INTERLEUKIN-4 RESPONSIVE DENDRITIC, MACROPHAGE/NEUTROPHIL CELLS ARE DISPENSABLE FOR HOST RESISTANCE AGAINST *LEISHMANIA MEXICANA* INFECTION IN MICE.

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Thesis submitted in partial fulfilment of the requirements for the Doctor of Philosophy degree in Clinical Science and Immunology

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6th June 2022

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I dedicate this thesis to all those who participated in my Ph.D journey, more especially to my daughters; Elizabeth Mokeira, Martha Sarange, and Florence Kemunto for their unquestionable love and patience.

DECLARATION

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LIST OF PUBLICATIONS

In addition to this work presented in this thesis, the author has made a significant contribution in the following publications

1. **Osero, B.O**., Cele, Z., Aruleba, R.T., Maine, R., Ozturk, M., Lutz, M. B., Brombacher, F and Hurdayal, R. (2022). Interleukin-4 responsive dendritic cells are dispensable to host resistance against *L. mexicana* infection. Front. Immunol. 12:759021. doi: 10.3389/fimmu.2021.759021.
2. **Osero, B.O**., R.T. Aruleba, F. Brombacher, and R. Hurdayal. 2020. Unravelling the unsolved paradoxes of cytokine families in host resistance and susceptibility to *Leishmania* infection. Cytokine X 2:100043. doi: 10.1016/j.cytox.2020.100043.
3. IL-4 mediated susceptibility of BALB/c mice to cutaneous leishmaniasis caused by *L. mexicana* is independent of IL-4Rα signaling via macrophage and neutrophil cells (manuscript in preparation)
4. Raphael Taiwo Aruleba, **Bernard Ong'ondo Osero** & Dr. Ramona Hurdayal. COVID-19 might have reversed the war against a serious parasitic disease. The Conversation Africa: article

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LIST OF ABBREVIATIONS AND SYNONYMS

ANOVA	Analysis of variance
Akt	Protein kinase B
APCs	Antigen-presenting cells
Arg-1	Arginase 1
ATP	Adenosine triphosphate
BM	Bone marrow
BMDCs	Bone marrow-derived dendritic cells
BMDMs	Bone-marrow-derived macrophage
CCR	CC-chemokine receptor
CD	Cluster of differentiation
$CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice	BALB/c mice with a deficiency of IL4Rα signaling on dendritic cells
cDCs	Conventional DCs
CL	Cutaneous leishmaniasis
CMP	Common myeloid progenitor
CPG	Cytosine and guanine separated by a phosphate
CTL	Cytotoxic lymphocytes
CXCR	CXC chemokine receptor
DCL	Diffuse cutaneous leishmaniasis
DCs	Dendritic cells
pLN	Popliteal lymph node
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcγR	FC gamma receptor
GM-CSF	Granulocyte colony-stimulating factor
GMP	Granulocyte-monocyte progenitor
GP	Glycoprotein
HSCs	Hematopoietic stem cells

IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IL-12Rβ2	Interleukin-12 receptor beta 2
IL-13 Rα1	IL-13 receptor alpha 1
IL-13 Rα2	IL-13 receptor alpha 2
IL-4Rα	IL-4 receptor alpha
iNOS	Nitric oxidase synthase
IRS	Insulin receptor substrate
JAK	Janus kinase
LCL	Localized cutaneous leishmaniasis
LCs	Langerhans cells
pLN	Popliteal lymph node
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LT-DCs	Lymphoid tissue DCs
$LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice	BALB/c mice with a deficiency of IL4Rα signaling on macrophages and neutrophils
Ly6G	Lymphocyte antigen 6 complex locus G6D
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MCL	Mucocutaneous leishmaniasis
MCP	Macrophage chemoattractant protein
mDCs	Migratory DCs
MDPs	Monocyte DC precursors
MHC	Major histocompatibility complex
MIP-3α	Macrophage inflammatory protein 3α
Mo-DCs	Monocyte-derived DCs
MPPs	Multipotent progenitors
MQS	Macrophages
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa B
NKC	Natural killer cells

NKT	Natural killer T cells
NO	Nitric oxide
NO-2	Nitrite
OX40L	TNF superfamily member 4
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid DCs
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear neutrophils
pre-DCs	Precursor DCs
ROS	Reactive oxygen species
RPMI media	Roswell Park Memorial Institute media
SEM	Standard error of the mean
SLA	Soluble *Leishmania* antigens
STAT	Signal Transducer and Activator of Transcription
Tip-DCs	TNF/iNOS-producing dendritic cells
T regs	T regulatory cells
TGF-β	Transforming growth factor-β
Th	T helper
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
VL	Visceral leishmaniasis
WHO	World Health Organization
WT	Wild type
CD3	Cluster of differentiation 3
Γc	Common γ chain

ABSTRACT

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Studies have shown that the protection against *L. mexicana* infection is potentiated by a type 1 response, driven by IL-12 production by dendritic cells. In contrast, IL-4 and IL-13 cytokines are associated with susceptibility to *L. mexicana* causing cutaneous leishmaniasis. This has been demonstrated in studies involving BALB/c mice deficient in IL-4, IL-13, and IL-4Rα infected with *L. mexicana*.

To determine the specific cell in IL-4Rα$^{-/-}$ BALB/c mice that contribute to the control of *L. mexicana* infections, studies on cell-specific IL-4Rα deficient mice need to be investigated. IL-4Rα-CD4^{+}T deficient mice revealed sex-dependent protection from *L. mexicana* infection, suggesting the critical role of non-lymphocyte cells in conferring protection against *L. mexicana* amastigote infection. Macrophage/neutrophil-specific IL-4Rα deficient mice are protected from *L. major* infection in the footpad. Surprisingly, this mouse strain infected in the base of the tail failed to control *L. mexicana* amastigote infection. Nonetheless, IL-4Rα-DC deficient mice were hyper-susceptible to *L. major* infection. This conundrum suggests that different *Leishmania* species, site of infection, and developmental stages of parasite dictate the outcome of the disease.

Here, mice with a deficiency of IL-4Rα signaling on DCs and macrophage/ neutrophil cells were subcutaneously infected with *L. mexicana* promastigotes in the footpad, and skin lesion progression was measured, and the clinical phenotype was evaluated by investigating both humoral and cellular immune responses. Mouse strains had similar footpad lesion progression, parasite loads, humoral responses, expansion of CD4^{+} and CD8^{+} T cells, their activation, memory phenotypes, and infiltration of DCs, macrophages, and neutrophils into the lymph nodes compared to their littermate IL-4Rα$^{-/lox}$ controls. Interestingly, IL-12p70 and IL-10 produced by BMDCs and BMDMs were similar. Nevertheless, nitrite/urea production was not affected.

Together, this study suggests that, unlike *L. major*, IL-4Rα signaling on DCs and macrophage/ neutrophil cells does not contribute to the susceptibility or resistance to BALB/c mice to infection with *L. mexicana*.

CHAPTER 1:

INTRODUCTION

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1.1 Literature review

1.1.1 Introduction and epidemiology

Leishmaniases are neglected tropical diseases caused by *Leishmania* spp; a vector-borne intracellular trypanosomatid protozoan parasite (Molyneux et al., 2017). The disease is divided into different clinical manifestations that range from self-healing skin lesions of localized cutaneous leishmaniasis (LCL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and visceral leishmaniasis depending on *Leishmania* parasite species and host immune response. Localized cutaneous leishmaniasis disease is manifested by a single dermal lesion or a few percentages of CL lesions do not heal without treatment (Bacellar et al., 2002; Scorza et al., 2017). A small percentage of LCL patients progress to MCL, which is characterized by a parasite invading the nasopharyngeal mucosa leading to injury and swelling (Torres-Guerrero et al., 2017). Diffuse cutaneous leishmaniasis is a rare form of CL that is characterized by the uncontrolled growth of parasites in lesions on various parts of the body (Hashiguchi et al., 2016). VL, also known as *kala-azar* is characterized by splenomegaly, hepatomegaly, and anemia and is fatal if left untreated (WHO, 2021).

According to WHO, approximately 1 million new cases of leishmaniasis and 65,000 deaths occur annually (WHO, 2021). Globally, the prevalence of leishmaniasis is estimated to be over 4.13 million with about 1.7 billion people at risk of infection in more than 98 countries worldwide distributed in American, European, Asiatic, African, and Australian continents (James et al., 2018; Pigott et al., 2014). CL mostly and widely occurs in tropical regions of Western and Central Asia, the Mediterranean basin, and America (WHO, 2021)

Leishmaniasis is associated with poverty, poor housing, civil war and conflicts, lack of proper nutrition, and a weak immune system. Besides, man-made activities like dam construction for irrigation schemes, deforestation, and urbanization can lead to the spread of leishmaniasis. Adaptation of sandflies to man-modified environments also contributes to increased transmission of leishmaniasis (Reithinger et al., 2007; WHO, 2021).

1.1.2 Life cycle of *Leishmania* parasites

Leishmania parasite is a dimorphic protozoan with a two-phase life cycle alternating between two developmental stages namely, promastigotes and amastigotes. Promastigotes have flagella and they reside inside the sand fly vector mainly the genus of *Phlebotomus* and *Lutzomyia* while amastigotes are round shaped with the absence of external flagella, thrive in human hosts as well as in dogs and rodents (Akhoundi et al., 2016; de Vries et al., 2015; Sunter and Gull, 2017). Out of eight hundred sand fly species identified so far, only 98 have been described as having potential for transmission of some *Leishmania* species (Maroli et al., 2013).

Infected female sand flies harbor metacyclic promastigotes in their saliva which can be deposited into mammalian host's skin during feeding (Bates, 2018) (**Figure 1.1**). Once in the skin, promastigotes are internalized by skin resident innate cells such as macrophages, dendritic cells (DCs), and neutrophils, which are specialized in phagocytosis (Gurung and Kanneganti, 2016; Martínez-López et al., 2018; Oualha et al., 2019). Importantly, resident macrophages are cells employed during infection to eliminate *Leishmania* parasites but paradoxically they provide an environment where promastigotes differentiate into amastigotes. Incidentally, *Leishmania* parasites can survive in the macrophages' phagolysosome-like organelle within the parasitophorous vacuoles (Alexander and Vickerman, 1975; Martínez-López et al., 2018; Rodríguez and Wilson, 2014). The amastigotes proliferate in parasitophorous vacuoles causing the rupture of macrophages, infecting neighbouring cells, and spreading to tissues depending on the *Leishmania* species and genetic background of the host (**Figure 1.1**) (Liévin-Le Moal and Loiseau, 2016).

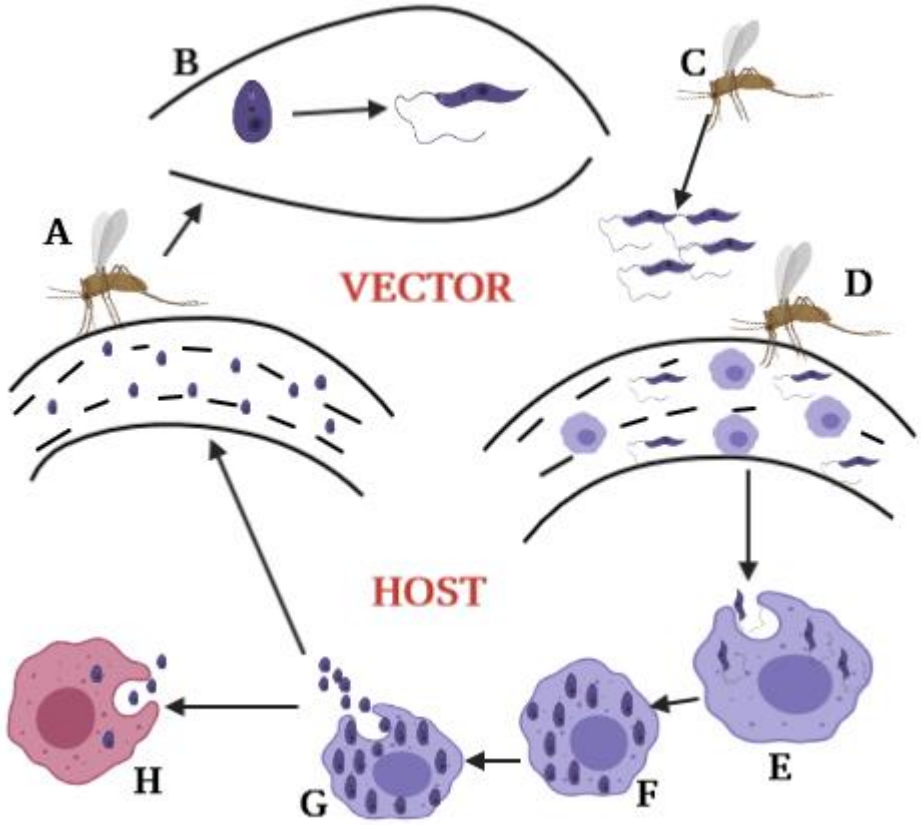

Figure 1.1: Life cycle of *Leishmania* parasite in vector (sandfly) and host (human). A) sandfly ingesting amastigotes from the skin of an infected human being, **B**) amastigotes differentiating into promastigotes in the midgut of the sandfly, **C**) mature metacyclic promastigotes are expelled into the saliva, **D**) infected sandfly feeding on skin of a human host and depositing promastigotes, **E**) promastigotes are phagocytosed by skin resident macrophages and neutrophils, **F**) promastigotes differentiate into amastigotes and proliferates, **G**) macrophages ruptures and releases amastigotes into circulation, H) amastigotes re-infect neighbouring macrophages (Adapted from Bates, 2018).

