *L. major* and collected draining lymph-nodes at day 3-post infection to asses the induction of dendritic cell subsets as well as the expression of markers of maturation. There was no difference in the percentage and absolute number of CD11c expressing cells (dendritic cells) present in the dLNs (Fig. 7A and 7B). Also the percentage and absolute number of $CD11c^+CD86^+$ (Figs. 7C and 7D) $CD11c^+CD40^+$ (Figs. 7E and 7 F), dendritic cells were not different between low-dose and high-dose infected groups. Interestingly, the percentage and absolute numbers of $CD11c^+MHCII^+$ dendritic cells in dLNs of high dose-infected mice were significantly ($p < 0.05$) higher than those from low dose-infected mice (Figs. 7G and 7H). In contrast numbers of $CD11c^+CD103^+CD8\alpha^+$ dendritic cells which are important in cross presenting exogenous antigens to $CD8^+$ T cells via MHC I [349, 350], was significantly ($p < 0.05$) higher in the low dose infected group compared to the high dose infected mice (Figs. 7I and 7J) early on in the infection. In addition and consistent with observations during early infection, the difference in absolute numbers of $CD11c^+$ dendritic cells expressing MHC II was still significantly ($p < 0.05$) higher in the high dose-infected mice that had healed their primary lesion compared to those that healed low dose infection (Figs. 7K and 7L). Together, data presented here indicate that antigen dose does not

affect the induction and maturation of dendritic cells however; it affects the differentiation of different dendritic cell subsets.

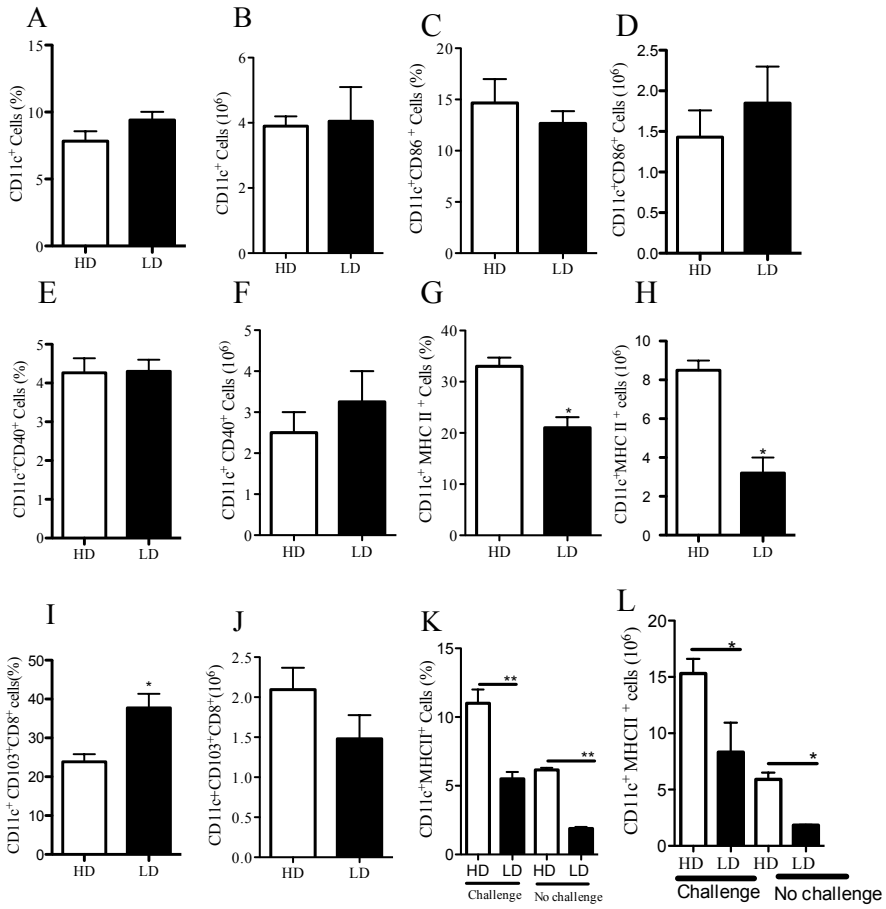

Figure 7: Induction and activation of dendritic cells after primary and secondary infection with low dose and high dose *L. major*.

Naïve C57BL/6 mice were infected with 10^3 low dose (LD) or $2x10^6$ high dose (HD) *L. major* parasites in their right hind footpad. Three days post-infection, mice were sacrificed and the draining lymph-node cells collected and digested with collagenase. The percetnage and absoute number of CD11c + cell (A-B) was determined by flow cytometry. Gating on CD11c, the percentage and absolute numbers of CD86 (C-D), CD40 (E-F), MHC II (G-H), CD103 (I-J) was determined by flow cytometry. In some expperiments, healed C57BL/6 mice were challenged or not with virulent *L. major*, three days after challenge, the expression of MHC II on CD11c positive cells (K-L) was determined by flow cytometry.* $P < 0.05$, ** $p < 0.01$

4.1.4.5 Differential Expansion of T cell Subsets in Low and High Dose Infected Mice Occurs *In vivo*

To confirm that the preferential activation of $CD8^+$ and $CD4^+$ T cell responses by low and high dose infections, respectively, occurs *in vivo* and is therefore physiologically relevant, we adoptively transferred CFSE-labeled whole spleen cells isolated from Thy1.2 mice that healed their low or high dose *L. major* infection into naïve Thy1.1 recipient mice. Recipient mice were then challenged 24 hr later with *L. majo*r and sacrificed after one week. At sacrifice, donor ($Thy1.2^+$) cells from the dLNs were assessed directly *ex vivo* for proliferation and cytokine (IFN-γ and TNF) production by flow cytometry. Similar to the *in vitro* findings, donor cells from low dose-infected mice contained significantly ($p < 0.05$) higher percentage of proliferating $CD8^+$ cells whereas those from high dose-infected mice had significantly ($p < 0.05$) higher percentage of proliferating $CD4^+$ cells (Fig. 8A). Also the percentage (Fig. 8B) of proliferating and IFN-γ and TNF-producing $CD8^+$ T cells were significantly ($p < 0.05$) higher in cells from low dose-infected donor mice compared to those from high dose-infected recipient donor mice (Fig. 8B). In contrast, donor cells from high dose-infected mice had significantly ($p < 0.05$) more proliferating and cytokine-producing $CD4^+$ cells than those from low dose-infected mice (Fig. 8C). Collectively, these results show that high and low dose *L. major* infections-induced differential T cell responses occur *in vivo* and create an imprint that is maintained over time during the course of infection.

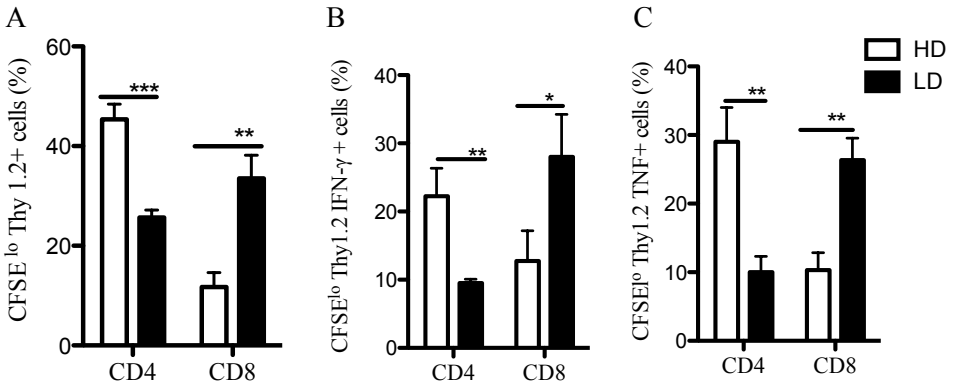

Figure 8. Differential expansion of CD4$^+$ and CD8$^+$ T cells following high and low dose infections with *L. major* occur *in vivo*. CFSE labelled whole spleen cells from Thy1.2 C57BL/6 mice that healed their primary 10^3 low dose (LD) or 2 x 10^6 high dose (HD) *L. major* infections were adoptively transferred to Thy1.1 congenic recipient mice. After 24 hr, the recipient mice were challenged with 5 million virulent parasites and sacrificed after 1wk. The percentages of proliferating donor (Thy1.2$^+$) cells (A), IFN-γ (B) and TNF (C)-producing cells were determined by flow cytometry. Results presented are representative of 2 independent experiments (n = 3-4 mice/group) with similar results. (*, $p < 0.05$. **$p < 0.01$; $p < 0.001$)

4.1.4.6 Primary Infections with Low and High Dose *L. major* Induce Comparable Protection Following Virulent Low and High Dose Challenge.

Although we found that the pattern of lesion development and parasite clearance are similar in mice infected with low and high dose *L. major*, it is possible that antigen dose may impact the quality of secondary anti-*Leishmania* immunity. To test the effect of antigen dose on secondary anti-*Leishmania* immunity, we generated healed low dose and high dose mice. When healed high and low dose-infected mice were challenged with high dose (5 million) virulent *L. major,* there were no significant differences in DTH response (Fig. 9A) and rapid parasite control in the challenged footpads (Fig. 9B). $CD44^{hi}$ $CD62L^{lo}$ central memory (Tcm) like T cells and $CD44^{lo}$ $CD62L^{hi}$ effector memory (Tem) like T cells are known to develop following primary infection with *Leishmania* major and are able to confer protection against challenge with virulent *L. major* in naïve mice [256]. Since antigen dose has been shown to influence the type and quality of memory cells in viral infections [351], we wished to characterize the subsets of memory cells in the lymph-nodes of mice that healed their primary *Leishmania major* infection. There was no difference in the percentage of $CD44^{hi}CD62L^{lo}$ Tcm like and $CD44^{lo}$ and $CD62L^{hi}$ Tem-like T cells in the draining lymph-nodes and spleen gated on both CD4 and CD8 T cells (Fig. 9C) of mice that healed their primary infection with low dose or high dose *L. major*. Together, these data suggest that both low and high dose infections induce qualitatively comparable infection-induced resistance that may be dependent on their ability to induce similar number of central and effector memory- like T cells.

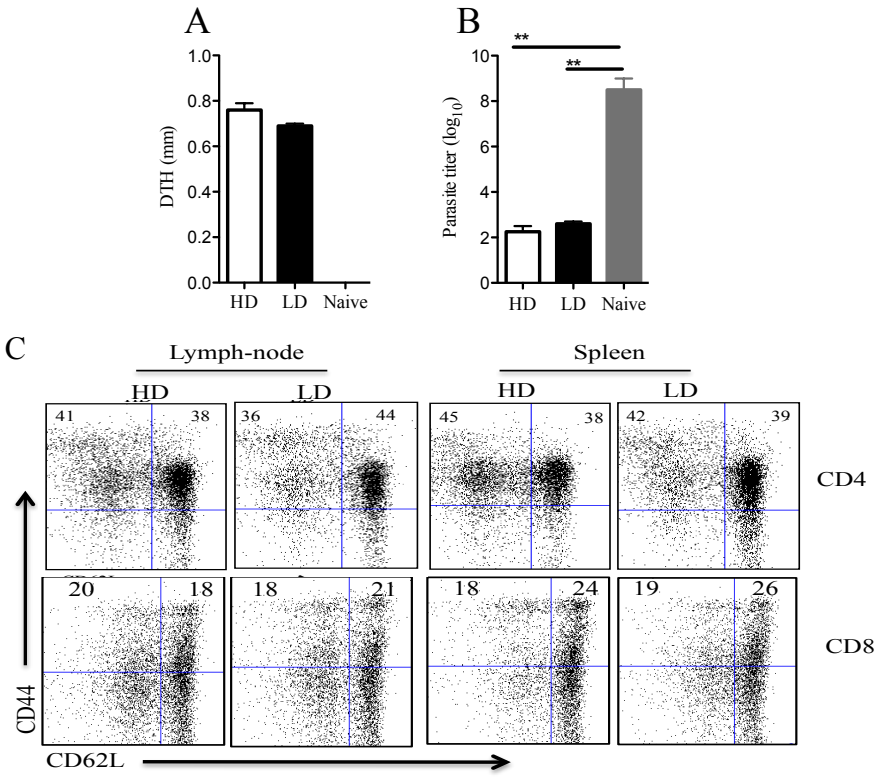

Figure 9. Low dose and high dose infection leads to comparable protection against virulent challenge.

C57BL/6 mice were infected with low dose (10^3) or high dose (2 x 10^6) *L. major* and allowed to completely resolve their lesions (> 12 wks.). Healed mice were then challenged with 5 x 10^6 parasites in the contra-lateral footpad and delayed-type hypersensitivity (DTH) response was determined at 72 hr post-challenge (A). Three weeks after challenge, mice were sacrificed and parasite burden in the challenge footpads was determined by limiting dilution assay (B).To determine if antigen dose affect memory T cells subsets, healed mice (>12 weeks post infection) were sacrifced and the percentage (C) of central ($CD3^+CD44^{hi}$ $CD62L^{hi}$) and effector memory ($CD3^+CD44^{hi}$ $CD62L^{lo}$) $CD4^+$(upper panel) and $CD8^+$ (lower panel) T cells in the draining lymph-nodes and spleens were determined by flow cytometry. Results presented are representative of 3 independent experiments (n = 3-4 mice/group) with similar results. **, $p < 0.01$.

4.1.4.7 $CD8^+$ T Cells are Dispensable for Protection Against Secondary *Leishmania major* Infection.

Although it was previously reported that $CD8^+$ T cells are important for optimal immunity to primary low dose *L. major* infection [352], it is not known whether they also contribute to secondary anti-*Leishmania* (infection-induced) immunity after low dose primary infection. We observed herein a preferential expansion of $CD8^+$ cells during recall responses in mice that healed their low dose *L. major* infection (see Figs. 5B and 6B), suggesting that $CD8^+$ T cells might also be critical for secondary anti-*Leishmania* immunity. We therefore, treated healed low and high dose-infected mice with anti-CD4 or anti-CD8 mAb (to deplete $CD4^+$ and $CD8^+$ cells, respectively, Fig. 10A) and challenged them with high *L. major* after 24 hr. Surprisingly, depletion of $CD8^+$ cells neither affected DTH response (Fig. 10B) nor rapid parasite control (Fig. 10C) in both low and high dose-infected mice. In contrast, $CD4^+$ T cell depletion in both low and high dose-infected mice significantly ($p < 0.05$) affected DTH response and parasite control compared to age-matched control-Ig treatment. Since CD8 T cells seems to play a role only in low dose primary infection [121], we then investigated whether, CD8 T cells will be important following low dose challenge. We show that just like in the high dose challenge study, depletion of $CD8^+$ cells did not affect DTH response (Fig. 10D) and rapid parasite control (Fig. 10 E) in mice that received both low and high dose primary infection. Collectively, these results indicate that although low dose *L. major* infection preferentially expands $CD8^+$ T cells, $CD4^+$ T cells are the major cells that mediate secondary anti-*Leishmania* immunity in mice. They further show that although $CD8^+$ T cells are important for optimal immunity to primary low dose infection [121], they are completely dispensable during a secondary *L. major* challenge.

