REGULATION OF IMMUNITY TO CUTANEOUS LEISHMANIASIS BY SEMAPHORIN 3E

BY

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ABSTRACT

Cutaneous Leishmaniasis is a disease of man and animals caused by the protozoan parasite *Leishmania*. *Leishmania major* is one of the major causative agents of cutaneous leishmaniasis. The host factors that regulate resistance to *Leishmania major* are not completely known but are influenced by the nature of innate and T helper cell responses. The limited understanding of the factors that influence resistance and susceptibility to the infection has delayed the development of vaccine and/or novel effective therapy against the disease. Semaphorin-3E (Sema3E), a mammalian membrane bound protein produced by several immune cells, has been reported to regulate cell migration and cell-mediated immune response, a critical arm of the immunity that controls resistance to intracellular pathogens such as *Leishmania.* However, the role played by Sema3E in immunity to cutaneous leishmaniasis has not been investigated before.

To investigate the role of Sema3E in immunity to cutaneous leishmaniasis, I assessed the level of Sema3E expression in *L. major*-infected mice and observed a significant increase in Sema3E at different time points. Furthermore, Sema3E-deficient (Sema3E$^{-/-}$) mice were more resistant to *L. major* infection than their WT counterparts as evidenced by significantly reduced lesion size and lower parasite burden, which was associated with significantly more IFN-γ and IL-17A production from CD4^{+} T cells in their draining lymph nodes than in WT mice. This was attributed to increased expression of costimulatory molecules and IL-12p40 by Sema3E$^{-/-}$ dendritic cells, increased frequency of Th1 and Th17 cells after polarization of Sema3E$^{-/-}$ CD4^{+} T cells, decreased frequency of T-regs and secreted IL-10 in the draining lymph node of Sema3E$^{-/-}$ mice compared to WT mice. Adoptive transfer of Sema3E$^{-/-}$ bone marrow cells to WT mice conferred better protection to the WT mice.

Collectively, the results of my study show that the absence of Sema3E improves resistance to *L. major* infection in mice by enhancing stronger $CD4^+$ Th1 and Th17 cell responses. These results indicate that Sema3E negatively regulates resistance to cutaneous leishmaniasis, suggesting that this pathway could be a potential drug target for controlling the disease.

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ABBREVIATIONS

Acronym	Definition
AmphB	Amphotericin B
ANOVA	Analysis of Variance
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guerin
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BSA	Bovine Serum Albumin
CCR-7	Chemokine receptor 7
CD	Cluster of differentiation molecules
CD103	Cluster Differentiation one hundred and three
CD11b	Cluster Differentiation eleven b
CD11c	Cluster Differentiation eleven c
CD25	Cluster Differentiation twenty-five
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 succinimidyl ester
CL	Cutaneous leishmaniasis
CpG	Cytidine-phosphateguanosine
CR3	Complement Receptor three
CTL	Cytotoxic T cell
CXCR	Chemokine, CXC Motif, Receptor
DC	Dendritic cell
DCL	Diffuse Cutaneous Leishmaniasis
dLNs	Draining Lymph nodes
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Foxp3	Forkhead box p3
GM-CSF	Granulocyte-macrophage colony stimulating factor
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-10	Interleukin ten

IL-12p40	Interleukin twelve p 40
IL-13	Interleukin thirteen
IL-4	Interleukin four
iNOS	Inducible Nitric Oxide Synthase
KO	Knock Out
LACK	*Leishmania* homologue of receptors for Activated C Kinase
LPS	Lipopolysaccharide
LST	Leishmanin Skin Test
MCL	Mucocutaneous Leishmaniasis
mDC	Myeloid dendritic cell
mg	Milligram
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
ml	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
ng	Nanogram
NK	Natural Killer
NO	Nitric Oxide
ODN	OligoDeoxynucleotide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

pg	Picogram
PKDL	Post-kala-azar dermal *leishmaniasis*
PMNs	Polymorphonuclear neutrophil
rIL-12	Recombinant Interleukin twelve
RPMI	Roswell Park Memorial Institute Medium
s.c.	Subcutaneous
sCD40L	Soluble Cluster Differentiation Forty Ligand
SD	Standard Deviation
Sema	Semaphorin
Sema3E	Semaphorin3E
SLA	Soluble Leishmania Antigen
T-bet	T box transcription factor
TCR	T Cell Receptor
TGF	Transforming growth factor
TGF-ß	Transforming Growth Factor Beta
Th	T helper cell
Th1	T Helper one
Th17	T Helper seventeen
Th2	T Helper two
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumour Necrosis Factor Alpha
Tregs	Regulatory T Cells

VL	Visceral *leishmaniasis*
WHO	World Health Organization
WT	Wild Type
µg	Microgram

CHAPTER 1

1.0 INTRODUCTION

1.1 BACKGROUND TO LEISHMANIASIS

Parasitic diseases are still a major global health problem affecting billions of people around the world. These diseases are capable of becoming chronic and result in high morbidity and mortality (1). Worldwide, there are millions of deaths which arises from parasitic protozoan diseases (2). Leishmaniasis a disease condition caused by over 20 species of *Leishmania* (a parasitic protozoan), is an important neglected parasitic disease. According to WHO estimates, about twelve million individuals are infected with *Leishmania* in about 98 countries, with approximately two million new cases occurring annually. This results in about fifty thousand deaths each year (196). Approximately 200 million people in Central and South America, Southern Europe, Asia and Africa reside in areas where the disease is endemic (196). The disease has also been reported in Canadian and American troops returning from Afghanistan (3).

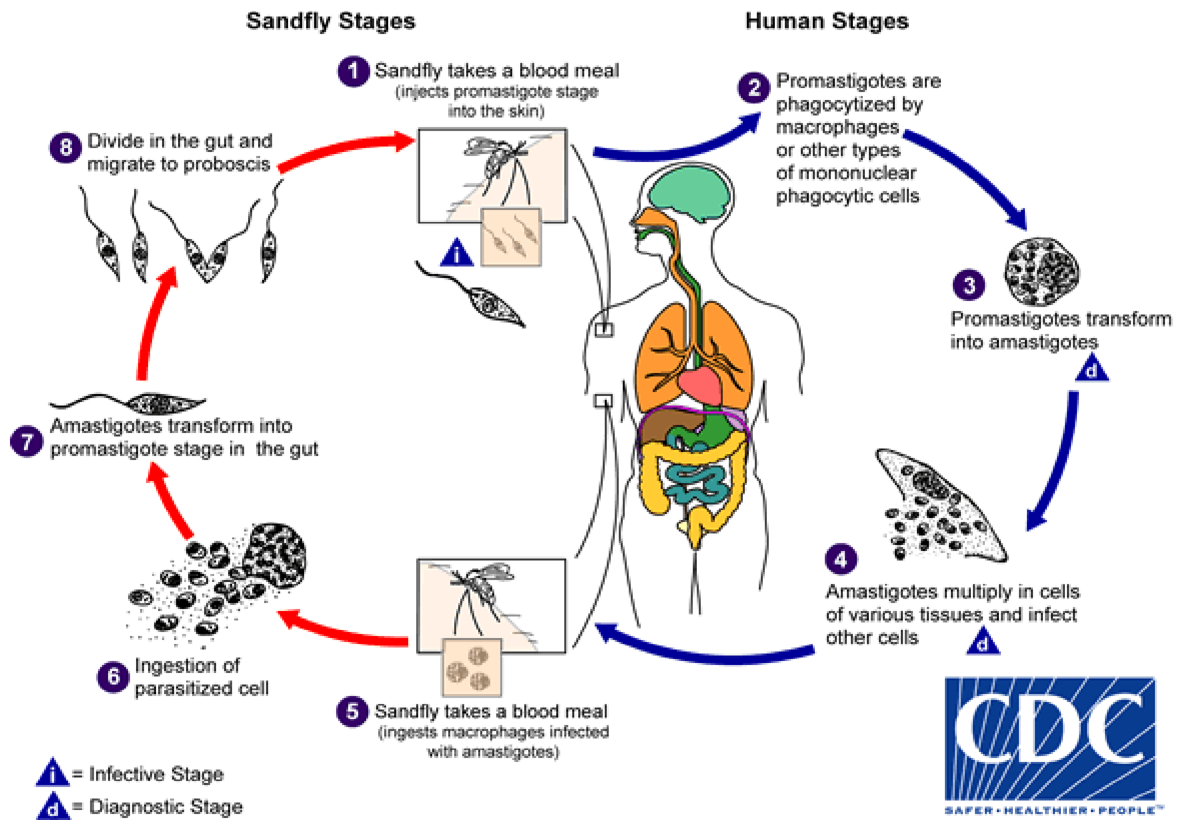
Leishmaniasis is a disease of humans and animals which occurs when an infected female *Phlebotomus* sandfly bites a host. The disease is mainly seen amongst the poorest people on earth and is associated with changes in the environment, poor nutrition and a weakened immune system (196). Infected individuals that go ahead to develop the disease are usually treated with pentavalent antimonial compounds administered parenterally. Although this treatment is effective against the disease, it has been reported to be very toxic to individuals following administration, hence it is important to develop novel therapeutic approaches against the disease (4).

1.2 THE VECTOR

The vectors for *Leishmania* parasites, are female *Phlebotomus* sand flies (in the "Old world") where as in the "new world" they are female sand flies of the genus *Lutzomyia.* There are nearly 30 species from over 500 species of sand flies which have been reported to be vectors for the parasites. The female sand flies are hematophagous in nature and thus require blood meals every 4-5 days, enabling them to develop their eggs for fertilisation. An infected female sand fly thus goes on to infect a mammalian host with the *Leishmania* parasite during a blood meal (5).

1.3 THE PARASITE LIFE CYCLE

Leishmania species exists as promastigotes (extracellular, flagellated and spindle-shaped) in the sand fly vector. The promastigotes develop near the endothelial cells of the sand fly's mid-gut (6). Because the sand flies are hematophagous, when they feed on a host's blood, the heavily parasitized sand flies injects a large quantity of their saliva which contains lots of parasites into the host (5). When a host is infected, the parasites are engulfed by dendritic cells (DCs) or epidermal macrophages which serve as antigen presenting cells. Within the phagolysosome of these APCs, the promastigotes are transformed to the amastigotes. The amastigote forms of *Leishmania* are round, immotile and non-flagellated. These amastigotes have the capacity to survive within macrophages, proliferate adequately and eventually rupture the infected cells. The parasites released from an already infected macrophage are taken up by other macrophages. Following a blood meal on an infected host, the sand fly will ingest the amastigote form. Within the midgut of the sand fly, the ingested *Leishmania* amastigote changes to a motile, flagellated spindle shaped form of the parasite called the promastigote. thus completing its life cycle (7)



Source ://www.d pd.cdc. gov/dpdx

There are 3 main forms of leishmaniasis namely Visceral Leishmaniasis, Mucocutaneous Leishmaniasis and Cutaneous Leishmaniasis.

1.4 VISCERAL LEISHMANIASIS.

Visceral Leishmaniasis (VL), which can be referred to as kala-azar, occurs in a host due to the bite of a female sand fly infected with *L. amazonensis, Leishmania donovani* (*L. donovani*), and *L. infantum.* (8). It is characterised by fever, anorexia, anaemia and weight loss accompanied with an enlarged liver and spleen. This form of the disease has been reported to be fatal if left untreated (196).

Visceral leishmaniasis has a yearly incidence of two hundred thousand to four hundred thousand worldwide (8). The incubation period for VL is mainly 2 to 6 months after which affected individuals show signs of infection. Some patients from East Africa, specifically Sudan, develop the post kalazar dermal leishmaniasis (PKDL). Individuals suffering from PKDL serve as sources for rapid spread of infection because the parasites are ubiquitous within the nodules (8).

1.5 MUCOCUTANEOUS LEISHMANIASIS.

The mucocutaneous form of Leishmaniasis (MCL) occurs in a host due to the bite of the phlebotomine sand fly infected with *L. panamensis*, *L. braziliensis,* and *L. guyanensis.* MCL is associated with the partial or complete damage of the oral, nasal and tracheal mucosa (196). It is characterised mainly by ulcerated lesions on the skin, usually on exposed body parts. This can result in serious disability and life-long scar formation. It is also estimated that between six hundred thousand to one million new cases of muco cutaneous leishmaniasis occur worldwide annually (196).

1.6 CUTANEOUS LEISHMANIASIS

Cutaneous Leishmaniasis (CL), widely regarded as the most common type of Leishmaniasis, occurs as a result of infection of a host with different species of *Leishmania* such as *L. major, L. mexicana and L. tropica*. Like the mucocutaneous form of Leishmaniasis, cutaneous Leishmaniasis can cause skin lesions, leave the individual with life-long scars and potentially lead to disability (9). Current treatment methods for cutaneous leishmaniasis are not very effective and have adverse effects (2). Currently, vaccine development against the disease has not been successful. This may be due to the complexity of the parasite and inadequate

knowledge of the factors and mechanisms that regulate immunity against it (196). There is an increased incidence of the disease in veterans that are deployed to areas where the disease is endemic. During the gulf war, the disease was not rampant. Although, in 2003 and 2004 an estimated proportion of twenty thousand, American soldiers who were returning from their mission in the middle east was diagnosed with cutaneous leishmaniasis (10). One of the initial symptoms of a host's infection would mainly be a minute reddish swelling (erythema) which develops at the site where an already infected sand fly bites the host. The red swelling transforms into a papule, and subsequently into a nodule. Furthermore, the nodule becomes ulcerated at about 2 weeks to 6 months and later becomes the pathognomonic lesion of local cutaneous leishmaniasis. The disease usually heals spontaneously in certain individuals thereby resulting in a life long protection against the disease in those individuals. The life long protection in these individuals could be limited to a *Leishmania* species or could extend to other *Leishmania* species (11)

1.6.1 Diagnosis of CL

The basis for the diagnosis of cutaneous leishmaniasis (CL) is mainly certain clinical symptoms usually backed up by various laboratory tests. There are various methods that have been described for the diagnosis of cutaneous leishmaniasis however they are not very accurate. CL can be diagnosed directly by microscopical parasitological examination, indirectly by testing the serum of suspected individuals or through the use of various molecular diagnostic techniques (12).

1.6.1.1 *Microscopy*

Cutaneous leishmaniasis can be diagnosed microscopically via visualization of *Leishmania* amastigotes in lesion smears stained with Giemsa. Microscopically, amastigotes are round bodies, with a diameter of approximately 2–4 μm, displaying prominent nuclei and kinetoplasts (13). One of the disadvantages of microscopical evaluations for the diagnosis of CL is that it may produce results that are non-specific or results with decreased sensitivity compared to other diagnostic techniques. (14).

1.6.1.2 *Parasite Culture*

One of the ways to culture *Leishmania* parasites is by introducing fluids acquired from a suspected lesion site on an individual or skin scrapings, and fresh skin biopsies from a suspected individual into Novy-MacNeal-Nicolle medium. However this technique is difficult, highly technical, time consuming and very prone to contamination (15). In addition, the parasite culture technique may have a diminished sensitivity coupled with a lot of variations (16). However, there are currently developed mini- and micro-culture equipments which have lower costs, are highly sensitive and much easier to use (15). This method is important because when it is successful, it serves as the source of *Leishmania* parasites that are used for subspecies identification through Polymerase Chain Reaction (PCR) (14).

1.6.1.3 *Serologic Tests*

Current serologic tests for diagnosis of CL include direct agglutination tests, lateral flow assay ELISA and western blot. Although, these tests are not commonly used for the diagnosis of CL due to their low sensitivity (12). Current studies based on rK39 (a *leishmania* antigen) and heat

shock protein (HSP83) indicates that the addition of some specific purified leishmania antigens or recombinant *leishmania* antigens to prepared parasite lysates would significantly improve the sensitivity of these serologic tests (17). De Vries *et al* (13) reported a novel diagnostic test which is presently under assessment (CL Detect™ Rapid Test). This is a membrane-based immunoassay that is developed to detect all species of *Leishmania* that has been reported to cause CL individuals.

1.6.1.4 *Leishmanin Skin Test*

Montenegro skin test (MST) or *Leishmania* intradermal skin test (LST) is a cellular reaction used to diagnose cutaneous leishmaniasis occasionally. This is because they are easy to use and are highly sensitive. For LST to be considered positive, the diameter of the resulting wheal should be ≥5 mm. Wheals that are <5 mm is reported as negative. Some of the major disadvantages of *Leishmania* intradermal skin test are; their reliance on culture facilities to produce the Montenegro skin test antigen, the various formulations of the antigen most times impacts on the sensitivity of the tests, and it cannot differentiate past infections from present infections (18).

1.6.1.5 *Polymerase Chain Reaction (PCR)*

There are several molecular techniques designed for the diagnosis of cutaneous leishmaniasis because they are believed to be more specific and sensitive compared to the other diagnostic methods. Molecular diagnostic methods involves the use of sampling methods that are not too invasive for diagnosis (19). PCR may be done on various biopsy smears, tissue scrapings, aspirated fluids and skin biopsies that are kept in waxed blocks (20). Currently, the PCR

protocols are highly automated thus ensuring the generation of faster, reliable results within a very short period of time (few hours) (21).

1.6.2 Treatment

The treatment of CL is necessary since it enhances cure, reduces scarring particularly at aesthetic sites, and avert the spread of the disease or relapse. Usually, treatment is administered for prolonged lesions of more than 6 months (22). Several treatment modalities are currently employed, and the efficacy and preference vary from region to region and between individuals. Generally, parenteral administration of antimony is the gold standard and has been very successful in the remedy of cutaneous leishmaniasis in all regions. However, the toxicity and excessive cost associated with the use of antimonials makes it very necessary for newer treatment regimens against cutaneous leishmaniasis to be developed (23).

1.6.2.1 *Physical Treatment*

A variety of physical methods have been used to treat cutaneous leishmaniasis in patients of different ages. Some of these physical methods include the administration of local heat, surgical excision, cryotherapy and cauterisation. Cryotherapy involves the use of cotton swab under moderate pressure to continually apply liquid nitrogen, up to 2 mm outside the lesion margin. A total time of 30–120 seconds is usually advised in cryotherapy and it involves 2-3 repeats of the process in a lesion (24)

1.6.2.2 *Chemical Treatments*

1.6.2.2.1 *Intralesional Antimonials*

The local infiltrations of cutaneous leishmaniasis lesion with pentavalent antimonials have been shown to be very effective in protecting the affected individual, and to have fewer side effects although the compounds applied by this method do not reach metastatic regions. The objective of a local pentavalent antimonial therapy is to fill the area affected during CL with antimonial compounds and it involves using a 25G needle to release the antimomials gradually as the needle moves deeper in the dermis. It is important to avoid releasing the antimonials into the subcutaneous space where they are absorbed and fail to reach the target sites. Two to five treatments of antimonials may be administered every 5–7 days. However, if the lesion is not cured following the fifth treatment, there should be a review of the disease within 1 month, and the physician may decide to administer systemic treatment to the patient (25).

1.6.2.2.2 *Parenteral Drug Therapy*

Amphotericin B; one of the major drugs used to eliminate systemic fungal infections has been employed by physicians as a reliable alternative of pentavalent antimonials- resistant VL. Amphotericin B is known to cause acute toxicity, and to avoid this issue, liposomal amphotericin B was developed. This liposomal amphotericin B has a better tolerance in host cells and is equally efficacious as the original amphotericin B. Liposomal amphotericin B is given intravenously in a dose of 3 – 10 mg/kg/day for 5 – 7 days (26). It is not used to a large extent in Old and New World leishmaniasis (22). In addition to VL, amphotericin B has also been reported to be a viable treatment option for cutaneous leishmaniasis (CL) (27). Wortmann *et al* reported that out of twenty patients that were suffering from CL, sixteen (84%) showed total recovery following the administration of liposomal amphotericin B. Although, three patients did not completely heal after initial treatment, they however showed total healing

following additional dosage of liposomal amphotericin B. As in VL, the use of liposomal amphotericin B for treating CL resulted in serious side effects such as renal toxicity (28). It is thus necessary for novel less toxic therapeutic approaches against CL to be developed.

1.6.2.2.3 *Oral Drug Therapy*

Clinicians who have decided to treat cutaneous Leishmaniasis via the oral administration of an anti-leishmanial drug have reported miltefosine as the primary drug of choice. Miltefosine affects the promastigote and amastigote stages of the parasite. Miltefosine interacts with lipids and causes apoptotic like cell death by inhibiting cytochrome c oxidase. Initially, miltefosine was registered in India for treating visceral leishmaniasis but now it is used to treat full range of clinical leishmaniasis. It is an effective recommended drug of choice in India and Ethiopia for VL, and Columbia and Bolivia for CL (29). The safety profile and efficacious quality of miltefosine as a therapeutic agent against zoonotic cutaneous leishmaniasis (ZCL) due to *L. major* has been investigated and compared against meglumine antimoniate in Iran. In the study, the total recovery was defined as 100% regeneration of epithelial cells at the sites of infection/lesion. In determining if the cure was successful or if it failed, both clinical and parasitological criteria were used (30).

1.7 EXPERIMENTAL MOUSE MODEL OF CUTANEOUS LEISHMANIASIS

Animals are often ideal for characterising the disease and studying their impact on humans. The major criteria used in choosing any animal model include physiological resemblance to humans, availability and easy handling. In studying the outcome and treatment of cutaneous leishmaniasis, mice and hamsters are the two most reliable models (31). Experimental

infections with different *Leishmania* species in mice present signs that are similar to the different kinds of human cutaneous leishmaniasis. Certain mouse strains, like BALB/c, are reported to be non-resistant to the *L. major* infection and this has been associated with their preference to produce a Th2 immune response after infection. The Th2 response is characterised mainly by IL-4 secretion, a cytokine associated with enhanced survival of intracellular *L. major* amastigotes (32). In contrast mice strains like C3H and C57BL/6, have been shown to be resistant to *L. major* infection, due to their capacity to produce an effective Th1 immune response, which is characterised by IFN-γ production that is necessary to halt the growth and proliferation of intracellular *L. major* amastigotes, thus leading to the development of self-healing lesions (33), (34). In further understanding the inability of BALB/c mice to resist CL and the ability of C57BL/6 to resist CL, one of the overarching questions in the field is what makes a $CD4^+$ T cell decide to become Th2 cell or Th1 cell, since a naïve $CD4^+$ T can become either following exposure to an antigen (35). Studies with C57BL/6 mice that do not produce IL-12 showed that IL-12 is necessary for inducing a naïve $CD4^+$ T cell's differentiation into Th1 subsets, and thus is critical for the development of resistance to *L. major* infection in C57BL/6 mice (36). However, for some reasons the BALB/c are reported to have a distinct T cell population (which recognizes a *Leishmania* antigen, known as *Leishmania* homolog of mammalian receptor for activated C kinase (LACK)- which bears a Vα8, Vβ4 T cell receptor) that mediates the initial production of IL-4 following *L. major* infection (37). Also, since lL-4 could drive IL-12 production in certain conditions, there by ensuing a consequent Th1 response (38), it is necessary that the role IL-4 plays in the immunopathogenesis of CL should be investigated further.

1.7.1 The Innate Immune Response in Cutaneous Leishmaniasis

The primary line of defence in a host is the innate immune response and it functions to protect a host against invading organisms. In the innate immune response to cutaneous leishmaniasis, cells like dendritic cells, macrophages, neutrophils and natural killer cells are associated with determining either the resistance/susceptibility of both murine and human species to *L. major* infection.