A naïve female sandfly during feeding on the skin of the infected mammalian host ingests the macrophage containing amastigotes. In the midgut of the sandfly, the amastigotes leave the macrophages and transform into infective metacyclic promastigotes that translocate to the saliva where they are ready to be injected into mammalian skin in the next cycle (Bates, 2007).

1.1.3 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) affects the skin and cartilages resulting in different clinical forms namely, localized cutaneous leishmaniasis (LCL), mucocutaneous leishmaniasis (MCL), diffused CL (Goto and Lindoso, 2010). In MCL, parasites penetrate the mucous membranes or other secondary sites forming metastatic lesions (Rossi and Fasel, 2018).

Leishmania parasites can be limited to the skin and lymphatic system causing LCL which is characterized by a pink papule that clinically may progress to either a nodule or plaque which ends up forming ulcers with an indurated border at the site of sandfly bite (Reithinger et al., 2007). Parasites can migrate deeper into tissues causing DCL which manifests as multiple nodular lesions of different sizes erupting in different sites away from that of sand fly bite (Hashiguchi et al., 2016; McGwire and Satoskar, 2014). Some cases of CL that are self-healing that may progress to MCL if not treated (David and Craft, 2009).

The CL is commonly caused by either old or new world *Leishmania* species including *L. major* and *L. aethiopica* (Karimkhani et al., 2016). These species are most prevalent in Mediterranean regions, most parts of Africa, the Middle East, and India whereas the new world species comprising of *L. amazonensis*, *L. mexicana*, *L. braziliensis*, *L. panamensis*, and *L. guyanensis* are endemic in Latin America (Goto and Lindoso, 2010; Hepburn, 2003). *Leishmania mexicana* majorly causes CL which can result in either LCL or DCL.

Old world *Leishmania* species cause self-resolving infections compared to new world species which are associated with chronic, non-resolving lesions (Akilov et al., 2007; Alexander and Kaye, 1985; Buxbaum et al., 2002; Goto and Lindoso, 2010). However, in some cases, viscerotropic *Leishmania* species can cause cutaneous lesions as well (Akilov et al., 2007).

Studies on the global burden of the disease reported that CL cases increased from 2.1 million in 2002 to nearly 4 million in 2015 (Bezerra et al., 2018; Mathers et al., 2007). Some countries in Asia, Africa and South America continents have continued reporting high CL cases of over 85% since 2018 (WHO, 2010). New cases of CL of between 690,900 to 1,213,300 have been reported worldwide annually, with estimated global mean age-standardized disability-adjusted life years for CL of 0.58 per 100,000 people (Alvar et al., 2012; Karimkhani et al., 2016).

The treatment of leishmaniasis using commonly known therapies like amphotericin B and sodium stibogluconate are often toxic, known to cause side effects, require parenteral administration, and thus lead to a long period of hospitalization (Ghorbani and Farhoudi, 2018; McGwire and Satoskar, 2014). Despite several promising studies on humans, no effective vaccine is yet available (Gillespie et al., 2016).

1.1.4 Adaptive immune responses to cutaneous leishmaniasis in experimental mice model

The mouse model of CL was established to investigate host mechanisms of resistance and susceptibility to experimental infection. However, immune responses involved in CL caused by *L. mexicana* and *L. major* infections differ significantly depending on the mouse strains (McMahon-Pratt and Alexander, 2004). For example, C57BL/6 mice develop progressive lesions during *L. mexicana* infection but are more resistant to *L. major* infection (Schroeder et al., 2013; Al-Mutairi et al., 2010). However, BALB/c mice develop cutaneous lesions caused by *L. mexicana* (Rosas et al., 2005). Both cytokine-mediated T cell and antibody immune responses have been implicated in resistance and susceptibility to CL disease

1.1.4.1 T cell-mediated immunity in CL

Studies on murine models of CL described the T helper (h)1/Th2 paradigm of resistance and susceptibility to *Leishmania* infection, respectively. These studies demonstrated that disease progression is associated with Th2 driven responses that downregulate protective Th1 immune responses (Heinzel et al., 1989; Mosmann et al., 1986) (**Figure 1.2**). Since then, this concept of the Th1/Th2 paradigm has changed and shown to be dependent on the parasite species initiating infection, in addition to involving other Th subsets such as Th17 (Gonçalves-de-Albuquerque et al., 2017) and T regulatory cells (Dayakar, 2018).

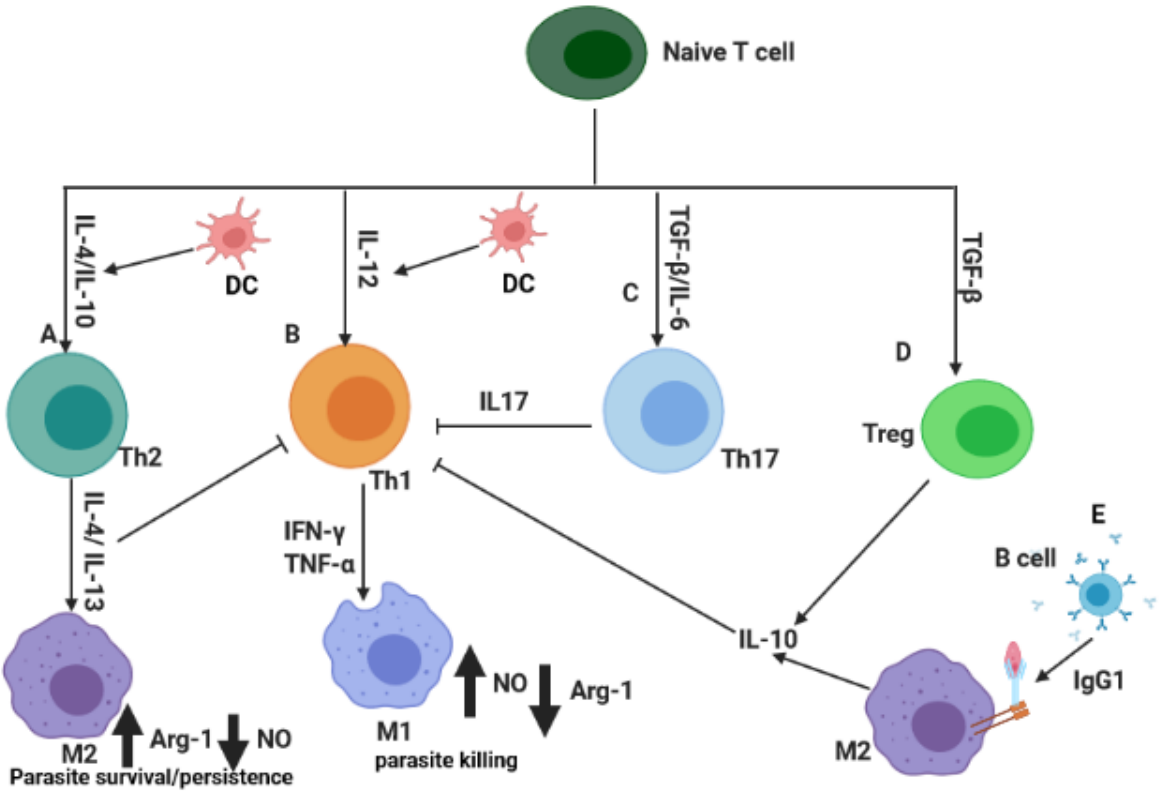

Figure 1.2: T helper (Th) cell-mediated immune responses during murine cutaneous leishmaniasis. A) Naïve T cells in presence of Interleukin (IL)-4/IL-10 from DCs, differentiate into T helper (Th) 2 cells facilitating M2 macrophage production of Arg-1 that favors parasite growth hence increasing disease severity. **B**) IL-4-DC instruction on IL-12 production drives expansion of Th1 cells characterized by Interferon (IFN)-γ and tumor necrosis factor (TNF)-α production. The synergy of the two cytokines induces classical macrophage activation leading to the production of NO that is toxic to the *Leishmania* parasite hence resolution of the disease. **C**) TGF-β in presence of IL-6 influences the expansion of Th17 cells that secrete IL-17 which directly inhibits Th1 responses or causes neutrophil influx into lesions, hence increasing pathology. **D**) Expansion of Tregs cells is mediated by TGF-β. Tregs secrete IL-10, an immunosuppressor/immunoregulatory cytokine E) B cell produces Immunoglobulin (Ig) G1 that forms an immune complex with amastigotes by opsonization. The immune complex interacts with FC gamma receptor (FcγR) III of macrophages inducing IL-10 production that suppresses Th1 immune responses. Modified from Osero et al., 2020.

Control of *L. mexicana* requires Th1 immune responses mediated by IFN-γ. Subsequently, IFN-γ stimulates macrophages to produce an inducible nitric oxidase synthase (iNOS) enzyme that mediates the release of nitric oxide (NO) that is toxic to parasites (Bogdan, 2015). It is therefore not surprising that *L. mexicana* infection affects Th1 cell proliferation and limits their IFN-γ producing ability (Hsu and Scott, 2007).

Conversely, *L. mexicana* can direct differentiation of T cells into a Th2 subtype that mediates susceptibility through IL-4 and IL-13 cytokine production (Alexander et al., 2002; Satoskar et al., 1995) (**Figure 1.2**). Th2 immune response downregulates Th1 immune response and IL-4/IL-13 signaling on macrophages leading to the increased arginase-1(Arg-1) production, an enzyme that competes with iNOS for arginine leading to decreased NO production hence, favoring the proliferation of parasites leading to severe CL disease (Muxel et al., 2017). The parasite killing by NO may be through stimulation of macrophage chemoattractant protein-1 (Kumar et al., 2010).

The immunology and outcome of *L. mexicana* and *L. major* are significantly different. For example, *L. major* infected C57BL⁄ 6 mice induce IL-12 driven Th1 immune responses characterized by sufficient IFN-γ producing $CD4^+$ T cells hence disease control (Stamm et al., 1999). Besides, IL-12 knockout C57BL⁄ 6 mice become susceptible to *L. major* infection (Park et al., 2002). Interestingly, C57BL⁄ 6 mice respond to *L. mexicana* infection with weak IFN-γ producing $CD4^+$ T cells that lead to chronic progressive lesions with concomitant higher parasite burden (Buxbaum and Scott, 2005). Importantly, early studies have shown that impaired IL-12R expression led to unresponsiveness to IL-12 in *L. major* and *L. amazonensis* but not in *L. mexicana* (Jones et al., 2000; Rodriguez-Sosa et al., 2001). BALB/c mice are susceptible to *L. major* infection characterized by low levels of IFN-γ and increased secretion of IL-4 and IL-10 (Hurdayal et al., 2013). In contrast, *L. major* infected BALB/c mice treated with a combination of extracts of *L. major* ribosomal proteins and CpG oligodeoxynucleotides, are protected from secondary infections due to increased levels of IFN-γ and reduced IL-4 and IL-10 production (Ramírez et al., 2010). However, *L. mexicana* and *L. amazonensis* infected $IL\text{-}10^{-/-}$ BALB/c mice fail to control lesion progression, but treatment with anti-IL-4 mAb leads to disease resolution (Padigel et al., 2003). Overall, these species-specific differences, experimental infection for CL requires the study of both *L. mexicana* and *L. major* parasite infections.

Interleukin-17 secreted by Th17 cells (**Figure 1.2**) attracts neutrophils into the site of *L. mexicana* infection leading to severe disease (Banerjee et al., 2016; Bettelli et al., 2008). Regulatory T cells (Tregs) are specialized in IL-10 that suppresses Th1 immune response in CL infection through inhibition of IL-12 production by DC, permeating the expansion of Th2 cell populations (Buxbaum, 2010; Buxbaum, 2005). In support, mice lacking IL-10 producing $CD4^+$ and $CD8^+$ T cells ($CD4^{cre}$ $IL\text{-}10^{fl/fl}$ mice) are protected from *L. mexicana*

infection (Buxbaum, 2015). In contrast, TGF-β produced by Tregs (**Figure 1.2**) together with IL-10 may prevent possible injuries caused by excessive inflammatory responses during infection (Maspi et al., 2016).

In clinical studies, healing CL patients have high quantities of IL-12 and IFN-γ in peripheral blood mononuclear cells (PBMCs) compared to non-healing patients, highlighting the vital role of Th1 immune response in protection from CL disease (Habibi et al., 2001; Shahi et al., 2013).