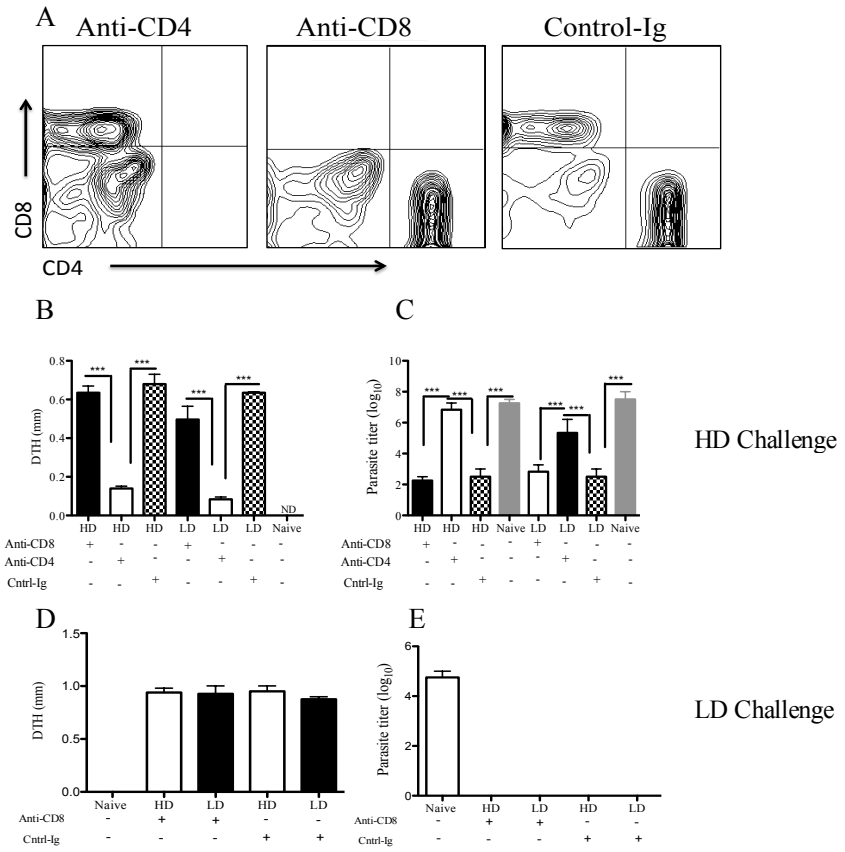

Figure 10. CD8$^+$ T cells are dispensable for secondary anti-Leishmania immunity following primary low dose *L. major* infection.

Mice that healed their primary low dose (LD) and high dose (HD) infection were treated with anti-CD8 or anti-CD4 mAbs to deplete CD8$^+$ or CD4$^+$ cells, respectively (A). Control-Ig or antibody treated mice were challenged with 5 x 10^6 *L. major* after 24 hr. and DTH response (B) was measured at 72 hr post-challenge.

Three weeks after challenge, mice were sacrificed and parasite burden in the challenged footpads was determined by limiting dilution (C). In some experiments healed mice were injected 100ug of anti CD8 antibody to depeleteCD8 T cells 24hrs before challenge with low dose (10^3) *L. major*. DTH response (D) was measured 72 hrs post challenge. Three weeks later mice were sarificed and parasite burden (E) was determined by limiting dilution asssay. Age-matched healed mice treated with control-Ig and naïve mice served as controls. Results presented are representative of 2 independent experiments (n = 3-4 mice/group) with similar results. *, $p < 0.05$; ***, $p < 0.001$.

4.2 Role of LIGHT (lymphotoxin like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) in Cutaneous Leishmaniasis

4.2.1 Introduction

The outcome of infection with the intracellular pathogen *Leishmania major,* the causative agent of cutaneous leishmaniasis, is dependent on the nature of $CD4^+$ helper T cell (Th) subset that is induced [15, 353-355]. Resistance is usually correlated with the development of strong IFN-γ-producing $CD4^+$ T helper type 1 (Th1) cells, which activate macrophages to produce nitric oxide, an effector molecule for killing intracellular parasites. In contrast, susceptibility has been associated with IL-4 and IL-10 production by Th2 cells, cytokines that deactivate macrophages and inhibit their intracellular parasite killing ability. A key factor that regulates the nature and quality of anti-*Leishmania* immunity in infected mice is the level of IL-12 production by dendritic cells and the degree of responsiveness of naïve $CD4^+$ T cells to this cytokine [140, 143, 356]. Thus, BALB/c mice are highly susceptible to *L. major* because their dendritic cells produce very low amounts of IL-12 and their $CD4^+$ T cells respond poorly to it due to the down regulation of IL-12Rβ expression [356-358]. In contrast, dendritic cells from the resistant C57BL/6 mice produce high levels of IL-12 and their $CD4^+$ T cells maintain IL-12 responsiveness throughout infection [356]. The pathways that influence early IL-12 production by dendritic cells during *L. major* infection are still poorly understood although the nature of activation and expression of various co-stimulatory molecules appear to be important [224, 226].

The tumour necrosis factor (TNF) super family of cytokines and their receptors play critical roles in the development of the immune system and in immune regulation, inflammation

and autoimmunity by regulating cell death, survival and differentiation [343, 359, 360]. There are four closely related members of this family, which include lymphotoxin (LT) alpha (LTα), LT beta (LTβ), TNF-α and LIGHT. Their cognate receptors include lymphotoxin β receptor (LTβ) and TNFR1, TNFR2 and herpes virus entry mediator (HVEM). Both LTα and LTβ are important for formation and structural integrity of peripheral lymphoid organs because mice with targeted deletion of these genes lack organized peripheral lymph nodes and their spleen architecture is structurally dysregulated [361-363]. In contrast, LIGHT deficient mice have normal peripheral lymphoid organs suggesting that unlike LTα and LTβ, its function may be more closely related to immune modulation [361, 362]. LIGHT is expressed on activated T cells, monocytes, granulocytes and immature dendritic cells [200-202] and binds to three receptors: LTβR, HVEM and in humans, the decoy receptor, DcR3/TR6 [200, 203, 204]. HVEM is expressed on resting T cells, NK cells, monocytes, immature DCs and endothelial cells [203, 205, 206], whereas LTβR is expressed mostly on stromal cells, DCs and endothelial cells but is absent on lymphocytes [207, 208]. The interaction of LIGHT with its receptors modulates several biological processes including cell survival, inflammation and up-regulation of intracellular adhesion molecule-1 (ICAM-1) leading to tumor eradication [364]. In addition, signals transmitted via LIGHT-HVEM interaction have costimulatory effects on T cells, enhancing their proliferation and cytokine production [200, 202]. Thus, LIGHT expression enhances cytolytic T lymphocyte-mediated tumor immunity and allograft rejection [202, 213] and its overexpression on T cells results in extensive T cell proliferative disorders that is characterized by massive polyclonal expansion of $CD4^+$ and $CD8^+$ T cells [214, 215]. It is known that blockade of LIGHT signalling results in severe impairment in anti-CD40-, LPS- and CpG-induced IL-12p40 production by macrophages and dendritic cells *in vitro* and *in vivo* and a

concomitant impairment in $CD4^+$ Th1 response and susceptibility to *L. major* in the otherwise resistant C57BL/6 mice [216]. However, no study has directly investigated the role of LIGHT in *Leishmania major* induced dendritic cell activation and IL-12 production, priming and maintenance of IL-12 production during *L. major* infection as well as the role of LIGHT in secondary anti-*Leishmania* immunity.

4.2.2 Hypothesis

Blockade of LIGHT will lead to impaired priming and maintenance of Th1 response and subsequent loss of secondary anti-*Leishmania* immunity following virulent challenge.

4.2.3 Objectives

a. Determine the role of LIGHT in *Leishmania major* induced maturation and IL-12 production of lymph-node dendritic cells.

b. Investigate the role of LIGHT in initiation and maintenance of primary and secondary immunity in experimental cutaneous leishmaniasis.

c. Determine if LIGHT interaction with its receptors is responsible for IL-12 production in the absence of CD40-CD40L interaction.

4.2.4 Results

4.2.4.1 LIGHT is Dispensable for Differentiation of Dendritic cells from Stem Cells but is Important for Maturation and IL-12 Production by Dendritic Cells.

It was previously shown that blockade of LIGHT by HVEM-Ig or LTβR-Ig affects production of IL-12 by bone marrow derived dendritic cells (BMDC) following LPS, CpG and anti-CD40 mAb stimulation *in vitro* [216]. To test whether LIGHT blockade globally affected DC maturation and cytokine production, we assessed the migration of cells into the draining lymph node and the number of dendritic cells in draining lymph-node following *L. major* infection. We also determined the expression of co-stimulatory molecules and IL-12 production by lymph node DCs in the presence or absence of HVEM-Ig, or LTßR-Ig in mice infected with *L. major*. As shown in Fig. 11A, HVEM-Ig impaired the global migration of cells into the draining lymph nodes. However, the number of $CD11c^+$ cells (DCs) in the lymph-node was not significantly different between mice treated with LTßR-Ig, HVEM-Ig or control-Ig (Fig. 11B), suggesting that LIGHT does not play a role in the migration of DCs into the lymph nodes draining the infection sites. Next, we determined the expression of costimulatory molecules on lymph node dendritic cells and data from this thesis show that the percentage of cells expressing CD40 and CD86 did not differ between cells treated with HVEM-Ig and or control-Ig (Appendix 1). However, the level of expression of CD40, and CD86 in the lymph-node dendritic cells as determined by the mean fluorescence intensity (MFI), were significantly ($p < 0.001$) reduced in the presence of HVEM-Ig and LTßR-Ig (Fig. 11C & Fig. 11D). Also, injection of HVEM-Ig and LTßR-Ig led to significantly less IL-12 production by DCs from mice infected with *Leishmania* compared to cells treated with the control-Ig at both day 3 (Fig. 11E) and 35 (Fig. 11F) post

infection. Taken together, these results show that although LIGHT is not important for the migration of DCs, it is important for their full maturation into competent antigen-presenting cells.

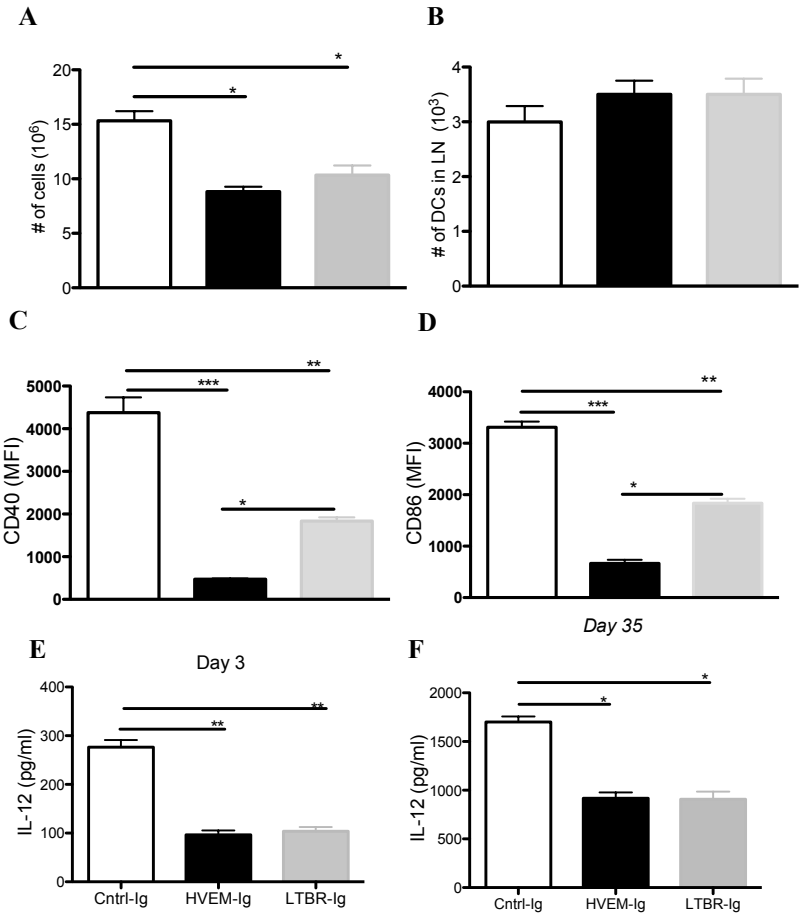

Figure 11. Blockade of LIGHT inhibits early cell migration; DC maturation and *Leishmania* induced IL-12 production *in vivo*. Wild type mice were treated with HVEM-Ig, LTBR-Ig or control-Ig 24hrs before being infected with *L. major*. Three days after infection, mice were sacrificed to isolate the dLN. Single cell suspensions from the dLN (A) were counted by trypan blue exclusion. The mean fluorescence intensity (MFI) for CD40 (B) and CD86 (C) were determined *ex vivo* by flow cytometry. Some of the cells were stimulated *in vitro* with SLA for 3 days and the production of IL-12p40 (D) was measured by ELISA. In some experiments, mice were sacrificed at day 35 post infection and the level of IL-12 p40 in 72-hour cell culture supernatant was determined by ELISA. The results presented are representatives of 2 different experiments (n = 4-5 mice per group). (* $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$)

4.2.4.2 LIGHT Signalling is Important for Both Priming and Maintenance of Anti-*Leishmania* Immunity

LIGHT signalling is important for IL-12 production by APCs, the development of Th1 cells and resistance to *L. major* [216]. Since IL-12 is required for both priming and maintenance of anti-*Leishmania* immunity [141, 144, 365], we were interested in investigating what stage during anti-*Leishmania* immune response that LIGHT signalling is required for resistance. To test the role of LIGHT in priming of *Leishmania* immunity, we blocked LIGHT before infection and to test its role maintenance of immunity LIGHT was blocked between 2-5 weeks after *L. major* infection. We show that blockade of LIGHT signalling before or at 2 weeks after *L. major* infection results in significantly ($p<0.01$-0.001) bigger lesion and higher parasite burden than in control-Ig treated group (Fig. 12A and 12B). This treatment also severely impaired the production of IFN-γ by $CD4^+$ T cells from the draining lymph nodes of infected mice upon SLA stimulation (Fig. 12C). The effect of this blockade was more prominent when the fusion protein was given prior to infection suggesting that while this pathway is important for both effective priming and maintenance of anti-*Leishmania* immunity, it is more important during the initiation phase of immune response.