1.7.1.1 *Dendritic cells*

Dendritic cells (DCs) are immune cells that develop from hemopoietic bone marrow stem cells, they are extensively distributed throughout the body of a host (39). Paul Langerhans had initially described dendritic cells over a 100 years ago, but their role as an important orchestrator of a host's immune system was established only a few decades ago. Dendritic cells are specialized in activating the adaptive immune system by taking up antigen, processing it and subsequently presenting it to T cells. Therefore, DCs generally can be regarded as the professional antigen presenting cells (40). Marovich *et al* reported that DCs serve as the major producers of IL-12 during the early phases of *L. major* infection (41). Currently, there are five different subsets of dendritic cells identified in the murine skin namely (dermal-epidermal Langerhans cells, $CD11b^{+}$ DC, $Langerin^{+}CD11b^{neg}$, dermal DC $XCR1^{+}CD11b^{neg}$ DC and $Langerin^{neg}XCR1^{neg}$ double-negative DC). These dendritic cells seem to function differently during the activation of T cell-dependent immunity. For example, the dermal dendritic cells promote the generation of Th1/Tc1-dependent immunity, whereas epidermal Langerhans cells mediate the production of parasite-specific regulatory T cells (42). The difference of the outcome of *Leishmania* infection in BALB/c and C57BL/6 is believed to be because of the

variation in T-helper cell responses stimulated by their respective DCs. In contrast, von Stebut *et al* reported that the DCs derived from the skin of the foetus of both BALB/c and C57BL/6 mice displayed comparable levels of IL-12p70 production as well as expression of costimulatory molecules after *L. major* infection (40). Studies revealed that Langerhans cells of the epidermis (MHC II ^{high}, CD11b^{low}, CD11c^{+}, CD8α, Langerin^{+} and CD205^{high}) have the ability to phagocytose *L. major* and migrate into the lymph nodes that drain the infection site to present antigens to antigen specific T cells (43). Dendritic cells express CD40 on their surface, which is one of the costimulatory molecules needed to ensure optimal secretion of IL-12 by dendritic cells. Dendritic cell secretion of IL-12 is important for a naïve CD4^{+} T cell's differentiation into Th1 cells, which in turn produce IFN-γ (44). Okwor *et al* reported that the use of monoclonal anti CD40 antibody to neutralize CD40 led to increased susceptibility to *L. major* infection due to a reduction in the secretion of IL-12 that resulted in a decrease in IFN-γ production (45).

CD80 (B7-1) and CD86 (B7-2) are costimulatory molecules that are expressed on the surface of the DCs. They are necessary for providing the critical second signal needed for naïve CD4^{+} T cells to differentiate into various subsets of CD4^{+} T cells (46). Their roles in shaping the outcome of *L. major* infection have been reported by several scholars. Hathcock *et al* reported that Th2 response in BALB/c mice is dependent on B7-2; also, the early Th1 immune response that confers protection to *L. major* in C57BL/6 relies on B7-2. On the other hand, B7-1 expression was upregulated in *L. major* infected mice and it was able to induce IL-4 and IFN-γ production from Th2 and Th1 cells respectively (47).

1.7.1.2 *Macrophages*

Macrophages are immune cells differentiated from monocytes as they migrate from the endothelium into tissues in response to different stimuli. They get to different tissues, reside

and help to modulate host immune responses. It was generally believed that macrophages are solely generated from bone marrow-derived monocytes but Mcgrath *et al* (48) reported that they are primarily derived from the yolk sac which is the primary site for haematopoiesis in mammals during embryonic development. The development of macrophages in the embryonic yolk sac occurs in two different phases. Firstly, macrophage progenitors appear in the yolk sac around embryonic day 7.25 from where they migrate to the brain when circulation commences (49). Secondly, multipotent erythro-myeloid progenitors are generated from embryonic day 8.25, serving as the main source for tissue resident macrophages (50).

Macrophages are important in mediating phagocytosis and elimination of micro-organisms in a host. The intracellular stage of the parasite infection in macrophages is crucial for *Leishmania* parasite replication and differentiation since *Leishmania* parasites are obligate intracellular pathogens. Shortly after infection, both macrophages and neutrophils migrate to the site of infection where they engulf the parasites however, neutrophils are better at phagocytosing the parasites compared to the macrophages (51). Neutrophils do not live for a long time, so macrophages are the cells that are finally infected by the parasites, so they are necessary for parasite survival and replication, but they also function as the cells necessary for effecting the elimination of the parasites. Phagocytosis of parasite promastigotes by macrophages is through interactions of surface receptors and various surface molecules of both macrophages and parasites. For the attachment of promastigotes to macrophages, the receptors involved are fibronectin receptor, the complement receptors CR1 and CR3 (Mac-1) and the mannose-fucose receptor (51). For amastigotes, Miles *et al* (52) demonstrated that parasites coated with host IgG can bind to FcγR on macrophages thus enhancing their uptake by macrophages. This process activates downstream signalling pathways which prevent parasite elimination, thus promotes intracellular parasite survival and proliferation. Upon phagocytosis, the promastigotes will undergo morphological changes to subsequently transform into amastigotes

inside the macrophages. Within the macrophages, the amastigotes proliferate inside phagolysosomes, rupture host cells and infect surrounding tissue macrophages.

Macrophages are one of the major effector cells that are needed for the clearance of the parasite. For an infected macrophage to have the capacity to effectively destroy parasites there is a need for the macrophages to receive adequate activating signals. These signals mediate the development of functionally distinct macrophage subsets necessary for either parasite proliferation or destruction. There are two types of macrophages that are activated during infection; classical activated macrophages (M1) and alternative activated macrophage (M2). Classically, the activation of macrophages is mediated by IFN-γ, an effector cytokine secreted by $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells. Upon IFN-γ stimulation, macrophages produce inducible nitric oxide synthase (iNOS) which breaks down *L arginine* to give rise to nitric oxide (NO) (53). Nitric oxide is vital for killing intracellular pathogens including *Leishmania*. iNOS is believed to be important in facilitating the clearance of parasites as Wei *et al* showed that the normally resistant (C57BL/6) mice that lost the capacity to synthesize iNOS lost its resistance to *L. major* infection although they maintained a strong IFN-γ type response (54). Apart from IFN-γ, some other cytokines like TNF, IFN-α, IFN-β and IL-1 have been linked with the activation of classical macrophages and the ensuing upregulation of iNOS expression. On the other hand, the alternatively activated macrophages are induced by Th2 cytokines like IL-4 and IL-13 (55). Kropf *et al* showed that polyamine, which is induced by IL-4, favours the survival of *L. major* parasites in macrophages (56). *Leishmania* parasites manipulate this pathway of macrophage activation as such they evade the killing by activated macrophages thereby favouring their survival. Dendritic cells produce IL-12 following *L. major* infection, which is critical for activating and differentiating naïve $CD4^+$ T cells to $CD4^+$ Th1 cells. Since IFN-γ is the major effector cytokine necessary for the clearance of the parasite within the macrophages, studies have reported that the suppression of IL-12 production will favour

parasite survival (57). This finding was corroborated when Reiner *et al* showed that the infection of macrophages (BMDMs) from both susceptible and resistant mice background with *L. major* promastigotes caused the suppression of IL-12 production while other pro inflammatory cytokines were unaffected (58). *L. major* parasites in an attempt to evade the host immune response induce the production of immunosuppressive cytokines. This was shown by Kane and Mosser when they demonstrated that macrophages infected by *Leishmania* produced suppressive cytokines like IL-10 and TGF-β, which have been shown to inhibit/deactivate macrophage functions (59).

1.7.1.3 ***Natural Killer Cells*** **(NK cells)**

NK cells are a subset of lymphocytes that produce pro-inflammatory cytokines and mediate cytolytic activity upon exposure to tumours and infections. In a host, NK cells constitute 10% of peripheral blood mononuclear cells (PBMC) and 0.4–5% of mononuclear cells in the blood and the secondary lymphoid organs respectively (60).

NK cells make up some parts of innate immune system. Their protective roles in the immune response to *Leishmania* infection have been studied *in vitro* and *in* vivo. Purified naïve NK cells from PBMCs of humans expand and secrete IFN-γ after exposure to *Leishmania* antigen (61). Studies with mice revealed that the depletion of NK cells seven days after infection caused a decreased in the production of IFN-γ that consequently lead to a significant increase in parasite burden (62), thus suggesting that NK cells are vital hosts cells that contribute to resistance of a host during the initial phase of *Leishmania* infection. NK cells were thought to mediate this early protection by producing IFN-γ which promotes macrophage activation hence leads to increased parasite killing. To support this, immunity against *L. major* infection was linked to NK cell-induced early IFN-γ production in the resistant HeN mice, while the non-resistant BALB/c mice showed a diminished NK activity (63). However, NK cells isolated *in*

vivo from the susceptible BALB/c mice cannot respond to parasites, due to a lack of proper activating stimuli (63). When appropriate stimuli such as chemokines like CXCL 10 and CCL 3 were provided, these NK cells became fully and functionally activated (63). In addition to being the major producer of IFN-γ, NK cells are also major cytotoxic lymphocytes. Indeed, it has been shown that activated NK can directly lyse *L. major*-infected macrophages or parasites thereby contributing directly or indirectly to parasite control following infection (64).

1.7.1.4 *Neutrophils*

When a host is bitten by a sand fly that is infected, neutrophils migrate through the vascular endothelium and are recruited primarily to the infection site. After approximately 2 days this influx of neutrophils is then ensued by a wave of macrophage infiltration (65). At the infection site, neutrophils phagocytose the parasites and destroy them by various mechanisms. Some of the mechanisms used by neutrophils to eliminate pathogens after infection of a host are secreted cytokines, various toxic granules released from infection site and the formation of neutrophil extracellular traps (NETs) (66). Various *Leishmania* species can induce the formation of NETs in neutrophils as demonstrated by *in vitro* studies. NETs are DNA structures that are coated with antimicrobial molecules which are secreted by neutrophils (67). The efficacy of neutrophil NETs varies depending on the *Leishmania* species that are involved. For instance, *L. amazonensis* promastigotes are defenseless against NETs where as *L. donovani, L. mexicana* and *L. infantum* promastigotes have various mechanisms through which they escape from NETs (66).

Leishmania parasites are phagocytosed and can survive within neutrophils for a period of time. Although most pathogens are easily killed within neutrophils, *Leishmania* parasites can survive and persist inside neutrophils (68). *L. major*-infected neutrophils showed delayed apoptosis with a corresponding increase in the chemokine MIP-1β. MIP-1β is a chemokine which has

been reported to function as a chemo-attractant for macrophages (69). Following the recruitment of macrophages to the infected area, the apoptotic neutrophils that are infected will be engulfed by macrophages. This concept of “silent” infection of macrophages (macrophages being infected passively) by phagocytosing infected apoptotic neutrophils, makes the neutrophils to be seen as “Trojan horses” (70). The uptake of infected neutrophils by macrophages has been shown to lead to the development of macrophages which produce high levels of IL-10 coupled with a diminished IL-12 production, thereby supporting parasite proliferation and survival (71). Recently, Peters *et al* showed *in vivo* and in real time by intravital microscopy that the initial cells which engulf the parasites following their inoculation (either by needle or sand fly challenge) into the host are neutrophils. They further showed that depletion of neutrophils resulted in lower parasite burden and reduced disease progression, suggesting that neutrophils increase pathogenesis and extent of disease severity (72).

A few days after infection, macrophages are predominantly the most parasitized host cells (72). Interestingly, it was shown that the infection rate of neutrophils and macrophages were similar at the infected sites during the early stages of infection although neutrophils have an increased phagocytic ability (72). There is contrasting views by various authors in the contribution of neutrophils in the immunity during *Leishmania* infection. Some studies suggest that they may contribute to protection while others suggest they enhance disease pathology following infection. The ability of neutrophil in influencing disease outcome after *L. major* infection depends on the parasite species, host, and stage of infection (71). For example, Novais *et al* showed that neutrophil depletion *in vivo* following *L. braziliensis* infection resulted in a remarkable increase in parasite burden of infected BALB/c mice and co-inoculation of live neutrophils and parasites ensued lower number of parasite in the site of infection as well as draining lymph node (73).

Neutrophil infiltration after infection occurs in 2 phases; early and late phase. The aftermath of *Leishmania* infection is to some extent influenced by the influx of neutrophils to the infected area. Alex *et al* reported that there was marked neutrophil infiltration during the first 6 hours of infection and the use of polyclonal antibodies or cyclophosphamide to deplete the neutrophils resulted in a decreased parasite burden although transient. A second wave of neutrophil infiltration 19 days post-infection resulted in deeper necrotic lesions with maximum parasite burdens. The use of cyclophosphamide to deplete the second infiltrating neutrophils resulted in lower parasite burden and smaller lesion sizes compared to their mock controls. There was a marked decrease of GM-CSF, ROS and NO produced by the neutrophils from the second wave compared to the first wave neutrophils. This suggests that the second wave neutrophils have a lower anti-leishmania capacity compared to their first wave counterparts (74). Lopez Kostka *et al* reported that the second wave of neutrophil infiltration was dictated by IL-17 and a decreased IL-17 levels at 4 weeks post-infection correlated with a lower number of neutrophils (75).

1.7.2 <u>The Adaptive Immune Response to Leishmaniasis in Mice</u>

Most infections are effectively controlled by an efficient innate immune response. However, when the innate immune system fails to effectively eliminate an invading pathogen, the adaptive immunity is initiated. Contrary to innate immunity that is believed to be "non-specific", adaptive immunity is highly specific, very specialized and would mostly respond in particular to the pathogen which induced/stimulated its response. One of the other major features of adaptive immunity is that it exhibits immunological memory. Simply put, the adaptive immune system of a host "remembers" the antigens/pathogens that it previously experienced and goes on to respond much faster and in a higher magnitude following re-

exposure to that particular pathogen/antigen. The Adaptive immunity is mediated primarily by T and B lymphocytes either through the effector proteins (cytokines and/or antibodies) they produce or by direct cytotoxicity of infected cells.

1.7.2.1 *T- Cells*

T cells are immune cells which are produced in the bone marrow but are educated in the thymus. They are pivotal in the immune response of a host against different pathogens. They form heterogeneous populations which consist of various subsets that play various roles during an immune response. The T cell subsets are $CD4^+$ T cells (which consist of other subsets described below in details) and $CD8^+$ T cells. Their roles include enhancing the anti-microbial activity of macrophages, enhancing the infiltration of other inflammatory cells toward the infection area, helping B cells to make antibodies and production of their various effector cytokines.

The T cell receptors are important for a T cell to recognize an antigen. TCRs do not recognize the complete antigen but have the capacity to recognize only short peptides. These peptides are formed following the phagocytosis and subsequent breakdown of an invading pathogen by antigen presenting cells. The APCs present these peptides to the T cells through some surface molecules know as MHC class I which is necessary for $CD8^+$ T cells antigen recognition and activation or MHC class II which $CD4^+$ T cells greatly relies on for their activation. MHC I molecules can be found on the surface of almost all somatic cells and are necessary for the recognition of an infected or diseased somatic cell by cytotoxic T cells. Unlike MHC I, class II MHC is specifically expressed extracellularly by the ‘professional’ APCs such as DCs, B cells and macrophages.

Almost all somatic cells in the organism have class I MHC molecules which allow cells that were infected or transformed to be recognized and destroyed by previously activated cytotoxic T (Tc) cells. Class II MHC molecules on the other hand are found only on the cell surface of so-called "professional" APCs. These molecules present their antigenic peptides to T (Th) cells. These APCs (DCs, macrophages, and B lymphocytes) have both classes of MHC molecules, necessary for priming both kinds of T cells (76).

T cells are the major components of a host's immune system responsible for cell mediated immunity. Cell mediated immunity is usually used by the host to clear obligate intracellular pathogens which generally can evade antibody mediated host responses.

As *Leishmania* parasites are obligate intracellular pathogens, T cells are very crucial in the resistance to *leishmaniasis* because the control of parasite proliferation and survival is largely dependent upon cell mediated immunity. The protective role of T cells in leishmaniasis has been properly established because mice that are usually not susceptible to leishmaniasis become highly susceptible when they cannot mount a T cell response and the subsequent adoptive cell transfer of functional donor T lymphocytes restores the lost resistance (77).

1.7.2.1.1 *$CD4^+$ T-helper 1 (Th1) and T-helper 2 (Th2) cells*

The ability of a naïve $CD4^+$ T cell to differentiate into different subsets is determined to an extent by cytokines in the immediate microenvironment together with the extent of the strength of T cell receptor and antigen interaction (78). Naïve $CD4^+$ T cells, following exposure to antigens presented by the MHC-II on the APCs (like DCs) and in the presence of optimal amounts of both IFN-γ and IL-12 secreted by APC, differentiate into Th1 cells. The resultant Th1 cells subsequently produce their effector cytokines such as IFN-γ (signature Th1 cytokine) and TNF. These two cytokines are known for their roles in cell-mediated immunity against intracellular microorganisms.

It is widely believed that IL-12 secreted by APCs like DCs and macrophages, induces the differentiation and expansion of Th1 cells through Stat-4 mediated expression of IFN-γ. IFN-γ subsequently stimulates Stat-1 to induce the expression of T-bet; a transcription factor that is widely identified as the major regulator of Th1 cells (79). The differentiation of naïve $CD4^+$ T cells to Th1 cells requires more than MHC Class II-peptide expression and cytokine production by APCs. One of the critical events that results in the activation and subsequent differentiation of naïve $CD4^+$T cells to Th1 cells is the effective interaction of CD28 and CD40L on the T cells with the costimulatory molecules (CD80, CD86, and CD40) on the APC respectively (80). Previously, it was thought that IL-4 was critical for Th2 cell differentiation via Stat-6 induction. However newer studies suggest that GATA 3, which is generally regarded as the main transcription factor for Th2 cells, is the major determinant for naïve $CD4^+$ T cell differentiation into $CD4^+$ Th2 cells (independent of IL-4 and Stat-6) (81).

Macrophages, the most parasitized host cell during leishmaniasis are targeted greatly by Th1 or Th2 cells/cytokines. IFN-γ, generally regarded as the 'signature' Th1 cytokine mediates the activation of M1 (classical) macrophages which results in the enhanced elimination of parasites (82). C57BL/6 mice is resistant to *L. major* because they mount a solid Th1 immune response against the parasites, while BALB/c mice on the other hand have a high susceptibility to *L. major* infection due to their increased IL-4 (Th2) response as opposed to IFN-γ response (7). In *L. major* infected BALB/c mice, IL-1α from DCs is also necessary in enhancing Th1 response, this was because the DCs of infected BALB/c mice produced significantly lower IL-1α and IL-1β compared to the DCs of C57BL/6 mice. The local administration of IL-1α in infected BALB/c mice brought about a marked reduction in the parasite burden which was interestingly associated with decreased Th2 response and increased Th1 response. This response, however, was dependent of IL-12 (83).

$CD4^+$ Th1 cells facilitates the elimination of parasites during cutaneous leishmaniasis via the production of their signature effector cytokine (IFN-γ) which binds to its receptor on parasitized macrophages. Within the macrophages, the binding of IFN-γ results in activation of the macrophages to upregulate the iNOS pathway, bringing about increased synthesis of nitric oxide which is used to effectively kill the intracellular *leishmania* amastigotes (7). Similarly, a $CD4^+$ T cell that is naïve can be effectively differentiated into IL-4 producing Th2 if exposed to certain stimuli such as OX40 ligand (OX40L) (84). Mast cells (85), basophils (86), eosinophils and NKT cells (87) are also examples of other cell types that have the capacity to secret IL-4 which also enhances the differentiation Th2 cells via autocrine signalling. Unlike Th1 cells, Th2 cells have been associated with the lack of resistant of BALB/c mice to CL, because they deactivate macrophages and suppress the iNOS pathway which is imperative for the destruction/ clearance of the intracellular parasite (7). The immunosuppressive effect of Th2 cells in BALB/c mice was reversed via depletion of neutrophils, which was shown to down regulate the primary IL-4 response and conferred more protection to the mice (88). However, further study on the role of neutrophils in Th2 induction *in vivo* should be done by the use of 1A8 clone of the neutrophil depletion monoclonal antibody since the RB6-8C5 clone can also deplete the LY6C positive monocytes (89). To further show the role of Th2 cells in inducing susceptibility to a host, SCID mice that received Th1 cells and Th2 cells became resistant and susceptible respectively (90). Also Th2 cells have been shown to suppress Th1 cell differentiation in susceptible mice, thereby helping parasites survive in the macrophages (91). Although it was previously believed as a fact that IL- 4 produced by Th2 cells only enhances susceptibility, emerging evidence otherwise shows that IL-4 could be needed to enhance DC functions and promote Th1 cell response (38). However, this will be properly discussed in the subheading 'Role of cytokines in cutaneous leishmaniasis'.

One of the major dilemmas in the development of Th1 and Th2 cells is the different effects of high or low antigen dose on Th1/Th2 development *in vitro* and *in vivo*. *In vitro* studies revealed that low antigenic doses enhance Th2 cell development while high dose antigens induce Th1 cell development (92). This finding was corroborated by Uzonna *et al* where they showed that low parasite doses in C57BL/6 mice favoured the induction of Th2 responses, whereas high parasite doses favoured the induction of Th1 immune response (93). The authors further reported that the Th2 cell response due to low dose of parasite in infected C57BL/6 mice was transient resulting in complete healing. This complete healing was attributed to the appearance of Th1 cells later on during infection. The appearance of Th1 cells following low dose parasite infection in mice was as a result of the activation of $CD8^+$ T cells that secretes IFN-γ. This is because when $CD8^+$ T cells were absent, Th2 response persisted in C57BL/6 mice following infection with low parasite dose (93). This study suggests that the discrepancy observed in the development of Th1 and Th2 cells both *in vitro* and *in vivo* with low antigen depends on the concomitant activation of IFN-γ secreting $CD8^+$ T cells.

In conclusion, further studies need to be done to fully understand the factors that induce/ influence naïve $CD4^+$ T cell differentiation into $CD4^+$ Th 2 cells. This is important so that this pathway of $CD4^+$ T cell differentiation could be manipulated to further protect a host against *Leishmania* parasites and by extension other intracellular pathogens that are supported by Th2 cell responses.

1.7.2.1.2 *$CD4^+$ T helper 17 cells*

Th17 cells, distinct from Th1 and Th2 cells, are another subset of effector $CD4^+$ T cells. They exert strong stimulatory effect on various immune cells and stromal cells. Th17 cell activity on cells such as macrophages and neutrophils results in an elevation of pro inflammatory cytokines secretion and cellular infiltration respectively (94). In both mice and humans, naïve $CD4^+$ T

cells differentiate to become Th17 cells in the presence of IL-6, IL-23, IL-1β and TGF-β (95). IL-6 and TGF-β enhance the development $CD4^+$ Th17 effector cells. Although TGF-β is believed to be crucial for the generation of Th17 cells classically, some studies have shown that Th17 cells can develop without TGF-β (96). Ghoreschi *et al*, reported that there are two classifications of Th17: the "classical" Th17 cells, which are formed when TGF-β is in abundance and IL-23 is limited and the "alternative" Th17 cells which are developed when IL-23 alone is present in the environment during Th17 differentiation. The "alternative" Th17 cells were found to be more pathogenic than the "classical" Th17 cells (96).

Transcription factors regulate the differentiation of naïve $CD4^+$ T cells into various subsets. The increased expression of the transcription factor, T-bet, results in Th1 differentiation, GATA 3 (Th2), RORγ-T (Th17) and Fox p3 (T-regs). RORγ-T^+ Th17 cells suppress the differentiation and proliferation of $Foxp3^+$ T-regs and vice versa (97). In a naïve $CD4^+$ T cell, the transcription factor Foxp3 signals when TGF-β is present resulting in the differentiation of the naïve $CD4^+$ T cells to T-regs. However, when IL-6 is abundant Foxp3 is suppressed and if the available IL-6 combines with TGF-β, there will be an upregulation of RORγ-T, which would result in Th17 differentiation. Therefore, it is conceivable that the consonance between $Foxp3^+$ T-regs and Th17 cells may be regulated by the interplay between the transcription factors Foxp3 and RORγ-T in a naïve $CD4^+$ T cell (97).

The role Th17 cells plays in the immunity to *Leishmania* infections seem to vary depending on the parasite species; this is because some studies show that Th17 cells mediate susceptibility to leishmaniasis after *L. major* infection (98) but mediate resistance to visceral leishmaniasis after *L. infantum* infection (99)/*L. donovani* infection (100). Gonzalez-Lombana *et al* reported that Th17 cells enhanced the susceptibility of a host after *L. major* infection by exacerbating the disease outcome due to excessive immune response as a result of a deficient IL-10 response which was necessary to modulate the host's immune response to the parasite, thereby

preventing immune pathology (98). Interestingly, IL-17A was associated with less pathogenicity in human *L. braziliensis* infection, due to the observed increase in the levels of IL-17 in individuals with subclinical cases of infection as against symptomatic patients (101). The increased expression of IL-27 (a regulator of IL-17) in *L. braziliensis* infected individuals showing symptoms of CL compared to the individuals with sub-clinal infection corroborates the notion that Th17 may be involved in elimination of parasites and decreased lesions, thus emphasizing the protective role of Th17 in cutaneous leishmaniasis (101).