1.1.4.2 Humoral immunity to CL in mice

B cells are antigen presentation cells (APCs) that can activate $CD4^+$ T cells during CL (Ronet et al., 2010). More significantly, they differentiate into plasma cells for antibodies production upon encounter with *Leishmania* antigens (LeBien and Tedder, 2008). The plasma cells proliferate in the germinal centre and isotype switch from IgD or IgM to IgG, IgA, or IgE (Allen et al., 2007). Antigen-specific IgG2a/b antibody isotypes are predominantly produced in mice protected from CL infection (Miles et al., 2005). In contrast,antigen-specific-IgG1 can opsonize *Leishmania* amastigotes forming an immune complex that binds to FcγRIII on macrophages leading to IL-10 production that subsequently enhances pathogenicity of CL caused by *L. mexicana* infection (Buxbaum and Scott, 2005; Thomas and Buxbaum, 2008; Chu et al., 2010; Rodríguez-Serrato et al., 2020) (**Figure 1.2**). Conversely, IgG2a/c have been shown to confer resistance to *L. mexicana* infection in IgG knockout BALB/c mice (Chu et al., 2010). IgE antibodies produced in a Th2 immune response have been demonstrated in CL infections suggesting that they can be used as a serum marker for the disease diagnosis (Al-Qadhi et al., 2015). Most importantly, BALB/c mice infected with *L. major* parasites display increased total IgE levels in their serum, hence highlighting the vital role of IgE in mediating susceptibility during CL (Hurdayal et al., 2013). Overall, findings from these studies suggest that Th1 associated antibody responses (IgG2a/b) and Th2 associated antibodies (IgG1/ IgE) promote protection and susceptibility to CL respectively.

1.1.5 Interleukin-4 receptor signaling

Interleukin-4 exerts its functions by interacting with two types of heterodimeric receptors; type I and type II receptors expressed on the cell surface. Type I receptors comprises of a 140 kDa binding chain called IL-4 receptor alpha (IL-4Rα) and common γ chain (γc) of the IL-2R

that is shared by other cytokines of the IL-2 family whereas type II receptor comprises of IL-4Rα and IL-13 receptor alpha 1(IL-13 Rα1) subunits (Ul-Haq et al., 2016).
Interleukin-13 signaling through type II receptor mechanistically binds to IL-13 Rα1 with high affinity forming a complex that subsequently recruits IL-4Rα whereas IL-4 first binds IL-4Rα with high affinity before associating with either γc or IL-13 Rα1 (LaPorte, 2008; Lupardus et al., 2010) (**Figure 1.3**).

IL-4 signaling through IL-4Rα on macrophages leads to differentiation of macrophages to M2 phenotype, an action associated with the activation of JAK/STAT6 signaling (Wang et al., 2014; Bhattacharjee et al., 2013; Czimmerer et al., 2018). Surprisingly, neutrophils have been implicated in priming macrophages into M2 during infections (Egholm et al., 2019). Besides, IL-4/IL-4 receptor signaling through STAT6 impairs NETs formation and chemotaxis of neutrophils (Impellizzieri et al., 2019; Woytschak et al., 2016). These findings suggest that IL-4R signaling on neutrophils may negatively impact host protection against *Leishmania* infection (**Figure 1.3**). IL-4/ IL-4Rα signaling stimulates the maturation of DC characterized with extensive dendrites and upregulation of MHCII, co-stimulatory molecules like CD86 and CCR7 receptors guiding effective migration of DC to lymphoid organs (Ahn and Agrawal, 2005; Saban, 2014). All these phenotypic markers are important in inducing DC effector functions. Interestingly absence of IL-4Rα signaling on inflammatory DCs impairs the production of iNOS, an enzyme that mediates the nitric oxide (NO) synthesis in myeloid cells important in killing intracellular parasites (Hurdayal et al., 2020). Under hypoxia conditions, IL-4Rα signaling inhibits the survival of neutrophils implying that during the early phase of infection, neutrophils may not be available to offer protection (Harris et al., 2019). Neutrophils are heterogeneous and in an environmental milieu with IL-4 and IL-13 cytokines promotes the shift to anti-inflammatory from pro-inflammatory neutrophils, hence negatively impacting host protection from *Leishmania* infection (Egholm et al., 2019).

Noteworthy, IL-4Rα is widely expressed in most cells, however, γc expression is low or absent in non-hematopoietic cells (McCormick and Heller, 2015). By extension, lymphocytes express low levels of IL-13Rα1 as opposed to γc. In line with this, lymphocytes express only type I receptors while myeloid cells express both type I and type II receptors (Junttila, 2018) (**Figure 1.3**). Comparatively, the IL-4Rα subunit is highly expressed in Th2 than Th1 cells explaining Th2 immune responses observed after IL-4 stimulation (Junttila, 2018).

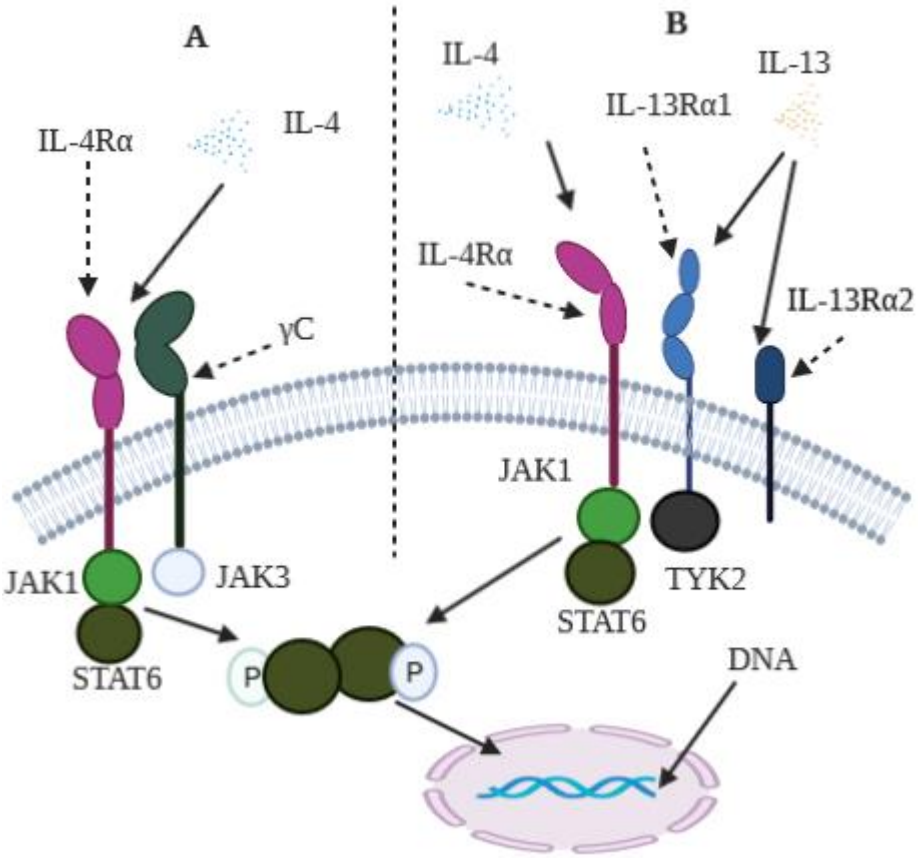

Figure 1.3: IL-4/IL-13 signaling through type I and type II receptors on the surface of cells. A) Type 1 receptor is constitutively expressed on most cells including myeloid and lymphocytes. B) Type 2 receptor is mainly expressed on the surface of myeloid cells and all non-hematopoietic cells. Both IL-4 and IL-13 can signal through the Type 2 receptor. Also, IL-13Rα2 can bind to IL-13 with high affinity. The cytokine-receptor complexes activate receptor-associated Janus Kinase proteins further activating signal transducer and activator of transcription (STAT) through phosphorylation. These proteins dimerize and translocate into the nucleus where they bind to transcription factors that bind promoter sites and transcribe genes downstream. The dashed arrow shows the receptor. (adapted from Junttila, 2018 and created by biorender).

The binding of IL-4 to IL-4Rα causes conformational changes in their intracellular domain that subsequently activates Jak kinase 3, JAK 1 or JAK 2 proteins associated with γc, IL-4Rα, and IL-13Rα1 (Morris et al., 2018). These proteins are capable of auto- or cross phosphorylating each other resulting in activation of either signal transducer and activator of transcription (STAT) 6 or Insulin receptor substrate (IRS) (Junttila, 2018). STAT6 forms dimers through Src Homology 2 domain, migrate into the nucleus and bind to the promoter sites on the DNA sequences causing Th2 expansion, IgE, and chemokine production (Chen and Jiang, 2013) (**Figure 1.3**). Importantly, the IL-4/STAT6 signaling pathway promotes

glucose metabolism a source of energy required for Th2 expansion (Maier et al., 2012; Ricardo-Gonzalez et al., 2010).

IL-13Rα2 is a monomer receptor that binds to IL-13 with high affinity hence making it unavailable in binding to dimeric IL-13Rα1 (Figure 1.3) (Lupardus et al., 2010). In the event IL-13 binds to IL-13Rα1, it forms a complex leading to the recruitment of IL-4Rα. The assembled IL-13/ IL-13Rα1/ IL-4Rα complex causes activation of STAT6 but not IRS (LaPorte, 2008).

1.1.6 Role of IL-4 and IL-13 cytokines during CL in an experimental mouse model

Interleukin (IL)-4 and IL-13 are the main cytokines involved in the expansion of Th2 cells involved in the severity of CL in BALB/c mice (Angkasekwinai and Dong, 2021; Hurdayal and Brombacher, 2014; Seyfizadeh et al., 2015). Besides Th2 cells, other sources of IL-4 and IL-13 include basophils, eosinophils, mast cells, and natural killer cells (Junttila, 2018; Akbari et al., 2003). IL-4 and IL-13 activate B cells to produce IgE and induce IgG1 class switching (Junttila, 2018). Moreover, IL-4 facilitates the migration of immune cells like T cells, monocytes to the site of inflammation by inducing vascular cell adhesion molecule 1 expression on vascular endothelium and reducing E-selectin expression (Andrews et al., 2006; LaPorte, 2008). IL-13 and IL-4 cytokines are vital in the polarization of macrophages to alternatively activate (M2) thus inhibiting the secretion of reactive oxygen and nitrogen species, TNF-α and IL-12 (Kolosowska et al., 2019; Sheikh et al., 2015). In contrast, early production of IL-4 protects the host from *Leishmania* infection via the instruction of DCs to produce IL-12 that drives Th1 immune responses (Hurdayal et al., 2013). This explains why IL-4 is regarded as a double-edged sword and it is an unsolved paradox in immune responses during cutaneous disease (Osero et al., 2020).

IL-4 indirectly controls the $CD8^+$ T cells proliferation and the generation of its long-term memory through the regulation of Fas and Bcl-2 expression (Riou et al., 2006). Besides, IL-4 also impairs the $CD8^+$ T protective response by negatively regulating the quality of their effector functions (Wijesundara et al., 2013).

During cutaneous infections, IL-4 signaling prevents the recruitment of neutrophils into the skin and their migration from bone marrow due to GM-CSF mediated upregulation of IL-4 receptors on neutrophils (Woytschak et al., 2016). Upon encounter with pathogens,

neutrophils engulf and neutralize the pathogen using intracellular granules and neutrophil extracellular traps (NETs), however, their phagocytic ability can be inhibited by the presence of IL-4 (Bober et al., 2000).

1.1.7 IL-4Rα signaling during CL infection in mice

Type 2-immune responses that mediate susceptibility in CL are modulated by both IL-4 and IL-13 (Hurdayal and Brombacher, 2017). Therefore, studies on the absence of IL-4, IL-13, and IL-4Rα in BALB/c mice have demonstrated the role of IL-4 and IL-13 in disease promotion during CL (Alexander et al., 2002; Kopf et al., 1996; Matthews et al., 2000; Mohrs et al., 2000; Mohrs et al., 1999; Satoskar et al., 1995). Paradoxically, other studies have demonstrated that IL-4 and IL-4Rα deficient mice remain susceptible to CL infection (Noben-Trauth et al., 1999). These differences in disease outcome may be due to other underlying factors like parasite substrain/species, developmental stage of parasite used, and site of infection.

Keen interest has been on studies involving IL-4Rα deletion on cell-specific to account for the different disease phenotypes exhibited during CL. IL-4Rα-mediated effects on $CD4^+$ T cells, macrophages, and neutrophils are involved in the promotion of CL disease caused by *L. major* (Hölscher et al., 2006; Radwanska et al., 2007). In contrast, IL-4Rα signaling on DCs is important in disease protection (Hurdayal et al., 2013). Whereas in BALB/c mice, IL-4Rα signaling on keratinocytes does not mediate protection in *L. major* infection (Govender et al., 2018). IL-4Rα-responsive B cells producing IL-4 mediates disease promotion via Th2 immune responses (Hurdayal et al., 2017). Whereas the BALB/c mice deficient of IL-4Rα signaling on macrophages and neutrophils intradermally infected with *L. mexicana* does not confer protection, IL-4Rα deficient $CD4^+$ T cell mice revealed sex-dependent disease protection (Bryson et al., 2011).