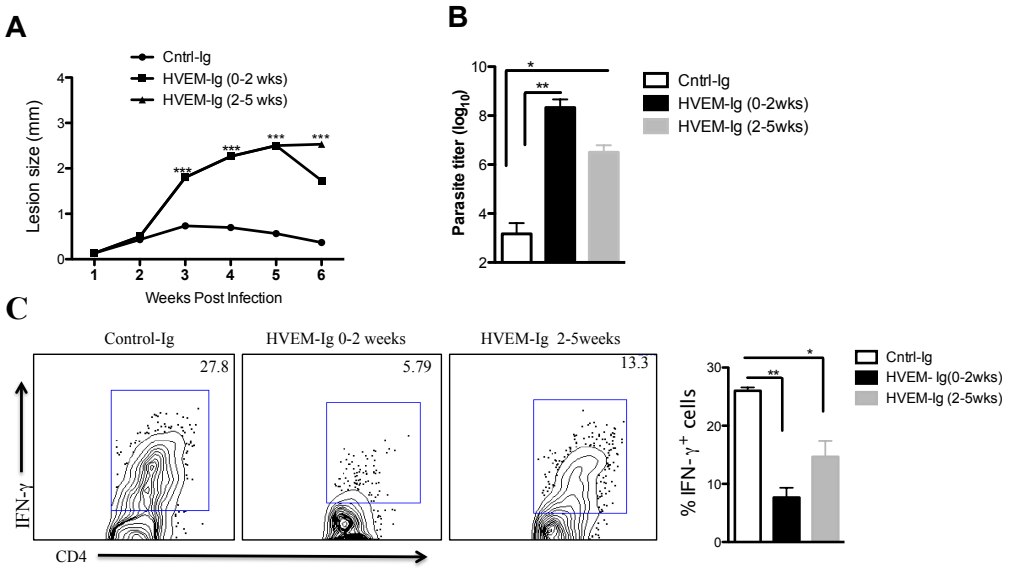

Figure 12. LIGHT is important for both priming and maintenance of anti-Leishmania immunity. C57BL/6 mice were injected with HVEM-Ig or control-Ig weekly starting at day –1 for 2 weeks (to test priming) or from 2 weeks after (to test maintenance) infection with *L. major* and the lesion size (A) was monitored weekly. Six weeks after infection, mice were sacrificed to estimate parasite burden (B). The percentages of IFN-γ producing T cells (C) were determined by flow cytometry. The results presented are representatives of 2 different experiments (n = 4-5 mice per group). (* $p < 0.05$; ** $p < 0.01$;***$p < 0.001$)

4.2.4.3 LIGHT is Dispensable During Secondary Response in Wild Type C57BL/6 Mice.

The preceding results show that LIGHT is critical for optimal immunity against *L. major*. To investigate whether LIGHT signalling is also important during secondary (memory) anti-*Leishmania* immunity, C57BL/6 mice were infected in the footpad with *L. major* and allowed to heal (i.e. to completely resolve their footpad lesion). Fourteen weeks after primary infection, healed mice were injected with HVEM-Ig or control-Ig one day before secondary challenge with *L. major*. The HVEM-Ig and control-Ig treated mice further received weekly injection thereafter for 2 weeks. Age-matched naïve mice mice were used as controls. Blockade of LIGHT signalling (by HVEM-Ig injection) before secondary challenge had no effect on DTH response (Fig. 13A) and control of parasite proliferation at the challenge site (Fig. 13B). Furthermore, whereas blockade of LIGHT significantly impaired IL-12 and IFN-γ production by dLN cells from infected naive mice (see Figs. 11 and 12); it has no effect on production of IL-12 and IFN-γ by cells from healed mice following secondary *L. major* challenge (Fig. 13 C and 13D). Taken together, these results suggest that while LIGHT signalling pathway is critical for effective primary immunity, it is dispensable during secondary anti-*Leishmania* immunity.

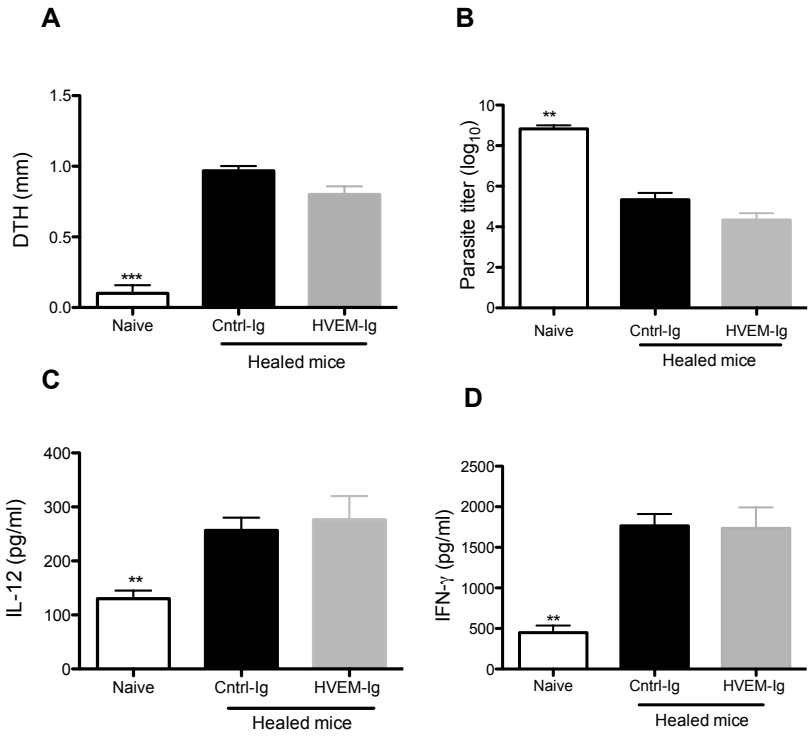

Figure 13. LIGHT is dispensable for secondary (memory) immune response to *Leishmania major*. C57BL/6 mice were infected with $1x10^6$ *L. major* and allowed to heal. Fourteen weeks after primary infection healed mice and uninfected age-matched controls were injected with HVEM-Ig or control-Ig (100 μg/mouse), challenged the next day in the contralateral feet with $5x10^6$ *L. major*. Delyayed Type hypersensitivity (DTH) response was measured after 72 hr. (A). At 3 weeks post-challenge mice were sacrificed to determine parasite burden (B). At sacrifice, dLNs were collected and cells were stimulated with SLA for 72 hr. and the level of IL-12 (C) and IFN-γ (D) in the cell culture supernatant was determined by ELISA. The results presented are representatives of 3 different experiments (n = 3-5 mice per group). (**, $p < 0.01$; *** ,$p < 0.001$

4.2.4.4 Treatment with rIL-12 Leads to Healing in the Absence of CD40-CD40L Interaction.

We compared primary and secondary immunity in CD40 and IL-12 deficient mice. Both CD40 and IL-12 KO mice were infected with *L. major* and then treated with recombinant IL-12 for the first two weeks. Treatment with rIL-12 lead to reduced parasite lesion size in both CD40 and IL-12 KO mice up to week 11 post infection (PI) Interestingly, from week 12 PI, the IL-12 KO displayed disease reactivation characterized by progressive increase in lesion size while the CD40 KO mice did not (Fig. 14 A). At 14 weeks post infection, both CD40 and IL-12 KO mice treated with rIL-12 were challenged. The CD40 KO mice displayed DTH response that was significantly ($p < 0.001$) higher than that observed in the IL-12 KO mice but comparable to the WT control mice (Fig. 14B). Also, CD40 KO mice treated with rIL-12 had comparable parasite burden with the WT mice and both (CD40 KO and WT) had significantly ($p < 0.001$) lower parasite burden compared to the IL-12 KO mice (Fig. 14C). Taken together, the data presented here supports the critical role of IL-12 in protection against *L. major* infection and further supports the notion that in CL, IL-12 can be produced independent of CD40-CD40L pathway.

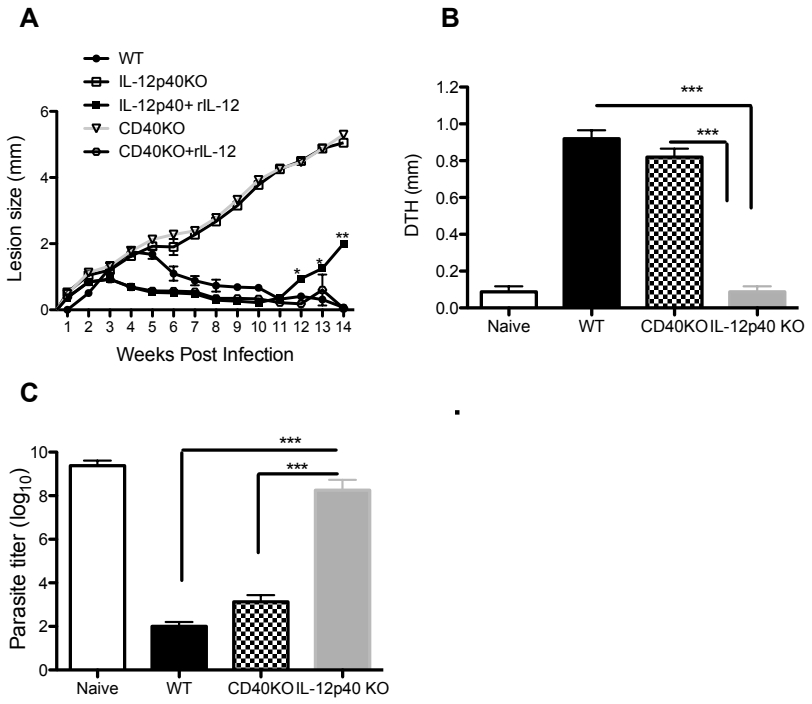

Figure 14. Treatment with rIL-12 leads to healing in *Leishmania major* infected CD40 KO but not IL-12 KO mice. IL-12 KO, CD40 KO and WT mice in C57BL/6 on background were infected with *Leishmania major*. The IL-12 KO and CD40 KO mice were treated with recombinant IL-12 or PBS for the first 3 wks. Lesion development (A) was monitored weekly. Eleven weeks post-infection mice were challenged with virulent *Leishmania major* and DTH response (B) was determined at 72hrs. by measuring footpad swelling. Parasite titre (C) was determined in the challenged feet by limiting dilution assay. Results presented are representative of 3 independent experiments (n = 3-4 mice per group) with similar results. (* $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$)

4.2.4.5 LIGHT is Critical for Secondary Immunity in CD40 Deficient Mice

Next we wondered whether the resistance in the CD40 KO mice is dependent on IL-12 production via the LIGHT pathway. We therefore generated healed CD40 KO mice by treating *L. major* infected CD40 KO, with rIL-12 (See Fig. 14). Healed CD40 KO mice were treated with 100μg of HVEM-Ig, LTßR-Ig or control-Ig 24hrs before challenge with virulent *L. major* along with groups of age-matched healed and naïve WT mice. We found that treatment with HVEM-Ig led to loss of immunity in healed CD40 KO mice (Fig. 15). HVEM-Ig treated CD40 KO mice had significantly ($p<0.01$) less DTH response compared to the control-Ig group (Fig. 15A) at 72 hrs. post-challenge. They also had significantly ($p < 0.01$) more parasites burden in the challenge feet when mice were sacrificed three weeks later (Fig. 15B). While the level of Th1 cytokines, IL-12 (Fig. 15C) and IFN-γ (Fig. 15D) were significantly ($p < 0.001$) lower in the CD40 KO mice that received HVEM-Ig compared to the control-Ig treated group, the level of the pathogenic Th2 cytokine IL-4 (Fig. 15E) was significantly ($p < 0.01$) higher in the HVEM-Ig group compared to the control-Ig group. It is important to note that there was no significant difference in DTH and parasite in healed CD40 KO mice treated with LTßR-Ig compared to the healed wild-type mice, suggesting that in the absence of CD40, IL-12 is produced through the LIGHT-HVEM pathway that is responsible for long-term healing and resistance to secondary challenge with virulent *L. major*.

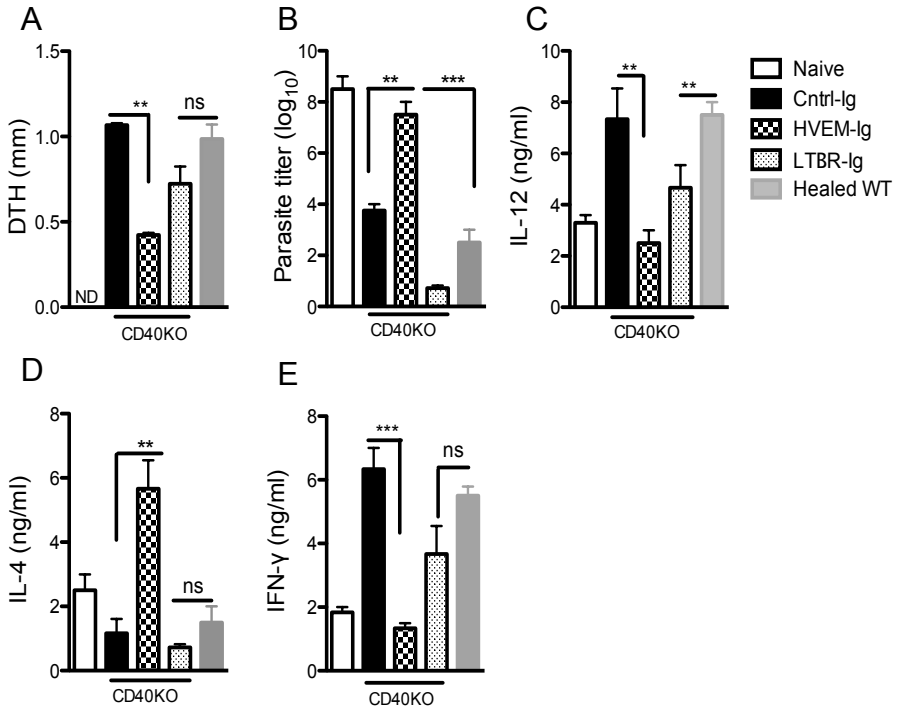

Figure 15. Blockade of LIGHT lead to loss of immunity in healed CD40KO mice.

Female CD40KO mice infected with *Leishmania major* were treated with recombinant IL-12 and lesion development was measured weekly untill healing. Healed mice were treated with HVEM-Ig, LTβR-Ig or control-Ig 24hrs before challenge with 5 million *L. major*. DTH response (A) was determined by measuring foot pad swelling in the challenge feet. Three weeks post challenge mice were sacrificed. Parasite titre (B) in the footpad and IL-12 (C), IFN-γ (D) and IL-4 (E) in the cell culture supernatant was determined by limiting dilution assay and ELISA respectively. Results presented are representative of 2 independent experiments with similar findings (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$).

4.3 **The Role of CD40 and CD40L Interaction in Immune Response to Experimental Cutaneous Leishmaniasis**

4.3.1 Introduction

CD40 and CD40L are transmembrane glycoproteins that play important costimulatory roles in the activation of T cells [366]. Early studies mainly focused on the role of CD40 and CD40L interaction in the regulation of B cell function such as activation, proliferation and isotype switching. However, it is now known that this pathway is also important at facilitating proper T cells activation [367]. Indeed, CD40L deficient mice could not produce functional cytotoxic T cells [368]. The CD40-CD40L pathway is particularly important in regulation of immunity to cutaneous leishmaniasis because it plays a critical in the production of IL-12 [344], an important cytokine that is required for priming and maintenance of optimal cell-mediated immunity [141, 142, 369].