Nascimento *et al* identified IL-17A as a critical host molecule in the immunity against *L. infantum*. In the study, IL-17R$^{-/-}$ C57BL/6 mice displayed increased susceptibility to the infection when compared to the WT C57BL/6 mice as there was significantly more parasite numbers than in their WT counterparts. The increased parasite burden in IL-17R$^{-/-}$ C57BL/6 mice was attributed to the significant increase in number of IL-10 secreting Fox p3^{+} regulatory T cells, coupled with a reduction in the number of CD4^{+} Th1 cells than in their WT counterparts (99). Th17 cells were reported to be associated with protection to *L. donovani* infections in mice. This protective effect exhibited by Th17 cells in *L. donovani* infections was attributed to their ability to induce chemokine secretion which attracts neutrophils and Th1 cells towards the infected areas (100).

Castilho *et al* reported that anti-inflammatory cytokines suppressed effector CD4^{+} Th1 and Th17 immune response during *L. panamensis* infection. They showed that the neutralisation of anti-inflammatory cytokines in mice infected with *L. panamensis* resulted in enhanced IL-17 and IFN-γ secretion, which were responsible for the enhanced parasite control and elimination (102). From this study, it is safe to speculate that the deficiency of suppressive cytokines like IL-4, IL-10 and IL-13 resulted in enhanced proliferation of Th17/Th1 cells simultaneously which could be associated to the elimination of parasites.

1.7.2.1.3 *Cytotoxic CD8$^+$ T cells*

CD8$^+$ T cells develop in the thymus and express CD8 molecules on their surface. They are activated by the presentation of antigens on MHC I of infected cells. Activated CD8$^+$ T cells are referred to as cytotoxic lymphocytes (CTLs) (Cerottini *et al* 1970), and respond by secreting effector molecules such as perforins, granzymes and cytokines like IFN-γ (104).

CD8$^+$ T cells play a vital role in the cell-mediated immune control of pathogens, especially intracellular pathogens such as viruses. However, their role in regulating a host's immune response to parasitic diseases is controversial. Although CD4$^+$ T cells are the key cells responsible for anti-*Leishmania* immunity, various studies have identified CD8$^+$ T cells that are specific to parasite antigen from *Leishmania* infected individuals (105). Since IFN-γ from CD4$^+$ T cells is crucial for macrophages to be activated to kill intracellular parasites, one would speculate that CD8$^+$ T cells will play a similar role in the resolution of *leishmaniasis* because they still produce IFN-γ like CD4$^+$ T cells. Contrary to this, some studies showed that mice without CD8$^+$ T cells or Class I MHC (which is obligatory for CD8$^+$ T cell activation), controlled primary *L. major* infections effectively (106). However, recent studies clearly demonstrates that CD8$^+$ T cells are crucial during low dose parasite infection or for an effective secondary immune response after re exposure to the parasite (105). Uzonna *et al* studied the mechanism of CD8$^+$ T cell-mediated protection during low dose infection (93). Their study demonstrated that a short lasting Th2 response was induced in naïve C57BL/6 mice following low parasite dose infection. Infecting CD8$^+$ T cells deficient C57BL/6 mice with low parasites resulted in persistent Th2 response. recombinant IL-12 administration or anti-IL-4 treatment resulted in an increased Th1 response in CD8$^+$ T cell deficient mice after low dose infection (93). Collectively, these observations suggest that CD8$^+$ T cells modulates CD4$^+$ T cell

function. As a further complication, some studies suggests $CD8^+$ T cell response in a host could be pathogenic as MHC class I deficient mice showed smaller lesions when compared to their WT counterparts following infection with *L. amazonensis* (107). Furthermore, RAG knockout mice showed decreased lesion size (although parasite burden was not controlled) after *L. major* infection. Adoptive transfer of $CD8^+$ T cells led to an increase in the development of lesions in those mice (108). In another study, BALB/c without $CD8^+$ T cells mice had decreased lesion size (108).

In contrast to primary infection, $CD8^+$ T cells function in vaccine-induced immunity is well-established. For instance, vaccination with LACK, resulted in $CD8^+$ T cell mediated protective immunity (109). In addition, some vaccine strategies have demonstrated the ability of $CD8^+$ T cells to protect against live parasites (110).

1.7.2.1.4 *Regulatory T cells*

Regulatory T cells (T-regs) are $CD4^+$ T cells which suppress the immune response of a host and thus are essential for maintaining homeostasis in the host's immune reactions and maintenance of self-tolerance (111). T-regs are classified into two groups: natural occurring T-regs (nT-regs) and induced T-regs (iT-regs). CD25 was solely used initially as nT-reg marker however, recent findings have identified Foxp3 to be a key marker for nTregs (112).

After a parasitic infection, it is necessary for the host to generate modulated immune response which identifies the pathogen and at the same time limits excessive host immune responses which could potentially harm the host. The regulation of the magnitude of immune response by the host following parasitic infection could be as a result of the host immune cells producing molecules that dampen the response, or due to the parasite actively producing molecules to

manipulate the host's immune system so as to enhance its survival within the host. To achieve this, the parasite could directly induce immunosuppressive cytokines like TGF-β and IL-10 that are produced by innate immune cells or T-regs cells (113). Pathogenic microorganisms manipulate the host's immune system to favour the infiltration and retention of natural T-regs. For adequate migration of natural T-regs to infected sites, chemokine receptor 5 (CCR5) expression is crucial (114). Also, the retention of nT-regs at *L. major* infected sites has been linked to their increased expression of CD103 (115). Although, regulatory T cells are overtly associated with susceptibility to *Leishmania* infection, uninfected C57BL/6 mice showed marked disease reduction after adoptive transfer of $CD4^{+}CD25^{+}$ T cells from the lesion areas (116).

Previous studies suggest that natural T-regs are localized at the infected area where they limit the function of effector cells through IL-10 independent or dependent mechanisms, thus ensures the parasite lasts longer in the host for a long time (117). This persistence of the parasite in a host has been reported to be beneficial to the host because it enhances the establishment of a lifelong immunity of the host to parasite re-infection. This model illustrates the importance of a host-parasite equilibrium which could be beneficial to both the host and the parasite (113). Although this model indicates that Tregs are beneficial, overexpression of regulatory T cells could harm the host by mediating unchecked parasite multiplication (113). In line with this, the development of lesions in mice infected with *L. major* was due to high number of IL-10 secreting regulatory T cells present at the infected regions (118). Also, the adoptive transfer of regulatory T cells isolated from acutely infected mice to mice infected for a long time resulted in the reoccurrence of the disease and disrupted the effector memory response (119). These studies suggest that following *Leishmania major* infection, regulatory and effector T cell immune balance may determine the aftermath of the infection.

1.7.2.2 *B cells*

B cells are lymphocytes that develop in the bursa of fabricus in chickens and in the bone marrow in other mammals (120). B cells that successfully rearrange their Ig genes are selected to continue their expansion within the follicles of the bursa. About 5% of B cells in the bursa survive and migrate to the periphery (121). B cells presents antigens to $CD4^+$ T cells because they express class II molecules needed for activating a naïve $CD4^+$ T cell (122). An activated B cell differentiates into plasma cells which are widely regarded as the effector B cells or the effector cells of the humoral immunity (123). Their major effector molecules are antibodies, which are mostly critical for a host's immune response to extracellular pathogens (124) Generally, it is believed that B cells do not contribute to protective immunity to cutaneous leishmaniasis. This belief is due to the fact that *Leishmania* parasites reside in the vacuole of macrophages (intracellular pathogens) and as such may not be easily accessed by antibodies (125). To demonstrate B cells are not protective in leishmaniasis, BALB/c mice that were sub-lethally irradiated showed resistance when the donor cells were $CD4^+$ T cells as against B cells (126), and serum transfer from healed mice did not confer protection (127). Cutaneous leishmaniasis in BALB/c mice, a very susceptible background, is characterised by the secretion of high amounts of *Leishmania*-specific antibodies; hence it is conceivable that B cells (antibodies) exacerbate the disease or enhance susceptibility (128). Miles *et al* (52) assessed the role of IgG in influencing the aftermath of leishmaniasis in BALB/c mice. They showed that IgG by mediating the enhanced secretion of IL-10 causes progression of the disease. Although most *L. major* studies demonstrated that B cells may be contributing to the exacerbation of the disease, Rodriguez-Pinto *et al* reported a contrary finding with *L. panamensis*. Their study reported that co-culture of B and T cells from infected individuals with *L. panamensis* antigen caused activation of the T cells and this was accompanied by enhanced IFN-γ and TNF-α secretion. They also showed that these B cells, after *in vitro*

stimulation with *L. panamensis,* have an increased expression of CD86, more than on dendritic cells (129). Likewise, Woelbing *et al* reported that IgG secreted by activated B cells in C57BL/6 mice after *L. major* infection increased antigen uptake capacity in dendritic cells via FC gamma 3 receptor (FCγR III) and thus increased the antigen presentation capacity of the dendritic cells which consequently resulted in increased Th1 response. B cell deficient C57BL/6 mice following *L. major* infection displayed a significant reduction in IFN-γ secretion which was reversed upon infection with IgG opsonized parasites (130).

1.8 THE ROLE OF CYTOKINES IN LEISHMANIASIS

Different cytokines have been shown to play various roles during the immune response to *Leishmania* infection. The outcome of *L. major* infection is determined, at least in part, by the type of cytokine response evoked by T cells of the infected host.

1.8.1 Interleukin-12

Interleukin-12 helps contribute to a host's protective immunity against *leishmaniasis*. The depletion of IL-12 with anti-IL-12 neutralizing antibodies caused a deficient Th1 immune response which resulted in an enhanced Th2 immune response during *L. major infection* thereby causing the mice to lose its resistance (131). Conversely, when recombinant IL-12 were administered to BALB/c mice that were previously susceptible to the infection, it led to an enhanced Th1 immune response, and conferred protection (34). Functional IL-12 (IL-12p70) is a heterodimer that is made up of p35 and p40 subunits. When either of these subunits are altered, there is marked susceptibility to *L. major* infection, confirming the importance of IL-12 in mediating Th1 immune response and establishing its importance for pathogen control (132). IL-12 is predominantly produced by dendritic cells and macrophages which are APCs

that are vital for activating a $CD4^{+}$ T cell. Macrophages have been reported to have an impaired IL-12 production following *Leishmania* infection/endocytosis (133). However, DCs retain the capacity to secrete IL-12(even when infected) thus encourage the increased production of IFN-γ (which has been associated with protection) after *Leishmania* infection (41).

1.8.2 Interferon-γ

Interferon-γ (IFN-γ) is the key cytokine required by parasitized macrophages to upregulate their nitric oxide (NO) production to effectively clear parasites (134). Furthermore, in an attempt to demonstrate the importance of IFN-γ in enhancing host protection to *Leishmania* infection, it was shown that the use of anti- IFN-γ antibodies to neutralize IFN-γ response in the resistant mice background resulted in enhanced Th2 immune response and a consequent loss of resistance (135). Similarly, recombinant IFN-γ when administered to BALB/c mice after *Leishmania* infection resulted in a delayed disease onset and significantly decreased the sizes of their lesions (32). Swihart *et al* showed that mice that were originally resistant to *L. major* but lacked the capacity to produce IFN-γ and/or express IFN-γ receptor lose their resistance to *L. major*. Although, the mice that lacked the capacity to secrete IFN-γ did not produce IFN-γ, the mice that were deficient in IFN-γ receptor expression still produced IFN-γ but could not activate their macrophages to produce NO (136). Various cells including $CD4^{+}$ Th1 cells (137), NK cells (138) and $CD8^{+}$ T cells (93) serve as sources of IFN-γ during leishmaniasis.

1.8.3 Tumour Necrosis Factor

Tumour necrosis factor (TNF) is an important cytokine secreted by activated macrophages, T cells and B cells in response to stimuli (53). Liew *et al* reported that TNF and IFN-γ collaborate in mediating the activation of macrophages, which results to nitric oxide-mediated anti-

leishmanial activity (53). In mice suffering from cutaneous leishmaniasis, the administration of recombinant TNF brought about a decreased lesion size coupled with lower number of parasites. Also, the neutralization of TNF with anti-TNF monoclonal antibodies in *L. major* infected mice resulted in a marked reduction in their lesion size and parasite burden (139). Although TNF is believed to be protective in leishmaniasis, some studies suggests that both TNFR1 nor TNFR2 are not necessary for the generation of a Thl response and by extension NO production (140). TNFR1$^{-/-}$ or TNFR2$^{-/-}$ mice displayed normal production of NO and eliminated the parasite (140). This suggests that there could be an alternative mechanism for priming macrophages *in vivo* to kill parasites (141). Although TNFR1$^{-/-}$ mice eliminated parasites, they could not resolve their lesions (142), perhaps indicating this receptor is vital for downregulating inflammatory responses after infection. Furthermore, Scott *et al* (143) hypothesised that TNFRI is needed to induce the death of cells in lesions, but when absent leads to accumulation of lymphocytes at the site of inflammation. Finally, these studies suggest that the function of TNF in cutaneous leishmaniasis may vary and so should be studied further.

1.8.4 Interleukin 4

Interleukin-4 (IL-4) is mostly produced by CD4^{+} Th2 cells. It interacts with its receptor on naïve CD4^{+} T cells and enhances its differentiation into Th2 cells (144). In addition, IL-4 also strongly inhibits IFN-γ secretion from CD4^{+} Th1 thus inhibits CD4^{+} Th1 differentiation and expansion (144). The uncontrolled leishmaniasis (increased susceptibility) found in infected BALB/c mice is attributed to the elevated numbers of CD4^{+} T cells which produce IL-4. These suppress the production of IFN-γ, the major cytokine required for macrophage activation and subsequent resistance to Leishmaniasis (145). Furthermore, susceptible BALB/c mice can become resistant following the administration of an antibody which neutralizes IL-4 (146). Notably, BALB/c mice which lack the ability to produce IL-4 (due to deletion of the IL-4 gene),

unexpectedly remained susceptible after *L. major* infection (LV39 sub strain) (147). On the other hand, IL-4 receptor α deficient BALB/c mice (IL-4R $\alpha^{-/}$) resisted *L. major* sub strain IR173 infection (148). These results suggested that the uncontrolled leishmaniasis (at least in BALB/c mice) may be mediated through another cytokine (other than IL-4) that signals through the IL-4Rα.

1.8.5 Interleukin-13

Interleukin -13 (IL-13) is a cytokine that disrupts the production of proinflammatory cytokines by T cells (149). Primarily, IL-13 regulates the outcome of intracellular parasitism, expulsion of intestinal worms and modulates airway hyperresponsiveness (AHR) (150). A clinical study demonstrated an elevation in the mRNA levels of IL-13 in localized cutaneous *leishmaniasis* lesions as well as in most visceral leishmaniasis patients (151). IL-13 transgenic C57BL/6 mice had increased size of lesions coupled with extended parasite survival which was linked to the overproduction of IL-4, thus suggesting a possible synergistic action between IL-4 and IL-13 (152). IL-13 influences disease outcome depending on the phase of infection. Thus, it is not clearly understood how IL-13 exacerbates the disease severity during the early stages of leishmaniasis (153) but enhances protection in the chronic phase (154). Interestingly, lymphocytes of mice have no detectable IL-13 receptors (155), this suggests IL-13 could be influencing murine immune responses by signalling through cells that are not lymphocytes. IL-13 impedes certain functions in macrophages such as the secretion of IL-12 (156), TNF (157) and iNOS (158).

1.8.6 Interleukin-10

Interleukin-10 (IL-10) is a cytokine formerly referred to as cytokine synthesis inhibitory factor (CSIF). It was believed previously that Th2 cells secretes IL-10 and uses it to suppress the

effector functions of Th1 cells (159). Formerly, IL-10 was stratified under Th2 cytokine because of its potent inhibitory actions on the expansion and functions of Th1 cells. However, it has now been shown that IL-10 have the capacity to suppress the activities of both Th2 and Th1 cells. IL-10 was also shown to be produced by regulatory T cells, mast cells, B cells, macrophages, NK cells and eosinophils (160). IL-10 has a large spectra of anti-inflammatory effects on DCs and macrophages which includes suppressing the secretion of various pro inflammatory cytokines like TNF and IL-12 (160). It was reported that IL-10 knockout mice or IL-10 receptor knockout mice developed enteritis spontaneously (161), suggesting that IL-10 is critical for suppressing excessive host immune response against normal gut microbial flora. In Leishmaniasis, IL10 deficient mice on BALB/c background are not susceptible to CL (59), indicating IL-10 negatively regulates pathogenesis of the disease. Wound healing during primary *L. major* infection has been associated with persistent parasites even in C57BL/6 mice that are considered to be resistant to the disease (162). However, some studies showed that IL-$10^{-/-}$ C57BL/6 mice totally eradicated parasites so they implicated IL-10 producing regulatory T cells as being responsible for parasite persistence during chronic infection (117). This suggests that IL-10, (particularly T-reg derived), is an important influencer of disease pathogenesis and parasite persistence. However, some studies suggest that IL-10 can determine the outcome of leishmaniasis depending on the strain of mice and infecting parasite species. In line with this, it was shown that C57BL/6 without the capacity to produce IL-10 cannot control *L. amazonensis* infection although they mounted a more robust Th1-response compared to their WT counterpart (163). Also, Kane and Mosser (59) reported that BALB/c mice without IL-10 production capacity do not control the disease progression after *L. amazonensis* and *L. mexicana* infection.

Collectively, these findings suggest that IL-10 significantly influences the development of immunopathology and persistence of leishmania parasites.

1.8.7 Interleukin-17

IL-17 is predominantly secreted by Th17 cells but can also be secreted by CD8^{+} T cells, neutrophils and macrophages (94). IL-17 is implicated in the upregulation and downregulation of pro inflammatory cytokines as well as some anti-inflammatory cytokines. In addition to regulating pro- and anti-inflammatory cytokine responses, IL-17 mediates the recruitment of neutrophils (94) and *Leishmania* infection induces its production (164). This was evidenced as there was increased quantity of IL-17 in individuals diagnosed of cutaneous leishmaniasis and mucocutaneous leishmaniasis (164) . IL-17A mediates its effector function by promoting the accumulation of neutrophils, secretion of IFN-γ from CD4^{+} Th1 cells (99), and increasing antimicrobial peptides secretion (165). A human study involving patients with visceral leishmaniasis (VL) suggests that IL-17 may be inducing protection. In the study, it was suggested that the protective role of IL-17A was due to its effect in increasing the levels of CXCL chemokines, that function as chemo-attractants for neutrophils and Th1 cells (100). In BALB/c model of *L. donovani* infection, increased production of IFN-γ, IL-17 and IL-23 were reported to mediate parasite clearance. The neutralization of IL-17 and IL-23 by their respective monoclonal antibodies, resulted in increased parasite burden in their spleen and liver (166). Conversely, the administration of recombinant IL-17 (but not IL-23) caused a marked increase in secretion of IFN-γ together with NO, stronger IL-17 response and enhanced clearance of parasite in both CL and ML (147). In these forms of leishmaniasis, the affected individuals develop excessive inflammatory responses that lead to tissue damage albeit the decreased parasites observed in the lesions (167). In IL-10 receptor deficient mice, the production of IFN-γ together with IL-17A were significantly increased and thus both cytokines were associated with the exacerbation of the disease (98). The blocking of IFN-γ response increased the quantity of IL-17A and enhanced pathology, this corroborates the opinion of

some scholars that believes IFN-γ may be crucial for the downregulation of both IL-17 and IL-10 responses C57BL/6 mice during leishmaniasis (98).

1.9 SEMAPHORINS

Semaphorins were previously described solely as axon guidance molecules in the nervous system (168). Currently, studies indicate they are expressed in other systems like the cardiovascular, endocrine, gastrointestinal, musculoskeletal, immune and respiratory systems (169). They contribute to the regulation of angiogenesis, morphogenesis, cell differentiation, cell adhesion, cell proliferation, and cell migration (170). Semaphorins were phylogenetically classified into 8 classes ranging from class 1-8. Semaphorins in class 1 and 2 are conserved in invertebrates while class 3, 4, 6 and 7 are specifically found in vertebrates. Class 5 is expressed by both vertebrates and invertebrates. Class 8 semaphorins are found in viruses. Among the semaphorins found in vertebrates, the class 3 semaphorins are the only known secreted form while classes 4, 5 and 6 are transmembrane and class 7 is glycophosphatidylinositol (GPI)-anchored (171).

The structure of semaphorins consists of an N-terminal portion which is referred to as the Sema domain and a short intracellular tail referred to as the basic domains. It is made up of ~500 amino acids (172). The Sema domain improves the receptor binding specificity of semaphorins with their respective plexin receptors (173).

1.9.1 Semaphorins and Infection

The roles of certain classes of vertebrate semaphorins in infection models have been reported. A study by Sultana *et al* reported that following the infection of WT mice with West Nile virus, there was a significant increase in the amount of Semaphorin 7A secreted at the cortical neurons (174). The increased secretion of Sema7A in the cortical neurons of the infected mice was accompanied by early death of the mice. Increased viral burdens and viral permeability through the blood brain barriers of the infected mice were associated with Sema7A induced upregulation of TGF-β and Smad6 signalling. They went ahead to show that the neutralization of Sema7A with monoclonal anti-Sema7A antibody resulted in increased survival, lesser viral burdens and reduced permeability of the West Nile virus through the blood brain barrier (174). Similar to Sema7A, Sema3E is also a vertebrate semaphorin that is secreted by some immune cells in the host; however, the role of Sema3E in infection models has not been reported so far.

1.9.2 Semaphorin 3E

1.9.2.1 *General overview*

Semaphorin 3E (Sema3E) was previously referred to as M-sema H, or SEMAH, and its gene was sequenced in 1998 from a tumor cell line by Christensen (175). As previously mentioned, semaphorins are guidance proteins expressed in various organs and they play important roles in disease pathology and homeostasis. Sema3E, typically referred to as “chemorepellent”, is specifically involved in angiogenic regulation, as well as in the regulation of cell proliferation and migration (176). It was first identified in metastatic cell lines and consists of a putative signalling sequence in the amino terminus followed by a large semaphorin domain (175).

Proteolytic cleavage of members of class 3 semaphorins is critical for their functional activity. This is predominantly mediated by furin-like pro-protein convertase enzymes which are capable of diminishing semaphorin signalling (177). This proteolytic processing also

downregulates the growth-repelling activities associated with class 3 semaphorins (178). However, in the case of Sema3E, this cleavage is responsible for its pro-metastatic activity in cancer cells, despite its structural similarities to the other members of the class (including tumour suppressive Sema3B and 3F).

1.9.2.2 ***Sema3E Holoreceptor Complex and Signaling***

Sema3E, like other semaphorins, interacts with plexins, which are transmembrane neuronal receptors to which the semaphorins bind directly or indirectly to induce their angiogenic, immune or neuronal axon guidance activities (179). Sema3E can bind directly and with high affinity to its receptor Plexin D1, as opposed to dependent binding occurring with other semaphorins involving neuropilins to form holoreceptor complexes with plexins (180). Activated B cells express Plexin D1 together with thymocytes although Plexin D1 is downregulated upon T cell maturation (181). Intracellularly, the Plexin D1 tail contains two domains, one of which is the SEMA/PLEXIN domain. This domain in turn includes two C RasGAP domains (consisting of GTPase-activating proteins and GTPases of the R-Ras subfamily (182). Plexin-D1 antagonizes PI3K which modulates migration signalling, and cell growth and survival (183) (184). Various functions of semaphorins are facilitated by STAT MAPK and PI3K signalling (185). Hung *et al* reported that a subset of flavoproteins known as molecule interacting with Cas ligand (MICAL) can sustain the reorganization of F-actins in the nerve cells which is mediated by semaphorin-plexin signalling (186). Understanding the signalling pathways of semaphorin-plexin interactions will be crucial in developing novel semaphorin based therapy.

1.9.2.3 *Different Roles of Sema3E in the Various Systems of Vertebrates*

Sema3E has been associated with different roles in various systems of vertebrates.

1.9.2.3.1 *Cardiovascular System*

Sema3E-Plexin D1 signalling is believed to play several crucial roles in cardiovascular development. Sema3E has been reported to repress the angiogenesis of the dorsal aortae by antagonizing vascular endothelial growth factor (VEGF) (187) and cell responsiveness to VEGF in retinal angiogenesis (188). Sema3E signalling has also been studied in cardiac morphogenesis and vascular patterning, with its impairment being associated with various human cardiovascular disorders (189).