Overall, IL-4 receptor signaling on myeloid cells plays an important role in modulating type 2 immune responses (Heeb et al., 2020; Hurdayal et al., 2020; Egholm et al., 2019; Weng et al., 2018). Hence, studies investigation of IL-4Rα signaling on DCs, macrophages, and neutrophils in response to *L. mexicana* in BALB/c mice will unravel their role in CL protection in global IL-4Rα$^{-/-}$ mice.

1.1.8 Myeloid cells

Myeloid cells consisting of neutrophils, macrophages, and DCs develop in the yolk sac during the embryological stage through myelopoiesis, migrating into bone marrow (BM) and eventually releasing cells into circulation to exert immune responses (De Kleer et al., 2014) (**Figure 1.4**).

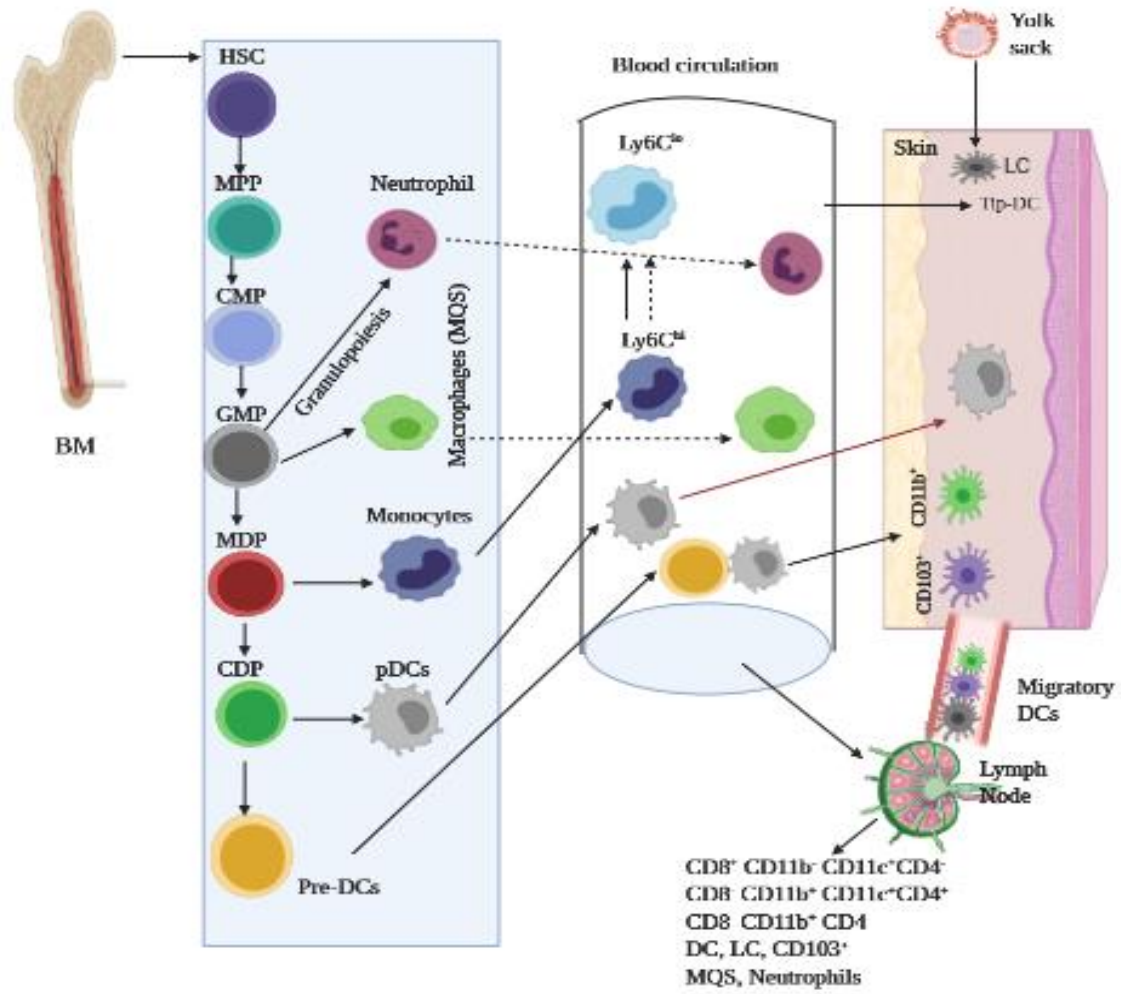

Figure 1.4: Ontogeny, classification, and migration of myeloid cells in mice during CL infection. Hematopoietic stem cells (HSCs) originating in the BM expand and mature giving rise to multipotent progenitors (MPPs). myeloid-lineage, common myeloid progenitor (CMP), and their progeny, granulocyte-monocyte progenitor (GMP) give rise to myeloid cells. Granulopoiesis gives rise to neutrophils whereas GMP commits to monocyte DC precursors (MDPs) that subsequently expand and mature to become a common DC precursor which gives rise to either Plasmacytoid DCs (pDCs) or precursor DC (pre-DCs). Pre-DCs move to the lymph node where they give rise to immature classical and conventional DCs (cDCs) which mature when activated by antigens delivered by Langerhans cells (LCs) from the peripheral tissues or through IL-4 signaling. Adapted from (De Kleer, 2014; Haniffa et al., 2013; Rosales, 2018; Takeuchi and Furue, 2007)

1.1.8.1 Ontogeny and classification of DCs

Dendritic cells play an important role in the regulation of immune responses through phagocytosis, antigen presentation to $CD4^+$T helper cells via MHCII molecules, and production of reactive oxygen species (ROS) for parasite killing (Fucikova et al., 2019; Savina, 2007). These cells are also capable of presenting immunogens to $CD8^+$ T helper cells via MHCI molecules (Bertholet, 2006; Joffre et al., 2012; Shen and Rock, 2006).

DCs originate from hematopoietic stem cells (HSCs) in the BM as either myeloid common precursors (MCP) or lymphoid common precursor (LCP) (Merad et al., 2013) (**Figure 1.4**). The myelopoiesis of DCs is a cascade of processes involving differentiation of MCP/LCP to common myeloid progenitors (CMPs), macrophage/DC progenitors (MDPs) common DC progenitors (CDPs), pre-DCs or plasmacytoid DCs (pDCs), and eventually to conventional DCs (cDCs) in the presence of GM-CSF (Lee et al., 2015; Naik et al., 2007; Onai et al., 2007; Fogg et al., 2006; Lutz et al., 1999).

Mouse cDCs can be categorized into cDC1 and cDC2 using the surface expression of CD8α and CD4. This include $CD11c^{hi}CD4^{-}CD8\alpha^{+}$ (cDC_1), $CD11c^{hi}CD4^{-}CD8\alpha^{-}$, and $CD11c^{hi}CD4^{+}CD8\alpha^{-}$ (cDC_2) (Guilliams and van de Laar, 2015; Zhou and Wu, 2017) (**Figure 1.4**). The $CD8^+$ cDCs can cross-present exogenous antigens through MHCI molecules hence playing a vital role in immune responses (Zanna et al., 2021). cDC1 are specialized in the production of IL-12p70 hence do not permit the proliferation of *Leishmania* parasites unlike cDC2 cells that encourage the survival of *Leishmania* parasites through differentiation of CD4 T cell to Th2 cells (Henri et al., 2002; Martinez-Lopez et al., 2015), cDC1 can induce tolerance and stimulate CTL responses (Bialecki et al., 2011; Hasegawa and Matsumoto, 2018).

During infection or inflammation, monocytes can differentiate into inflammatory monocytes also known as monocyte-derived DCs (MoDCs)/ inflammatory DCs/ TNF-iNOS DCs (Tip DCs) (**Figure 1.4**). These cells express Fc gamma receptor 1 (FcγR1) that mediate target cell elimination through antibody-dependent cellular cytotoxicity (Mildner et al., 2013; Stewart et al., 2014). Myeloid and lymphoid DCs have also been described in humans with differences depending on precursor, anatomical localization, function, and outcome of the immune response (Merad et al., 2013).

1.1.8.2. Role of DCs in immune responses during CL

Immature DCs found in circulation are in a constant survey of foreign particles or antigens and due to their high phagocytic and endocytic ability, they engulf the antigens (Sallusto, 1995). Noteworthy, phagocytosis of antigens activates DC maturation depending on the presence of IL-4 or IL-13 in their environmental milieu (Cook et al., 2012; Vento-Tormo et al., 2016). Mature DCs lose their phagocytic ability and gain MHC-II and co-stimulatory molecules, a process triggered by inflammatory stimuli such as IL-1β and TNF-α) and with the aid of CCR7 molecules, these cells migrate into draining LN where they present antigens to naïve T cells (Platt et al., 2010; Liu et al., 2021) (**Table 1.3**). Alternatively, LCs may interact with resident memory T cells in the skin (West and Bennet, 2017). DC drives the proliferation and differentiation of T cells to either Th1 or Th2 immune responses by secreting IL-12 and IL-4 or IL-10 cytokines respectively (Hurdayal et al., 2013; Schülke, 2018; Biedermann et al., 2001) (**Figure 1.2**).

Table 1.1: Major difference between mature and immature DCs

Immature DCs	**Mature DCs**
Round and spherical	Rough and boundless membranes with longer dendrites (Kim and Kim, 2019)
Upregulated endocytic and phagocytic receptors like mannose receptor, DEC-205, Fcγ I (CD64), and II (CD32)	Loss of endocytic and phagocytic receptors (Platt et al., 2010)
Downregulation of antigen-presenting molecules such as MHC II/I, CD86, CD40	Upregulation of antigen-presenting and co-stimulatory molecules such as MHCI/II, CD40, CD58, CD80 & CD86 (Kim and Kim, 2019)
Upregulated adhesive molecules such as L-selectin	Loss of adhesive structures such as L-selectin (Ye et al., 2017)
Downregulated migratory chemokines such as CCR6 and MIP-3α	Acquisition of high cellular motility molecules such as CCR6 and homing CCR7 (Liu et al., 2021)

Leishmania parasites can alter DC biology, favoring their persistence in the infected host (Martínez-López et al., 2018). Studies have demonstrated that *Leishmania* parasites inhibit

the differentiation of DCs from monocytes and several intracellular signaling pathways involved in the disease pathogenesis (Contreras et al., 2014).

Infection of C57BL/6 mice with *L. mexicana* limits the recruitment of Mo-DCs without affecting T cell proliferation in the LN (Hsu, 2007; Petritus, 2012). Therefore, regulation of DC recruitment to the infected skin and LN could also be used as a mechanism of immune evasion by *L. mexicana* parasites (Feijó et al., 2016). Monocyte-derived DCs play an important role in the development of protective immunity (Auffray, 2009; Rivollier, 2012; Serbina, 2008; Shi and Pamer, 2011). In some inflammatory settings or during infections by viral, bacterial, fungal, or parasitic agents, Mo-DC differentiates into tumor necrosis factor (TNF) and Tip-DC with potent antimicrobial effector functions also known to be crucial in killing *Leishmania* parasites (Aldridge, 2009; Bain, 2013; Serbina, 2003; Tamoutounour, 2012). In addition to NO production, Mo-DCs migrate into the pLN where they stimulate antigen-specific Th1 T cell responses. Indeed, in *L. major* infection, Mo-DCs induce LN hypertrophy promoting additional recruitment and differentiation of naïve T cells into the Th1 enhancing the protective response (Carvalho, 2012a). In contrast, *L. mexicana* amastigotes downregulate the activation of mitogen-activated protein kinase but activate PI3K and AKT thereby inhibiting apoptosis of infected Mo-DCs (Contreras et al., 2014; Vázquez-López et al., 2015).

Downregulation of apoptosis, which is a key host-dependent response mechanism has been exhibited in *L. mexicana* infected Mo-DCs induced by treatment with camptothecin, a known inducer of apoptosis in all nucleated mammalian cells (Valdés-Reyes et al., 2009). This suggests that *L. mexicana* has the capacity of delaying apoptosis in the infected Mo-DC hence increasing disease severity (Gutiérrez-Kobeh et al., 2013).

Dendritic cells have surface molecules that are a target of *Leishmania* parasites. These include OX40-Ligand (OX40L) and programmed cell death protein-ligand 1/2 among others (Autenrieth et al., 2015; Kong et al., 2019). OX40L regulates IL-10 production from regulatory T cells (Ito, 2006). Furthermore, OX40L-OX40 signaling promotes the expression of inflammatory mediators and T cell proliferation (Lei et al., 2018), and consequently, $Ox40L^{-/-}$ mice become susceptible to *L. major* infection (Akiba et al., 2000; Tuladhar et al., 2015). *L. mexicana* promastigotes are easily phagocytosed by DC-specific intercellular adhesion molecule-3-grabbing non-integrin receptor to alter cytokine production thus impacting the virulence (Argueta-Donohué et al., 2016; Rivera-Fernández et al., 2019).

Overall, these findings indicated that modulation of DC surface molecules can alter the disease outcome.