Macrophage 1 antigen, also known as CD11b/CD18 and complement receptor 3, is a surface receptor integrin that also serves as a pattern recognition receptor (PRR) for some pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide [370]. Mac-1 is expressed on different cell types including monocytes, granulocytes, macrophages and natural killer cells [371], and plays a role in different cellular processes including cell adhesion, migration, chemotaxis, cell activation, cytotoxicity and phagocytosis [372, 373]. Mac-1 has also been shown to contribute to infection-induced inflammatory responses following Ross River virus infection [374]. In addition, *Leishmania* parasites have been shown to bind indirectly to

Mac-1 via antibody-mediated opsonisation [236] or directly through interaction with *Leishmania* lipophosphoglycan (LPG) [240].

Several studies have investigated the role of CD40 and CD40L interaction in the development of protective immunity in leishmaniasis with conflicting results. Deficiency of CD40L renders the usually resistant mice highly susceptible to infection with *Leishmania amazonensis*, which was related to failure of the infected mice to produce IL-12 resulting in impaired Th1 response [226]. However, a later study by Padigel et al [230, 231] showed that CD40L deficient mice are capable of producing IL-12 and mounting a protective Th1 response after infection with low dose *Leishmania major*. Although the discrepancy between these studies may be related to differences in parasite dose and strains, it is conceivable that an alternate pathway exists for IL-12 production that is independent of CD40-CD40L interaction. For example, a recent report shows that in the absence of CD40, macrophage antigen 1 (Mac-1) can bind to CD40L and mediate inflammatory responses by producing proinflammatory cytokines [235]. It is conceivable that in the absence of CD40, CD40L interaction with Mac-1 could lead to IL-12 production leading to resistance to *L. major* infection. In this section of the thesis, I assessed the role of CD40-CD40L interaction in cutaneous leishmaniasis by comparing disease progression and immune response in CD40 and CD40L deficient mice infected with *L. major* under identical experimental conditions. In addition, I investigated whether CD40L-Mac-1 interaction could provide an alternative pathway for IL-12 production in the absence of CD40 molecule.

4.3.2 Hypotheses

I hypothesize the following:

1. There are differences in the outcome of *L. major* infection in CD40 KO and CD40L KO mice following rIL-12 treatment.

2. CD40L-Mac-1 interaction is important for sustained IL-12 production and maintenance of anti-*Leishmania* immunity in the absence of CD40 molecule.

4.3.3 Objectives

a. To characterize and compare disease progression and immune response to *Leishmania major* in CD40 and CD40L deficient mice.

b. To determine whether Mac- 1-CD40L interaction leads to IL-12 production in the absence of CD40.

c. To investigate the role of Mac-1-CD40L interaction in maintenance of anti-*Leishmania* immunity in CD40 deficient mice made resistance by treatment with rIL-12

4.3.4 Results

4.3.4.1 Impaired Th1 Response and Disease Reactivation in CD40L Deficient mice Treated with Recombinant Interleukin -12

To fully understand the role of CD40-CD40L interaction in experimental cutaneous leishmaniasis, we infected CD40 KO, CD40L KO and WT mice in the resistant C57BL/6 background with *L. major*. Some CD40 KO and CD40L KO mice were treated with rIL-12 (0.3µg, 3x per week) for the first two weeks to facilitate healing.). As shown in Fig. 16 A and 16B and consistent with previous reports [226], CD40 and CD40L KO mice infected with *L. major* were highly susceptible as exemplified by the development of non-healing lesion. Treatment with rIL-12 led to initial healing in both groups of mice, suggesting that the inability to heal in the untreated mice was related to impaired IL-12 production as previously reported [226]. Interestingly, by week 7 post-infection, lesion reactivation occurred in the CD40L KO whereas the CD40 KO and WT mice continued to resolve their lesions and were completely healed by 12 weeks post-infection (Fig. 16 A & 16B). Some mice from each group (CD40 KO, CD40L KO and WT) were sacrificed at 3 and 7 weeks post infection to assess immune response as well as parasite burden. There was no difference in parasite burden and immune response at 3 weeks post-infection. However, by 7 weeks, (which corresponds to the onset of disease reactivation), CD40L KO mice displayed significantly ($p < 0.01$) higher parasite burden in the footpad compared to the WT and CD40 KO mice (Fig. 16 C). This increase in parasite burden corresponded with significantly ($p < 0.01$-0.001) lower IL-12 (Fig. 16D), and IFN-γ (Fig. 16E) and significantly ($p < 0.001$) higher IL-4 (Fig. 16F) production by dLN cells from infected CD40L KO compared to the CD40 KO and WT mice. It is important to note that there was no difference in lesion size, parasite or immune response between WT and CD40KO treated with

rIL-12. Taken together, these results are in agreement with previously published data that showed that CD40L KO mice are highly susceptible to *Leishmania* infection. However, they further show that there are differences in the immune response and disease outcome in CD40 and CD40L KO mice following *L. major* infection. Most importantly, they suggest that the existence of alternate pathway(s) for IL-12 production in the *L. major*-infected CD40 KO mice, which is/are absent in the infected CD40L KO mice.

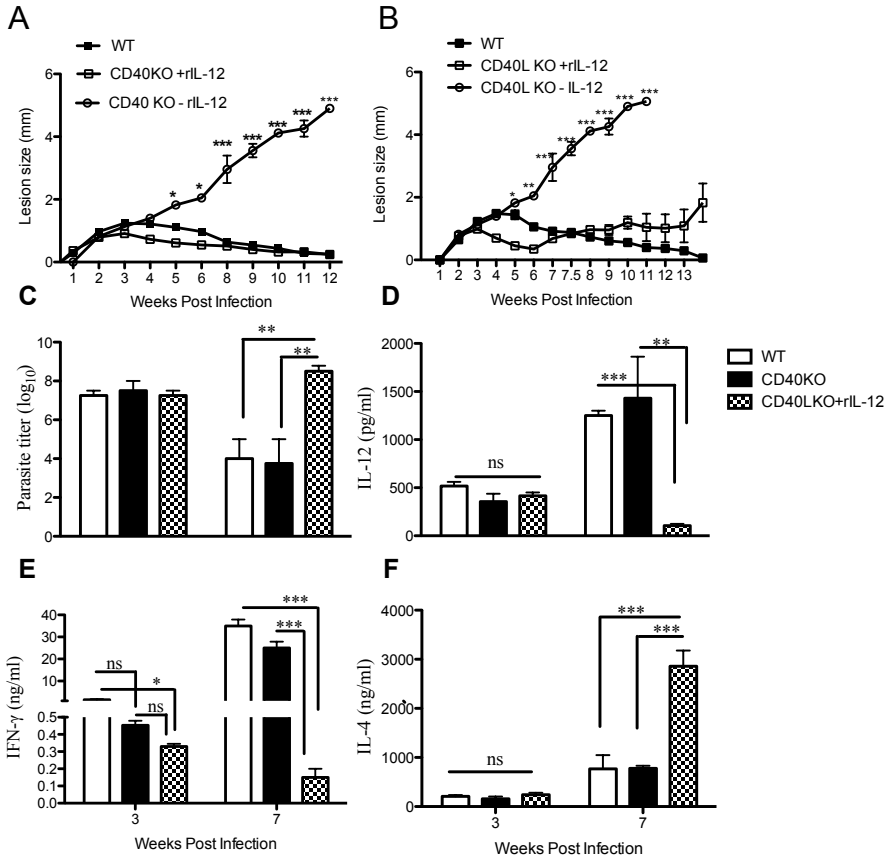

Figure 16: Healing in CD40KO treated with recombinant IL-12 but not CD40LKO deficient mice.

Wildtype, CD40KO and CD40L KO mice in the C57BL/6 background were infected with $1x10^6$ in the hind footpad. The CD40 KO and CD40L KO mice were treated with 0.3ug recombinant IL-12 three times per week for the first two weeks in the footpad. Lesion size (A and B) were measured weekly with vernier caliper. At indicated times some mice were sacrificed and the number of parasite burden (C) in the infected footpad was determined by limiting dilution assay. The levels of IL-12 (D), IFN-γ (E) and IL-4 (F) were determined by ELISA results and are representative of three independent experiments with similar results. (n= 20 mice/group) (* p< 0.05; ** p<0.01;***p<0.001).

4.3.4.2 Dendritic Cells and Macrophages from CD40 KO mice produce IL-12 following stimulation with Soluble CD40L.

Recent reports show that in the absence of CD40, CD40L interacts with Mac-1 and mediates inflammation [235], leukocyte recruitment and atherogenesis [375]. Since IL-12 is important for initiation and maintenance of protective immunity to cutaneous leishmaniasis, we considered the possibility that the interaction of CD40L and Mac-1 is responsible for IL-12 production that maintains immunity in CD40 KO mice. To test this, we first assessed the ability of dendritic cells and macrophages from CD40 KO mice to produce IL-12 following addition of soluble CD40L (sCD40L) and whether this could be blocked by the addition of anti-Mac-1 monoclonal antibody (mAb). We generated BMDMs and BMDCs from WT, CD40 KO and CD40L KO and treated them with sCD40L in the presence or absence of anti-mac 1 antibody. We found that stimulation of BMDMs and BMDCs with sCD40L leads to IL-12 production in all groups including CD40 KO mice (Figs. 17A- 17F). Interestingly, this ability of sCD40L to induce IL-12 production was significantly ($p < 0.001$) impaired in the presence of anti-mac-1 antibody in the CD40 KO (Figs. 17B & E) but not the WT (Figs 17A & D) or CD40L KO (Figs. 17C & F). To validate the above results, we assessed the ability of splenic $CD11b^+$ or $CD11c^+$ cells from WT, CD40 KO and CD40L KO mice to produce IL-12 in the presence of sCD40L. We observed that the ability of sCD40L to induce IL-12 production in the presence of anti-mac-1 antibody was significantly ($p < 0.001$) impaired in the splenic $CD11b^+$ and $CD11c^+$ cells from CD40 KO (Figs. 17H & 17K) but not WT (Figs. 17G & 17J) or CD40L KO (Figs. 17I and 17L)

mice. Taken together, these results show that in the absence of CD40, Mac-1 can interact with CD40L leading to IL-12 production, which could account for the difference in the disease out come between the CD40 KO and CD40L KO mice infected with *L. major.*

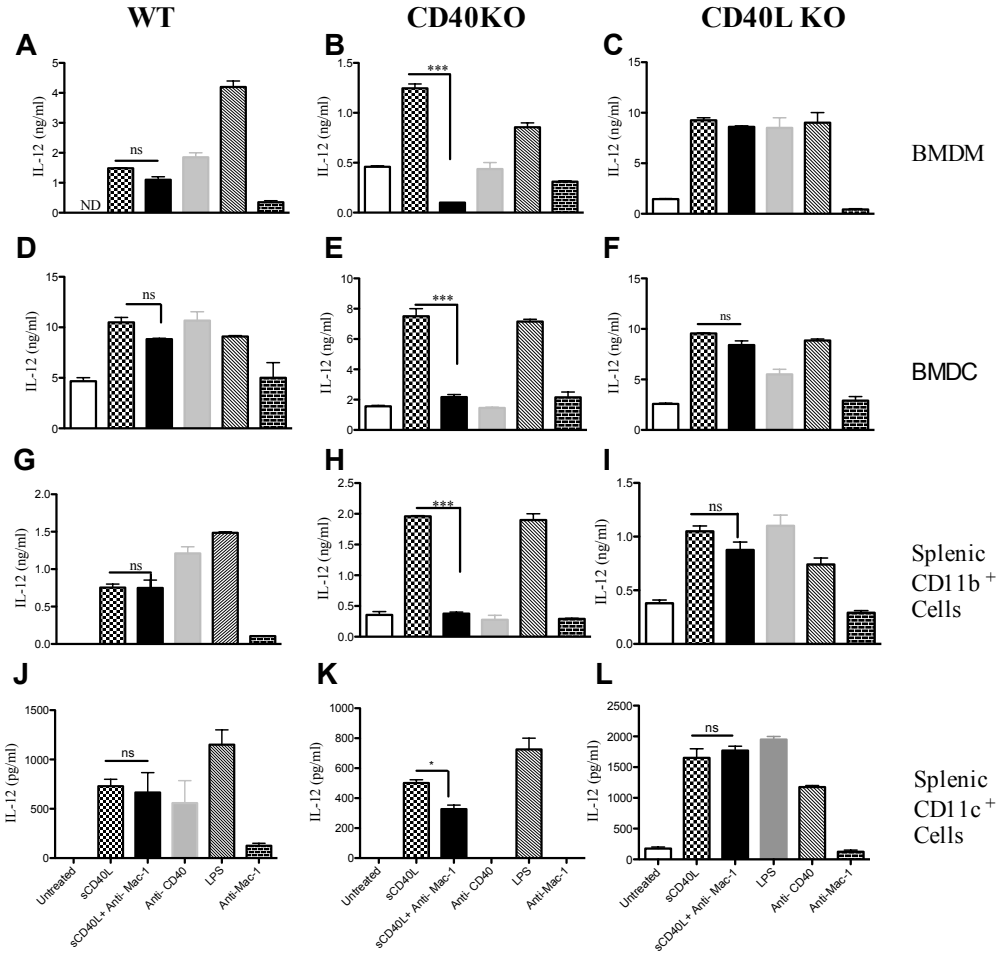

Figure 17. Treatment with macrophage antigen 1 blocking antibody leads to impairment in sCD40L induced IL-12 production *in vitro*. Macrophages and dendritic cells were generated from bone marrow or isolated from spleens of WT, CD40KO and CD40LKO mice and stimulated with 2ug of sCD40L in the presence or absence of mac-1 blocking antibody for 48 hours. IL-12 level in bone marrow derived macrophages (First Row A-C), bone marrow derived dendritic cells (Second row D-F), splenic CD11b+ cells (Third row G-I) and Splenic CD11c+ cell (Fourth row J-L) culture supernatant was determined by ELISA. Results are representative of three independent experiments with similar results. (* $p< 0.05$; ** $p<0.01$;***$p<0.001$).

4.3.4.3 Treatment with Mac-1 Blocking Antibody Leads to Disease Reactivation in Healed CD40 Deficient Mice.

We observed that CD40 deficient dendritic cells and macrophages produce IL-12 when stimulated with sCD40L via a Mac-1 dependent pathway (Fig. 17). To determine whether the sustained resistance observed in CD40 KO mice treated with rIL-12 is dependent on IL-12 production through CD40L-Mac-1 interaction, we treated healed CD40 KO and WT mice with anti-Mac-1 blocking antibody or control-Ig and assessed disease reactivation by measuring lesion recrudescence and parasite burden at the primary infection site. As early as one week following anti-Mac-1 treatment, there was a significant ($p < 0.05$-0.001) increase in lesion size (Fig. 18A) and parasite burden (Fig. 18B) in CD40 KO (but not WT) mice treated with anti-Mac-1 mAb. This was associated with significantly ($p < 0.01$-0.001) lower IL-12 (Fig. 18C) and IFN-γ (Fig. 18D) production by dLN cells from anti-Mac-1-treated CD40 KO mice compared to their WT controls. In contrast, the production of IL-4 was significantly higher in the healed CD40 KO treated with mac-1 blocking antibody compared to their WT controls (Fig. 18E). It is important to note that treatment of healed WT mice with Mac-1 blocking antibody did not have any impact on parasite burden or immune response (Fig. 18). Together, these observations show that the maintenance of resistance in *L. major*-infected CD40 KO mice is dependent on IL-12 production through the Mac-1-CD40L pathway.