1.9.2.3.2 *Asthma*

Studies by Movassagh *et al* have shown that Sema3E is expressed in the airways of asthmatic patients and mice experimentally induced to develop airway allergic inflammatory response (190). As a key regulator in allergic asthma, Sema3E inhibits the proliferation of airway smooth muscle cells and impedes their migration by modulating the pathways for Akt, ERK1/2 and Rac1 signalling (191). Genetic ablation of Sema3E in mice increases the deposition of collagen, exacerbates the responsiveness of airway smooth muscle cells, enhances Th2/Th17 mediated inflammation of the lungs and extends lung granulocytosis after house dust mite exposure. (191). A downregulated asthma response was observed after intranasal recombinant Sema3E was administered to allergen-sensitized mice (190), thus suggesting that Sema3E ameliorates lung inflammation. The severe inflammation observed in Sema3E deficient mice was attributed to the higher number of Th17 cells and $CD11b^+$ dendritic cells within the lung tissues in both allergic and steady state conditions compared to wild-type control mice (192). Thus, it is

conceivable that Sema3E signalling via Plexin-D1 negatively regulates Th17 differentiation and/or migration of CD11b^{+} DCs to the lungs. These findings are in agreement with a previous study which showed that Sema3E inhibits migration and proliferation of human ASM cell (191).

1.9.2.3.3 *Immune Regulation*

In vitro studies show that Sema3E acts as a potent inducer of macrophage migration and expression of proinflammatory cytokines (193). The signals generated following binding of Sema3E to Plexin D1 leads to enhanced expression of markers of classically activated macrophages, and thus leads to increased proinflammatory cytokine responses in the macrophages. (193). Dendritic cells from Plexin D1 deficient mice secreted higher levels of IL-12/IL-23 p40 than their WT counterpart mice (194). In addition, differentiated Th2 cells express significantly more Sema3E than other immune cells including Th1 cells (194). Given this finding that Th2 cells express Sema3E, it is conceivable it could be suppressing Th1 cell development thereby promoting an environment favourable for Th2 development. A recent report showed that NK cells displayed enhanced migration when exposed to the conditional media obtained from LPS and Poly I: C stimulated immature dendritic cells from Sema3E$^{-/-}$ compared to their similarly treated WT counterpart cells (195). The enhanced migration of the activated NK cells was abrogated when recombinant Sema3E was added to the conditional medium from Sema3E$^{-/-}$ DCs (195). This study suggests that Sema3E limits the migration of NK cells towards immature DCs in the NK/DC cross talk.

CHAPTER 2

2.0 RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1 RATIONALE

The host factors that regulate resistance to *Leishmania major*, a causative agent of cutaneous leishmaniasis (CL), are not completely known but are influenced by the nature of innate and adaptive (particularly T helper cell) responses (143). There is currently no vaccine for CL due to the complex biology of the causative parasites and inadequate knowledge of the factors and mechanisms that regulate immunity infection and disease outcomes (WHO, 2016). In addition, the current treatment methods for CL are not very effective and have significant side effects (197). Therefore, there is need to discover novel therapeutic approaches to combat the infections. Understanding the principles that determine whether a host resists or succumbs to the infection is critical for the development of vaccine and/or effective therapy against the disease. Recent reports show that a host protein, Semaphorin 3E (Sema3E) and its receptor Plexin–D1, are displayed on the surface of immune cells that are important in the immunopathogenesis of *Leishmania* infection, like macrophages, T cells and dendritic cells (198). In addition, Th2 cells which mediate susceptibility to *L. major* infection (143), were reported to have more Sema3E on their surface than the protective Th1 cells (194). Sema3E was reported to dictate immune response and affect cell migration (172) which are critical events in immunity to CL.

Collectively, these findings indicate that Semaphorin 3E might influence the host's immunity to CL. Hence, I seek to study the role of Semaphorin 3E in regulating the outcome of *L. major* infection in mice with the aim of possibly identifying a novel therapeutic target against *L. major* infection.

2.2 OVERARCHING AIM OF STUDY

To investigate the role of Sema3E in regulating immune response and disease outcome during *Leishmania major* infection.

2.3 HYPOTHESIS

Semaphorin 3E contributes to a host's susceptibility to *L. major* infection by downregulating dendritic cell functions and suppressing $CD4^+$ Th1 and Th17 immune responses.

2.4 OBJECTIVES OF STUDY

Objective 1: To determine the outcome of *L. major* infection on the expression of Sema3E in mice.

Objective 2: To assess the outcome of *L. major* infection in Sema3E deficient mice.

Objective 3: To investigate the effect of the deficiency of Sema3E on dendritic cells and $CD4^+$ T cells infected with *L. major*

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 ANIMALS, HOUSING AND BREEDING

Six to eight-week-old C57BL/B6 mice were acquired from the Genetic Modeling Centre (GMC), in the University of Manitoba. Semaphorin 3E knockout (Sema3E$^{-/-}$) on the B6 background were produced in house by GMC by crossing homozygous males and female knockout mice originally obtained from Dr. Chengua GU of Harvard Medical School, Boston, Massachusetts USA. All mice were housed at the University of Manitoba CACS in the absence of pathogens and were utilized based on the laid down guidelines of the Canadian Council for Animal Care.

3.2 PARASITES

Leishmania major (L. major) Fredlin (MHOM/80/Fredlin) strains was used throughout the study. The parasites (promastigotes) were artificially grown in the incubator at 26°C with M199 medium (Hyclone, Logan, UT) reconstituted with 20% heat-inactivated fetal bovine serum (FBS) (Cansera, Mississauga, ON, Canada), and 100 U/ml penicillin/streptomycin.

3.3 ANIMAL INFECTION

Promastigotes obtained from the incubator after 7 days were washed thrice with sterile phosphate buffered saline (PBS) at 3000 revolutions per minute for 15 minutes. After which parasites were then kept in sterile PBS at 40×10^6 per ml. 50 µL of the solution contained 2×10^6 parasites that were carefully injected subcutaneously into foot pad of the left feet. In other studies where intradermal ear infection was done, mice were infected with 10^6 in 10 µL of sterile PBS.

3.4 LESION SIZE MEASUREMENT AND PARASITE QUANTIFICATION

Digital Vernier calipers was used every week to measure the developing lesions. After sacrificing the infected mice, the parasite burden in the infected feet was assessed by limiting dilution assay as has been described formerly (199). It involves the sacrificing of mice by isoflurane and cervical dislocation, and the infected feet were cut off with a razor blade and placed into a beaker containing PBS complemented with 2% Pen/Strep (PBS/Penstrep) and kept on ice. Initially, the feet were transferred into 70% EtOH for 5 minutes, after which they were transferred to chlorhexidine disinfectant for additional 5 minutes. From the disinfectant, the feet were further transferred into a second 70% EtOH for 5 minutes, after which they were rinsed in PBS/Penstrep. Thereafter, the feet were trimmed with a razor blade to cut off toe nails. An incision was made on the footpad and forceps were used to peel off the skin. The feet were cut into smaller pieces, placed inside a grinder containing 2 ml of PBS/Penstrep and then ground. Homogenized tissue was transferred to a conical tube (B-D falcon tube). The tissue grinding tool was cleaned further by pouring 2 ml PBS/Penstrep into the conical tube after which the whole contents of the conical tube was spin down for 5 minutes at 600 rpm. Thereafter, the resultant fluids were transferred into a new 15ml conical tube and later were spinned for 15 minutes at 3000rpm. The resultant fluids were discarded and the pellets (containing parasites) were dissolved in 2 ml of complete Schneider media (Hepes, FBS and Glutamate). 20 μL of the suspension were put into the first wells of 96-well plate containing 180 μL of complete Schneider media (to produce a 1:10 dilution). The wells were mixed (by pipetting up and down 10 times) and 20 μL from the wells were transferred to the second and serially diluted until the last row. The plates were covered with foil and put in the incubator set at 27°C for 7 days after which parasite numbers were quantified using a light microscope.

3.5 ISOLATION OF DENDRITIC CELLS AND MACROPHAGES FROM BONE MARROW CELLS.

To generate dendritic cells from bone marrow cells (BMDCs), the tibia and femur of WT and Sema3E knockout (Sema3E$^{-/-}$) C57BL/6 were used. The BMDCs were isolated as described by Lutz (200). Briefly, muscle tissues were removed from the long bones in the hind limb of the sacrificed mice with razor blades and the two epiphyses of the bones were cut with razor. The contents of the bone marrow were removed (using 18-gauge needle attached to a syringe) with 5 ml RPMI medium into 15 ml polypropylene tubes. The cells were vortexed to disperse marrow tissues and centrifuged at 1200 rpm for 5 minutes. ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) was used to decontaminate the RBCs in the sediment for 5 minutes, and the resultant cells later cultured in 100 x 15mm Petri dish (BD falcon) at 2 x 10^5/ml in 10 ml of complete RPMI media containing 20 ng/ml of GMCSF (Peprotech, Indianapolis, IN) at 37 ^oC. After 3 days, 10 ml of freshly prepared supplemented RPMI medium containing 20 ng/ml of GMCSF (Peprotech) was put into the cultured cells. On the sixth day of the cell culture, 10 ml of the culture medium was taken away from the growing cells carefully to minimize disturbing the settled cells. The collected culture medium was spinned down for 10 mins at 1000 rpm after which the cell pellets were resuspended in fresh 10ml 20 ng/ml GM-CSF RPMI medium and put back into the Petri dish. On the 8 day, the BMDCs were ready for use and the percentage purity was determined by flow cytometry with PE conjugated anti-CD11c antibody that showed 90-95% purity.

To isolate macrophages, bone marrow cells (obtained as above) were cultured in 100 x 15mm Petri dish (BD Falcon) at 4 x 10^5/ml in 10 ml of complete RPMI media containing 30% L929 cell culture supernatant at 37^oC. After 3 days, additional 10 ml of complete RPMI media containing 30% L929 cell culture supernatant was added to the cells. On day 7, the differentiated macrophages (which are adherent to the petri dishes) gently washed with 10 ml

sterile PBS and detached gently with the use of a cell scraper. The macrophages were spinned for 5 mins at 1200 rpm in a 4°C centrifuge. The supernatant fluids were discarded, and the cells were later put in 10 ml of complete RPMI and later kept on ice until they were ready for use.

3.6 *IN VITRO* INFECTION WITH *L. MAJOR* AND STIMULATION

Macrophages (BMDMs) and dendritic cells (BMDCs) isolated from the bone marrow of WT and Sema3E$^{-/-}$ mice were derived as described above. They were counted, and their stock concentration determined. *L. major* promastigotes cultured in M199 medium (Hyclone, Logan, UT) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Cansera, Mississauga, ON, Canada), and 100 U/ml penicillin/streptomycin were poured into 50ml falcon tubes and centrifuged for 15minutes at 3000 rpm. After 15 minutes of centrifugation, the supernatants were discarded, and the parasite pellets were placed in 10 ml of sterile 1x PBS. The parasites were further centrifuged twice for an additional 15 minutes at 3000 rpm. After the second centrifugation, parasites were diluted 20 times and counted. *L. major* promastigotes were used to infect the BMDMs and BMDCs *in vitro* in a polypropylene tube in the ratio of 1 cell:5parasites. The free parasites (parasites that did not infect the cells) were discarded by washing the tubes thrice with complete RPMI after 6 hours of infection. In some studies, the cells were further activated *in vitro* with LPS (100 ng/ml, Sigma-Aldrich) and CpG (1µg/ml, Sigma-Aldrich). The infected and/or stimulated cells were cultured at 37°C for various times (ranging from 6 to 72 hours) and assessed for parasite infectivity together with costimulatory molecules expression via microscopy or flow cytometry respectively. ELISA was used to quantify the cytokines in the culture fluids.

3.7 CFSE STAINING PROCEDURE

The cells from the spleens and surrounding draining lymph nodes were counted with haemocytometer and centrifuged for 5 minutes at 1200 rpm. The cells were later placed in

sterile warm PBS at 10^8/ml. The stock solution of CFSE (5 mM in DMSO) was diluted 2000 times with warm PBS, and later added to the cell suspensions at 1:1 ratio. The tubes containing the cells were wrapped with aluminium foil and rocked gently for 5 minutes. Thereafter, CFSE labeling activity was quenched by the addition of 5 ml of FBS. The cells already labelled with CFSE were centrifuged for 5 minutes at 1200 rpm and the cells were later kept in complete media, counted and stored on ice until used.

3.8 CO-CULTURE EXPERIMENTS

$CD4^+$ T cells were derived (by positive selection) from the spleen of PEPCK-specific T cell receptor transgenic (TCR Tg) mice based on the protocol of the manufacturer (Stem Cell). Flow cytometry was later used to determine the purity of the cells to be 90-92%. The isolated T cells were stained with CFSE as has been previously explained above. BMDCs were derived from the WT C57BL/6 and Sema3E knockout mice as previously described. 100 ng/ml LPS were used to stimulate BMDCs overnight and they were later co-cultured with the CFSE labeled T cells in the ratio of 1 DC:100 T cells for 4 days in the presence of PEPCK peptide (5 µM). Thereafter, the proliferation of the T cells and intracellular expression of IFN-γ were analyzed by flow cytometry.

3.9 DIRECT EX VIVO INTRACELLULAR CYTOKINE STAINING AND *IN VITRO* RECALL RESPONSE.

At different time points following infection of the mice, single cells of the spleens and draining lymph nodes were derived by the use of cell strainers. Cells were later stained to check for the expressions of CD3, CD4, CD25, IFN-γ, Foxp3, CD11c, IL-17A, F4/80, TNF-α and IL-4 via flow cytometry. Briefly, the cells were put inside flow cytometry tubes (BD Falcon) and later washed with FACS buffer which is comprised of 0.1% new calf serum and 0.1% sodium azide

in PBS. 100 μL of Fc receptor blocker (2.4G2 Hybridoma supernatant) was put inside the tubes containing the cells and were later incubated for 5 minutes on ice. The surface markers of interest were stained with fluorochrome-labelled antibodies (eBioscience) after which cells were kept on ice for 25 minutes. In order to detect some intracellular cytokines, PMA (20 ng/ml), ionomycin (1μM), and Brefeldin A (10 μg/ml) (all from Sigma) were used to separately stimulate the cells for 4-6 hours. At the end of the desired time (4-6 hours) the stimulated cells are stained for surface markers of interest and fixed for 15 minutes with 0.5 ml 2% paraformaldehyde (Sigma). For the permeabilization of the cell membrane, saponin was dissolved in FACS buffer and was incubated alongside the cells on ice for 15 minutes. The Cells were later washed and stained intracellularly for cytokines like TNF-α, IL-4, IFN-γ and IL-17A) using Fluorochrome-conjugated antibodies (eBioscience; each at 0.5 μL per tube). After staining the cells, they are incubated on ice for 30 minutes. FACS buffer mixed with saponin was used to wash the stained cells after which cell membranes were closed by washing with FACS buffer alone. The staining for the expression of Foxp3 was done with commercially available T-regs staining kit (eBioscience). Foxp3 fixation/permeabilization concentrate and diluent together with Foxp3 permeabilization buffer were used in accordance with the manufacturer's protocols. After the staining procedure, the cells were kept in FACS buffer. The samples were acquired and later analysed respectively with FACS Canto II flow cytometer (BD Bioscience, Mississauga, ON, Canada) and FlowJo software (TreeStar). For the *in vitro* recall response, the cells of the spleens and draining lymph nodes were obtained at various times post infection as previously described and stimulated with 50 μg/ml SLA after which they were kept for 3 days in the incubator at 37°C. The derived supernatant fluids were kept in -20°C fridge until they were analysed for the quantities of various cytokines by ELISA.

Table1: List of fluorochrome conjugated antibodies used for flow cytometry

Serial number	Antibody	Clone	Fluorochrome
1	CD3	145-2C11	FITC
2	CD4	GK 1.5	Pacific Blue
3	CD25	PC61	PE
4	CD11C	N418	Pacific blue
5	F4/80	BM8	FITC
6	Foxp3	FJK-16s	APC
7	IL-10	JES5-16E3	PE, APC
8	IL-4	11B11	PE
9	IL-17A	eBio 17B7	APC
10	CD40	1C10	PE, PercCp/cy5.5
11	CD80	16-10A1	APC, PE
12	CD86	GL1	FITC
13	MHC II	M5/114.15.2	APC, PE, eflour 450
14	IFN-γ	XMG1.2	PE, Pacific blue, Percp/cy 5.5
15	TNF-α	MP6-XT22	PE, APC
16	Ki67	SolA 15	APC, eflour 450

3.10 SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

ELISA was used to quantify the different cytokines analysed in this study. Primary antibodies (100 µl/well) were added to the ELISA plates (Immulon VWR, Mississauga, ON) at a concentration previously optimized in our lab (table 2) in bicarbonate coating buffer (pH of 9.6) and later kept overnight at 4°C. Later the next day, the plates were rinsed 5 times with

ELISA wash buffer that is made up of PBS, 0.05% Tween 20 with a pH of 7.4. The wells in the ELISA plates were blocked for 2 hours at 37ºC with ELISA blocking solution (5% heat inactivated FBS in PBS) 200 µl/well to prevent nonspecific binding of analytes or their secondary antibodies to the plates. Thereafter, the plates were rinsed 5 times with the wash buffer and later diluted recombinant cytokine standards (all from Peprotech) were added (100 µL /well) and titrated in a 2-fold serial dilution to generate standard curves (see Table 2). Samples were added (100 µL /well) and diluted in assay diluent (5% heat inactivated FBS in PBS) and titrated serially in 2-folds and later kept on 4ºC overnight. The following day, the plates were washed 8 times using ELISA washing buffer and 100 µl of the biotin conjugated detection antibody (Bio legend) was added to all wells. After 2 hours of incubating the plates at 37ºC, they were washed 8-10 times, and streptavidin linked horseradish peroxidase (1:1000 BD Pharmingen, San Diego, CA) was added to the whole wells and kept for 30 minutes in 37ºC. The plates were read at 405 nm (Spectra Max) following the observed colour change.

Table 2: List of cytokines, starting recombinant standard dilution, sample dilution and sensitivities of the sandwich ELISA

Cytokine	Standard (pg/ml)	Sample dilution	Sensitivity pg/ml
IFN-γ	5000	1:5	30.14
IL-17	5000	1:5	35.3
IL-4	2000	1:5	31.2
IL-10	2000	1:5	15.12
TNF-α	4000	1:5	31.18
IL-12p40	5000	1:5	29.14

3.11 REAL TIME PCR ASSAY

1 day, 2 days and 7 days after infection, the infected ears were harvested and evaluated for Sema3E expression. The samples were homogenized and reversely transcribed to cDNA using the RT-transcription kit (Gibco-BRL, Gaithersburg, MD). Ear tissue samples were placed inside 1.5ml Eppendorf tubes containing 1ml of Trizol (Thermofisher ™) reagent on ice, mixed properly and kept for 5minutes at room temperature. 0.2 ml of chloroform was added into the Eppendorf tubes containing a mixture of Trizol and digested ear tissue samples. Tubes were mixed vigorously for 15 seconds, left at room temperature for about 3minutes and later centrifuged for 15minutes at 12000 x g. The aqueous phase of the solution was decanted into fresh RNase free tubes, after which 500 µl of isopropyl alcohol was added. Furthermore, the tubes were incubated for 10minutes at room temperature and later spinned for 10 minutes at 11000 x g.

The supernatants were discarded and replaced with 75% alcohol. The resultant RNA pellets were dissolved in RNase free water and stored at -20°C. The RNA was reverse transcribed into cDNA by dropping 10 µL of RNA into the designated wells in a 96-well plate containing 10 µL of reverse transcription master mix (25X dNTP + Nuclease-free water + 10x RT Random primers + RNase inhibitor + Multiscribe Reverse Transcriptase +10X RT Buffer). The thermal cycler was programmed according the manufacturers protocols and the reverse transcription ran. The primers used were Sema3E forward AAAGCATCCCCAACAAACTG and Sema3E reverse GTCCAGCAAACAATTCACTACCA (Invitrogen ™).

3.12 POLARIZATION OF T CELL SUBSETS

Single Cells from the spleens of WT and Sema3E knockout mice were stained with CFSE as described above. After the CFSE labelling, the cells were resuspended in complete DMEM medium at 2 x 10^{6} cells/ml such that 100µL contained 2 x 10^{5} cells. 100 µL of the culture media

containing 2 x 10^5 CFSE stained cells were seeded into each designated well in the presence of 100 µL of the different polarization cocktails to give the final concentration as follows:

Table 3: List of cytokines and antibodies used for polarizing naïve CD4⁺ T cells into various CD4⁺ T cells subsets.

Th	Anti-CD3	Anti-CD28	20ng/ml r IL-12	10ng/ml r TGFβ	100ng/ml r IL-6	10ug/ml Anti-IL-12	10ug/ml Anti-IFN-γ	10ug/ml Anti- IL-4
Th0	+	+	-	-	-	-	-	-
Th1	+	+	+	-	-	-	-	+
Th17	+	+	-	+	+	+	+	+

3.13 BONE MARROW CHIMERA EXPERIMENTS

Bone marrow cells of donor WT and Sema3E $^{-/-}$ mice were prepared into single cell suspensions and resuspended in such sterile PBS such that 10 million cells are contained in 150 µL. The recipient WT and Sema3E$^{-/-}$ mice were deprived of food overnight (but provided water) and then lethally irradiated (900 rads for 2 minutes). Following the irradiation, the mice were reconstituted with 10 million bone marrow cells of the donor (were administered through the tail vein). The mice that received bone marrow cells from the donor were later infected with *L. major* promastigotes after they were fully reconstituted by the donor cells by 8weeks (as assessed by flow cytometry).

3.14 STATISTICAL ANALYSIS

The results of this study were presented as the mean ± SEM. For comparison of parasite burden, quantity of secreted cytokines and sizes of lesions between the different mice groups, two-

tailed student T test and one way or two-way analysis of variance (One way or Two way ANOVA) was used. In this study, a result is considered to be significant if $p \leq 0.05$.

CHAPTER 4

4.0 RESULTS

4.1 *L. MAJOR* INFECTION REGULATES SEMA3E EXPRESSION AT CUTANEOUS SITE

Susceptibility to *L. major* infection is associated with an elaborate Th2 immune response (7). Holl *et al* reported a higher expression of Sema3E in $CD4^+$ Th2 cells and dendritic cells compared to some immune cells like $CD4^+$ Th1 cells and B cells (194). Since Sema3E is expressed on cells like T cells, dendritic cells and macrophages (194), that are known to be critical in regulating host immune response to *Leishmania* infection (40), I sought to investigate whether *L. major* infection affects the expression of Sema3E. To address this, wild type (WT) C57BL/6 mice were infected with 2 x 10^6 in the ears, while the control group were injected with PBS. Ear samples were harvested on days 1, 2 and 7 post-infection, and qPCR was used to quantify the expression level for Sema3E mRNA. I observed that Sema3E mRNA expression in the *L. major* infected group at 1- and 2-days post-infection, was significantly increased compared to their PBS treated counterparts. However, the expression of Sema3E mRNA at 7 days post-infection was decreased significantly when compared to the PBS injected group (Fig. 1). This result suggests that *L. major* induces the expression of Sema3E mRNA in host cells at early time points (Days 1 and 2) after infection, perhaps in a bid to possibly suppress hosts effector responses against it. However, at the later time points after infection, the host may be attempting to suppress Sema3E expression in order to mount a stronger effector response against the parasite.

Figure 1: Sema3E expression is regulated by *L. major* Infection

C57BL/6 mice were infected with 2 x 10^6 *L. major* parasites and control mice (uninfected) were injected with PBS in their ears respectively. At indicated time points, the mice were sacrificed, and their ears harvested and analyzed for Sema3E mRNA expression by qPCR. (n = 3 mice per group) **, $p < 0.01$; ***, $p < 0.001$

4.2 DEFICENCY OF SEMA3E ENHANCES RESISTANCE TO EXPERIMENTAL *L. MAJOR* INFECTION

Since I observed that *L. major* infection lead to a change in the expression of Sema3E, I assessed the role of Sema3E in host resistance to experimental cutaneous Leishmaniasis. Wild type (WT) and Sema3E knockout (Sema3E$^{-/-}$) C57BL/6 mice were infected with 2 x 10^6 *L. major* on the foot pad of their right feet and monitored for 11 weeks. I observed a significantly reduced lesion size in Sema3E$^{-/-}$ mice compared to their WT counterparts (Fig. 2A). At week 5 and 8 post-infection, I also observed a significantly lower parasite burden in the foot pad of Sema3E$^{-/-}$ mice compared to their WT controls (Fig. 2B). The above results show that the deficiency of Sema3E during *L. major* infection enhances host resistance to the parasite, suggesting that Sema3E may negatively regulate host protective immunity to *L. major*. This observation is in line with my earlier speculation that the reduction in Sema3E level in the infected WT mice at day 7 (Fig. 1) after *L. major* infection could be an attempt to bring about a higher effector T cell response against the parasite.