1.1.8.3 Role of IL-4Rα in maturation and activation of DCs during CL infection

Interleukin-4 and IL-13 cytokines are involved in the maturation of DCs (Webb et al., 2007; Ahn et al., 2005). Surprisingly, during the early phase of *L. major* and *L. mexicana* infections, the expression of MHCII or co-stimulatory molecules, such as CD80, CD86, and CD40 on DCs are unchanged between IL-4Rα deficient BMDCs and IL-4Rα sufficient BMDCs in culture (Osero et al., 2022: Hurdayal et al., 2013). These findings are indicative of the little role of IL-4Rα signaling on DC in their maturation and activation in the acute phase of CL infections. On the hand, in the late phase of *L. major* infections, $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice have reduced MHCII expression on DCs, contrary to *L. mexicana* infections suggesting that the expression of MHCII and co-stimulatory molecules on DCs is species-specific dependent in the late phase of CL (Hurdayal et al., 2020; Osero et al., 2022). In contrast, $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice immunized with rIL-4/CPG/*Leishmania major* antigen conditioned IL-4Rα deficient BMDCs cannot either induce the maturation/activation of DCs Th1 immune responses, consequently leading to uncontrolled lesion development and parasite proliferation during *L. major* infections (Masic et al., 2012). This demonstrates that mice clinically protected from *L. major* infections, possess high populations of mature and activated DCs.

1.1.8.4 Ontogeny and classification of neutrophils

Neutrophils are generated through granulopoiesis (**Figure 1.4**), a process that is dependent on cytokines such as; IL-23, IL-17A, and granulocyte colony-stimulating growth factors (Borregaard, 2010; Stark et al., 2005). Granulopoiesis involves several stages including myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear in an environment rich in CCAAT/enhancer-binding protein α that facilitate the differentiation of granulocytes (Hirai et al., 2006). From bone marrow, neutrophils migrate into circulation with the aid of CXCR4 or CXCR2 chemokines (Eash et al., 2010).

Neutrophils may comprise two distinct phenotypes during inflammation named Polymorphonuclear (PMN)-1 and PMN-2. The PMN-1 and PMN-1 are specialized in IL-2 and IL-10 production respectively (Yang et al., 2019; Tsuda et al., 2004)

1.1.8.5 IL-4/IL-13 signaling on neutrophils during CL in mice

Neutrophil cells are the most abundant leukocytes in blood circulation (50-70%) and are rapidly recruited to the site of infection (Amulic et al., 2012; Mayadas et al., 2014; Regli et al., 2020). They respond to infections by employing mechanisms that lead to killing or inactivating the pathogen through phagocytosis, degranulation, production of ROS, and NETs formation (Egholm et al., 2019; Guimarães-Costa et al., 2014). However, during CL, neutrophils can kill or provide a safe niche for the entry of parasites, a process that is *Leishmania* spp dependent. More importantly, IL-4 and IL-13 signaling through IL-4 receptors on neutrophils inhibit these effector functions (Heeb et al., 2020). In support, mice treated with IL-4 during cutaneous infections worsen the disease due to decreasing CXCR2 expression, hence impairing neutrophil migration. On contrary, these actions are absent in IL-4Rα-deficient mice (Woytschak et al., 2016).

IL-4Rα-deficient mice have increased neutrophil infiltration and worsened inflammatory diseases however they are protected from *L. major* infections (Panda et al., 2020; Schmid et al., 2018; Cao et al., 2007; Mohrs et al., 2000). Similarly, mice with IL-4Rα-macrophage/neutrophil-specific deletion demonstrated the control of *L. major* infection (Hölscher et al., 2006). In contrast, the same mouse strain was not protected from *L. mexicana* when infected in the base on the tail (Bryson et al., 2011). So far, no studies involving footpad infection of mice with IL-4Rα deficiency in macrophage/neutrophil cells with *L. mexicana*.

1.1.8.6 Role of neutrophils in cutaneous leishmaniasis

Neutrophil interacts with DCs preventing DC activation depending on the infecting pathogen (Schuster et al., 2013). In some infections, neutrophils release TNF-α, IL-4, and IFN-γ cytokines that can induce DC and T cell maturation both *in vitro* and *in vivo* experiments (Gideon et al., 2019)

Phagocytosis of apoptotic neutrophils by DCs may lead to downregulation of their co-stimulatory molecules hence affecting T cell differentiation (Clayton et al., 2003). However, in some pathogen infections like *M. tuberculosis* and *L. major* apoptosis of neutrophils is delayed affecting antigen uptake by DCs consequently causing a delay in T cell activation (Aga et al., 2002; Blomgran et al., 2012).

After ingesting *Leishmania* parasites, neutrophils secrete secondary granules containing lactoferrin and gelatinase that exert antimicrobial activity through the initiation of inflammatory responses (Mantovani et al., 2011; Nathan, 2006). The engulfed pathogens can also be trapped and killed by histones and/or antimicrobial granule-derived proteins released by NETs (Brinkmann, 2004; Rosales, 2018) (**Figure 1.5**).

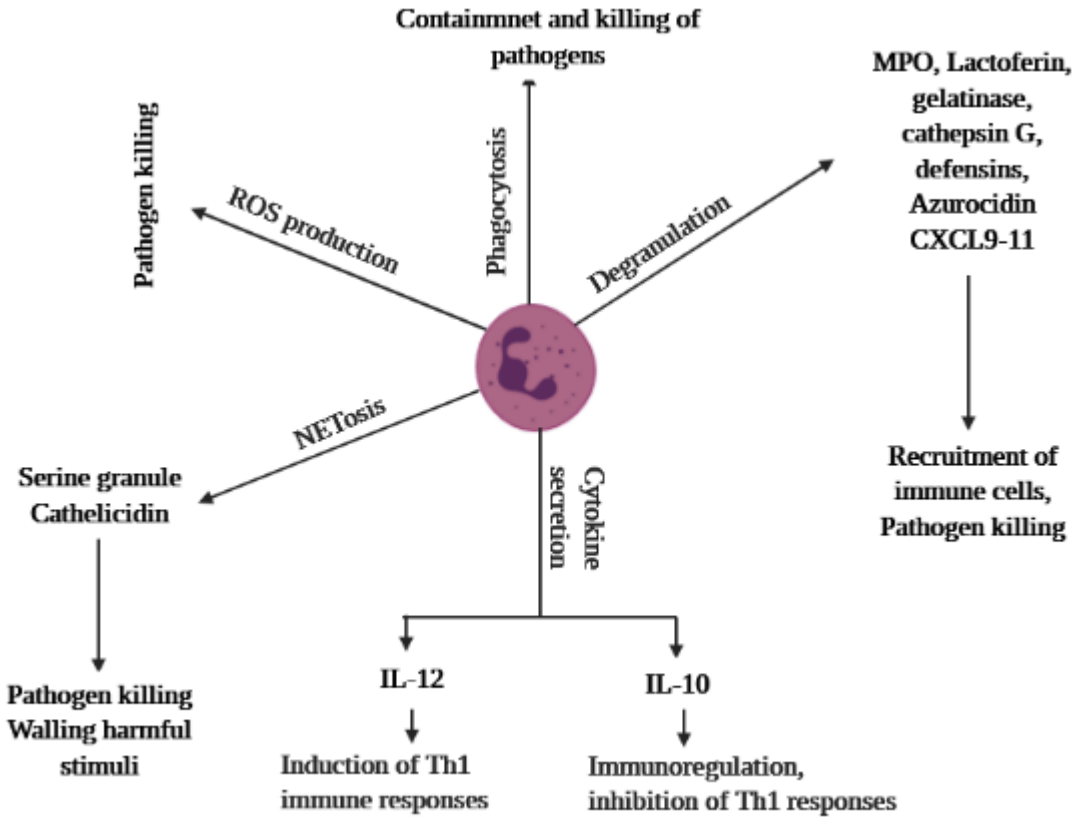

Figure 1.5: Effector functions of neutrophils during *Leishmania* infection. Neutrophils secrete cytokines secretion (IL-12), NETosis, degranulation, and ROS production that leads to the killing of pathogens. Secretion of IL-10 by neutrophils inhibits Th1 immune responses and prevents the production of reactive nitrogen species by macrophages. More interestingly, during CL, neutrophils can kill or provide a safe niche for the entry of parasites, a process that is *Leishmania* spp dependent (Adapted from Mayadas et al., 2014).

Upon *Leishmania* infection, neutrophils efficiently kill the parasites via ROS production and formation of NETs in a process called NETosis (Mayadas et al., 2014). Neutrophils also release cytokines and chemokines that activate and/or recruit macrophages, marginal B cells, and DCs thus may greatly contribute to shaping the adaptive immune response during *Leishmania* infections (Boaventura et al., 2010; Puga et al., 2011; Schuster et al., 2013). However, most *Leishmania* spp have devised mechanisms that enable them to escape the killing by neutrophils (Carlsen et al., 2015a). Early neutrophil recruitment, in *L. mexicana*

infection, contributes to the development of nonhealing lesions with uncontrolled parasite growth due to the absence of a protective immune response (Hurrell et al., 2015). Mechanistically, the uptake of *L. mexicana* amastigotes by neutrophils had no impact on its activation and apoptosis but had increased IL-10 levels leading to parasite persistence hence qualifying as reservoirs (Hurrell et al., 2017) (**Figure 1.5**). Besides, chronic inflammation in the late phase of *L. mexicana* infected BALB/c mice is associated with excessive neutrophils recruitment together with Th17 cell differentiation (Pedraza-Zamora et al., 2017).

More importantly, neutrophils can produce cytokines that regulate macrophages consequently modulate inflammation (Chen and Jiang, 2013). For example, Polymorphonuclear (PMN)-1 leukocytes can play a vital role in host protection against CL by producing IL-12/IFN-γ which classically activates macrophages whereas PMN-2 secrete IL-10 that aid the differentiation of M2 macrophages phenotype leading to CL disease severity (Tecchio et al., 2014).

1.1.8.7 Ontogeny of macrophages

Macrophages are derived from embryonic progenitors that have been described as either yolk sac or fetal monocytes. More importantly, the yolk sac embryonic precursors are proposed to be the main precursors for tissue-resident macrophages (Gomez Perdiguero et al., 2015; Perdiguero and Geissmann, 2015). Nevertheless, during embryonic hematopoiesis, the embryonic yolk sac (YS) gives rise to erythroblasts, megakaryocytes, and macrophages (Bertrand et al., 2005).

1.1.8.8 Macrophages polarization in immune responses during CL

Macrophages play a sentinel role against pathogens with efficient pathogen recognition receptors that make them efficient for phagocytosis, induction of inflammatory cytokines, and antimicrobial molecules (Arango Duque et al., 2014). Macrophages are involved in pathogen surveillance, recycling of nutrients, and organismal homeostasis (Cairo et al., 2011; Flannagan et al., 2010; Sieweke and Allen, 2013). Surprisingly, macrophages can cause tissue damage and contributes to disease pathology (Bashir et al., 2016; Burdo et al., 2015).

Macrophages can be activated into two different states namely, M1 and M2 (Abdelaziz et al., 2020) (**Figure 1.6**). The direction of polarization of either phenotype depends on the

mediators in their environmental milieu. For instance, in the presence of IFN-γ, IL-12, LPS, and GM-CSF, macrophages undergo classical activation whereas IL-4/IL-13 (through IL-4Rα), IL-10, TGF-β, favor alternatively activated macrophage (Jaguin et al., 2013; Martinez and Gordon, 2014; Sica and Mantovani, 2012; Zhang et al., 2017).

Classically activated macrophages (M1) exhibit high phagocytic ability and strong microbicidal properties. M1 cells are stimulated by IFN-γ through iNOS and NADPH oxidase to produce reactive oxygen and nitrogen intermediates including nitrite (NO_2^-), Dinitrogen dioxide, nitrate, and oxoperoxonitrate toxic to intracellular pathogens such as bacteria and *Leishmania* (Carneiro et al., 2016; Horta et al., 2012; Hostetter et al., 2005). Nitric oxide is toxic to intracellular pathogens through multiple mechanisms involving inhibition of synthesis, oxidation, and degradation of DNA. Nitric oxide also through S-nitrosylation of proteins, tyrosine nitration, deprivation of iron kills parasites (Pourbagher-Shahri et al., 2021).

Leishmania induces alternatively activated macrophages to activate arginase-1, which mediates the production of l-ornithine essential for the synthesis of polyamines and urea, and at the same time suppress iNOS thereby favoring the growth and survival of the parasites (Kropf et al., 2005; Muxel et al., 2017) (**Figure 1.6**). M2 phenotypes are specialized in the production of IL-13, IL-10 and TGF-β, anti-inflammatory cytokines, that are involved in host susceptibility to *Leishmania* infection (Jaguin et al., 2013; Tarique et al., 2015).