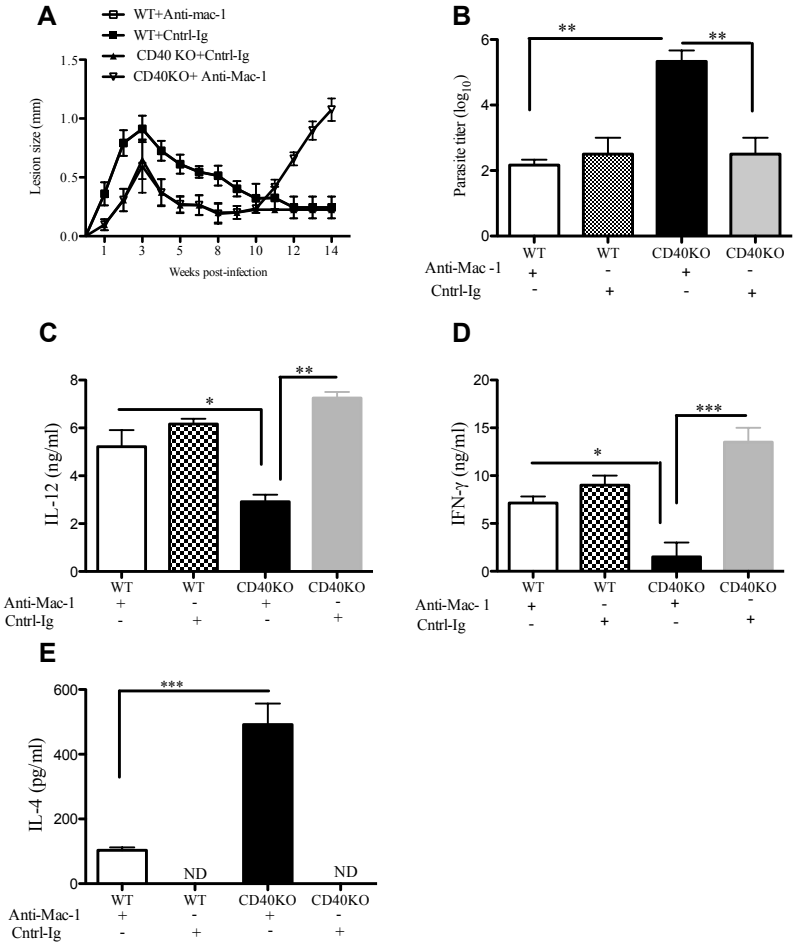

Figure 18. Blockade of macrophage antigen 1 leads to spontaneous disease reactivation in healed CD40KO mice.

Healed Wildtype and CD40KO mice in the C57BL/6 background were treated with mac-1 blocking antibody (100ug/mouse) twice per week intraperitoneally for one week. Lesion development (A) in the primary infection was measured weekly with a vernier caliper. Three weeks after treatment, mice were sacrificed and parasite burden (B) in the primary infection footpad was determined by limiting dilution assay. Draining lymphnode cells were cultured with SLA for 72 hours. The levels of IL-12 (C), IFN-γ (D) and IL-4 (E) in the cell culture supernatant were measured by ELISA. Results are representative of two independent experiments with similar results. (n= 3-4 mice/group) (* $p< 0.05$; ** $p<0.01$;***$p<0.001$).

4.3.4.4 Loss of Established Infection-induced Immunity in CD40KO Mice Following Blockade of Macrophage Antigen-1

Next we investigated the impact of blocking CD40L-Mac-1 interaction in secondary immunity to *L. major*. Healed CD40 KO and WT mice were treated with Mac-1 blocking antibody 24 hours before challenge with virulent *L. major* and delayed-type hypersensitivity (DTH) response and parasite burden were determined after 3 and 21 days, respectively. In contrast to the healed WT or CD40 KO mice treated with control-Ig, treatment of healed CD40 KO mice with Mac-1 blocking antibody led to significantly ($p < 0.001$) lower DTH response (Fig. 19A) and significantly ($p < 0.01$) higher parasite burden (Fig. 19B). In addition, the levels of IL-12 (Fig. 19C) and IFN-γ (Fig. 19D) in the supernatant fluids of dLN cells from CD40 KO treated with Mac-1 blocking antibody were significantly ($p < 0.01$) lower than the control-Ig treated group. Collectively, these results indicate that the interaction of Mac-1 with CD40L is responsible for maintenance of Th1 response and subsequent protective secondary immune response following virulent *L. major* challenge.

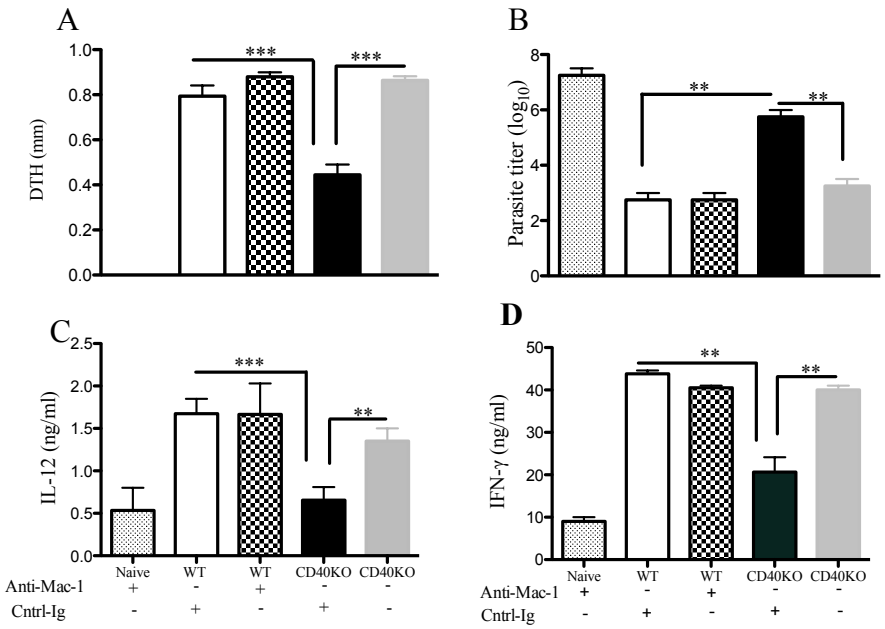

Figure 19. Blockade of Mac-1 leads to loss of immunity following virulent challenge in healed and resistant CD40KO mice. C57BL/6 and CD40KO mice were infected with 1 million *L. major* and allowed to heal. Twelve weeks after primary infection (when lesion was completely resolved), healed mice and uninfected age-matched controls were injected with anti-Mac-1-Ig or control-Ig (200 μg/mouse) and challenged the next day in the contralateral feet with 5 million *L. major*. DTH response was measured after 72 hr. (A). The mice further received weekly injections of anti-Mac-1-Ig and at 3 weeks post-challenge mice were sacrificed to determine parasite burden (B). The levels of IL-12 (C) and IFN-γ (D) in the cell culture supernatant were measured by ELISA. Results are representative of two independent experiments with similar results. (n= 3-4 mice/group) (** $p<0.01$;***$p<0.001$).

5. DISCUSSION

5 .1 Effect of Antigen Dose in Primary Immune Response after Infection with Low and High Dose *L. major*

Clinical observations and experimental studies suggest that the development of an effective cell-mediated immunity is essential for protection against leishmaniasis. Although $CD4^+$ T cells are critical for protective immunity in cutaneous leishmaniasis [289], $CD8^+$ T cells have also been shown to be essential in certain situations, particularly in low dose infections [121, 376]. During low dose *L. major* infection, $CD8^+$ T cells were shown to contribute to lesion resolution and parasite control by producing IFN-γ that augment optimal $CD4^+$ Th1 response [121, 376]. However, whether $CD8^+$ T cells also contribute to secondary anti-*Leishmania* immunity following resolution of primary infection is unclear. In addition, no study has investigated the impact of parasite dose on early T cell expansion and secondary anti-*Leishmania* immunity. To address these issues, we compared disease progression and immune response in mice infected with low and high dose *L. major*. Data from this thesis show that although the pattern of lesion development and parasite burden were similar in both high and low dose infections, the quality of the immune response was very different. Whereas high dose infection induced strong $CD4^+$ T cell proliferation and Th1 cytokine response, low dose infection predominantly activates proliferating and Th1 cytokine producing $CD8^+$ T cells both at early and later time point in disease progression. I used the Thy1.2 – Thy1.1 congenic adoptive transfer system to test whether the differential activation of T cells by low and high dose *L. major* occurs *in vivo*. We demonstrated that this differential activation of $CD4^+$ and $CD8^+$ T cells by high and low dose infections, respectively, occurs *in vivo* following *L. major* challenge. Interestingly, while depletion of $CD4^+$ T cells in mice that healed both high and low dose infections led to loss of

immunity following secondary *L. major* challenge, depletion of $CD8^+$ T cells had no effect both in the low and high *L. major* challenge. Taken together, the results presented in this section of the thesis show that although low dose *L. major* infection preferentially activates $CD8^+$ T cells that contributes to optimal primary immunity, they are completely dispensable for resolution of secondary *L. major* challenge.

Previous studies showed that $CD8^+$ T cells produce large amounts of IFN-γ following *L. major* infection and were critical for optimum primary immunity [121, 376]. In addition, earlier studies suggest that $CD8^+$ T cells are activated following secondary *L. major* challenge and contributes to secondary anti-*Leishmania* immunity [377, 378]. However, we show that $CD8^+$ cells are completely dispensable for protective secondary anti-*Leishmania* immunity. Depletion of $CD8^+$ T cells in mice that healed their low dose or high dose infections before virulent challenge were as resistant as those treated with control-Ig. In contrast, depletion of $CD4^+$ T cells led to complete loss of infection-induced immunity. Collectively, these observations suggest that the role of $CD8^+$ T cells is limited to providing help for optimal activation of $CD4^+$ Th1 cells during primary infection. As such once effective $CD4^+$ Th1 response is induced during primary low dose infection, $CD8^+$ T cells are no longer required, thus making them dispensable during a secondary response. We believe that differences in animal models, parasite strain and experimental design could account for the discrepancy between our findings and the studies that found a role for $CD8^+$ T cells in secondary immunity. For example, in those studies, splenocytes were first depleted of $CD4^+$ cells and then cultured *in vitro* for extended period of time before assaying for IFN-γ production by $CD8^+$ T cells [377, 378]. Such *in vitro* culture conditions could potentially influence the magnitude of $CD8^+$ T cell responses that otherwise would not be seen in short-term and/or bulk whole cell cultures as performed in our study.

One must wonder why high and low dose infections differentially activate $CD4^+$ and $CD8^+$ T cells, respectively? It is conceivable that this may be related in part to differences in activation threshold for $CD4^+$ and $CD8^+$ T cells. It has been shown that naïve $CD4^+$ T cells require at least 6 hours of contact with APCs presenting their cognate peptides in order to acquire optimum signals leading to activation, proliferation and cytokine production [379]. In contrast, naïve $CD8^+$ T cells require less than 2 hours of antigenic stimulation to acquire enough signals required for their activation, proliferation and cytokine release, suggesting that the requirements for activating $CD8^+$ T cells are less stringent [380]. Hence, low dose infection provides lower antigen availability and may mimic short duration required for activation thus favouring the activation of $CD8^+$ T cells. In contrast, high infectious dose provides high antigen load that could overcome the need for longer contact thus favouring expansion of $CD4^+$ T cells. In addition, high dose infection was associated with strong up regulation of MHC class II molecules on dendritic cells (Fig. 5G), which would potentially favour activation of $CD4^+$ T cells. Furthermore, $CD103^+CD8\alpha^+$ dendritic cells have the ability to cross present exogenous antigens on MHC I to $CD8^+$ T cells [349, 350]. The fact that we found the percentage of $CD103^+CD8\alpha$ population of dendritic cells to be significantly ($p<0.01$) higher in the low dose infected mice compared to the high dose group could explain the preferential expansion of $CD8^+$ T cells in LD infected mice. Although differences in co-stimulatory molecules expression has been associated with differences in activation of $CD4^+$ and $CD8^+$ T cells [250], it is unlikely that it contributed to differential induction of $CD4^+$ and $CD8^+$ T cells in our model system. This is because we did not observe any difference in expression of CD40 and CD86 molecules on dendritic cells from the draining lymphnodes following low and high dose infections, suggesting that the effect of antigen dose is mostly restricted to TCR-peptide interaction and not on co-stimulation.

Following recovery from natural *L. major* infection (which is usually self-resolving), a long-term (sometimes lifelong) immunity develops to reinfection. This observation is the basis for leishmanization, which is the deliberate inoculation of lesion-derived virulent parasites into hidden parts of the body in order to prevent a more serious visible cutaneous disease. Leishmanization is the oldest and only effective preventive practice against human cutaneous leishmaniasis [381]. The usual practice in leishmanization is to employ a relatively high dose inoculum because it is believed that such high dose is able to induce strong inflammatory and immune responses necessary for protection against subsequent reinfections. As a result, some leishmanized individuals develop large (sometimes non-healing) lesions that require medical treatment. In some cases, exacerbated chronic skin disease and/or immunosuppression have been reported [292]. Due to the effectiveness of leishmanization, recent efforts have focused on ways to make the practice safer; including suggestions to include killed parasites in the inoculum [293] or to use genetically engineered attenuated parasites [296]. Whether high doses of parasites during leishmanization (as is currently practiced) is required for protection is unclear. By comparing response to high and low dose *L. major* infections side by side, this thesis show that despite inducing comparatively lesser inflammatory responses (smaller lesion sizes), low dose infection induced secondary immunity and protection following virulent *L. major* challenge comparable to high dose infection. Indicating that vaccination with large dose of live virulent parasite is not necessary to achieve protection, thus suggesting that leishmanization with low dose inoculum could be a more viable alternative practice as it would lead to smaller lesion at the inoculation site that is less prone to ulceration and secondary bacteria infection. In line with this, it has been shown that low dose infection of the highly susceptible BALB/c mice leads to resistance and protection against virulent *L. major* challenge [382]. Collectively, our study shows

that parasite dose influences the magnitude of expansion of $CD4^+$ and $CD8^+$ T cells following *L. major* infection. While low dose infection preferentially activates $CD8^+$ T cells, high dose infection leads to preferential activation of $CD4^+$ T cells. Surprisingly, despite the strong activation of $CD8^+$ T cells and their importance in primary immunity following low dose infection, secondary immunity in mice that healed their low dose *L. major* infection was completely dependent on $CD4^+$ (and not $CD8^+$) T cells. See appendix 5 for the graphical summary of this section.