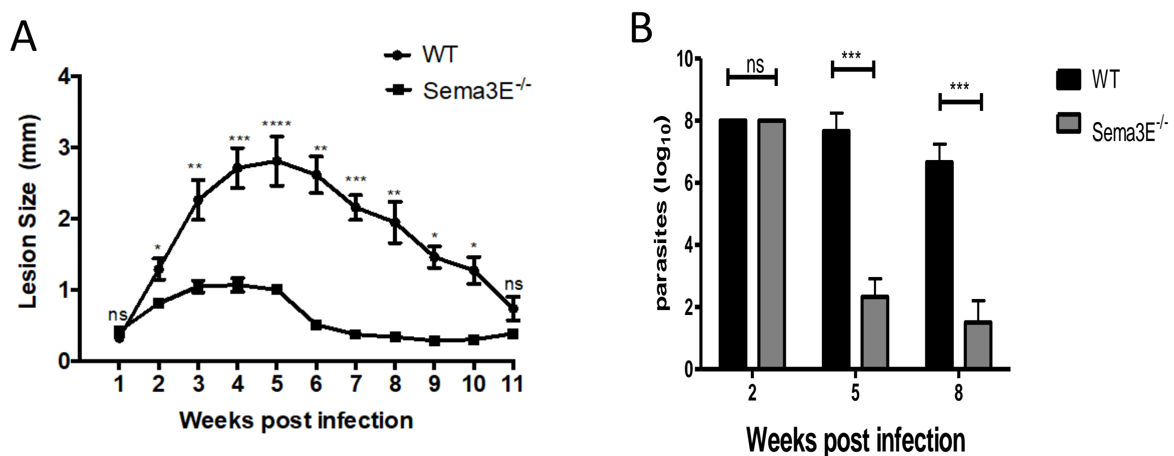


Figure 2: Deficiency of Sema3E leads to enhanced resistance to experimental *L. major* infection.
WT and Sema3E$^{-/-}$ C57BL/6 mice were infected with 2 x 10^6 *L. major* in their right foot pad and the disease progression was measured weekly with digital callipers (A). At 2, 5- and 8-weeks after infection, mice were sacrificed, and parasite burden of their infected footpad was quantified by limiting dilution assay (B). Results are representative of 2 different experiments (n = 12 mice per experiment) with similar outcome. ns, not significant. **, $p < 0.01$; ***, $p < 0.001$.

4.3 ENHANCED RESISTANCE OF SEMA3E DEFICIENT MICE TO *L. MAJOR* INFECTION IS MEDIATED BY HEMATOPOIETIC CELLS

Apart from immune cells, Sema3E is expressed by epithelial cells and retinal ganglion cells (198). Since I observed an increase in resistance to *L. major* infection in the Sema3E$^{-/-}$ mice, I sought to investigate whether this resistance was conferred by Sema3E deficient bone marrow-derived (immune cells) or structural cells. To address this, I adopted the bone marrow chimera approach. WT and Sema3E$^{-/-}$ mice were irradiated at a sub lethal level and bone marrow cells were transferred from WT to Sema3E$^{-/-}$ mice and vice versa. After 8 weeks, the recipient mice (which now had their immune cells fully repopulated with the donor bone marrow cells as assessed by flow cytometry) were infected with *L. major* subcutaneously in their right foot pad. I observed an enhanced resistance to *L. major* infection in the WT mice which received bone marrow cells from Sema3E$^{-/-}$ mice. This was evidenced by smaller lesion size (green line in Fig. 3A) and decreased parasite burden (Fig. 3B). I also observed a loss of resistance to *L. major* infection in the Sema3E$^{-/-}$ mice which received WT bone marrow cells. (Fig 3 And B). This result confirms that the enhanced resistance to *L. major* infection observed in Sema3E$^{-/-}$ mice is due to Sema3E deficiency in immune cells since the sub lethally irradiated Sema3E$^{-/-}$ mice with WT bone marrow cells (Red line in Fig. 3A) had competent Sema3E$^{-/-}$ stromal cells but still could not control the infection (Blue line in Fig. 3A).

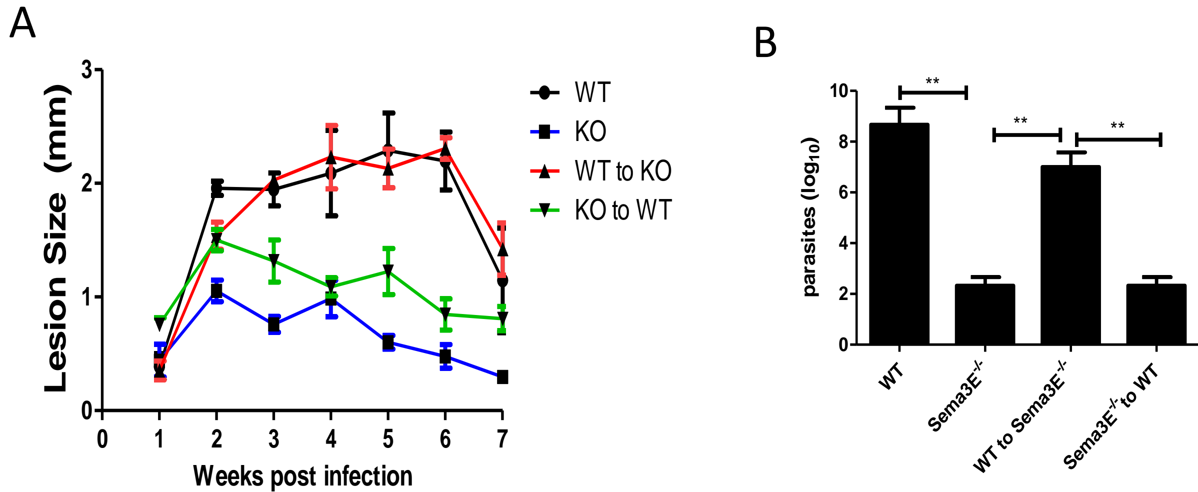


Figure 3: Sema3E deficient bone marrow cells enhance resistance to *L. major* infection in WT mice.

Bone marrow cells from Sema3E $^{-/-}$ C57BL/6 mice and WT mice were transferred to WT C57BL/6 mice and Sema3E $^{-/-}$ C57BL/6 mice respectively. After 8 weeks (when the mice immune cells were fully reconstituted as assessed by flow cytometry), the recipient mice were infected with 2 x 10^6 *L. major* in their right foot pads and the disease progression was measured weekly with digital callipers (A). At 5 weeks post-infection, mice were sacrificed, and parasite burden of their infected footpad was quantified by limiting dilution assay (B). Results are representative of 2 different experiments (n = 12 mice per experiment) with similar outcome. **, $p < 0.01$;

4.4 SEMA3E DOES NOT DIRECTLY SUPPRESS THE PARASITE CLEARANCE CAPACITY OF MACROPHAGES

Results from the bone marrow chimera study indicated that the enhanced resistance in the Sema3E$^{-/-}$ mice was mediated by immune cells. Therefore, I decided to investigate the precise immune cell(s) involved. Since *L. major* preferentially reside in macrophages during infection (40), it is possible that the enhanced resistance to *L. major* infection in the Sema3E$^{-/-}$ mice is as a result of the absence of Sema3E/Plexin-D1 signalling in the infected Sema3E$^{-/-}$ macrophages which may cause these macrophages to resist parasitic infection (infectivity) or directly inhibit their growth/proliferation. To address this, I infected WT and Sema3E$^{-/-}$ BMDMs mice with *L. major* (1 cell :5 parasites) and at different time points, I assessed the infectivity of the macrophages by Giemsa staining and counting the number of both infected cells and number of parasites inside each cell. At all designated time points, both the percent infection (number of infected macrophages per 100 cells, data not shown) and the number of parasites per infected cell between the WT and Sema3E$^{-/-}$ macrophages were not significantly different (Fig. 4A and B). In addition, I used the Griess assay to measure nitrite concentration (an indirect way of measuring nitric oxide production by infected cells), which is an indication of the parasite clearing capacity of the macrophages. I observed that the amount of nitric oxide produced by infected WT and Sema3E$^{-/-}$ macrophages was not significantly different (Fig. 4C). This result suggests that the enhanced resistance (lower parasite burden and smaller lesions) to *L. major* infection observed in Sema3E$^{-/-}$ mice is not as a result of the intrinsic enhanced anti-leishmanial capacity of their macrophages.

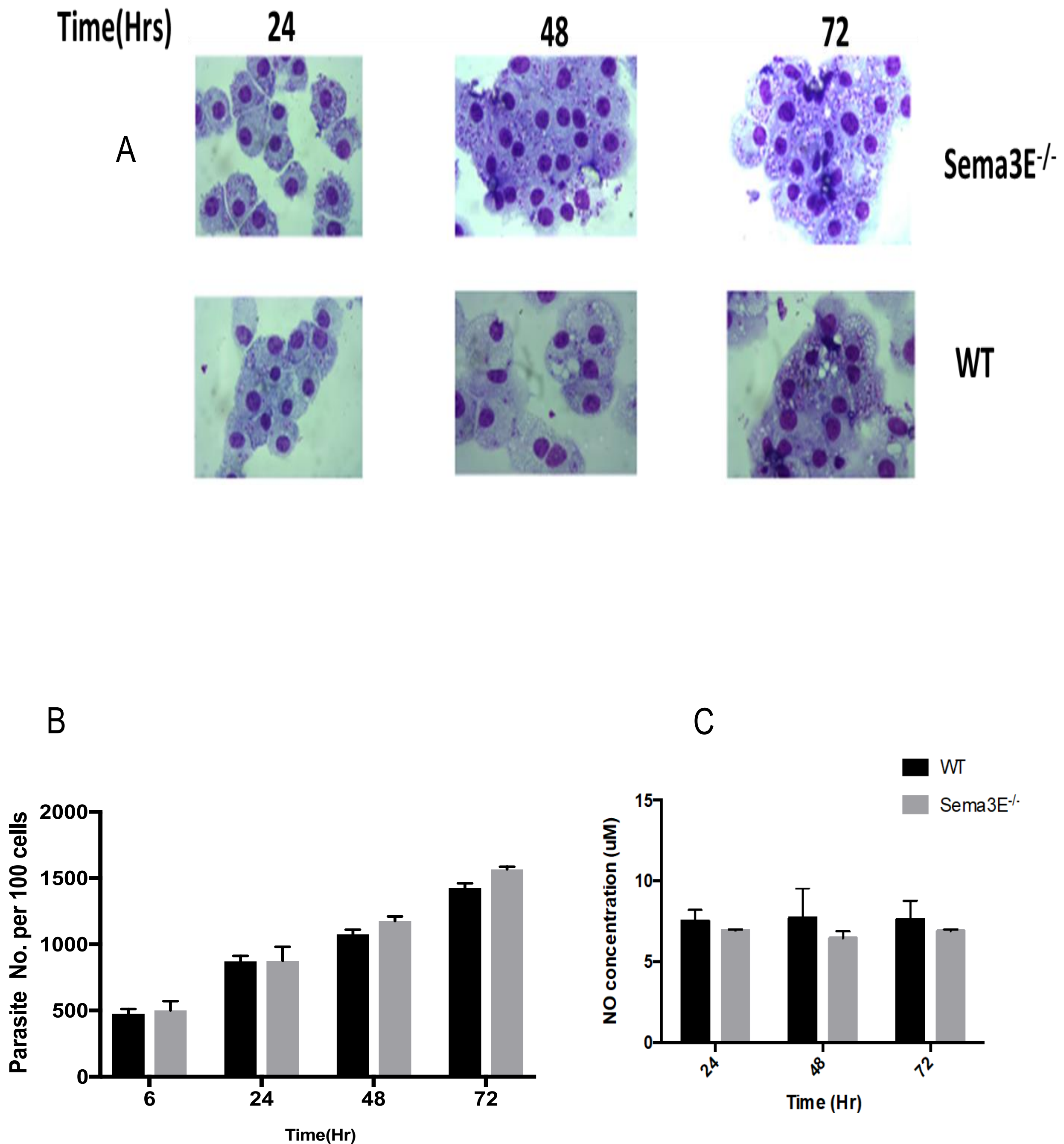


Figure 4: Macrophages from Sema3E deficient mice do not have increased ability to control parasite proliferation *in vitro*.

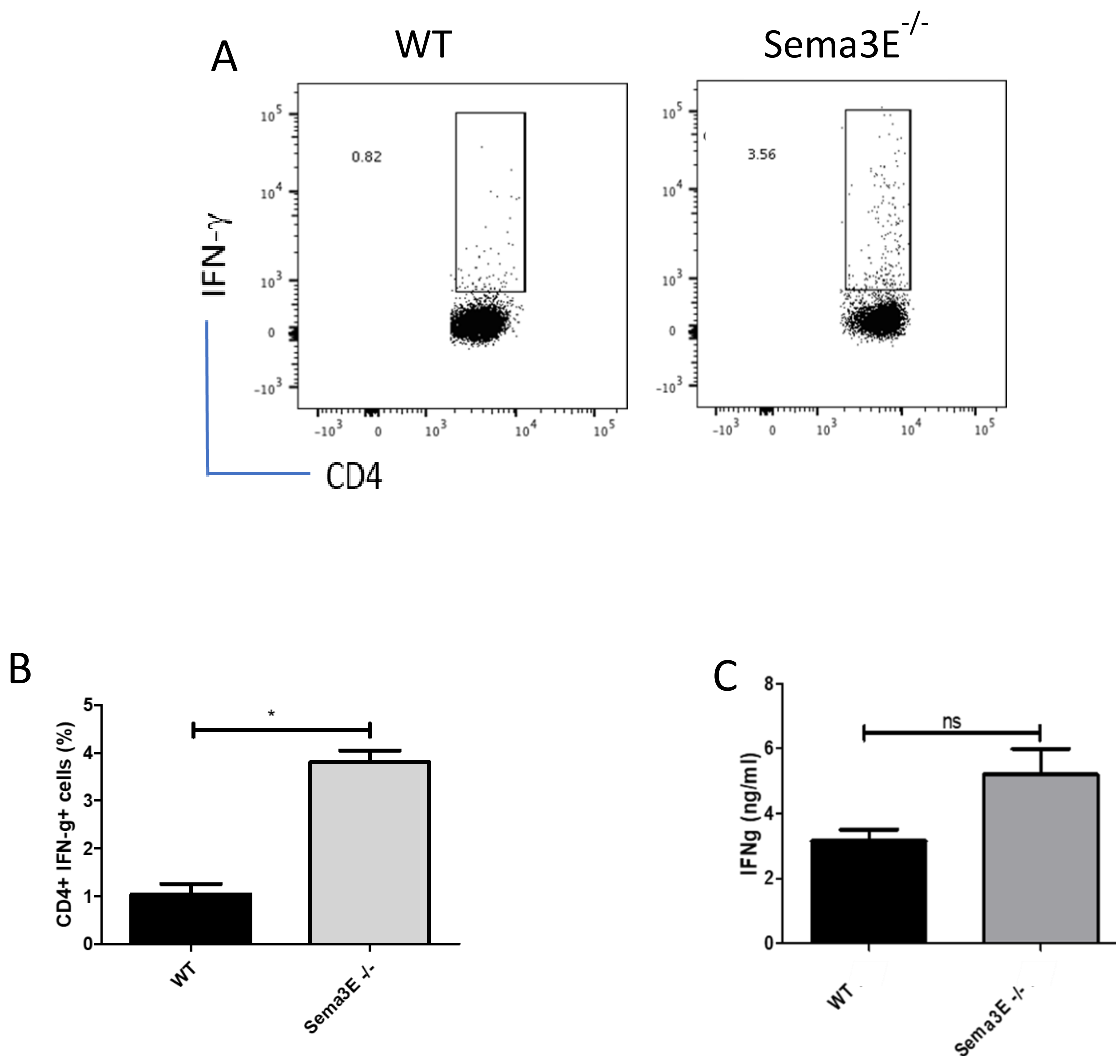
WT and Sema3E$^{-/-}$ bone marrow-derived macrophages (BMDMs) were infected with *L. major*. At set time points, the infected cell tubes were cytospined and stained with Giemsa after which the number for parasites inside the cells were analysed by microscopy (A and B). The resulting supernatant fluids were assayed for nitric oxide production by Griess assay (C). Results are representative of 2 different experiments with similar outcomes. ns, not significant.

4.5 DEFICIENCY OF SEMA3E AFFECTS CD4$^+$ T CELL RESPONSE DURING *L. MAJOR* INFECTION

Since there was no evidence of parasite clearing capacity of Sema3E$^{-/-}$ BMDMs being enhanced intrinsically, I then postulated that Sema3E$^{-/-}$ mice display greater resistance to *L. major* infection probably because of the increased effector function of the different subsets of CD4$^+$ T cells (Th1 and Th17) widely associated with aiding parasite clearance in macrophages. Therefore, I assessed the effect of Sema3E on the induction of various subsets of CD4$^+$ T helper cells during *L. major* infection. I focused on CD4$^+$ Th2 Th17 and Th1 cells because these CD4$^+$ T cell subsets are known to influence the outcome of *L. major* infection. A major cytokine produced by CD4$^+$ Th1 cells is IFN-γ, which is critical for macrophage activation and subsequent parasite clearance. In contrast, IL-4 produced by CD4$^+$ Th2 cells, is associated with macrophage deactivation and reduced parasite clearance (32). The role of CD4$^+$ Th17 cells (whose effector cytokine is IL-17A) in *L. major* infection is a bit controversial because some studies shows it enhances susceptibility of a host to *L. major* infection (201) while other studies have shown it helps to confer resistance to a host infected with *L. major* (202). To investigate the impact of Sema3E on the induction of different subsets of CD4$^+$ T cells in mice infected with *L. major*, WT and Sema3E$^{-/-}$ mice were infected with *L. major* and at different time points, CD4$^+$ T cell responses were evaluated in the draining lymph nodes. Two weeks after infection, there was a significant increase in the frequency of CD4$^+$ IFN-γ$^+$ T cells (Th1) in the draining lymph nodes of infected Sema3E$^{-/-}$ mice (Fig. 5A and 5 B) compared to their infected WT controls. However, the quantity of secreted IFN-γ in the draining lymph node of WT and Sema3E$^{-/-}$ mice at 2 weeks post-infection was not significantly different (Fig. 5C).

Furthermore, at 5 weeks post-infection, I observed an increased frequency of CD4$^+$ IFN-γ$^+$ T cells (Th1) in the draining lymph node of Sema3E$^{-/-}$ mice compared to their WT counterparts

(Fig. 5D and 5 E). Interestingly, the quantity of secreted IFN-γ (Fig. 5F) and IL-17 (Fig. 5I) in the draining lymph node of Sema3E$^{-/-}$ mice were significantly increased compared to their WT counterparts. I also observed that the frequency of CD4^{+} IL-4^{+} T cells (Th2) in the draining lymph node of Sema3E$^{-/-}$ mice was significantly reduced compared to their WT counterparts (Fig. 5G and 5H). Collectively, these results suggest that in *L. major* infected mice, Sema3E suppresses the frequency of the anti-*leishmania* Th1 cells and Th17 cells while enhancing the frequency of pro-*leishmania* Th2 cells. They further suggest that the increases in resistance to *L. major* infection in Sema3E$^{-/-}$ mice could be because of the increased frequency of Th1 cells and increased secretion of IFN-γ and IL-17 that could result in a more robust activation of the infected macrophages thus leading to enhanced parasite clearing capacity of the macrophages.



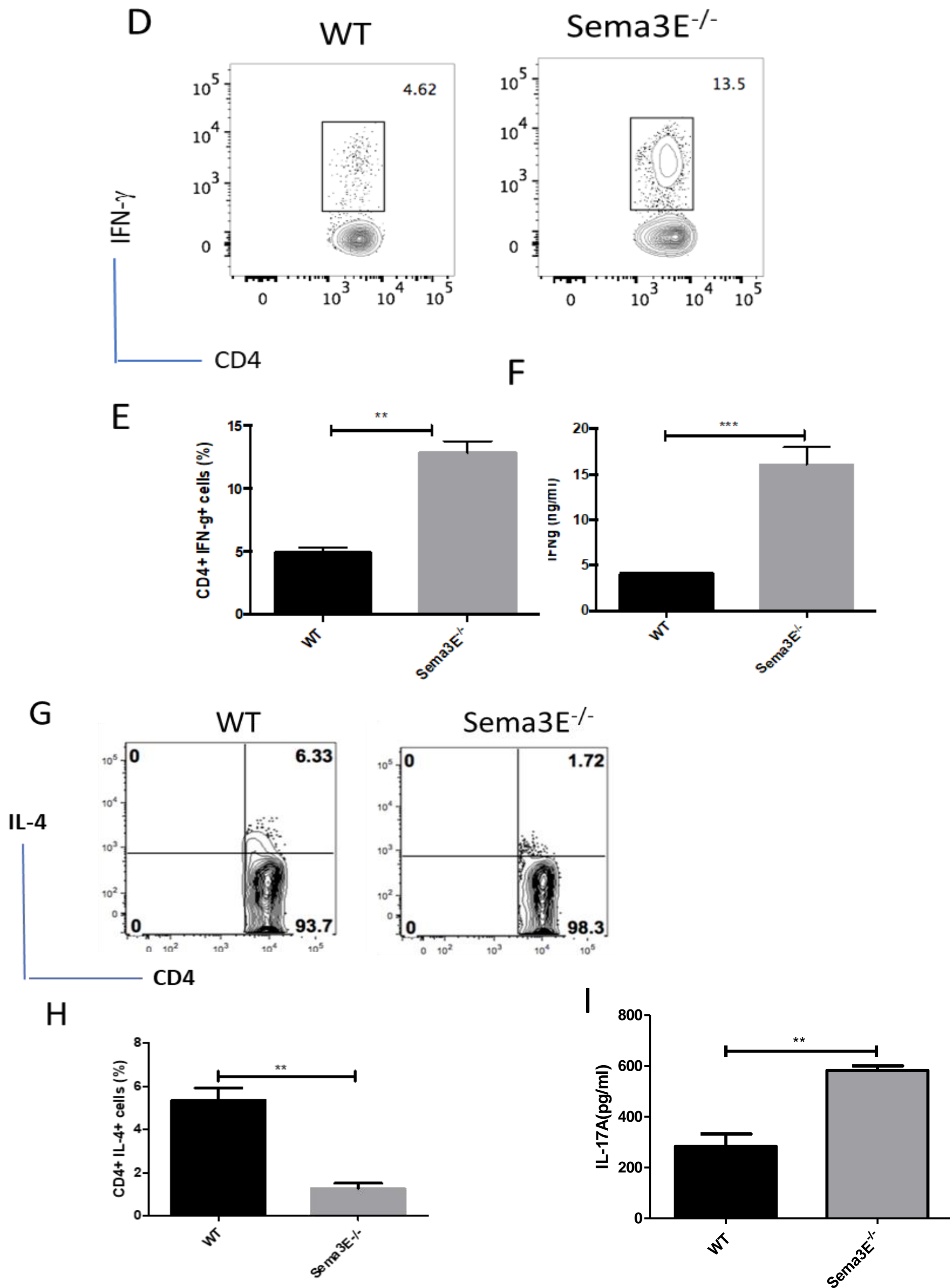
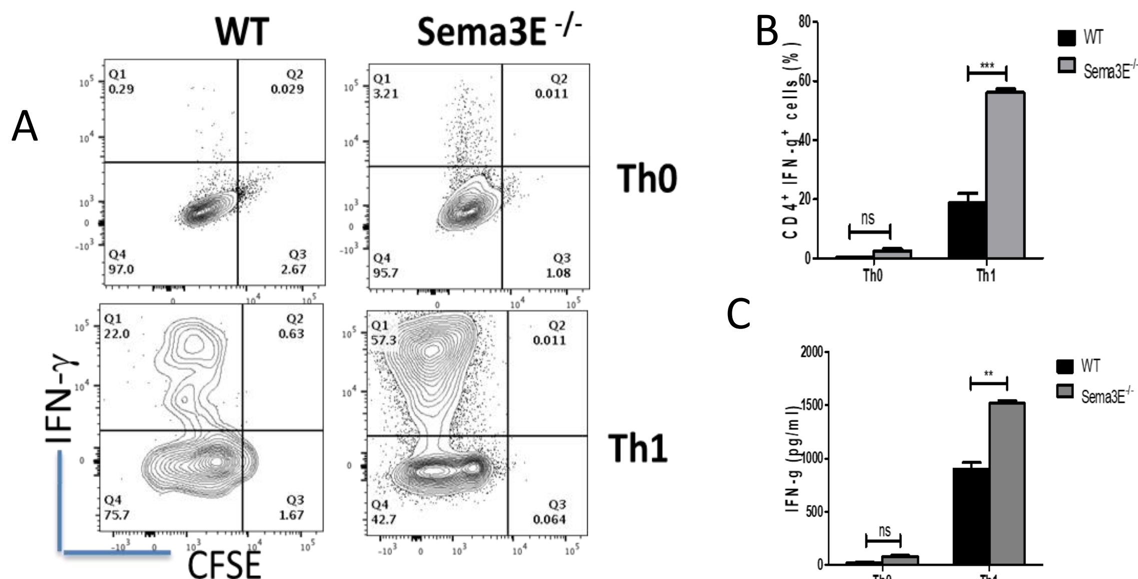


Figure 5: Deficiency of Sema3E in C57BL/6 mice leads to enhanced Th1 response, IL-17A production and reduced Th2 response

L. major infected WT and Sema3E$^{-/-}$ C57BL/ 6 mice were sacrificed at 2- and 5-weeks post-infection. The frequency and quantity of IFN-γ (A-F), IL-4 (G and H) producing CD4^{+} T cells and IL-17A secretion in the draining lymph nodes were determined directly *ex vivo* by flow cytometry and ELISA. Results are representative of 2 different experiments (n = 12 mice per experiment) with similar outcome. ns, not significant. **, $p < 0.05$; ***, $p < 0.001$.