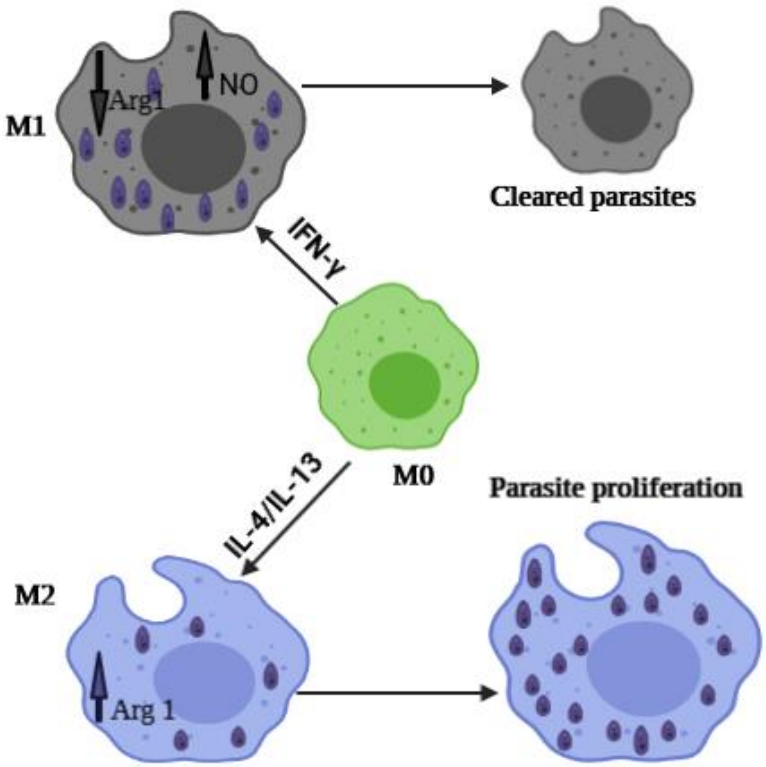

Figure 1.6: Polarization of macrophages into different phenotypes during Leishmania infection. Polarizing signals like IFN-γ induce classically activated (M1) macrophages that produce significantly high amounts of NO, toxic to *Leishmania* parasites hence clearance of parasites controlling the infection. In contrast activation by either IL-4 or IL-13 or both results in alternatively activated (M2) macrophages that produce significantly increased arginase 1 (Arg 1) and decreased NO, hence survival and proliferation of parasites leading to disease severity (adapted from Abdelaziz et al., 2020).

Mechanistically, Fc gamma receptors (FcγR) expressed on the surface of M2 macrophages bind the Fc portion of IgG1 antibodies facilitating the uptake of *L. mexicana* and *L. amazonensis* leading to IL-10 secretion suppressing Th1 immune responses (Buxbaum and Scott, 2005). Moreover, *Leishmania* killing is also enhanced by the activation of macrophage immunoproteasome involved in antigen processing and presentation to lymphocytes through MHC molecules (Olivier et al., 2005). Therefore, the balance between M1 and M2 is critical in regulating inflammatory responses, maintenance of immune homeostasis, and wound healing in CL as demonstrated in other infections (Boothby et al., 2020; Han et al., 2021; Krzyszczyk et al., 2018; Mosser et al., 2021).

Leishmania mexicana parasites maximally utilize the nutrient available in the phagolysosome of macrophages by downregulating glucose transport and increasing the catabolism of fatty acids (Burchmore and Barrett, 2001; Burchmore et al., 2003)). Moreover, depletion of

Leishmania iron transporter 1 in *L. amonzonensis* abolishes the differentiation of promastigotes to amastigotes (Mittra et al., 2013).

Lipophosphoglycan (LPG) permits the survival of *Leishmania* parasites by inhibiting macrophage phagolysosomal biogenesis (Franco et al., 2012). Disruption of phagocytosis, maturation of phagosomes, and proteins that aid vesicle trafficking affects the survival of *Leishmania* parasites (Arango Duque et al., 2013).

Studies on *L. braziliensis* and *L. amazonensis* have shown increased macrophage superoxide dismutase expression that favor parasite survival (Khouri et al., 2009). Incidentally, *L. mexicana* uses macrophages as reservoirs and attenuates the expression of IFNγ-R thus preventing the production of NO by macrophages (Abu-Dayyeh et al., 2010; Bhardwaj et al., 2005). Additionally, *L. mexicana* exosomes enable parasite survival in macrophages by dampening immune response specifically by decreasing the microbicidal activity and MHC-1 and CD86 expression (Soto-Serna et al., 2020).

Overall, the survival or killing depends on the macrophage phenotype and how the parasite employs mechanisms that enable them to evade the macrophage effector functions.

1.1.8.9 IL-4Rα signaling on macrophages in CL

Interleukin-4 and IL-13 signaling via IL-4Rα facilitates the differentiation of macrophages to M2 phenotype (Jaguin et al., 2013). Mice with a deficiency of IL-4Rα expression on macrophages/neutrophils have suppressed M2-related genes like Mrc1 and Arg1 but increased iNOS mRNA (Weng et al., 2018). In addition, the global knockout of IL-4Rα in mice (IL-4Rα$^{-/-}$ mice) suppresses the expression of mannose receptors by macrophages (Linehan et al., 2003). Overall, these findings consistently demonstrate that IL-4Rα regulates the polarization of macrophages to the M2 phenotype. BALB/c mice deficient of IL-4 and IL-4Rα have been shown to control CL caused by *L. major* (Kopf et al., 1996; Mohrs et al., 1999), paradoxically in some studies, these mouse strains were unable to suppress parasite proliferation and survival at the site of infection (Noben-Trauth et al., 1999; Noben-Trauth et al., 1997). However, this discrepancy is believed to occur due to the sub-strain and virulence of *Leishmania* parasites used (Hurdayal and Brombacher, 2017). Hence, the use of BALB/c mice with cell-specific IL-4Rα deficiency e.g macrophages/neutrophils-IL-4Rα deficient

mice) provides an experimental model that can be used to interrogate how IL-4Rα signaling on macrophages influences the outcome of CL disease.

Therefore, BALB/c mice deficient of IL-4Rα signaling on macrophages and neutrophils were protected from *L. major* infection in the footpad, characterized with NO producing M1 macrophage phenotypes, despite a type 2 immune response rich environment (Hölscher et al., 2006). In contrast, a similar mouse strain subcutaneously infected in the rump did not control CL disease following infection with *L. mexicana* amastigotes (Bryson et al., 2011).

Overall, it is now clear that CL is dependent on several factors ranging from parasite species, site of infection, the dosage of infecting parasite, and developmental stage of parasites.

1.1.9 Factors affecting immune response and disease outcome in CL

Factors such as the developmental stage, dose, species, strain, and sub-strain of *Leishmania* parasites used, and route/site of infection plays a critical role in directing the immune responses and disease outcome in experimental models of CL (Mahmoudzadeh-Niknam et al., 2007; Sacks and Noben-Trauth, 2002; Tripathi et al., 2007). In addition, host genes and pathways in relation to the aforementioned factors also modulate disease outcome, as shown in IL-4-deficient mice (Mohrs et al., 2000; Mohrs et al., 1999).

1.1.9.1 Route of infection

The skin is a natural route of infection equipped with myeloid cells namely DCs, macrophages, and neutrophils, all with potent immunomodulatory effects (Quaresma, 2019). Subcutaneous and intradermal injections of *Leishmania* parasites into the footpad and ear respectively have been known to initiate infection in experimental mouse models of cutaneous leishmaniasis (Belkaid et al., 1998). In support, a study revealed increased pathogenicity in BALB/c/c mice subcutaneously infected in the footpad with *L. major* compared to those infected intradermally (Sarreshteh et al., 2017). In addition, BALB/c and B6 mice infected in the rump become susceptible to *L. amazonesis* when compared to those infected in the footpad (Felizardo et al., 2012). On contrary, IL-4 deficient B6 mice infected with *L. amazonesis* in the rump are protected but IL-4 deficient BALB/c mice infected in the same site have severe disease (Felizardo et al., 2012). DBA/2 mice infected on the ear control cutaneous disease caused by *L. major* but become susceptible when infected in the base of the tail (Baldwin et al., 2003).

Overall, it is important to investigate whether the route/site of infection influences the CL disease outcome during *L. mexicana* infection to determine their role in protection in IL-4Rα$^{-/-}$ BALB/c mice.

1.1.9.2 Parasite dose

Mice infection with a particular dose of parasite inoculum may influence the protection or progression of CL. Low dose (100 metacyclic promastigotes) of *L. major* infection into the ear dermis of C57BL/6 cause non-healing lesion when compared to high doses ($2x10^6$ metacyclic promastigotes) (Ribeiro-Gomes et al., 2014). This may be due to excessive antigens present in high dose infection are taken up by other cells including dendritic cells, that produce IL-12 in response to *L. major* infection resulting in expansion and activation of Th1 cells (Konecny et al., 1999; Moll, 2003; von Stebut et al., 1998).

Surprisingly, the kinetics of lesion development and the onset of disease protection in IL-13$^{-/-}$ mice are dependent on the infecting parasite dose. Additionally, infection of IL-13$^{-/-}$ mice with a high inoculum dose of *L. mexicana* amastigotes cause parasite persistence and enhanced lesion development (Alexander et al., 2002). Following high dose infection with *L. major Seidman*, BALB/c mice develop more severe pathology compared to a low dose (Kropf et al., 2003).

Altogether, these studies suggest that the ability of the dose of inoculum of the parasite to influence the course of infection is augmented by parasite species, their developmental stage, and the genetic background of the host mice.

1.1.9.3 Developmental stage of *Leishmania* parasites

Under laboratory conditions, the two major cell morphologies of *Leishmania* parasites, exemplified by promastigotes and amastigotes, can be used to initiate disease in mice to mimic natural infection by sand flies. High expression of tryparedoxin peroxidase isoform present in amastigotes of *L. amazonensis*, but absent in promastigotes, shown to antagonize host-derived antimicrobial products hence increasing the disease severity (Barr and Gedamu, 2003; Henard et al., 2014; Jirata et al., 2006). Additionally, amastigotes can survive in the phagolysosome by avoiding NO activity as highlighted during *L. pifanoi* and *L. mexicana* infections (Pham et al., 2005; Wilkins-Rodríguez et al., 2010).

Differential microbicidal activity of neutrophils against promastigotes and amastigotes feature during chronic cutaneous, diffuse cutaneous, and mucocutaneous infections (Boaventura et al., 2010; Daboul, 2010; Dantas et al., 2014). Briefly, *L. amazonensis* amastigotes are resistant to the microbicidal activity of neutrophils leading to severe disease as compared to infection with promastigotes (Carlsen et al., 2013; Carlsen et al., 2015a). In addition, *L. mexicana* amastigotes are more virulent due to the high expression of cysteine proteinase b compared to metacyclic promastigotes (Abu-Dayyeh et al., 2010; Souza, 1992).

Leishmania amastigotes constitutively express potential virulence factors such as cysteine protease, glycosyl inositolphospholipids, and zinc-metalloprotease glycoprotein (GP)[63] and LPG compared to promastigote form suggesting the difference in disease severity (Schneider et al., 1993; Winter et al., 1994). Most importantly, their differential alteration of NF-κB may also be the reason for their virulence pattern (Abu-Dayyeh et al., 2010).

1.1.9.4 *Leishmania* species, strains, and sub-strains

The strain of *Leishmania* parasite species can play a vital role in influencing the severity of the disease. The severity of CL may be due to different strains or species of *L. major* (Musa et al., 2019; Lemos de Souza et al., 2000). For instance, C57BL/6 mice are traditionally known to control *L. major* infection but susceptible to *L. mexicana* infection (Bhardwaj et al., 2005; Loeuillet et al., 2016).

To demonstrate how different parasite species influence CL outcome, IL-4 deficient mice become protected against *L. amazonensis* infection even after treatment with exogenous IL-12 but protected from *L. major* and *L. mexicana* infections (Jones et al., 2000; Satoskar et al., 1995). Notwithstanding IL-$4^{-/-}$ mice are unable to control *L. major* strain LV39 suggesting the strain of parasite critically influences disease outcome (Kropf et al., 2003; Noben-Trauth et al., 1996).

Comparatively, studies using amastigotes of *L. major* and *L. tropica* have also complemented the parasite species-specific dependent disease outcome in CL. In support, BALB/c mice become susceptible to *L. major* amastigotes when infected in the base of the tail, but resistant to *L. tropica* infection (Spotin et al., 2014).
Overall, using different species of *Leishmania* that cause CL disease in an experimental mouse model, will comparatively provide knowledge of how different cell types influence the

disease outcome. More importantly, their dose and site of infection together with the aspect of the developmental stage of the parasite can be incorporated in experimental murine mouse studies to investigate their role in determining the outcome of CL disease.

1.1.10 Rationale

Interleukin-4 and IL-13 share a common receptor, IL-4Rα, whose effects in CL have been widely studied in *L. major* infection and to some extent in *L. mexicana*. More importantly, IL-4/IL-13 modulates the maturation and activation of DCs hence regulating the Th1 immune responses via IL-4 instruction on DC-IL-12 production (Hochrein et al., 2000). In addition, IL-4Rα signaling on macrophages mediates a switch of M1 towards M2 macrophages phenotype polarization (Weng et al., 2018). Control of CL infection is aided by Th1 immune responses, whereas effector functions of the M2 phenotype permeate parasite survival-promoting the infection. Hence studies targeting IL-4Rα signaling on these myeloid cells will provide insight into their role in CL. The phenotypic difference of IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ from WT BALB/c mice after infection with *L. mexicana* revealed the control of the infection with characteristic decreased lesion development and parasite burden (Alexander et al., 2002; Satoskar et al., 1995).