5.1.2 Missed Opportunities and Limitations

Although subcutaneous infection in the footpad is a well-established model of experimental cutaneous leishmaniasis, the results from this study could have been strengthened if the ear infection model was also used for primary and secondary (challenge) experiments. This is important since Sandfly vectors often transmit disease by depositing *Leishmania* parasites in the dermis of exposed part of the body, intradermal infection in the ear may represent a more natural model of infection and could make the data more physiologically relevant to human disease. There are very few studies comparing subcutaneous (foot pad) and intradermal (ear) infection models, but it is possible that there could be immunological differences between subcutaneous infection in the footpad and intradermal infection in the ear. In line with this, it was recently reported that the early inflammatory cell recruitment differs significantly in mice infected with *Leishmania* by intradermal and subcutaneous injection of *Leishmania* [115].

5.2 Role of LIGHT (*lymphotoxin like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes*) in Cutaneous Leishmaniasis

It was previously reported that LIGHT signalling is critical for optimal IL-12 production and Th1 immune response development in mice and its blockade either by using HVEM-Ig fusion protein or targeted gene deletion of LIGHT or HVEM results in susceptibility to *L. major* infection in the otherwise resistant strain of mice [216]. However, this study did not directly investigate whether LIGHT signalling is important for early and/or sustained IL-12 production in *L. major*-infected mice *in vivo* as well as whether LIGHT is required for secondary anti-*Leishmania* immunity was not investigated. Data from this thesis corroborate the importance of LIGHT in primary anti-*Leishmania* immunity and further show that its blockade with HVEM-Ig or LTβR-Ig significantly impaired maturation and expression of costimulatory molecules on dendritic cells as well as their production of IL-12 following *L. major* infection *in vitro* and *in vivo*. It further demonstrates that LIGHT signalling is required at both priming and maintenance stages of primary anti-*Leishmania* immunity but is completely dispensable during secondary anti-*Leishmania* immunity in WT mice. Interestingly and in contrast to the data from WT mice, blockade of LIGHT signalling in the absence of CD40-CD40L interaction led to lower DTH and IFN-γ responses, impaired parasite control and higher Th2 response following secondary *L. major* challenge. Thus, LIGHT is critical for priming and maintenance of Th1 response during primary immune response but is dispensable during secondary anti-*Leishmania* immunity in the presence of functional CD40 but becomes critical in the absence of CD40 signalling. This section of the thesis identifies LIGHT as an important molecule that regulates the initiation and maintenance of IL-12-dependent anti-*Leishmania* immunity in mice.

Previous studies showed that direct engagement of LIGHT on T cells has costimulatory effects and plays a crucial role for optimal T cell activation by acting as a costimulatory molecule for T cells [202, 203, 383, 384]. In this regard, it was shown that blockade of LIGHT-HVEM interaction *in vitro* inhibits polyclonal T cell proliferation [203, 383], and purified T cells from LIGHT$^{-/-}$ mice showed decreased responses to anti-CD3 stimulation *in vitro* [385, 386]. In addition its direct effect on T cells activation, LIGHT has been shown to indirectly influence T cell activation and differentiation by inducing DC maturation and IL-12 production [387, 388]. Addition of anti-LIGHT antibodies into T-DC cultures inhibited IL-12 production by dendritic cells [216. , 217]. This report suggests that binding of LIGHT expressed on T cells to HVEM on dendritic cells or vice versa may be the mechanism involved in LIGHT-mediated enhancement of IL-12 production. I found that blockade of LIGHT with fusion protein results in significant impairment in *Leishmania* specific IL-12 production and expression of costimulatory molecules on dendritic cells leading to impairment of Th1 response *in vitro* and *in vivo*. In concordance with this, a recent publication showed that LIGHT-HVEM interaction is critical for IL-12, TNF and IFN-γ producing T cells that are responsible for controlling hepatic infection in *L. donovani* infected mice [217]. Taken together, these results suggest that direct interaction of LIGHT with its ligands on antigen presenting cells (such as dendritic cells) is an important pathway for LIGHT-mediated IL-12 production, T cell activation and resistance to *Leishmania major* in mice.

It has been suggested that the effects of signals transmitted via HVEM on the immune system can provide both activating and inhibitory signals depending upon the ligand it interacts with. Thus, while LIGHT-HVEM and LIGHT-LTβR interactions have costimulatory effects on T cells thereby enhancing their proliferation and cytokine production [200, 202]. The interaction of

HVEM with BTLA results in profound immune deactivation [389, 390]. Stanley et. al [217] suggest that blockade of LIGHT-HVEM interaction allows more LIGHT to bind to BTLA thus leading to inhibitory signals (disease enhancement) however, when interaction of HVEM to LTα is blocked, it allows more LIGHT to bind to HVEM leading to activation (protective immunity). This is consistent with our finding that the blockade of LIGHT significantly impaired the expression of costimulatory molecules and IL-12 production by DCs *in vitro* and *in viv*o following *L. major* infection. The significant reduction in CD40 expression in the absence of LIGHT suggests that LIGHT may also play a role in the regulation of CD40 expression. Thus this thesis is consistent with HVEM playing a dual (stimulatory and inhibitory) role in T cell responses depending on which cognate ligand it interacts with [390].

IL-12 is a critical cytokine for the development and maintenance of Th1 cells and resistance to *L. major* [139, 140, 143]. LIGHT has been shown to be important for IL-12 production during primary immune response to *L. major* [216]. However, whether LIGHT is required at the initiation or maintenance stage of IL-12 was unclear. Hence, this thesis investigated the stage of anti-*Leishmania* immune response that LIGHT signalling is required for resistance. Blockade of LIGHT signalling before (to test priming) or after (to test maintenance) 2 weeks of infection results in significantly bigger lesions and higher parasite burden than in the controls (Fig. 12A- 12B), and also severely impaired the production of IFN-γ by cells from the and draining lymph nodes of infected mice (Fig. 12C). The data show that LIGHT is required at both the initiation and maintenance stages of anti-*Leishmania* immunity.

Interestingly, we found that while treatment with HVEM-Ig or LTβR-Ig at both the priming and maintenance phases of anti-*Leishmania* effector responses impairs the development of primary anti-*Leishmania* immunity, it had relatively no effect in controlling secondary (memory)

immunity to *L. major*. Treatment with HVEM-Ig or LTβR-Ig fusion protein had no effect on IL-12 production, induction of DTH response and accelerated parasite clearance (Fig. 13). It is likely that the requirements for recalling memory cells during secondary challenge are different from those needed for differentiation of naive cells into effector cells, with the latter being more dependent on LIGHT-mediated IL-12 production than the former. It has been shown that the conditions for activating memory cells are relatively less stringent than those for naive cells with memory cells requiring lesser costimulation, antigen, TCR-peptide avidity and cytokines than naïve cells [391, 392].

Treatment of infected IL-12 deficient mice with rIL-12 leads to resolution of cutaneous lesion and control of parasite proliferation [141, 144]. However, recrudescence occurs following cessation of rIL-12 treatment, suggesting that the continuous IL-12 is important for initiation and maintenance of resistance in *L. major*-infected mice [141]. However, we have found that unlike IL-12 deficient mice, treatment of CD40 deficient mice with rIL-12 leads to resistance which is maintained indefinitely even after cessation of recombinant cytokine treatment (Fig. 14). This suggests that CD40-CD40L dependent IL-12 production is needed for initiation but is dispensable for maintenance of established anti-*Leishmania* immunity. We therefore tested whether LIGHT signalling compensates for IL-12 production in the absence of CD40-CD40L interaction during secondary immune response and we found that blockade of LIGHT in healed CD40KO mice led to impaired DTH response and parasite clearance as well as reduced protective Th1 and enhanced Th2 responses. This is consistent with previous findings that showed that signalling via other costimulatory pathway (TRANCE-RANK) could compensate for IL-12 production during primary *L. major* infection [393]. However, the blockade of TRANCE-RANK pathway did not did not lead to loss of immunity in healed CD40 deficient

mice (Appendix 2). As such the data presented here suggests that CD40-CD40L interaction is not the only pathway that can lead to IL-12 production that in the absence of CD40, IL-12 production through the LIGHT pathway is enough to induce protection against virulent challenge.

There is a significant amount of redundancy and ligand sharing between members of the TNFSF. For instance LIGHT binds to HVEM and LTβR in mice as such it is difficult to determine which receptor LIGHT signals through to mediate its effect. This thesis did not clearly determine whether LIGHT mediates its effect through HVEM or LTβR pathway. I tried to address this issue in this thesis by using both LTβR-Ig and HVEM –Ig. However, blockade of LIGHT using both HVEM-Ig and LTβR-Ig produced similar results. This could be because both fusion proteins act as decoy receptors and have comparable ability to mop up LIGHT. However, because previous published data from our lab using LTβR KO mice showed that LTβR KO mice infected with *L. major* developed chronic disease [210] unlike mice that received HVEM-Ig [216], which developed a more severe ulcerating lesions. We therefore believe that the protective effect of LIGHT is through the interaction of LIGHT with HVEM and not LTβR.

This thesis has demonstrated that LIGHT is important for DC maturation and IL-12 production following infection with *L. major* and blockade or disruption of this pathway leads to impairment in $CD4^+$ Th1 development and IFN-γ production. Furthermore, we provide evidence that LIGHT is required at both the priming and maintenance stages of anti-*Leishmania* immunity but is dispensable during secondary (memory) response in an intact mouse but become relevant in the absence of CD40-CD40L signalling. Data presented in this thesis corroborate our initial report and further suggest that LIGHT could provide a new therapeutic target for regulation of IL-12

production *in vivo* and for controlling various $CD4^+$ Th1-mediated autoimmune and inflammatory diseases such as arthritis, diabetes, colitis etc.

See appendix 6 for the graphical summary of this section.

5.2.1 Missed Opportunities and Limitations

LIGHT binds to HVEM and LTβR in mice as such it is difficult to determine which receptor LIGHT signals through to mediate its effect. This thesis did not clearly determine whether LIGHT mediates its effect through HVEM or LTβR pathway. Data from this thesis could have been a lot stronger if I used antibodies that can specifically block one pathway without impacting the other to tease out which receptor LIGHT binds to mediate its effect. Also it would have been nice to use LIGHT/CD40 double knock out mice to address the role of LIGHT in secondary immunity to *Leishmania major* in the presence or absence of CD40. Even though CD40 deficient mice are commercially available, LIGHT deficient mice is not. Unfortunately, my several attempts to obtain LIGHT deficient mice were not successful.

5.3 Role of CD40 and CD40L Interaction in Immune Response to Experimental Cutaneous Leishmaniasis

The primary aim of this part of this thesis was to further determine the role of CD40-CD40L interaction in primary and secondary immune response to *L. major.* In addition, I also wished to investigate the contribution of Mac-1-CD40L interaction in secondary immune response to *L. major* in the absence of CD40 signalling. The data here show that there are differences in disease outcome and immune response between CD40 and CD40L KO mice infected with *L. major* following treatment with rIL-12; while CD40 KO mice treated with rIL-12 completely heal their footpad lesion and remained resistant to secondary challenge, CD40L KO mice reactivated disease following cessation of rIL-12 treatment. This differential disease outcome in CD40L KO mice was associated with impaired Th1 and enhanced Th2 responses. Blockade of Mac-1 signalling using blocking anti-Mac-1 mAb in healed CD40 KO led to impaired IL-12 production, disease reactivation and impaired Th1 response, suggesting that CD40L-Mac-1 interaction is responsible for IL-12 production and sustained immunity in CD40 KO mice.

There have been conflicting reports on the role of CD40-CD40L interactions in immunity to experimental leishmaniasis. While a study showed that CD40L deficient mice heal their low dose *L. major* infection [231], another study showed that these mice are highly susceptible to *L. amazonensis* infection [226]. We found that both CD40 KO and CD40L KO mice were highly susceptible to *L. major* infection and this susceptibility was associated with impaired IL-12 and IFN-γ and a concomitant increase in IL-4 production by cells from infected mice. Interestingly, treatment of both CD40 and CD40L KO mice with exogenous IL-12 lead to different outcomes following *L. major* infection: complete healing with no lesion reactivation and resistance to

secondary challenge with *L. major* in CD40 KO mice. In contrast, exogenous rIL-12 treatment led only to a short-term lesion resolution in the CD40L KO mice which was followed by disease reactivation a few weeks after rIL-12 treatment was stopped. This disease reactivation in CD40 KO mice was associated with enhanced Th2 and reduced Th1 response in contrast to the CD40 KO mice that maintained a sustained Th1 response. The finding of striking differences in immune response and disease outcome between infected CD40 and CD40L KO mice following cessation of rIL-12 treatment is in agreement with previous finding by Padigel et. al [230] that showed that CD40-CD40L pathway is not the sole pathway for IL-12 production in leishmaniasis.

IL-12 is a critical cytokine for both the development of primary immunity and maintenance of effector Th1 cells that mediate secondary resistance to *L. major.* It is widely believed that CD40-CD40L interaction is the primary pathway required for IL-12 production for optimal anti-*Leishmania* immunity. However, other alternate pathways such as TRANCE-RANK [393], LIGHT (Fig. 15) and Mac-1 (Fig. 19) for maintenance of IL-12 production that mediates sustained immunity and protection during secondary *L. major* challenge have been identified. This raises the question as to which of the different pathways is most critical during secondary/memory response to *L. major.* I believe that while all the three pathways may be important for optimal secondary immune response to *L. major,* the current observations support the idea that Mac-1-CD40L interaction may be the most important. This is supported by the fact that neither blockade of TRANCE-RANK interaction nor inhibition of LIGHT signalling leads to spontaneous disease reactivation and loss of immunity in healed CD40 KO mice (Fig. 15, Appendix 3). In contrast, blockade of Mac-1 led to both spontaneous disease reactivation (akin to

IL-12 KO mice) and loss of infection-induced immunity in CD40 KO that healed their primary *L. major* infection.

Although our *in vitro* and *in vivo* studies strongly suggest that Mac-1 interaction with CD40L is responsible for sustained IL-12 production in mice lacking functional CD40, it is plausible that other as yet untested pathways may be involved. For example, it has also been shown that sCD40L can bind to another integrin, $\alpha 5\beta 1$ [394] which is a primary receptor for fibronection, a key molecule that plays an important role in regulating inflammatory cytokine production by some cells. However, we believe that Mac-1-CD40L interaction is responsible for the bulk of IL-12 produced in this system since blockade of Mac-1 using anti-Mac-1 mAb completely abolished IL-12 secretion by dendritic cells from CD40 KO mice (Fig. 18). Even though Sanders et. al [395] reported that stimulation of neutrophils with soluble CD40L led to their increased expression of Mac-1, we did not observe any difference in the level of CD11b expression on cells stimulated with sCD40L. This could be because the cells (macrophages) already expressed very high levels of CD11b (Appendix 4) and so it was difficult to further increase their expression. de Oliviera and colleagues [396] recently showed that serum levels of sCD40L increases in VL patients that are responding positively to treatment and they concluded that sCD40L could be used a biomarker for monitoring treatment outcome in VL. Whether the serum level of sCD40L is increased following experimental *L. major* infection in both WT and CD40KO mice is unknown. Given that the interaction of sCD40L and Mac-1 is critical for maintaining anti-*Leishmania* immunity in CD40 KO mice, it is conceivable that that the level of sCD40L in the serum or expression of CD40L on T cells is high in healed CD40KO mice. However, more studies are needed to address this question. See appendix 7 for the graphical summary of this section.