4.6 DEFICIENCY OF SEMA3E ENHANCES CD4+ Th1 AND Th17 POLARIZATION IN VITRO

Next, I investigated the possible mechanisms involved in enhanced Th1 and increased IL-17A production in the draining lymph node of *L. major*-infected Sema3E$^{-/-}$ mice compared to their WT counterparts. I investigated the effect of Sema3E on influencing the differentiation of purified CD4^{+} T cells into various CD4^{+} T cell subsets. I isolated CD4^{+} T cells from the spleens of WT and Sema3E deficient mice and stimulated them with anti-CD3 and anti-CD28 mAbs coupled with various cytokine and antibody cocktails known to enhance Th1 and Th17 differentiation *in vitro*. I observed a higher frequency of CD4^{+} IFN-γ^{+} T cells and increased secretion of IFN-γ from Sema3E$^{-/-}$ T cells compared to their WT counterparts (Fig.6A-C). In addition, the frequency of CD4^{+} IL-17A^{+} T cells and IL-17A produced by Sema3E$^{-/-}$ cells were significantly increased compared to their WT counterparts (Fig.6D-F). Thus, the deficiency of Sema3E signalling enhances increased differentiation of naïve CD4^{+} T cell to Th17 and Th1 cells, suggesting that Sema3E signalling on CD4^{+} T cells (as in WT CD4^{+} T cells) impedes the differentiation of a naïve CD4^{+} T cell to Th17 and Th1 cells. This may be one of the possible mechanisms that accounts for the enhanced Th1 and Th17 response in *L. major* infected Sema3E$^{-/-}$ mice.



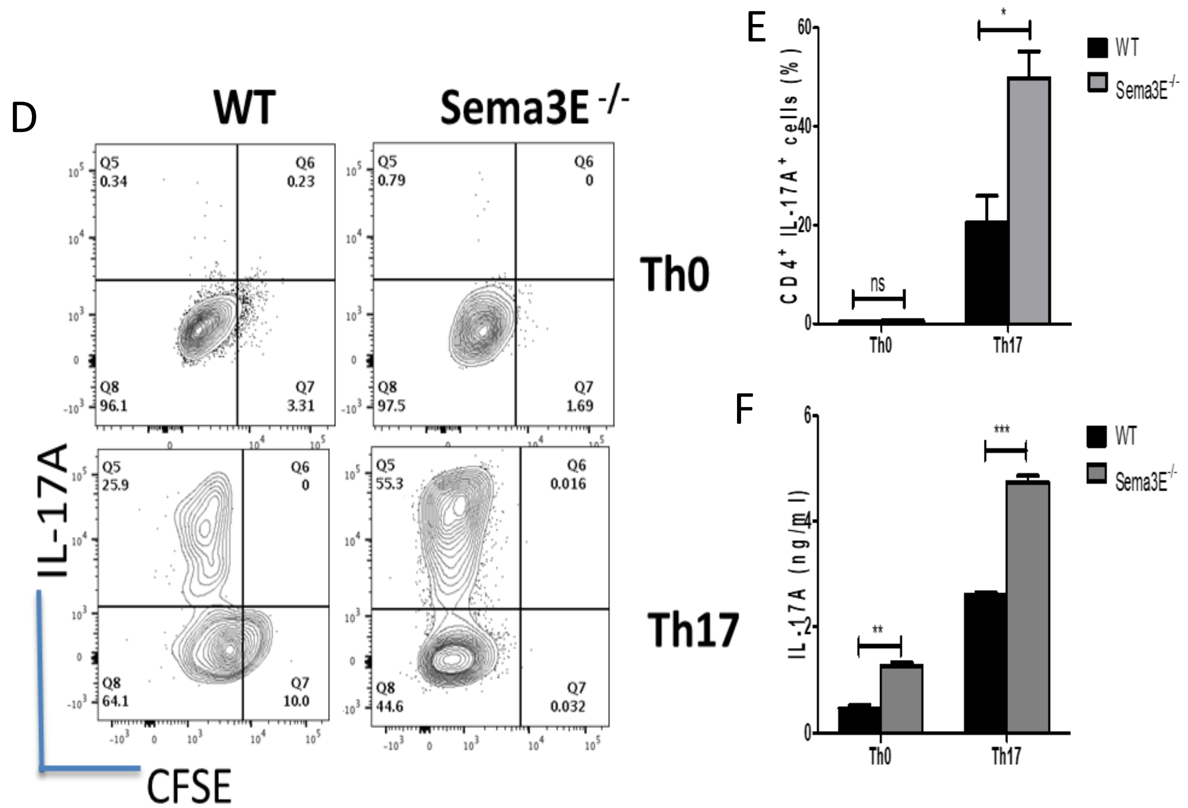
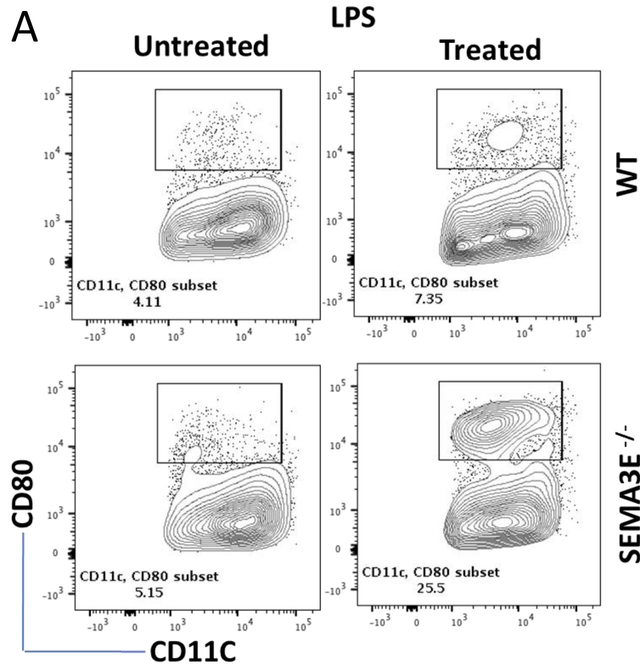


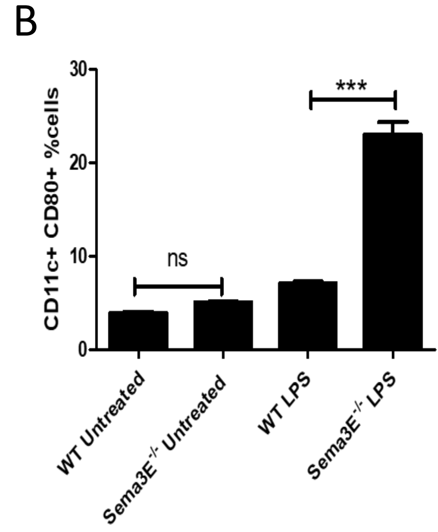
Figure 6: Deficiency of Sema3E enhances CD4^{+} Th1 and Th17 polarization in naïve CD4^{+} T cells

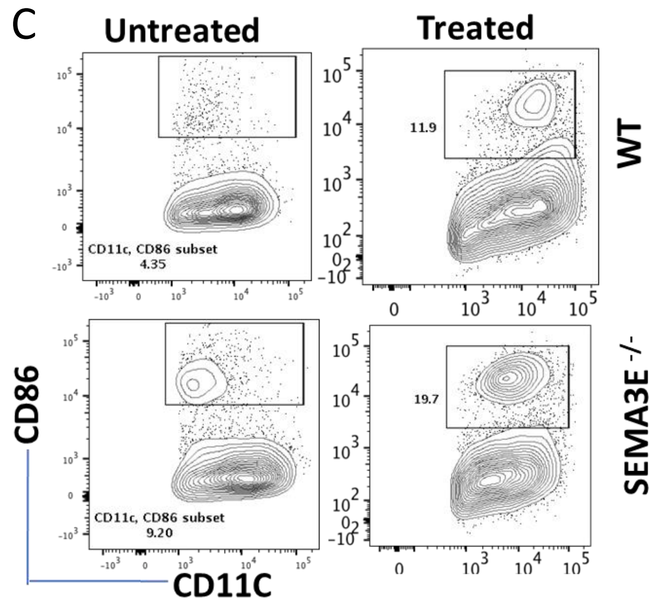
CD4^{+} T cells were sorted (> 97% purity) from splenocytes of WT and Sema3E$^{-/-}$ C57BL/6 mice, stained with CFSE dye and stimulated *in vitro* with anti-CD3 and anti-CD28 mAbs under Th1 and Th17 differentiating conditions. After four days, the frequency of IFN-γ^{+} (Th1, A and B) and IL-17A^{+} (Th17, D and E) cells were determined by flow cytometry. The quantity of secreted IFN-γ (C) and IL-17A (F) were assessed by ELISA. Results are representative of 2 different experiments with similar outcome. ns, not significant. **, $p < 0.01$; ***, $p < 0.001$.

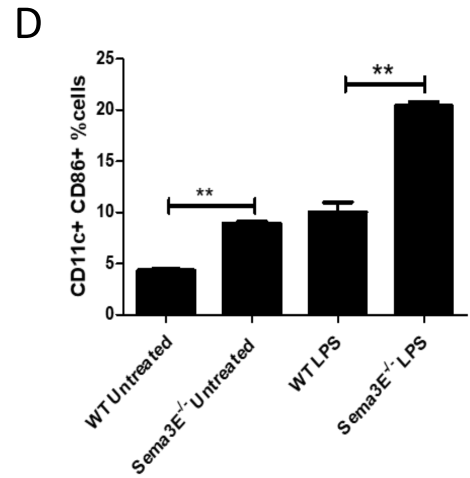
4.7 SEMA3E DOWNREGULATES COSTIMULATORY MOLECULE EXPRESSION AND IL-12p40 SECRETION BY DENDRITIC CELLS

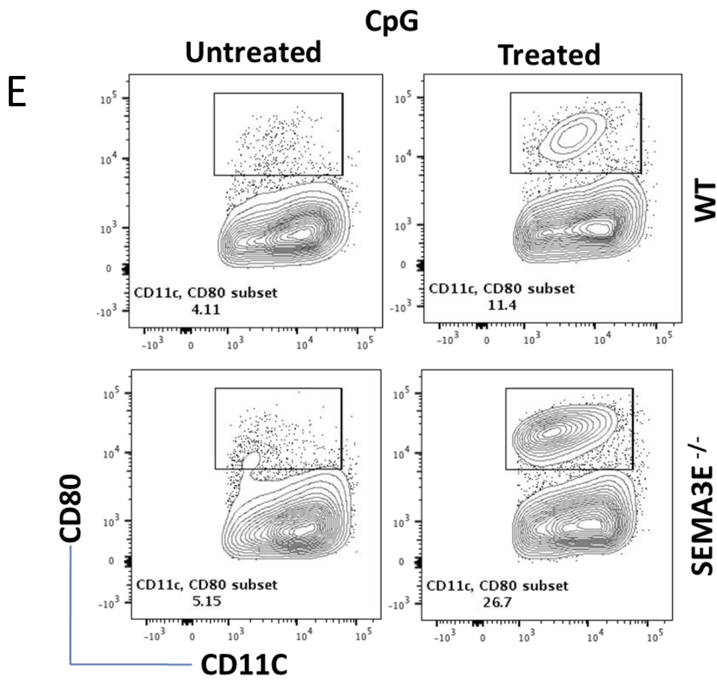
DCs are necessary for presenting antigens for activating naïve $CD4^+$ T cells to Th1 cells. They employ costimulatory molecules such as CD80 and CD86 coupled with cytokines like IL-12p40 and IL-23p40 to drive Th1 (44) and Th17 (97) immune response respectively. Dendritic cells express Sema3E and its receptor Plexin-D1 (194). Holl *et al* reported that the deficiency of Plexin-D1 on dendritic cells resulted in increased production of IL-12p40 and IL-23p40 following the stimulation of the dendritic cells with LPS for 6 hours (194). I therefore sought to examine the possible the role of dendritic cells in the enhanced Th1 and Th17 response observed in the *L. major* infected $Sema3E^{-/-}$ mice. BMDCs from WT and $Sema3E^{-/-}$ mice were stimulated with LPS (Toll-like receptor 4 agonist), CpG (Toll-like receptor 9 agonist), or infected with *L. major* for 24 hours, after which the expression of key costimulatory molecules was assessed by flow cytometry and IL-12p40 secretion was measured by ELISA. $Sema3E^{-/-}$ BMDCs displayed a significant increase in the expression of CD80 and CD86 following LPS (Fig. 7A-D) and CpG stimulation (Fig. 7E-H) or *L. major* infection (Fig. 7I-L) compared to their WT counterparts. Also, I observed that $Sema3E^{-/-}$ BMDCs produced significantly more IL-12p40 following LPS and CpG stimulation, or infection with *L. major* compared to WT BMDCs (Fig. 7M-O). These results suggest that Sema3E suppresses costimulatory molecules expression and IL-12p40 production by dendritic cells and may subsequently suppress the ability of dendritic cells to mediate the activation and differentiation of a naïve $CD4^+$ T cell to Th1 and Th17 cells.

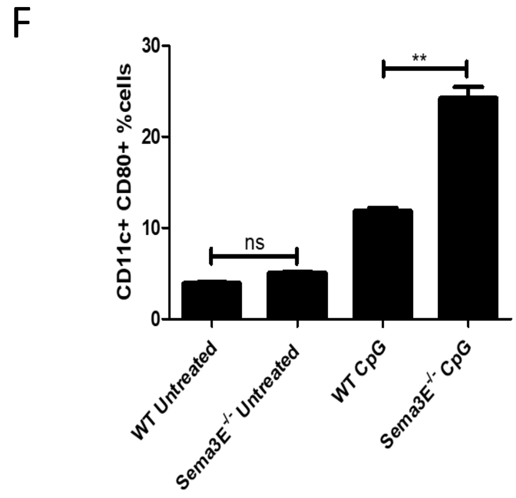

A
LPS
Untreated
Treated
WT
SEMA3E -/-
CD11c, CD80 subset
4.11
7.35
5.15
25.5
CD80
CD11C

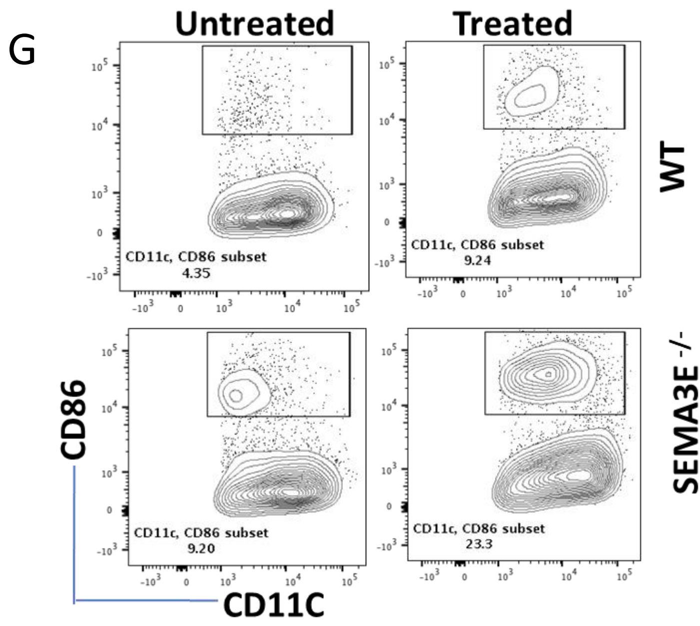

B
CD11c+ CD80+ %cells
ns
***
WT Untreated
Sema3E-/- Untreated
WT LPS
Sema3E-/- LPS

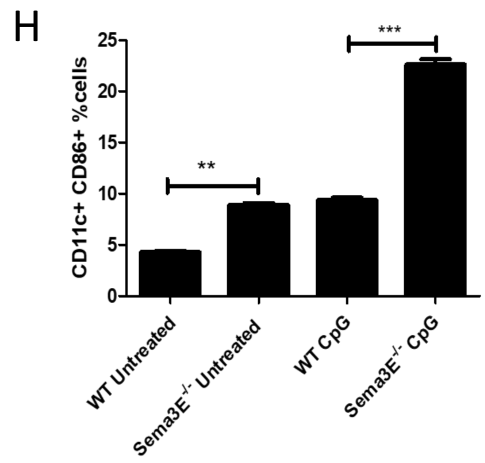

C
Untreated
Treated
WT
SEMA3E -/-
CD11c, CD86 subset
4.35
11.9
9.20
19.7
CD86
CD11C


D
CD11c+ CD86+ %cells
**
**
WT Untreated
Sema3E-/- Untreated
WT LPS
Sema3E-/- LPS


E
CpG
Untreated
Treated
WT
SEMA3E -/-
CD11c, CD80 subset
4.11
CD11c, CD80 subset
11.4
CD11c, CD80 subset
5.15
CD11c, CD80 subset
26.7
CD80
CD11C


F
CD11c+ CD80+ %cells
ns
**
WT Untreated
Sema3E-/- Untreated
WT CpG
Sema3E-/- CpG


G
Untreated
Treated
WT
SEMA3E -/-
CD11c, CD86 subset
4.35
CD11c, CD86 subset
9.24
CD11c, CD86 subset
9.20
CD11c, CD86 subset
23.3
CD86
CD11C


H
CD11c+ CD86+ %cells
**
***
WT Untreated
Sema3E-/- Untreated
WT CpG
Sema3E-/- CpG

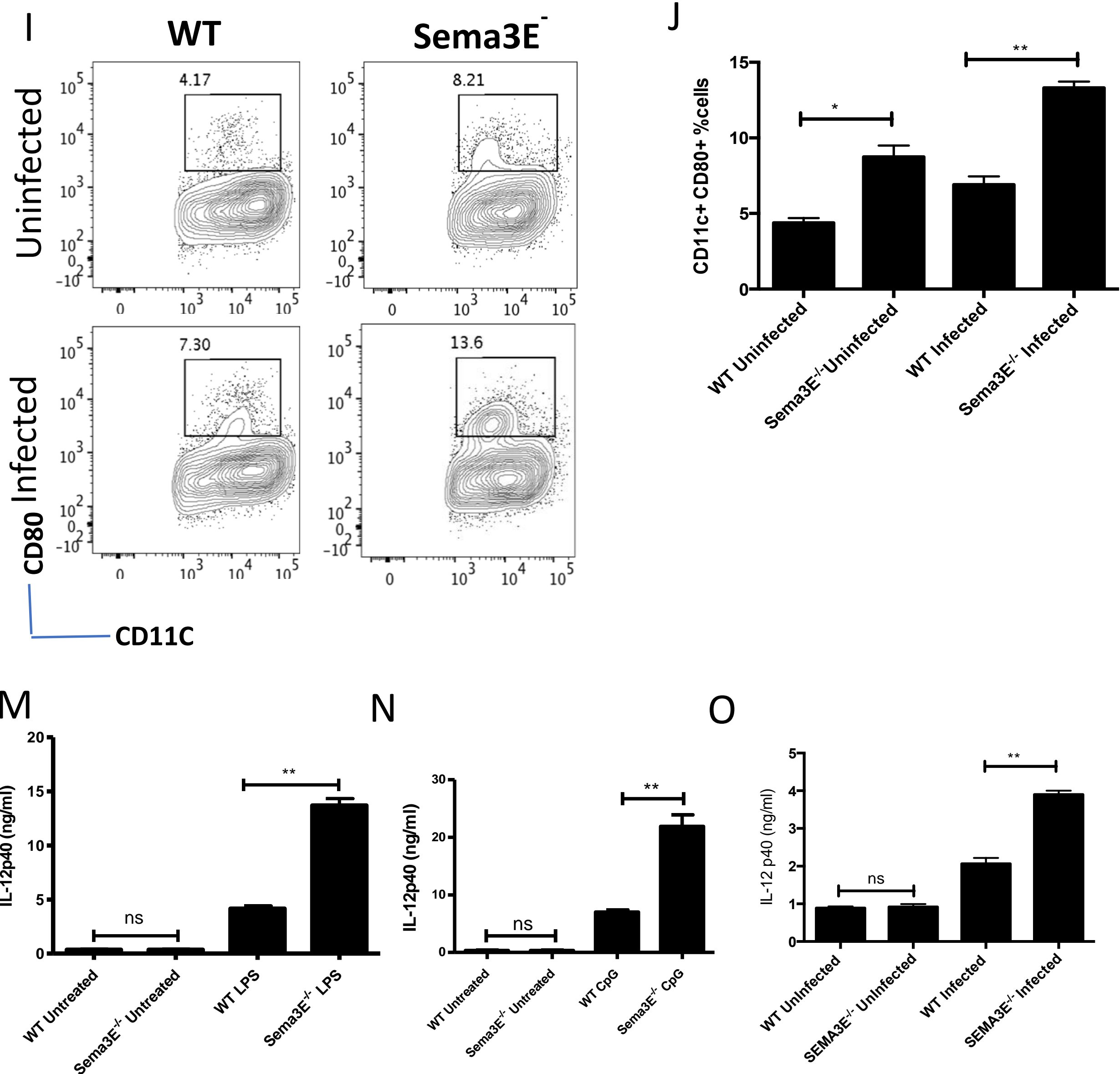


Figure 7: Sema3E deficiency enhances CD80, CD86 expression and IL-12p40 secretion by BMDCs.

Bone marrow-derived dendritic cells (BMDCs) from Wild type (WT) and Sema3E$^{-/-}$ C57BL/6 mice, were stimulated with either LPS (A-D) CpG (E-H) or infected with *L. major* (I-L) for twenty-four (24) hours and CD80 and CD86 expression were determined by flow cytometry. The cultured supernatant fluids were assayed for IL-12p40 (M-O) by ELISA. Results are representative of 3 different experiments with similar outcomes. ns, not significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.8 SEMA3E DEFICIENT DENDRITIC CELLS ARE IMPAIRED IN THEIR ABILITY TO INDUCE THE DIFFERENTIATION OF NAÏVE CD4$^+$ T CELLS INTO CD4$^+$ Th1 AND Th17 CELLS

I demonstrated that Sema3E$^{-/-}$ BMDCs display higher CD80, CD86 expression and increased secretion of IL-12p40 following *L. major* infection and stimulation with other toll-like receptor agonists. Kelsall *et al* and Korn *et al* reported that IL-12p40 secretion and costimulatory molecule expression by dendritic cells are signals necessary for activating naïve CD4$^+$ T cells to Th1 (44) and Th17 cells (97). Therefore, I decided to investigate the impact of enhanced costimulatory molecule expression and IL-12p40 secretion by Sema3E$^{-/-}$ dendritic cells on the differentiation of naïve CD4$^+$ T cells into Th17 and Th1 cells. I performed a coculture experiment between WT and Sema3E$^{-/-}$ BMDCs and CFSE-incorporated naïve CD4$^+$ T cell from PEPCK T cell receptor transgenic (TCR Tg) mice. These mice have been genetically engineered such that approximately all their CD4$^+$ T cells are only responsive to an epitope on PEPCK, an immuno dominant antigen of *L. major* discovered by Dr. Uzonna's Lab (203). The cocultured cells were stimulated with PEPCK peptide (NDAFGVMPPVARLTPEQ) and after 4 days, the cells were checked for proliferation and cytokine (IL-17A and IFN-γ) production. I observed a significant increase in the frequency of CD4$^+$ IFN-γ$^+$ T cells and quantity of secreted IFN-γ following exposure of CD4$^+$ TCR Tg T cells to Sema3E$^{-/-}$ dendritic cells as opposed to their WT DC counterparts (Fig.8A-C). Although, I did not observe any significant difference in the frequency of CD4$^+$ IL-17A$^+$ T cells (Fig. 8D and E), the quantity of IL-17A secreted into the culture supernatant fluid following exposure of CD4$^+$ TCR Tg T cells to Sema3E$^{-/-}$ dendritic cells were significantly increased compared to their WT DC counterparts. (Fig. 8F). Thus, deficiency of Sema3E on DCs leads to enhanced antigen-specific Th1 and Th17 responses by T cells. These results taken together, suggest that the enhanced Th1 and Th17 response observed in the *L. major* infected Sema3E$^{-/-}$ mice is related to increased capacity of Sema3E$^{-/-}$

dendritic cells to stimulate the activation and differentiation of naïve CD4^{+} T cells to Th1 and Th17 cells.