Recently, studies on the deficiency of IL-4Rα signaling on CD4 T cells reveal resistance of mice to *L. major* infections but sex-dependent in *L. mexicana* infected BALB/c mice; with controlled infection in female mice but persisted infection in adult male mice (Bryson et al., 2011). This study, therefore, demonstrated that IL-4Rα signaling on non-lymphocyte cells may be involved in protection in IL-$4R\alpha^{-/-}$ mice. Contrary to this, IL-4Rα deficiency on macrophages/neutrophils did not render BALB/c mice resistant to *L. mexicana* amastigote infection in the rump (Bryson et al., 2011). However, CL studies in *L. major* infected BALB/c mice have reported a protective role of IL-4Rα signaling in macrophages/neutrophils characterized with increased leishmanicidal activity despite a normal Th2 immune response (Hölscher et al., 2006).

Further studies have shown that BALB/c mice lacking IL-4Rα signaling in DCs become hyper susceptible to cutaneous *L. major* infection in the footpad (Hurdayal et al., 2013). These mice had upregulated Th2 responses and impaired macrophage killing capacity, pointing to the critical role of IL-4Rα signaling on DCs in protection against CL caused by *L. major*. Hence, it could be possible that the absence of IL-4Rα signaling on DCs in IL-$4R\alpha^{-/-}$

mice infected with *L. mexicana* could have contributed to disease susceptibility. However, differences in the outcome of CL have been shown in BALB/c mice depending on the infecting *Leishmania* species, the developmental stage of the parasite, and the site of infection (Spotin and Parvizi, 2016; Spotin et al., 2014). Owing to this, the current study sought to investigate the importance of IL-4Rα signaling on DCs, macrophages/neutrophils in the control of *L. mexicana* infection in BALB/c mice. To this end, this study utilized BALB/c mice with a specific deletion of the IL-4Rα on DCs and macrophages/neutrophils (Hölscher et al., 2006; Hurdayal et al., 2013; Nieuwenhuizen et al., 2018). Herein, the clinical phenotype, humoral and cellular responses of BALB/c mice with IL-4Rα deficiency on DCs and macrophage/neutrophil cells during experimental footpad infection with *L. mexicana* promastigotes are described.

1.1.11 Study objectives

1. To investigate on the impact of IL-4Rα signaling on DCs on the disease phenotype and immune response to *L. mexicana* infection.
2. To investigate on the impact of IL-4Rα signaling on macrophages/neutrophils on the disease phenotype and immune response to *L. mexicana* infection.

CHAPTER 2:

MATERIALS AND METHODS

CHAPTER 2:

MATERIALS AND METHODS

2.1 Mice

Six to 8-week old male or female IL-4Rα$^{-/-}$ (Mohrs et al., 1999), CD11c^{cre}IL-4Rα$^{-/lox}$ (Hurdayal et al., 2013), LysM ^{cre}IL-4Rα$^{-/lox}$ (Hölscher et al., 2006) BALB/c mice with age and sex-matched IL-4Ra$^{-/lox}$ littermates were used for *L. mexicana* challenge experiments. CD11c^{cre}IL-4Rα$^{-/lox}$ mice were generated from CD11c^{cre}C57/BL6 mice inter-crossed with IL4Rα$^{flox/flox}$ BALB/c mice (Herbert et al., 2004) and homozygous IL-4Rα$^{-/-}$ BALB/c mice and backcrossed to a BALB/c background for nine generations. CD11c^{cre}IL4Rα$^{-/lox}$ mice have a deficiency of IL-4Rα signaling on DCs. Transgene-bearing CD11c^{cre}IL4Rα$^{-/lox}$ were identified by PCR genotyping as described by Hurdayal et al., 2013. Similarly, macrophages/neutrophils IL-4Rα-deficient BALB/c mice were generated using LysM^{cre} mice, an F_9 BALB/c mouse (Clausen et al., 1999), were intercrossed with conditional IL-4Rα$^{flox/flox}$ BALBL/c mice and further mated with complete IL-4Rα$^{-/-}$ BALB/c mice to generate LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice (Hölscher et al., 2006).

Hemizygous littermates (IL-4Rα$^{-/lox}$) mice with functional one allele of IL-4Rα were used as controls in all experiments. On analysis of IL-4Rα expression using flow cytometry, IL-4Rα was efficiently depleted in DCs and macrophages/neutrophils of the lymph nodes when compared with IL-4Rα$^{-/lox}$ littermate controls (Hurdayal et al., 2013; Hölscher et al., 2006). These mice were bred and housed in a temperature-controlled room under a 12-hour light/12-hour dark cycle and maintained under specific-pathogen-free conditions in the animal breeding facility of the University of Cape Town (UCT).

All experiments were done as approved by the Institutional Faculty of Health Sciences, Animal Research Ethics Committee (AEC) (AEC number: 019/001). Animal suffering was minimized as indicated in the protocol

2.2 Parasite culture

Leishmania mexicana parasites from liquid nitrogen were de-frozen and placed in an aseptic biosafety cabinet cleaned with 70% ethanol or 1% varlkon. Parasites were transferred into a 50ml centrifuge tube and washed by resuspending in 1ml of filtered 1xPBS and finally topped to 10ml of 1x phosphate buffer saline (PBS) and centrifuged at 3000 rpm at 4^{o}C for

10min. the pellet was resuspended in 1ml of 1xPBS before topping up to 10ml with 1xPBS. The process of washing was repeated three times and the final pellet was resuspended in 1xPBS and added into a 25cm^3 culture flask containing fresh filtered M199 media with 10% Fetal calf serum (FCS) and 0.5% penicillin/streptomycin antibiotics. The flask was placed in an incubator at 27ºC for 5-7 days until they fully differentiated into stationary promastigotes. The parasites were passaged/split in 3-4 times for good yields.

2.3 Growth curve for parasite

Parasites from the incubator were transferred from the culture flasks into centrifuge tubes and centrifuged at 3000 rpm for 5min at 25ºC. The pellets were resuspended in 10ml of 1xPBS and again centrifuged as above. This procedure was repeated two times and finally, the pellet was resuspended in 1ml of 1xPBS, and parasites were counted using a Neubauer chamber in ratio 1:10. Briefly, 10µl of parasite suspension were mixed with 90µl of paraformaldehyde. Of the counted parasites 10x10^6 parasites were calculated and the equivalent amount of parasite suspension was added into freshly prepared 10ml of M199 media with 10% FCS and 0.5% Penicillin/streptomycin antibiotic. The parasites were left to grow in a 27ºC incubator while counting daily until they reached the infecting stationary phase signaling mature and infective promastigotes. Stationary phase promastigote growth was reached 5–6 days after initiating promastigote culture from a starting concentration of 10^6 promastigotes per/ml.

2.4 Preparation of parasites for mice infection

Leishmania mexicana parasites at the plateau phase are transferred into the 50ml centrifuged tube and washed with 1xPBS by centrifuging at 3000rpm for 5min at 25ºC. the pellets were washed by resuspending in 10ml of 1xPBS and centrifuged at 3000rpm for 5min at 25ºC. the above procedure was repeated three times and parasites were counted using a Neubauer chamber as described in **2.3**. Of the counted parasites an equal amount of parasite suspension representing 50µl was used for infection

Mice were anesthetized with an intraperitoneal injection of Xylazine (10mg/kg) + Ketamine (100mg/kg) using a 30G needle of a 1ml insulin syringe. Mice were monitored for 5 minutes, and anaesthesia was confirmed by palpebral reflexes, rate, depth of respiration, and absence of any muscular reflex after pinching of the foot toes. Mice were injected subcutaneously with 50 µl of 2× 10^6 parasites using a 30-gauge needle into the footpad of anesthetized mice.

2.5 Evaluation of disease clinical outcome

Mice were monitored daily for discomfort/stress, activity, movement, piloerection, isolation, hunched posture of the mice, and their lesion development in the footpad was measured to assess disease clinical outcome. The thicknesses of the footpad were measured by a dial-gauge caliper weekly for 8 weeks. Weights of mice were also measured weekly to assess their health status.

2.6 Quantification of Parasite load using limiting dilution assay

At 8 weeks post-infection, mice were euthanized using halothane and parasite quantification in the infected footpad, and popliteal lymph nodes (pLNs) were performed using a limiting dilution assay. The homogenates of footpad tissues were prepared and resuspended in M199 media, whereas lymph nodes cell suspensions were prepared by mechanical digestion of pLNs and passing through 40µm cell strainers. LN cells were washed through with 10% FCS DMEM supplemented with 5ml HEPES, 500ul 1x β-mercaptoethanol, and 2.5ml of pen/strip (100 U/ml/100 µg/ml, respectively. 200ul of footpad homogenates and lymph node cell suspensions were added into respective neat wells for each mouse in a 96-well plate containing 100ul of complete DMEM. A serial 1:2 dilution was made for footpads and lymph node homogenates and the plates were incubated at 26 ˚C for 7 days after which the lowest dilution at which parasites were observed (2^n) was determined using a microscope.

2.7 Analysis of myeloid and lymphoid cells by flow cytometry

Single-cell suspension of 1×10^6 cells from the pLNs was stimulated with 200µg/ml phorbol myristate acetate (PMA) and 1mg/ml ionomycin for 2 hours followed by 4 hours incubation with 2mM monensin to inhibit protein trafficking through the Golgi. The cells were stained with 50 µl FACs buffer containing 1% rat serum, 1% FC-γ blocker, and a cocktail of fluorophore-conjugated antibodies for 30 minutes at 4^{o}C in the dark. Antibodies used included surface extracellular staining markers: Ly6C-PerCPCy5.5 (Clone AL-21), CD11b-V450 (Clone M1/70), AF700-MHCII (clone, M5/114), CD11c-APC (clone, HL3), PE-Cy7-F4/80 (clone, BM8), CD44-FITC (Clone IM7), CD4-V500 (Clone RM4-5), CD3-AF700 (Clone 500A2), CD62L-V450 (Clone MEL-14), CD19-PerCPCy5.5 (Clone 1D3), CD8-APC (Clone 53-6.7). For intracellular cytokine staining, single-cell suspensions were fixed in 4%paraformaldehyde and permeabilized with 0.5% saponin buffer and stained with IFN-γ-

AF700 (clone XMGL2), IL-4-APC (clone 11B11), IL-10-PE (clone eBio13A), and IL-13 PEcy7 (Clone BI6). 200,000 events were read on a BD Fortessa (BD flow Biosciences). Data were analyzed using FlowJo software version 10 (TreeStar). Data were obtained as a percentage of the total cells acquired. Absolute cell numbers were calculated as the product of the cell percentage and the total number of cells in the isolated pLN.

2.8 Lymph node cell stimulation and cytokine detection

Cytokine staining was performed on antigen-stimulated LN T cells. LN cells were re-stimulated ex vivo with αCD3 and heat-killed *L. mexicana* metacyclic parasites. A 200µl of 20ug/ml αCD3 was coated in a 48 well plate and incubated at 37˚C with a 5% CO_2 incubator for 30 mins. The supernatant was removed by aspirating and 400ul DMEM media was added followed by 100ul of LN cell sample and incubated for 72hrs at 5% CO_2 and 37˚C. $8x10^6$/ml *L. mexicana* metacyclic parasites were incubated at 56ºC water bath for 30min to lyse parasites to release antigens. 100ul of LN cell suspension was added into wells with 150ul DMEM media followed by the addition of 250ul of the heat-inactivated *L. mexicana* metacyclic parasites into the designated wells and incubated for 72hrs at 5% CO_2 and 37 ˚C.
IFN-γ, IL-4, IL-13, IL-10, TGF-β levels were detected in the supernatants by capture ELISA. Briefly, an anti-cytokine antibody was diluted in carbonate coating buffer (PH 9.5) and 50µl was coated in a 96 well plate and incubated at 4ºC overnight. Unbound anti-cytokine antibody was removed by washing with 200µl of washing buffer. Followed by overnight blocking of plates with blocking buffer. 50 µl of sample and specific cytokine standards were added accordingly after washing with washing buffer and incubated at 4ºC overnight then followed by the addition of 50 µl of a biotin-labeled secondary antibody and incubated for 3hrs at 37ºC. 50 µl of diluted alkaline phosphatase-labeled streptavidin or HRP-streptavidin conjugate was added and incubated for 1hr at 37ºC followed by detection with addition of 50 µl of diluted substrate P-Nitrophenol-phosphate (detected at absorbance 405) or a mixture of an equal volume of TMB peroxidase and peroxidase substrate (detected at absorbance 450nm) using a SOFTmax PRO.