5.3.1 Missed opportunities and Limitations

This thesis has discovered a novel role for CD40L-Mac-1 interaction in immunity to *L. major* in the absence of CD40 signalling. Mac-1 is expressed on different cell types such as macrophages, monocytes, granulocytes and natural killer cells and this thesis did not investigate the contribution of these other cell types in the interaction with sCD40L and the eventual disease outcome in CD40KO mice infected with *L. major*. The data in this section of the thesis could have been strengthened if the role of these other cells types was investigated. For example, NK cells are good producers of IFN-γ (a cytokine that is important in the control of leishmaniasis) and these cells have been shown to contribute to immunity to experimental cutaneous leishmaniasis. It would therefore be highly informative to determine whether the contribution of NK cells to immunity is related in part to their ability to interact with CD40L via Mac-1.

5.4 Implications for Vaccine Design, Vaccination and Treatment Strategies

Leishmanization is the only known "vaccine" that has been shown to be effective against human leishmaniasis. It involves the injections of large doses of virulent *Leishmania* parasites into humans however a significant number of vaccinated individuals develop non-healing lesion [397]. Data from this thesis show that in comparison to the high dose ($2x10^6$) infected mice, mice infected with low dose (10^3) developed significantly smaller lesions. Interestingly and despite the smaller lesion size, mice that received primary infections of low dose parasites were protected against virulent challenge just like mice that received high dose (5 million) *L. major* parasites. This has significant implications in vaccine design and vaccination strategies in leishmaniasis. Even though leishmanization is not an approved vaccination strategy against leishmaniasis, it is the only known effective vaccine against the disease and is used to protect against the disease in endemic areas. Data from this thesis shows that it is possible to achieve protection (at least in mice) with a relatively small dose of parasites and it further suggests that leishmanization with low dose parasites in endemic areas could reduce the possibility of developing large non-healing lesions by vaccinated individuals usually associated with leishmanization with large doses of *Leishmania* parasites.

The control of infectious diseases could be by prevention of infection using vaccines or effective treatment of infected individuals with drugs (chemotherapy) and/or the use of immune modulating strategies (immunotherapy). There are chemotherapeutic agents for treatment of leishmaniasis, but they are associated with severe side effects and are expensive and compounding this problem is the development drug resistant *Leishmania* parasites. Thus there is need for the development of new drugs as well as treatment strategies. I found that LIGHT did

not play a significant role in secondary immunity in WT mice suggesting that targeting LIGHT as a vaccination strategy in CL may not be a viable option. However, since I found that blockade of LIGHT lead to enhanced diseases in normally resistant mice indicates that LIGHT plays an important role during primary immune response and support the idea that LIGHT may be a good therapeutic target for leishmaniasis. This can be achieved by different methods such as treatment with recombinant LIGHT, use of viral vectors or other molecules that enhance and/or activate the LIGHT pathway either used alone or in conjunction with conventional chemotherapy to reduce the duration of treatment and /or the amount of drugs prescribed to patients.

Mice studies suggest that IL-12 is critical for resolution of CL and this raises the question as to why is recombinant IL-12 is not been currently used as treatment for cutaneous leishmaniasis? This could be because, treatment with rIL-12 has been used in cancer treatment with very disappointing results; cancer patients that received systemic injection of rIL-12 developed severe side effects and even died in some cases [150]. Since LIGHT interaction with its receptors leads to IL-12, it is plausible that the idea of indirectly activating IL-12 through the LIGHT pathway could be a better alternative to treatment with rIL-12 since it will allow the host initiate and regulate the induced IL-12 response.

Leishmania lipophosphoglycan can bind directly to CD11b as such CD11b has been speculated to be the major route for *Leishmania* uptake by macrophages [240, 398]. Interestingly, the absence of CD11b did not alter the resistant phenotype in C57BL/6 mice infected with *Leishmania* as both WT and CD11b KO mice were able to control primary and secondary infection. On the other hand, CD11b did play a minor role in susceptibility in the susceptible Balb/c mice strain; CD11b KO mice in the Balb/c mice developed slightly bigger lesions following infection. Interestingly, there was no difference in Th1 and Th2 immune

response in between both WT and KO mice in the two backgrounds [241]. Taken together, previously published data suggests that the role of CD11b is in the phagocytosis of *Leishmania* parasites by macrophages in wild type mice. However, data from this thesis describes a previously unknown ability of Mac-1 to interact with other ligands with functional implications in experimental CL since the blockade of CD11b (Mac-1) led to reduction in IL-12 production by cells from both WT and CD40 deficient mice. As such this thesis opens the door for more studies looking at this Mac-1-CD40L interaction as possible vaccination and immunotherapeutic targets in cutaneous leishmaniasis (Discussed in section 6).

5.4 Major findings

5.4.1 Impact of Parasite Dose on Initial T Cell Expansion, Primary and Secondary Anti-*Leishmania* Immunity.

Data from this thesis showed that initial dose of infection, plays a role in determining the type of early and late T cell response in experimental cutaneous leishmaniasis. This is because initial antigen dose affected the subset of antigen presenting cells that is activated which subsequently determined the type of T cell response made during primary and secondary immune response to *Leishmania major. i.e* high initial antigen dose activated mostly MHC-II dendritic cells and $CD4^+$ T cells while low initial antigen dose activated more $CD103^+CD8\alpha^+$ expressing dendritic cells and $CD8^+$ T cells. The most intriguing and surprising data from this thesis is the finding that despite the differential activation of CD8 T cells at both early and late infection times by low dose infection they (CD8 T cells) did not play any vital role in maintaining immunity after secondary challenge with *L. major*.

Furthermore, these data were recently published in peer review journal. "**Ifeoma Okwor**, Ping Jia, Zhirong Mou, Nonso Onyilagha and Jude Uzonna. *$CD8^+$ T cells are preferentially activated during primary low dose Leishmania major infection but are completely dispensable during secondary anti-Leishmania immunity.* PLoS Negl. Trop. Dis. 2014 Nov 20;8(11):e3300. doi: 10.1371/journal.pntd.0003300. eCollection 2014"

5.4.2 Contribution of LIGHT in Primary and Secondary Immune Response to *Leishmania major*.

In this objective of this thesis, I showed that just like what was observed with bacterial DNA (CpG) and toxin (LPS) LIGHT is also important in *Leishmania major* induced maturation and cytokine production by dendritic cells. This thesis corroborated previous finding that LIGHT is

important for optimal primary immune response to *L. major*. It also explored the previously unknown role of LIGHT in secondary anti-*Leishmania* immunity. Surprisingly and in stark contrast to what was observed during primary infection with *L. major*, LIGHT was completely dispensable during memory response to *L. major* in WT mice with functional CD40-CD40L signalling. It was particularly interesting to find that LIGHT plays a protective role in experimental CL in the absence of CD40-CD40L interaction. The blockade of LIGHT led to loss of infection –induced immunity exemplified by the significantly, more parasite burden and enhanced Th2 response in normally resistant healed CD40 KO mice. Overall, work from this thesis uncovered a previously unknown compensatory pathway for IL-12 production in the CD40 deficient mice and suggests that LIGHT could be a potential therapeutic target for cutaneous leishmaniasis.

Findings from this section of the thesis are currently in press for the Journal of Immunology. ***Ifeoma Okwor,*** *Gui-Lian Xu, Yang-Xin Fu and Jude E Uzonna. Deficiency of CD40 Reveals an Important Role for LIGHT in Secondary Anti-Leishmania Immunity.*

5.4.3 Role of Macrophage Antigen 1 In Primary and Secondary Immune Response To Experimental *Leishmania Major* Infection.

Data from this thesis reveal that there are differences in immune response to *Leishmania major* in the CD40 deficient and CD40L deficient mice. While treatment with exogenous IL-12 can lead to healing and long-term resistance in CD40 deficient mice, the CD40L deficient mice reactivated disease when rIL-12 treatment was stopped. Furthermore, this thesis uncovered a novel role for CD11b in cutaneous leishmaniasis. CD11b (previously thought to be important only in phagocytosis of *Leishmania*) can interact with CD40 ligand and this interaction can lead

to IL-12 production by bone marrow derived and splenic macrophages and dendritic cells *in vitro*. Since IL-12 is critical for initiation and maintenance of primary anti-*Leishmania* immunity it was very exciting to find that macrophage antigen-1 has a major contribution in the maintenance of immunity in healed CD40KO mice. The blockade of Mac-1 with monoclonal antibody in healed CD40KO mice led to spontaneous disease recrudescence/reactivation as well as loss of established infection-induced immunity following virulent challenge.

5.5. Hypotheses and Conclusions

There are three main hypotheses that were tested in this thesis:

The first hypothesis *"Based on the observation that primary immunity to low dose L. major is dependent in $CD8^+$ but not $CD4^+$ T cells, I hypothesized that primary infection with low and high dose L. major will induce different cell mediated immune response leading to differences in memory response to L. major"*

We found that low and high dose *L. major* infection induced mostly CD8 and CD4 T cells, respectively however, both low and high dose *L. major* infection induced comparable type and quantity of memory-like T cells and comparable protection following virulent challenge. We proved the first aspect of the hypothesis, however, we disapproved the second aspect of the hypothesis. We therefore conclude that despite their ability to activate different T cell subsets, both low and high dose *L. major* has comparable ability to protect mice against homologous virulent challenge. This has major implication in vaccine design (discussed in section 5.4).

For the second hypothesis *"Since LIGHT is critical during primary immune response to L. major, I hypothesized that LIGHT will also play a critical role in secondary immunity to L. major as such blockade of LIGHT will lead to loss of infection–induced resistance following virulent challenge in normally resistant healed C57BL/6 mice"*

I found that the blockade of LIGHT did not play a significant role during secondary immune response in the WT mice but is critical in secondary immunity in the absence of CD40; I therefore disapproved the first part of the hypothesis and conclude that the role of LIGHT in

secondary immunity to *L. major* is dependent on the presence or absence of functional CD40-CD40L signalling. With LIGHT being dispensable in the presence of functional CD40 and critical in the absence of functional CD40-CD40L signalling.

The third hypothesis *"Since the interaction of CD40 and CD40L is important or IL-12 production, I hypothesized that there will be comparable immune response and disease outcome in CD40 and CD40L deficient mice infected with L. major and treated with recombinant interleukin 12"*. I found that there are differences in disease outcome and immune response in CD40 KO and CD40L KO infected with *Leishmania major.* Utilizing both *in vitro* and *in vivo* studies I found that Mac-1-CD40L interaction is responsible for the observed difference between CD40 and CD40L KO mice infected with *L. major.* As such blockade of Mac-1 in healed CD40KO mice led to spontaneous disease reactivation as well as loss of immunity. I therefore disapproved my hypothesis and accept the null hypothesis that disease outcome and immune response between CD40KO and CD40L KO mice infected with *L. major* are different.

6. **FUTURE DIRECTIONS**

6.1 Impact of Parasite Dose on Initial T cell Expansion, Primary and Secondary Anti-*Leishmania* Immunity

The finding that low dose infection can confer protection that is equivalent to high dose infection is very interesting and exciting. However, the major drawback is that it is highly unlikely that leishmanization with virulent *Leishmania* parasites will be a universally accepted vaccination strategy. Studies utilizing high dose avirulent genetically modified parasites have shown that these avirulent parasites are able to confer protection in mice. It will be interesting to test if low dose of these mutant parasites will be able to confer protection just like virulent parasites. During natural infection, Sandflies inject *Leishmania* parasites intradermally into the host. So it will be interesting to investigate whether low dose intradermal infection can protect mice against virulent challenge.

6.2 Contribution of LIGHT in Primary and Secondary Immune Response to *Leishmania major*

Using chimeric protein that prevents the interaction of LIGHT with its ligands, data from this thesis have shown that LIGHT plays an important role in primary immune response to *L. major*. Studies by Stanley et. al [217] showed that the level of LIGHT increases with *L. donovani* infections and was associated with resistant phenotype. Whether there are differences in the level of LIGHT following *L. major* infection both in the resistant and susceptible mice is not known. So it will be interesting to investigate what happens to the level of LIGHT following *L. major* infection in both the susceptible and resistant mice strains. Secondly, if increase in LIGHT is associated with resistance, it will be interesting to test if treatment with recombinant LIGHT will rescue the highly susceptible Balb/c mice after virulent *L. major* infection.

Also LIGHT binds to two receptors, HVEM and LTβR; thus raising the question, of which receptor LIGHT bind to in order to mediate its effect? There is evidence that the effect of LIGHT could be dependent on the receptor it interacts with [217]. Studies utilizing antibodies that can target each receptor without affecting the action of the other is necessary to fully answer the question of which receptor through which LIGHT mediates its protective effect. Also in order to conclusively understand the role of LIGHT in secondary immune response to *L. major* in the absence of CD40 signalling, studies utilizing CD40 and LIGHT double KO mice is necessary. If LIGHT is critical then the CD40/LIGHT double deficient mice infected with *L. major* and then treated with rIL-12 should reactivate disease upon cessation of rIL-12 treatment.

6.3 Role of Macrophage Antigen 1 in Primary and Secondary Immune Response to Experimental *Leishmania major* Infection

This thesis showed that blockade of Mac-1 leads to decrease IL-12 production, disease reactivation and loss of infection-induced immunity in CD40KO mice. The role of Mac-1-CD40L interaction in experimental cutaneous leishmaniasis needs further characterization. As such it will be interesting to compare immune response and disease outcome in CD40 deficient, CD11b deficient and CD40/CD11b double deficient mice that received primary infection with *L. major*. Four key questions to address are i) does CD11b/CD40 heal like CD40 KO after treatment with recombinant IL-12; ii) do they (double KO mice) reactivate disease when IL-12 treatment is stopped; iii) if they heal, how long do they remain healed and are they able to resist secondary challenge with virulent *L. major*; iv) what cell types are involved in the Mac-1 – CD40L interaction?