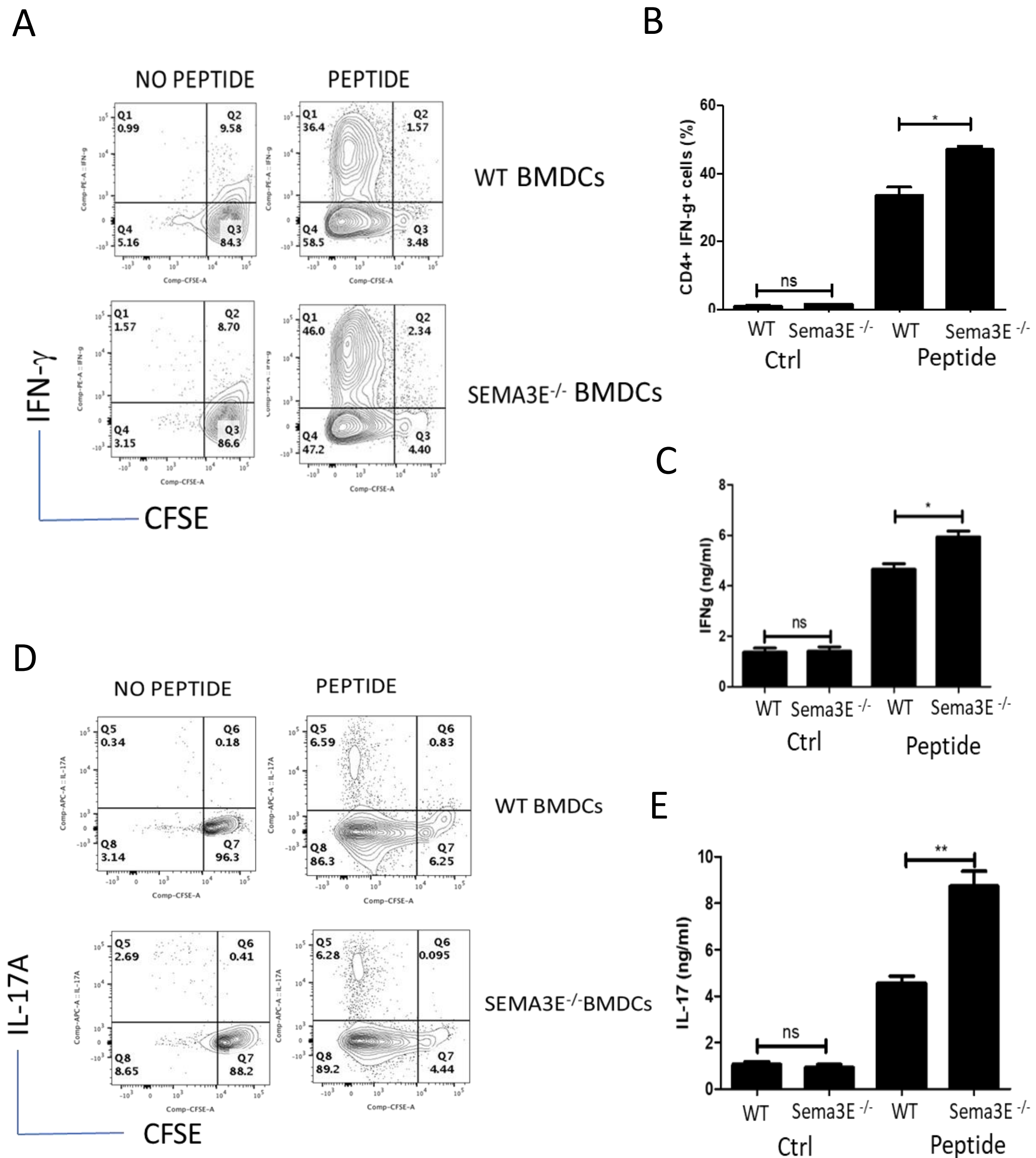


Figure 8: Sema3E deficient dendritic cells enhances Th1 and Th17 differentiation. Peptide pulsed WT and Sema3E$^{-/-}$ dendritic cells were cocultured with CFSE stained TCR Tg CD4^{+} T cells in a DC: T cell ratio of 1:100. After four days, the frequency of IFN-γ^{+} (Th1, A and B) and IL-17A^{+} (Th17, D) cells were analyzed via flow cytometry. The quantity of IFN-γ (C) and IL-17A (E) were determined by ELISA. Results are representative of 3 different experiments with similar outcome. ns, not significant. **, $p < 0.05$; ***, $p < 0.001$.

4.9 SEMA3E PERMITS THE INCREASE IN THE SECRETION OF IL-10 AND POPULATION OF REGULATORY T CELLS BEFORE AND DURING INFECTION IN WT MICE

T-regs are identified as $CD4^+$ $Foxp3^+$ $CD25^+$ T cells (111). The increase in the number of regulatory T cells (T-regs) in the draining lymph nodes of an *L. major*-infected mice has been associated with decreased resistance to the disease (204). This is due to T-regs-mediated suppression of effector $CD4^+$ Th1 cells, which are necessary for the elimination of the parasites (204). Interleukin 10 (IL-10) is an immunosuppressive cytokine secreted by T-regs which suppresses effector $CD4^+$ Th1 activity (205). In this study, I observed $Sema3E^{-/-}$ mice are highly resistant to *L. major* infection and this was accompanied by an increase in the frequency of $CD4^+$ Th1 cells and quantity of secreted IFN-γ and IL-17A in their draining lymph nodes. Korn *et al* reported that an upregulation in RORγT (the transcription factor for Th17 cells) results in a downregulation of FoxP3 (transcription factor for T-regs) in T cells (97). Since I observed an increase in the differentiation of naïve $Sema3E^{-/-}$ $CD4^+$ T cells to Th17 cells (due to possible upregulation of RORγT in $Sema3E^{-/-}$ T cells), I speculated that perhaps there would be a downregulation of FoxP3 in $Sema3E^{-/-}$ T cells (decreased T-regs), and this could also be a possible mechanism that causes the enhanced IFN-γ and IL-17A secretion in *L. major* infected $Sema3E^{-/-}$ mice. Therefore, I assessed the effect of Sema3E deficiency on the frequency of T-regs and IL-10 secretion in the draining lymph nodes during *L. major* infection. I observed a significantly reduced frequency of $CD25^+$ $FoxP3^+$ cells (T-regs) in the draining lymph nodes of both naïve and *L. major* infected $Sema3E^{-/-}$ mice compared to their WT counterparts (Fig 8A and B). In addition, I observed a significant decrease in the amount of secreted IL-10 in the draining lymph node of the infected $Sema3E^{-/-}$ mice (Fig. 8C). These results suggest that Sema3E signalling in a host may support the increase in the frequency of T-regs with or without *L. major* infection. This decrease in the frequency of immuno suppressive T-regs in $Sema3E^{-/-}$

mice may also be another mechanism utilised by Sema3E$^{-/-}$ mice to facilitate the enhanced resistance to *L. major* infection

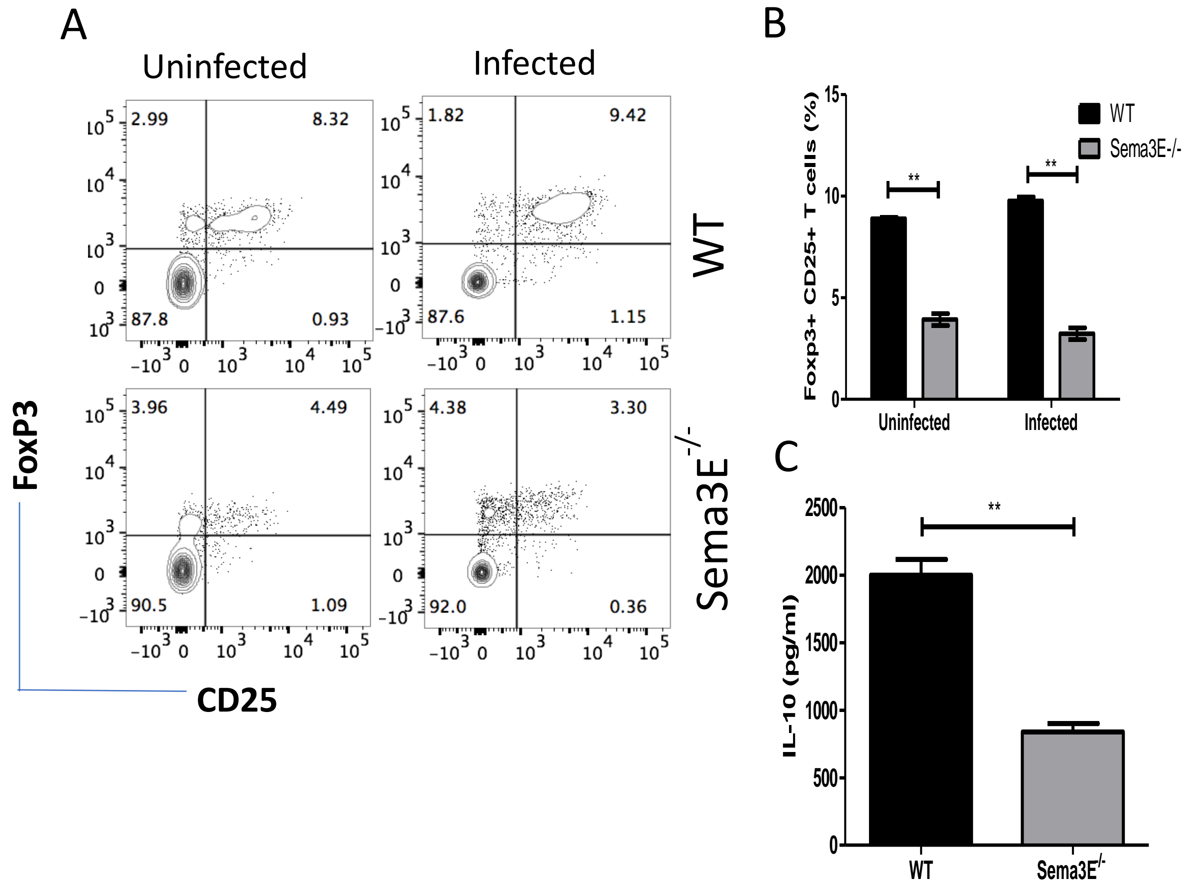


Figure 9: Sema3E deficiency leads to reduced numbers of regulatory T cell and IL-10 production by draining lymph node cells from *L. major*-infected mice
The frequency of CD4^{+} CD25^{+} Foxp3^{+} T cells in the lymph nodes of naïve (uninfected) and infected (5 weeks post-infection) of WT and Sema3E$^{-/-}$ mice were assessed by flow cytometry (A and B). The amount of IL-10 secreted in cultures of draining lymph node cells from infected WT and Sema3E$^{-/-}$ mice was assessed by ELISA (C). Results are representative of 3 different experiments (n = 12 mice per experiment) with similar outcome. **, $p < 0.01$.

CHAPTER 5

5.0 DISCUSSION

Protective immunity to cutaneous leishmaniasis is mediated by IFN-γ secreting $CD4^+$ Th1 cells**.** IFN-γ binds to its receptor on parasitized macrophages resulting in their activation, upregulation of nitric oxide production and elimination of parasites within the macrophages. This study investigated the role of Sema3E in the host immunity to *L. major* infection in mice. I observed a significant increase in Sema3E expression at the infection sites at different following *L. major* infection. I further found that $Sema3E^{-/-}$ mice were highly resistant to *L. major* infection when compared to their WT counterparts this was evidenced by significantly ($p < 0.05$-0.01) reduced lesion sizes and lower parasite burdens at different times after infection. The enhanced resistance of $Sema3E^{-/-}$ mice was associated with significantly ($p < 0.05$) more IFN-γ and IL-17A production by $CD4^+$ T cells from the draining lymph nodes near the infection site than those from infected WT mice. In line with this, $CD4^+$ T cells from $Sema3E^{-/-}$ showed increased capacity to be differentiated into Th1 and Th17 cells *in vitro*. Dendritic cells from $Sema3E^{-/-}$ mice displayed increased expression of costimulatory molecules and produced significantly more IL-12p40 following *L. major* infection or stimulation with CpG compared to their WT counterpart mice. Furthermore, I found that deficiency of Sema3E was associated with significantly reduced frequency of T-regs in the lymphoid organs of both infected and uninfected mice, which was associated with significant reduction in IL-10 production. These findings identify Sema3E as a negative regulator of immunity to cutaneous leishmaniasis.

Sema3E is expressed by somatic cells and immune cells. Among immune cells, dendritic cells and $CD4^+$ Th2 cells are reported to express the highest levels of Sema3E (194). In addition, $CD4^+$ Th2 cells have been associated with enhancing a host's susceptibility to *L. major*

infection (35). These findings stimulated my interest to investigate whether *L. major* induces Sema3E expression. Therefore, I assessed the outcome of Sema3E expression during *L. major* infection and observed that following infection in WT C57BL/6 mice, mRNA expression Sema3E was increased significantly at 1 day and 2 days post infection compared to the PBS treated group (control). However, at day 7 post-infection, I observed a that the mRNA expression of Sema3E in the *L. major* infected group was reduced significantly. This reduction in the mRNA expression of Sema3E at day 7 post-infection suggested that the infected mice in a bid to mount a stronger anti-Leishmania immune response, downregulated Sema3E expression which I speculate at that point to be favouring a pro *Leishmania* immune state.

To further examine the role of Sema3E in determining the outcome of *L. major* infection, I obtained Sema3E deficient (Sema3E$^{-/-}$) mice from our collaborator, Dr. Soussi Gounni at the University of Manitoba. I infected WT and Sema3E$^{-/-}$ C57BL/6 mice with *L. major* promastigotes and monitored the progression of the disease by measuring the size of the lesions and quantifying parasites. The results showed that the lesion size and parasite burden of the infected Sema3E$^{-/-}$ mice were significantly reduced when compared to their WT counterparts. Since Sema3E is expressed by immune cells (194), I was also interested in investigating whether the enhanced resistance in Sema3E$^{-/-}$ mice is due to deficiency of Sema3E in immune cells. I conducted a bone marrow chimera study where Sema3E deficient bone marrow cells were transferred to sub-lethally irradiated WT mice. The recipient WT mice were subsequently infected with *L. major* parasites. Interestingly, I observed that the WT mice that received Sema3E deficient bone marrow cells showed similar disease outcomes as Sema3E$^{-/-}$ mice as they both displayed significant reduction in their lesion sizes and parasite burdens. This result showed that the increase in resistance to *L. major* infection observed in the Sema3E deficient mice was due to Sema3E deficiency in immune cells.

Based on my findings, I became interested in investigating the specific immune cell(s) responsible for driving enhanced resistance to *L. major* infection in Sema3E$^{-/-}$ mice. Macrophages are the cellular hosts of *L. major*, where their promastigotes metamorphose into amastigotes (57). Macrophages also express both Sema3E and its receptor Plexin D1 (194). Therefore, I wanted to determine if the enhanced resistance and reduced parasite burden observed in the infected Sema3E$^{-/-}$ mice were due to the inability of the parasite to infect and/or proliferate within their macrophages. To address this, I infected bone marrow derived macrophages from WT and Sema3E$^{-/-}$ mice with *L. major* parasites *in vitro* and counted the number of parasites per cell and number of infected cells per field under the light microscope at designated time points (from 6-72 hours). There was no significant difference in both the number of cells infected with *L. major* and the number of parasites per infected cell in the WT and Sema3E$^{-/-}$ macrophages. This result suggested that the enhanced resistance observed in the Sema3E$^{-/-}$ mice after infection may not be due to the ability of the parasites to infect and/or proliferate in Sema3E$^{-/-}$ macrophages thereby implying that Sema3E does not directly regulate the susceptibility of macrophages to *L. major* infection. It has been reported by Park *et al* that *Leishmania* parasites infect macrophages and suppress their ability to produce nitric oxide which is the effector molecule necessary for the killing the parasites (57). Therefore, I assessed whether Sema3E directly influences the ability of macrophages to produce nitric oxide after infection. Results from my study showed similar levels of secreted nitric oxides in WT and Sema3E deficient macrophages, thereby suggesting that Sema3E does not affect parasite-mediated suppression of nitric oxide production in infected macrophages. Also, the comparable number of proliferated parasites within the WT and Sema3E deficient macrophages supports our conclusion that Sema3E deficiency does not directly enhance the parasite clearance capacity of macrophages.

Dendritic cells, which also express Sema3E, have been associated with protective immune response to Leishmaniasis. Unlike macrophages, dendritic cells are not suppressed by the presence of parasites within them (132). Thus, they function as the major antigen presenting cells in the host immune system during Leishmaniasis. Dendritic cells phagocytose *L. major*, process and present their antigenic peptides to naïve $CD4^+$ T cell in a class II MHC dependent manner. In addition, dendritic cells upregulate their costimulatory molecules like CD40, CD80 and CD86, which bind their ligands present on naïve $CD4^+$ T cells. Dendritic cells also secrete proinflammatory cytokines like IL-12p40 which facilitate the differentiation of naïve $CD4^+$ T cells into IFN-γ secreting $CD4^+$ Th1 cells (206). Therefore, I decided to assess the effect of Sema3E on the expression of costimulatory molecules (CD40, CD80, and CD86) on dendritic cells. I stimulated WT and $Sema3E^{-/-}$ bone marrow derived dendritic cells (BMDCs) with toll-like receptor agonists like LPS, CpG, and *L. major* promastigotes for 24 hours. The expression of CD80 and CD86 as well the secretion of Il-12p40 was significantly increased in BMDCs from $Sema3E^{-/-}$ mice compared to their WT counterparts. CD80, CD86 and IL-12p40 are necessary for dendritic cells to mediate the differentiation of naïve $CD4^+$ T cell into $CD4^+$ Th1 cells (which are the effector cells necessary for anti-leishmania immunity). These findings suggested that Sema3E signalling (as seen in WT DCs) could be downregulating the proinflammatory pathway in dendritic cells thereby suppressing their ability to activate and differentiate naïve $CD4^+$ T cells into $CD4^+$ Th1 cells. The implication of this finding is that the enhanced protection against *L. major* infection observed in $Sema3E^{-/-}$ mice could be because their DCs have an increased capacity to induce naïve $CD4^+$ T cell differentiation into the protective $CD4^+$ Th1 cells.

Furthermore, I wanted to assess the immune response in the draining lymph nodes of infected WT and $Sema3E^{-/-}$ mice. I sacrificed infected WT and $Sema3E^{-/-}$ mice at various time points after infection and observed that in the resistant ($Sema3E^{-/-}$) group, there was a significant

increase in the frequency of IFN-γ secreting $CD4^+$ Th1 cells in the draining (popliteal) lymph node. I also quantified the effector cytokines present in the lymph node. Interestingly, I observed that the $Sema3E^{-/-}$ mice have a significantly higher quantity of secreted IFN-γ and IL-17A in their draining lymph node compared to their WT counterparts. IFN-γ and IL-17A are effector cytokines secreted by $CD4^+$ Th1 cells and Th17 cells respectively. Scott *et al* reported that IFN-γ is the major effector cytokine required for the protection against *L. major* infection (143). To demonstrate the importance of IFN-γ, studies have shown that the normally resistant/self-healing mice (C57BL/6) that are engineered to become deficient in IFN-γ lose their resistance and become very susceptible to *L. major* infection, displaying increased lesion sizes and higher parasite burden compared to the WT C57BL/6 control group (136). Furthermore, Heinzel *et al* reported that the normally *L. major* susceptible mice strain BALB/c will become resistant to infection following exogenous administration of recombinant IFN-γ or recombinant IL-12, which is a cytokine renowned for its necessary role in the differentiation of naïve $CD4^+$ T cells into $CD4^+$ Th1 cells (207).

The role played by IL-17A in determining the outcome of cutaneous leishmaniasis is controversial. This is because some evidence shows that $CD4^+$ Th17 immune responses are associated with immunopathology during *L. major* infection in mice (98). However, a recent study from our lab demonstrated that pentraxin-3 deficient mice were highly resistant to *L. major* infection and that this resistance was associated with increased IL-17A secreting $CD4^+$ Th17 cells. The neutralization of IL-17A with monoclonal anti-IL-17A antibody abolished the resistance observed in the $PTX3^{-/-}$ mice (208). In addition, Novoa *et al* associated the increase in IL-17A to subclinical human cutaneous leishmaniasis. They demonstrated that patients with the clinical form of leishmaniasis have lower levels of IL-17A at the sites of infection (101). Movassagh *et al* also reported that following house dust mice stimulation, $Sema3E^{-/-}$ mice had a significant higher frequency of $CD4^+$ Th17 cells and quantity of secreted IL-17A in their

broncho-alveolar lavage fluid (BALF) compared to the WT controls (209). These findings stimulated my interest in investigating the outcome of $CD4^+$ Th17 cells response in Sema3E$^{-/-}$ mice following *L. major* infection. Interestingly, I found that the quantity of IL-17A in the draining lymph nodes of Sema3E$^{-/-}$ mice was significantly increased compared to their WT counterparts.

I have shown that the enhanced resistance to *L. major* infection observed in Sema3E$^{-/-}$ mice is associated with increase in IL-17A and IFN-γ levels in their draining lymph nodes. However, the mechanism for the increase in IL-17A and IFN-γ due to Sema3E deficiency remains unknown. Various toll-like receptor agonists or *L. major* stimulated Sema3E$^{-/-}$ and WT dendritic cells (BMDCs) resulted in enhanced expression of costimulatory molecules and IL-12p40 secretion by the Sema3E$^{-/-}$ BMDCs compared to their WT counterparts. Therefore, I proceeded to investigate the implication of the enhanced costimulatory molecule expression and IL-12p40 secretion by stimulated Sema3E$^{-/-}$ BMDCs with regards to the differentiation of naïve $CD4^+$ T cell to $CD4^+$ Th1 and Th17 cells. I cocultured *Leishmania* peptide-pulsed WT or Sema3E$^{-/-}$ BMDC with naïve CFSE labelled $CD4^+$ TCR Tg T cell. After 4 days of coculture, I observed that the quantity of secreted IFN-γ and frequency of $CFSE^-$ $CD4^+$ IFN-γ^+ TCR Tg T cells (proliferated Th1 cells) that were cocultured with peptide pulsed Sema3E$^{-/-}$ BMDCs were significantly increased compared to their WT co cultured counterparts. Although there was no significant difference in the frequency of $CFSE^-$ $CD4^+$ IL-17A^+ TCR Tg T cells, the quantity of secreted IL-17A by the $CFSE^-$ $CD4^+$ IL-17A^+ TCR Tg T cells that were cocultured with peptide pulsed Sema3E$^{-/-}$ BMDCs was significantly increased compared to their WT cocultured counterparts. These results suggested that the increased frequency of $CD4^+$ Th1 cells and quantity of effector cytokines IFN-γ and IL-17A observed in *L. major* infected Sema3E$^{-/-}$ mice may be due to the increased costimulatory molecule expression and IL- 12p40 secretion of Sema3E$^{-/-}$ dendritic cells. Furthermore, this suggests an increase in the capacity of Sema3E$^{-/-}$

dendritic cells to facilitate the differentiation of naïve $CD4^+$ T cells to $CD4^+$ Th1 and Th17 subsets. To confirm this, it is important that further studies such as the adoptive cell transfer of $Sema3E^{-/-}$ dendritic cells to sub-lethally irradiated WT mice are carried out.