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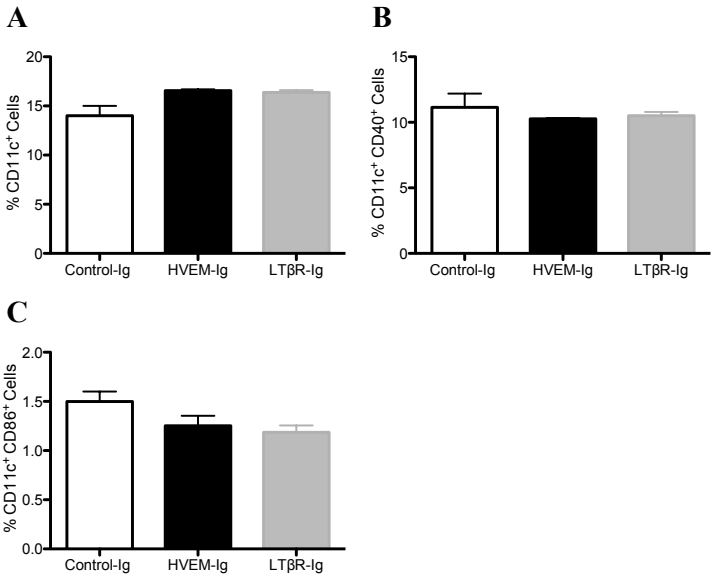
8.0 APPENDICES

8.1 Blockade of LIGHT does not Affect the Percentage of CD11c cells Expressing CD40 and CD86 Followoing *L. major* Infection



Appendix 1. Blockade of LIGHT does not affect the percentage of CD11c cells expressing CD40 and CD86 followoing *L. major* infection. Wild type mice were treated with HVEM-Ig or control-Ig 24hrs before being infected with *L. majo*r. Three days later mice were sacrificed to isolate the dLN. The percentage of $CD11c^+$ cells (A) and $CD11c^+$ $CD40^+$ (B) and $CD11c^+CD86^+$ (C) dendritic cells determined ex vivo by flowcytometry.

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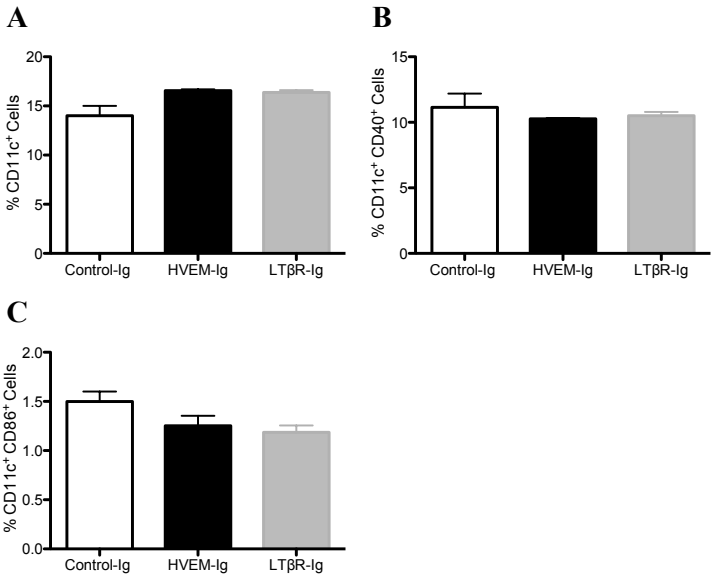

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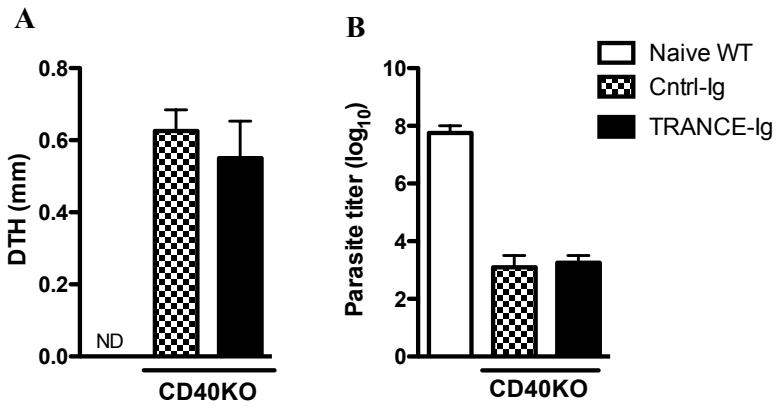
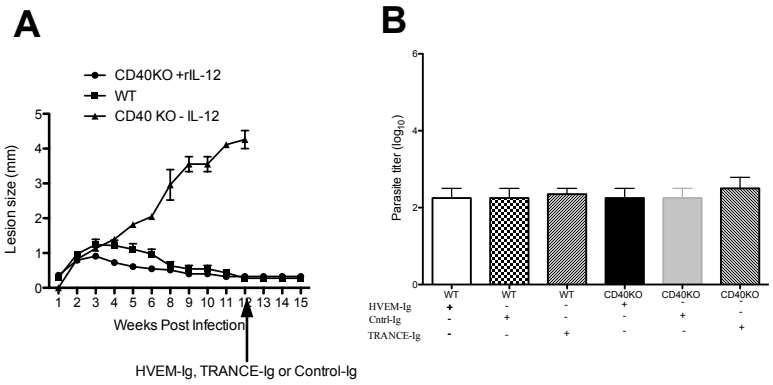
8.2. TRANCE - RANK Interaction is not Required for Secondary Immunity in Healed CD40KO Mice.



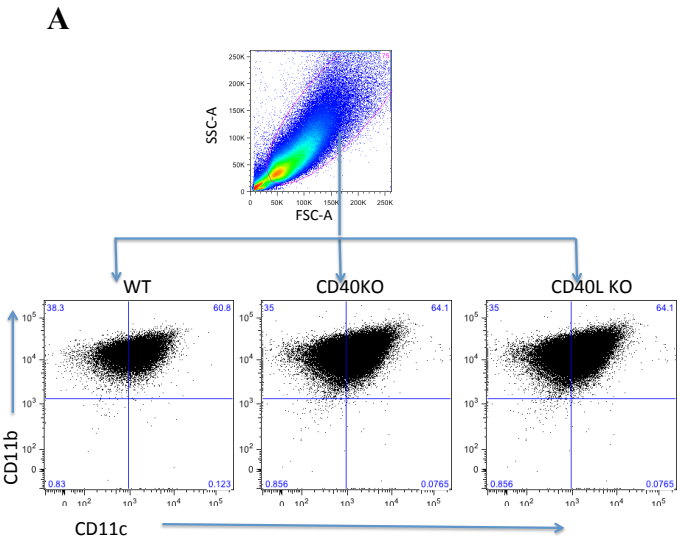
Appendix 2. Healed female CD40KO mice were treated with TRANCE-Ig fusion protein or control-Ig 24hrs. before challenge with 5 million *L. major* and DTH response (A) was determined by measuring foot pad swelling in the challenge feet 72 hrs post challenge. Three weeks post challenge mice were sacrificed and parasite titre (B) in the footpad.was measured. Results presented are representative of 2 independent experiments with similar findings.

8.3 Blockade of LIGHT with HVEM-Ig do not Lead to Spontaneous Disease Reactivation in Healed CD40KO Mice



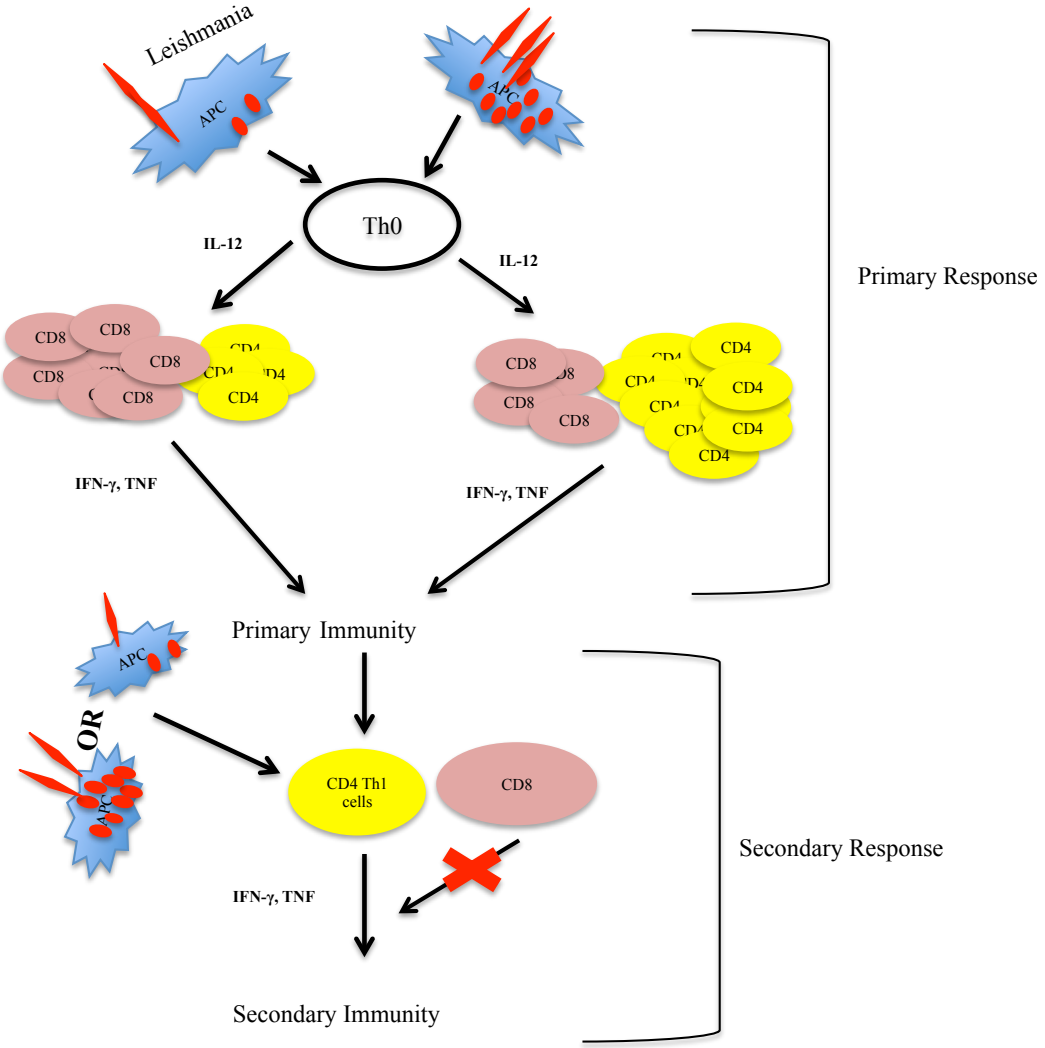
Appendix 3. Blockade of LIGHT with HVEM-Ig do not lead to spontaneous disease reactivation in healed CD40KO mice.
C57BL/6 and CD40KO mice were infected with 2 million *L. major* and allowed to heal. Twelve weeks after primary infection (when lesion was completely resolved), healed WT and CD40KO mice were treated with HVEM-Ig, TRANCE-Ig or Control-Ig (100ug/mouse) once per week intraperitoneally for two weeks. Lesion development (A) in the primary infection was measured weekly with a vernier caliper. Three weeks after treatment, mice were sacrificed and parasite burden (B) in the primary infection footpad was determined by limiting dilution assay. Results are representative of two independent experiments with similar results. (n= 3-4 mice/group).

8.4 Expression of CD11c and CD11b on Antigen Presenting Cells.



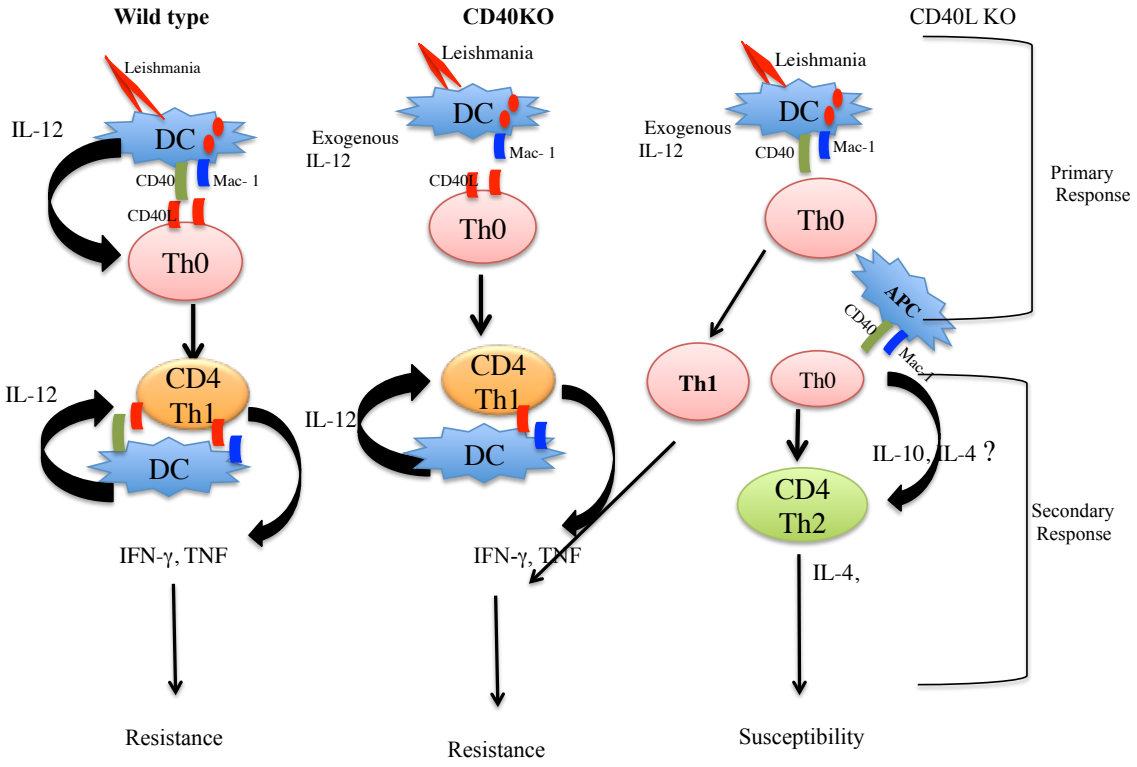
Appendix 4. Expression of CD11c and CD11b on Antigen Presenting cells.
Bone marrow macrophages were generated from naive WT, CD40 KO and CD40L KO mice in C57BL/6 background. Cells were first gated on live cells based on foward and side scatter characteristics and the expression of CD11c and CD11b were analysed by flow cytometry.

8.5. Graphical abstract of aim 1



Appendix 5. Effect of dose on expansion of T cells. During primary immune response low dose (LD) 10^3 or high dose (HD) $2x10^6$ *Leishmania major* parasites differentially expand CD8 and CD4 T. During secondary response in both LD and HD infected mice, only CD4+ T cells are required for resistance against virulent challenge.

8.7. Graphical abstract of aim 3



Appendix 7. Role of CD40-CD40L interaction in immune response against *Leishmania major*. In wild type mice during primary immune response *Leishmania major*, IL-12 is produced through CD40-CD40L pathways leading to activation of naïve T cells and protective primary immunity. During secondary exposure, IL-12 production through the CD40-CD40L activates antigen specific T cells leading to resistance.

In CD40 KO mice, during primary response exogenous IL-12 treatment leads to the induction of protective primary immune response. During secondary exposure to *Leishmania* parasites, IL-12 production through the Mac-1-CD40L activates antigen experienced T cells leading to protective secondary immune response.

In CD40L KO during primary response, exogenous IL-12 treatment leads to the induction of protective primary immune response. During secondary exposure to *Leishmania* parasites. In the absence of CD40L, no IL-12 is produced either through the CD40-CD40L or Mac-1-CD40L interaction and my lead to the activation of the pathogenic CD4 Th2 cytokine and susceptibility.