Holl *et al* have shown that Sema3E and its receptor Plexin-D1 are also expressed by T cells and $CD4^+$ Th2 cells express Sema3E more than $CD4^+$ Th1 cells (194). It is conceivable that Sema3E and Plexin-D1 signalling on naïve $CD4^+$ T cells could regulate their differentiation into different subsets of $CD4^+$ T cells. Consequently, I assessed the effect of Sema3E on the propensity of a naïve $CD4^+$ T cell to be polarized into $CD4^+$ Th1 and Th17 cells (since infected $Sema3E^{-/-}$ mice showed enhanced IFN-γ and IL-17A response). To achieve this objective, I isolated WT and $Sema3E^{-/-}$ $CD4^+$ T cells and cultured them in Th17 and Th1 polarizing conditions. Interestingly, I observed that naïve $Sema3E^{-/-}$ $CD4^+$ T cells were polarized to $CD4^+$ Th17 and Th1 cells more than their WT counterparts. The differentiation of a naive $CD4^+$ T cell to $CD4^+$ Th17 or $CD4^+$ Th1 is to some extent determined by the upregulation of transcription factors Rorγ-T and T- bet respectively (210). I speculate that Sema3E/Plexin D1 signalling in the naïve WT $CD4^+$ T cells suppresses the expression of Rorγ-T (Th17) and T-bet (Th1) in the polarized cells thus allowing increased proliferation and differentiation of $Sema3E^{-/-}$ naïve $CD4^+$ T cells to $CD4^+$ Th17 and Th1 cells. Coupled with the increased frequency of $CD4^+$ Th17 and Th1 cells in the polarized $Sema3E^{-/-}$ group, I observed that the amount of secreted IL-17A and IFN-γ from $Sema3E^{-/-}$ polarized $CD4^+$ T cells were significantly increased compared to the WT polarized $CD4^+$ T cells. These results in general suggests that following exposure to *L. major* in a WT mouse, Sema3E/Plexin D1 signalling could suppress the propensity of a naïve $CD4^+$ T cell to differentiate into Th17 and Th1 cells by suppressing the expression of Rorγ-T and T-bet respectively. This could be a possible pathway shut down in the $Sema3E^{-/-}$ mice during *L. major* infection thus accounting for the enhanced resistance which

was associated with increased frequency of CD4$^+$ Th1 cells and quantity of IFN-γ and IL-17A (Leishmanicidal cytokines) in their draining lymph nodes.

Interleukin-10 (IL-10) has been shown to contribute to increase in the susceptibility of mice to *L. major* infection (117). Studies have reported that T-regs which promote the susceptibility of a host to *L. major* infection, utilizes two possible mechanisms to supress effector CD4$^+$ T cells. One mechanism acts in a contact-independent manner via the secretion of cytokines such as IL-10 which are known to be immunosuppressive in nature. The secreted IL-10 binds to the IL-10 receptor on the surface of CD4$^+$ Th1 and Th17 cells where they mediate the downregulation of the secretion of effector cytokines like IFN-γ and IL-17A (160). Due to the immunosuppressive role of IL-10 in *L. major* infection, I assessed the quantity of IL-10 in the culture supernatant fluids of draining lymph node cells of Sema3E$^{-/-}$ mice. I observed that the quantity of IL-10 secreted by the cells in the draining lymph nodes of *L. major*-infected Sema3E$^{-/-}$ was significantly less compared to their WT counterparts. This result suggests that the enhanced Th1 and Th17 response in the draining lymph node of Sema3E$^{-/-}$ mice, which were associated with the enhanced resistance of the mice to *L. major* infection, could be because of the reduced production of IL-10 (an immunosuppressive cytokine) in their draining lymph nodes. However, the use of IL-10 reporter Sema3E$^{-/-}$ mice may be necessary to further investigate this.

Although I did not show the cell populations that accounted for the reduced IL-10 production observed in the Sema3E$^{-/-}$ mice, T-regs are reported to serve as a major source of IL-10 in *L. major* infection (117). Therefore, I assessed the populations of T-regs in the draining lymph nodes of Sema3E$^{-/-}$ mice. Interestingly, I observed that the frequency of FoxP3$^+$ CD25$^+$ T-regs in the draining lymph node of Sema3E$^{-/-}$ mice were significantly reduced compared to their WT counterparts during *L. major* infection. This result suggests that in addition to a decrease in the quantity of secreted IL-10, the reduced frequency of T-regs in the draining lymph nodes

of Sema3E$^{-/-}$ mice may be associated with the observed increase in the frequency of effector $CD4^+$ Th1 and Th17 cells (204). This allows for a greater number of effector Th1 and Th17 cells that could be utilized by the Sema3E$^{-/-}$ mice to fight against the infection. Studies have reported that FoxP3 (the transcription factor for T-regs) and Rorγ-T (the transcription factor for Th17 cells) compete within a naïve $CD4^+$ T cell to determine the fate of the T cell. If Foxp3 expression is favored, naïve $CD4^+$ T cells tends to become T-regs. On the other hand, if the expression of Rorγ-T is dominant, naïve $CD4^+$ T cells would become Th17 cell (97). Therefore, I investigated whether the increased propensity of naïve Sema3E$^{-/-}$ $CD4^+$ T cell to polarize to Th17 cells (due to a possible increased expression of Rorγ-T in the naïve $CD4^+$ T cell) would result in a reduction in the frequency of T-regs (decrease in $Foxp3^+$ T-regs). Interestingly, I observed that uninfected Sema3E$^{-/-}$ mice had significantly reduced frequency of $FoxP3^+$ $CD25^+$ T-regs in their lymph nodes compared to their WT counterparts thereby suggesting that Sema3E/Plexin D1 signalling may be influential in determining and regulating the expression of Foxp3 or Rorγ-T in a naïve $CD4^+$ T cell. Furthermore, the adoptive transfer of Sema3E$^{-/-}$ T-regs to sub-lethally irradiated WT mice would help provide more insight on the role of Sema3E deficient T-regs in the enhanced resistance to *L. major* infection. Alternatively, the use of IL-2/anti-IL-2 mAb immune complex to expand T-regs populations in Sema3E$^{-/-}$ would also provide a considerable level of evidence to determine whether the reduced frequency of T-regs observed in Sema3E deficient mice contributes to enhanced resistance observed in these mice after *L. major* infection.

Weinkopff *et al* reported that dermal vasculature remodelling occurs following *L. major* infection in C57BL/6 mice. They attributed disease exacerbation to the increased blood vessel formation induced by the parasite (211). Sema3E/Plexin-D1 signalling has been shown to be a critical regulator of neovascularization in ischemic retinas (212). Since Sema3E/Plexin-D1 signalling is important for neovascularization, which is one of the possible ways *L. major*

parasites causes immunopathology, it is safe to speculate that the enhanced resistance to *L. major* in Sema3E$^{-/-}$ mice could be because of the possible disruption in the neovascularization pathway in Sema3E$^{-/-}$ mice during *L. major* infection. To validate this thought, studies will be carried out in the future to ascertain wound healing induced by physical injuries in Sema3E$^{-/-}$ mice.

5.1 LIMITATIONS OF STUDY AND MISSED OPPORTUNITIES

In this study, I showed through quantitative PCR that Sema3E expression is regulated by *L. major* infection. Detection of secreted Sema3E in infected tissue homogenates by ELISA would have strengthened my observations because there are some post transcriptional and translational regulations that sometimes results in the failure of expressed mRNA being translated to proteins. I did not pursue this validation process because previous studies in allergic asthma model showed that the current anti-Sema3E antibodies are not at effective at detecting Sema3E levels in tissue samples (such as lung homogenate) due to low sensitivity.

In this study, Sema3E$^{-/-}$ mice were highly resistant to *L. major* infection and this was associated with increase in CD4^{+} Th1 and Th17 response in their draining lymph nodes. Although IFN-γ secreting CD4^{+} Th1 cells have been reported to be the major effector cytokines that mediate parasite killing inside the macrophages (32), the studies here did not directly show that the enhanced resistance were due to this increased Th1 response and IFN-γ production in these mice. I could have tested this by attempting to reduce the numbers of CD4^{+} Th1 cells in Sema3E$^{-/-}$ mice to the level observed in the WT mice. This would definitively determine if the superior resistance to *L. major* infection observed in Sema3E$^{-/-}$ mice is due to their enhanced Th1 cell response. However, this may not be easy to accomplish because the neutralization of IFN-γ or reduction in the numbers of IFN-γ-secreting CD4^{+} Th1 cells in the resistant C57BL/6

WT mice background would result in loss of resistance to the infection. Notwithstanding, I attempted to adoptively transfer splenocytes from Sema3E$^{-/-}$ to sub lethally irradiated WT mice to see if the recipient WT mice would display the T cell phenotype observed in Sema3E$^{-/-}$ mice. Unfortunately, the results of this study did not turn out as expected because in this particular experiment, the donor cells were rapidly lost. I speculate this to be because the donor Sema3E$^{-/-}$ mice were not purely of the recipient C57BL/6 background.

Alternatively, the use of IFN-γ reporter Sema3E$^{-/-}$ mice would further demonstrate the effect of Sema3E on IFN-γ response in a mouse during *L. major* infection.

5.2 FUTURE DIRECTIONS

5.2.1 Determine the Role of Regulatory T-Cells and IL-10 in the Enhanced Resistance Observed in Sema3E Deficient Mice During *L. Major* Infection

T-regs are known for enhancing parasite survival and host's susceptibility to *L. major* infection (117). Since, I observed that Sema3E$^{-/-}$ mice have decreased frequency of CD25^{+}Foxp3^{+} T-regs (compared to their WT counterpart mice) in their lymph nodes (Fig. 9A and 9B). It is conceivable that this lower frequency of T-regs may be responsible for their protection against *L. major*. If this is the case, forced expansion of T-regs population in Sema3E$^{-/-}$ mice (up to the levels in WT mice) with IL-2/anti-IL-2 immune complex should cause them to lose their enhanced resistance and behave like WT mice following *L. major* infection. Also, I demonstrated that there is a significant decrease in the secreted IL-10 of draining lymph node cells from infected Sema3E$^{-/-}$ mice (Fig. 9C). IL-10 have been reported to be a major immunosuppressive cytokine during the effector phase response following *L. major* infection. Since Sema3E$^{-/-}$ mice demonstrate a diminished IL-10 secretion in their draining lymph node during *L. major* infection, treating these mice with recombinant IL-10 (to increase the quantity

of IL-10) could give a better insight to the role of the decreased IL-10 observed in Sema3E$^{-/-}$ mice during *L. major* infection.

5.2.2 Assess the Roles of IL-17A in Sema3E$^{-/-}$ Mice During *L. major* Infection

Infected Sema3E$^{-/-}$ mice showed increased resistance to *L. major* infection and this was accompanied by increased quantity of IFN-γ and IL-17A secreted by cells from their draining lymph nodes. It is conceivable that IL-17A may induce and/or augment IFN-γ producing Th1 cells in the regional lymph nodes of Sema3E$^{-/-}$ mice (99). To further examine the role of IL-17A in Sema3E$^{-/-}$ mice during *L. major* infection, treating Sema3E$^{-/-}$ mice with IL-17A neutralizing antibody to should be carried out and the disease outcome assessed. Demonstrating the role of IL-17A in the enhanced resistance to *L. major* infection observed in Sema3E$^{-/-}$ mice could facilitate the understanding of possible mechanisms used by Sema3E deficient immune system to adequately control *L. major* infection in Sema3E$^{-/-}$ mice.

5.2.3 Assess Sema3E Regulation of the Expression of Th1 and Th17 Transcription Factors in a Naïve CD4^{+} T Cell

This study showed that Sema3E$^{-/-}$ mice are resistant to *L. major* infection and this was associated with increased secretion of IFN-γ (Th1 effector cytokine) and IL-17A (Th17 effector cytokine) (Fig. 5F and 5I) and increased frequency of CD4^{+} Th1 cells in their draining lymph nodes (Fig. 5D). Since CD4^{+} T cells express Sema3E and its receptor Plexin D1(194), it is conceivable that Sema3E/Plexin D1 signalling may affect the differentiation of a naïve CD4^{+} T Cell to CD4^{+} Th17 and Th1. *In vitro* polarization of isolated CD4^{+} T cells from the spleens of Sema3E$^{-/-}$ mice showed that their naïve CD4^{+} T cells differentiate into Th17 and Th1 cells more than their WT counterparts (Fig. 6E and 6B). One of the possible mechanisms for this outcome could be that Sema3E/Plexin D1 signalling in a naïve CD4^{+} T cell suppresses the expression of RORγT (Th17 transcription factor) or T-bet (Th1 transcription factor) during the

differentiation of the naïve $CD4^+$ T cell to Th17 and Th1 cells respectively. Quantitative real time PCR could be used to quantify the levels of RORγT and T-bet expression in WT and $Sema3E^{-/-}$ $CD4^+$ T cells after polarization to Th17 and Th1 cells. If Sema3E suppresses the expression of T-bet and RORγT, it would be expected that $Sema3E^{-/-}$ $CD4^+$ T cells will show increased levels of Tbet and RORγT as they display increased differentiation to Th1 and Th17 cells when compared to their WT counterpart $CD4^+$ T cells. As a proof of concept, $Sema3E^{-/-}$ $CD4^+$ polarization will also be done in the presence of recombinant Sema3E to assess the comparability of the outcome to WT $CD4^+$ T cell polarization.

5.2.4 <u>Assess the Effect of Sema3E Deficiency on the Expression of IFN-γ Receptor on Macrophages</u>

Macrophages are the major immune cells preferentially parasitized by *L. major* (51). During *L. major* infection, IFN-γ secreted by $CD4^+$ Th1 cells bind to IFN-γ receptor on parasitized macrophages to effect the killing of the intracellular amastigotes via the upregulation of iNOS pathway which eventually leads to increased secretion of nitric oxide that kills the parasites (32). $Sema3E^{-/-}$ mice displayed an enhanced resistance to *L. major* infection as shown by the significantly lower parasite burden compared to their WT counterparts (Fig.2B). Although I have demonstrated that $Sema3E^{-/-}$ mice have increased frequency of IFN-γ secreting Th1 cells in their draining lymph nodes, it is possible that $Sema3E^{-/-}$ macrophages may also have increased expression of IFN-γ receptor when compared to their WT counterparts. To address this possibility, dermal macrophages at lesion sites will be assessed for IFN-γ receptor during *L. major* infection by flow cytometry in both WT and $Sema3E^{-/-}$ mice.

5.2.5 Assess the Role of Dendritic Cells in Sema3E$^{-/-}$ Mice During *L. major* Infection *In Vivo*

Dendritic cells are renowned for their antigen presentation, co-stimulation and cytokine secretion necessary for the differentiation of naïve CD4^{+} T cells to anti *leishmania* Th1 cells (41). Holl *et al* reported that dendritic cells express Sema3E and plexin D1 and that the deficiency of Plexin D1 caused increased secretion of IL-12p40 and IL-23 following the stimulation of the dendritic cells with LPS (194). I showed that similar to Plexin D1$^{-/-}$ dendritic cells (194), Sema3E$^{-/-}$ dendritic cells displayed increased IL-12p40 secretion following stimulation with LPS (Fig. 7M), CpG (Fig. 7N) and *L. major* infection (Fig. 7O). I showed that the co-culture of Sema3E$^{-/-}$ dendritic cells with naïve CD4^{+} TCR Tg caused increased frequency of differentiated Th1 cells (Fig. 8A), increased IFN-γ (Fig. 8C) and IL-17A (Fig. 8E) secretion from naïve CD4^{+} TCR Tg compared to the co culture with WT dendritic cells. This result suggested that the increased IFN-γ (Fig. 5F) and IL-17A (Fig. 5I) secretion observed in Sema3E$^{-/-}$ mice during *L. major* infection, may be due to their dendritic cells having a possibly enhanced capacity to mediate the activation and differentiation of naïve CD4^{+} T cells to Th1 cells and Th17 cells. Although I have demonstrated this concept *in vitro*, it will be important to see if this replicates *in vivo*. The use of dendritic cell specific Sema3E$^{-/-}$mice will elaborate the contribution of Sema3E$^{-/-}$ dendritic cells to the enhanced Th1 and Th17 response observed in Sema3E$^{-/-}$ mice during *L. major* infection. Although, one major draw back of inducible cell specific knockout studies is the possibility of inducing the desired gene knockout in the wrong cells (off target effects).

5.2.6 <u>Induce Sema3E Deficiency in WT Mice After *L. major* Infection as an Immunotherapeutic Approach</u>

This study revealed that complete genetic knockout of Sema3E in mice resulted in better outcomes of disease. This makes it necessary to evaluate the outcome of *L. major* infection in WT mice following the use of molecules to prevent Sema3E and cell surface plexin D1 signalling in immune cells during infection. Although there are no reported neutralizing antibodies against murine Sema3E, Kermarrec *et al* reported that soluble plexin D1 can be used to competitively scavenge Sema3E and prevent it from binding to cell surface Plexin D1 (213) thus creating a "Sema3E deficient" environment and subsequently preventing Sema3E/ plexin D1 signalling. In the future I plan to use recombinant soluble Plexin D1 to prevent Sema3E from binding to cell surface plexin D1 and create a Sema3E deficient-like environment. It is conceivable that the use of soluble plexin D1 to prevent Sema3E plexin D1 signalling in immune cells will result in disease outcomes comparable to what I observed in genetically derived Sema3E deficient mice.

5.3 OVERALL SIGNIFICANCE

This study has identified and demonstrated Semaphorin 3E as a novel host molecule that enhances the susceptibility of a host to *L. major* infection. Since our study has demonstrated that Sema3E is a negative regulator to the immune response of a host against *L. major* infection, the immunotherapeutic neutralization of Sema3E expression in an infected individual may result in better outcomes of disease.

5.4 CONCLUSION

In conclusion, I have been able to show that Sema3E, a host protein which is induced after *L. major* infection, is a possible host molecule that allows the parasite to survive and proliferate

in the host. This is the first report showing the role of Semaphorin 3E in the pathogenesis of cutaneous leishmaniasis caused by *L. major*. I have shown that Sema3E is involved in causing a decrease in the ability of dendritic cells to produce IL-12p40 (a critical cytokine that is necessary for causing naïve CD4$^+$ T cells to differentiate into CD4$^+$ Th1 cells). Sema3E is also involved directly in suppressing the ability of a naïve CD4$^+$ T cell to be polarized to CD4$^+$ Th1 cells and Th17 which are cells that are necessary to protect a host from *L. major* infection. And finally, Sema3E enhances the expansion and increase in the frequency of regulatory T cells (which are immunosuppressive in nature) during *L. major* infection. I have been able to identify three possible ways Sema3E helps *L. major* parasites to cause increased pathology to an infected host. However, more work needs to be done to find out which of the three pathways is being utilized by the parasite to evade a host immune response to *L. major* infection.

GRAPHICAL ABSTRACT

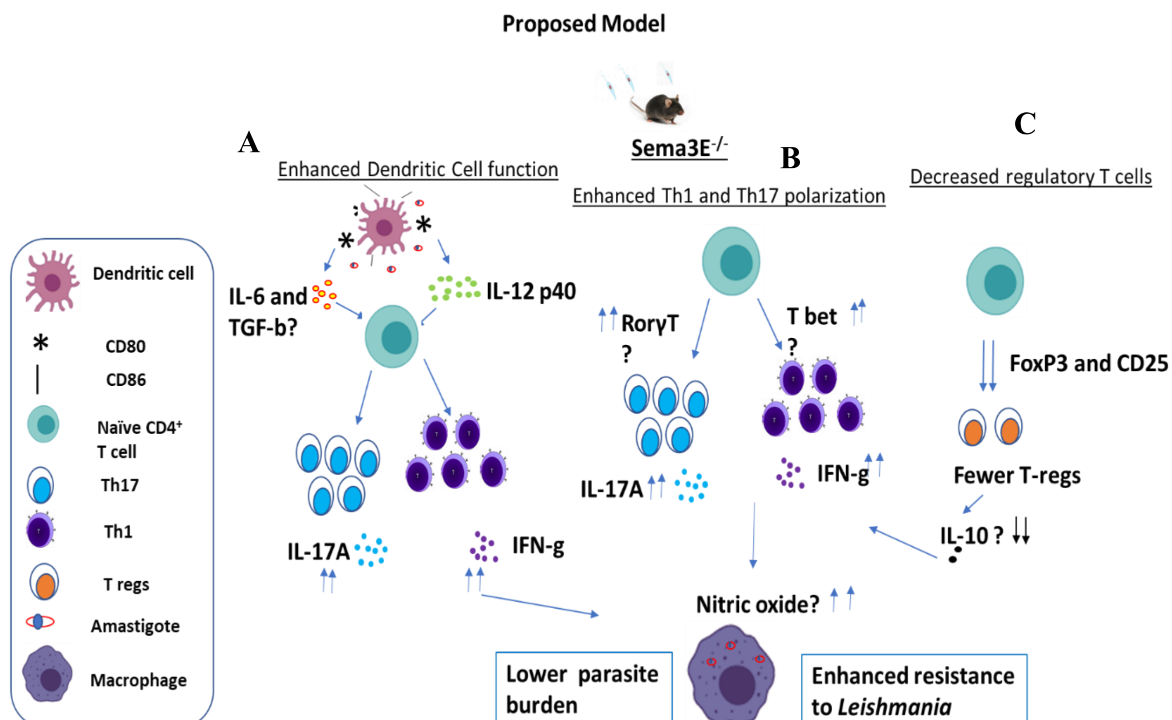


Figure 10: Graphical representation for the possible mechanisms that could be mediating enhanced resistance to *L. major* infection in Sema3E$^{-/-}$ mice.

(A) After infection of a mouse deficient in Sema3E production, dendritic cells upregulate their costimulatory molecules (CD80 and CD86) as well as increase their IL-12p40 secretion, which possibly mediates the increase in the differentiation of naïve $CD4^+$ T cells to Th1 cells. Although my current study did not show it, it is also conceivable that infected $Sema3E^{-/-}$ dendritic cells also produce increased amounts IL-6 which could drive enhanced Th17 response observed in the draining lymph node of these mice. Further studies need to be carried out to determine if this is occurring in $Sema3E^{-/-}$ mice during *L. major* infection. This mechanism was demonstrated by the infection DCs *in vitro* coupled with the co culture experiment of the DCs and TCR Tg $CD4^+$ T cells, where $Sema3E^{-/-}$ DCs caused the increased differentiation of naïve TCR Tg $CD4^+$ T cells to Th17 and Th1 cells when compared to naïve TCR Tg $CD4^+$ T cells exposed to WT DCs.

(B) The second possible mechanism for the enhanced resistance observed in $Sema3E^{-/-}$ mice is the probability that during *L. major* infection, Sema3E deficiency results in enhanced frequency of Th17 and Th1 cells due to a possible upregulation of Rorγ-T and T-bet in a naïve T cell. This speculation is based on the observations showing that $Sema3E^{-/-}$ $CD4^+$ T cells differentiate into Th17 and Th1 cells more than their WT counterparts (Fig. 6E and 6B). In this study, transcription factors for Th1 (T-bet) and Th17 (Rorγ-T) have not been assessed, although this was captured as one of the future plans for this study.

(C) The third possible mechanism for the enhanced resistance to *L. major* infection in $Sema3E^{-/-}$ mice, is the probability that Sema3E is critical for differentiation and/or expansion of Tregs. This is based on the finding that during *L. major* infection, the deficiency of Sema3E results in a significant reduction in the frequency of regulatory T cells in the draining lymph nodes (Fig. 9A and 9B) which may result in the observed reduction in the quantity of IL-10 (Fig. 9C) and subsequently cause an enhanced Th1 and Th17 immune response in $Sema3E^{-/-}$ mice.

These models are by no means mutually exclusive, and Model A, B and C may all work collectively to cause increased nitric oxide production within the infected Sema3E$^{-/-}$ macrophages, thus resulting in marked decrease in parasite load at the infection site in Sema3E$^{-/-}$ mice.

Further studies need to be carried out to particularly ascertain the mechanism for the enhanced resistance to *L. major* infection due to a deficiency in Sema3E production during *L. major* infection.

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