2.9 Detection of *Leishmania mexicana* specific -IgG1, IgG2a, IgG2b, and total IgE

L. mexicana specific-IgG1, IgG2a, and IgG2b were detected in the serum of infected mice by ELISA, as previously described (Govender et al., 2018). Briefly, 96 well flat-bottomed plates were coated with 50µl of 10µg/ml of *Leishmania mexicana* SLA in coating buffer (pH 7.5)

overnight at 4°C. Samples were serially 3-fold diluted and 50µl added to each well accordingly, followed by overnight incubation at 4°C. Bound *Leishmania*-specific antibodies were detected with a 3hour incubation at 37°C with secondary AP conjugate isotype of goat anti-mouse IgG1 IgG2a and IgG2b. The 50µl of DNPP substrate was then added to the wells and, following color development, absorbance was measured at 450/490nm using a SOFTmax PRO. Total IgE was detected in the blood of infected mice by capture ELISA as previously described (Hurdayal et al., 2013), using capture IgE mAb and biotinylated rat anti-mouse IgE.

2.10 Generation of bone marrow-derived DCs

2.10.1 Preparation of murine bone marrow cells

Mice were euthanized using halothane and the femurs and the tibia were dissected by cutting using scissors. The bones were placed into a 50ml falcon tube containing sterile PBS containing 3 % FBS on ice and transported to the tissue culture lab.

In a tissue culture hood, the bones were placed in 5ml of sterile RPMI 164010% FCS, penicillin/streptomycin (100 U/ml/100 µg/ml, respectively), and flesh was removed and flushed with a syringe with a 27-gauge needle filled with RPMI 1640 to extrude bone marrow into a 50 ml falcon tube. Clusters within the BM suspension were disintegrated by vigorous pipetting (Lutz et al., 1999).

Cell suspensions were centrifuged for 5 minutes at 300× *g* at 4°C and gently resuspended in 5ml of RPMI 1640 media. Viable cells were counted by Trypan blue exclusion method, using a ratio of 1:10 (10µl of the cell suspension to 90µl trypan blue solution).

2.10.2 Generation of DCs from bone marrow cell culture with GM-CSF

Generation of BMDCs was done as described by Lutz et al.,1999. Briefly, 2×10^6 of BM cells/ml were seeded in 100 mm diameter bacteriological Petri dishes with 10ml of RPMI1640 media supplemented with 10% FCS, 100 U penicillin/100µg/ml streptomycin and 50% GM-CSF (R10 media) and incubated at 37°C in a 5% CO_2 atmosphere. 5ml of fresh R10 media was added to the cells at days 3 and 6 of incubation. The culture was incubated at 37°C, 5% CO_2 until day 10. Non-adherent cells were gently pipetted from the petri dish into a 50ml falcon tube and used for the subsequent analyses (Hurdayal et al., 2013). Maturation

and activation of BMDCs were assessed by flow cytometry using fluorochrome-conjugated antibodies.

2.10.3 Maturation, activation, differentiation, and infection of DCs

Cells were washed with RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin and centrifuged at 300g 4ºC for 5min. The pellet was resuspended in fresh media, cells were counted in presence of trypan blue to confirm viability. 5×10^5 cells were seeded in 48 well plates and stimulated with 1µg/ml of LPS or *L. mexicana* promastigotes at the ratio of 10:1 (i.e 5×10^6 parasite to 5×10^5 cells) in the presence or absence of 1000U/ml recombinant mouse IL-4 incubated at 37ºC, 5% CO_2 for 48hrs. The samples were gently pipetted to dissociate DC clusters and to promote contact between parasites and DCs. Infected cells and uninfected cells (controls run in parallel) were placed at 37°C in a 5% CO_2 incubator for 48 hours (Prina et al., 2004). The culture was centrifuged at 1500rpm for 5min at 4ºC and the supernatant was gently pipetted and collected for iNOS assay activity and cytokine assay (IL-12p70 and IL-10) using ELISA, whereas the pellets in the wells were kept for arginase 1 enzyme activity assay.

2.10.3.1 Nitrite Assay

The level of nitrite (NO_2) reflects NO synthesis (Klinger, 2016). Culture supernatants were assayed for NO_2 using the Griess reaction. Briefly, 50 µL of culture supernatant was incubated with 50 µL of 1% sulfanilamide (reagent 1) at room temperature in the dark for 10 min followed by 5-10min incubation with 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% orthophosphoric acid at room temperature in the dark. Absorbance was measured at 540 nm using a versa max microplate reader and the micromolar concentrations of NO_2 were determined by interpolation from a $NaNO_2$ standard curve run together with samples and blanks.

2.10.3.2 Determination of concentration of urea arginase activity

Cells in the wells were resuspended with 100 µl of 0.1% triton X and 50 µl was transferred into 96 well plates and the cells were activated by adding 50 µl of arginase activation buffer and was block heat at 55ºC for 10min. 25 µl of the activated mix was transferred to a 96 well plate and incubated with 25 µl of 0.5M arginine for 60min at 37ºC and reaction stopped by

adding 200 µl of the acid mix before adding 12.5 µl of 9% α-isonitrosopropiophenone. This was followed by block heating at 100ºC for 45min before incubating in dark for 10min. The OD was read at 540nm using a versa max microplate reader.

2.10.4 Bone marrow-derived macrophages generation, stimulation, and infection

Cells suspension of $10x10^6$ harvested from the bone marrow as described above were incubated in Petri dishes with 50 ml Plutznik media (complete DMEM containing 50% L929 cell supernatant, 10% FCS, 2 mM glutamine, 500µl of mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin) (**Appendix 1**), at 37ºC, 5% CO_2. On days 3 and 6, the culture was supplemented with 25ml of complete DMEM media (as shown above). On day 8, the supernatant was gently removed, and cells were washed 2 times with complete 1x PBS Five milliliters of warmed complete DMEM was added and cells were removed by gently scraping with a rubber policeman. The cells were collected in 50 ml tubes and centrifuged at 450 x g for 10 min. Viable cells were counted by Trypan blue exclusion method.
$5x10^5$ macrophages were seeded in 48-well plates and left to stay overnight at 37ºC, 5% CO_2 incubator. Non-adherent macrophages were washed twice with 1xPBS. The macrophages were infected with *L. mexicana* promastigotes at MOI of 10:1 and incubated for 24 hours. Designated wells were incubated with 1000 U/ml and 100 ng/ml of recombinant IL-4 and IFN-γ respectively for 48hours before pre-incubated with 100 U/ml recombinant murine IFN-γ and 10ng/ml of LPS for 16 hours. The supernatants were collected and assayed for the accumulation of IL-12p70, IL10 (as described above), and iNOS activity (Nitrite assay).

2.11 Statistical analysis

Statistical analysis for samples from more than two groups was performed using a one-way ANOVA Bonferroni test significant difference test. Statistical analysis for two groups was compared using the student t-test. Means± standard errors of the means of footpad thickness, percentage cell surface markers, NO_2, urea concentrations, and cytokine positive cells were calculated. The analysis was performed using GraphPad Prism 5.0 for MacIntosh. Each experimental group was compared to littermate controls (IL-4Rα$^{-/lox}$). Statistical values of *$p < 0.05$ and **$p < 0.01$ and ***$p < 0.0001$ were considered significant.

CHAPTER 3:

RESULTS

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RESULTS

3.1 Results

3.1.1 Lack of IL-4Rα signaling on dendritic cells and macrophage/neutrophils does not attenuate footpad swelling and parasite growth when compared to littermate control BALB/c mice following *L. mexicana* infection.

To determine the effects of the absence of IL-4Rα on dendritic cells (DCs) and macrophages/neutrophils in the host defense following *L. mexicana* infection, IL-4Rα-DCs deficient mice ($CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$), mice with IL-4Rα-deficiency on macrophages/neutrophils ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$), $IL\text{-}4R\alpha^{-/lox}$ (littermate controls), and global $IL\text{-}4R\alpha^{-/-}$ BALB/c mice were infected with 2×10^6 stationary promastigotes, and footpad swellings were measured weekly using vernier caliper for 8 weeks to assess clinical outcome. The difference in swellings to 2mm base measurements was recorded.

Upon subcutaneous infection of the animals with 2×10^6 metacyclic promastigotes of *L. mexicana*, LV4, into the left hind footpad, global $IL\text{-}4R\alpha^{-/-}$ controlled footpad swelling as previously described (Alexander et al., 2002). In contrast, both female and male $CD11c^{cre}$ $IL\text{-}4R\alpha^{-/lox}$ mice developed disease progression with increasing non-healing footpad swelling that persisted for the 8week period (**Figures 3.1A, B**). Since there was no difference in footpad swelling phenotype between $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ at week 2-6 weeks pi, no in-depth analysis on immune responses was necessary at week 2 or 6 post-infection (**Figure 3.1A**, **B**). Owing to this observationck of IL-4Rα expression on DCs may not play a vital role during early phase of CL caused by *L. mexicana* infection. Importantly, no significant difference was observed between $CD11c^{cre}$ $IL\text{-}4R\alpha^{-/lox}$ male and female mice (**Figure 3.1C**). Besides, the littermate $IL\text{-}4R\alpha^{-/lox}$ female mice had similar non-healing footpad swelling compared to wild-type (WT) BALB/c mice (**Figure 3.1D**), demonstrating that deficiency in one allele of IL-4Rα on DCs does not also affect the outcome of CL disease caused by *L. mexicana* infection.

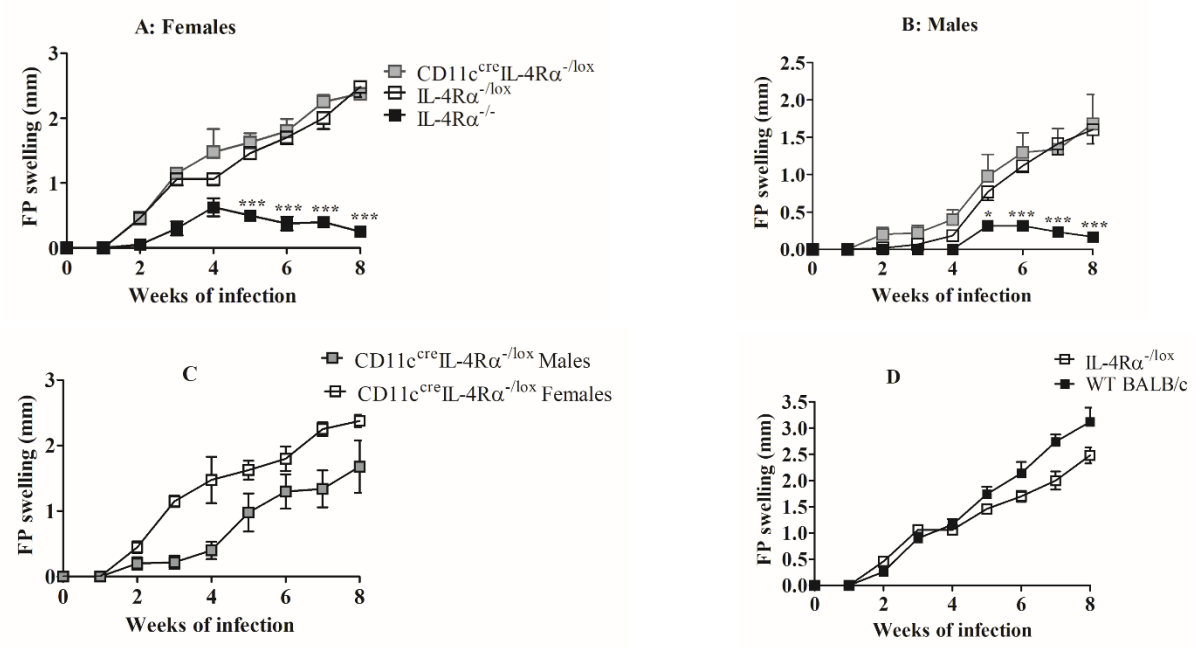

Figure 3.1: The Footpad swelling of CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice subcutaneously infected with *L. mexicana* in the hind footpad. CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and littermate control IL-4Rα$^{-/lox}$ BALB/c mice were infected with 2x10^6 *L. mexicana* promastigotes into the hind footpad. Footpad swelling was measured at weekly intervals in female (**A**) and male (**B**) mice. Fp swelling was compared between male and female CD11c^{cre}IL-4Rα$^{-/lox}$ mice during *L. mexicana* infection (**C**). Similarly, FP swelling was compared between littermate IL-4Rα$^{-/lox}$ female mice and WT BALB/c mice following *L. mexicana* infection (**D**). Swelling for CD11c^{cre}IL-4Rα$^{-/lox}$ were compared to IL-4Rα$^{-/lox}$ mice using one-way ANOVA (Bonferroni post-test), defining differences to IL-4Rα$^{-/lox}$ mice as significant (*p≤0.05; ***p≤0.001) (n=4-6).

Enumeration of parasites in the footpad and lymph node was done using a limiting dilution assay. Both IL-4Rα-DC specific-deficient female (**Figure 3.2A)** and male **(Figure 3.2B)** mice had similar numbers of parasites in their lesions and LN compared to their littermate controls at week 8 p.i. In contrast, the global IL-4Rα$^{-/-}$ female mice had significantly decreased parasite burdens in both FP and LN compared to IL-4Rα$^{-/lox}$ female mice, 8 weeks p.i following *L. mexicana* challenge (**Figure 3.2A**). A similar trend was observed in parasite dissemination into the LN of male mice (**Figure 3.2B**). Surprisingly, in male mice, there was no difference in parasite burden in the footpad amongst the three strains of mice (**Figures 3.2B**).

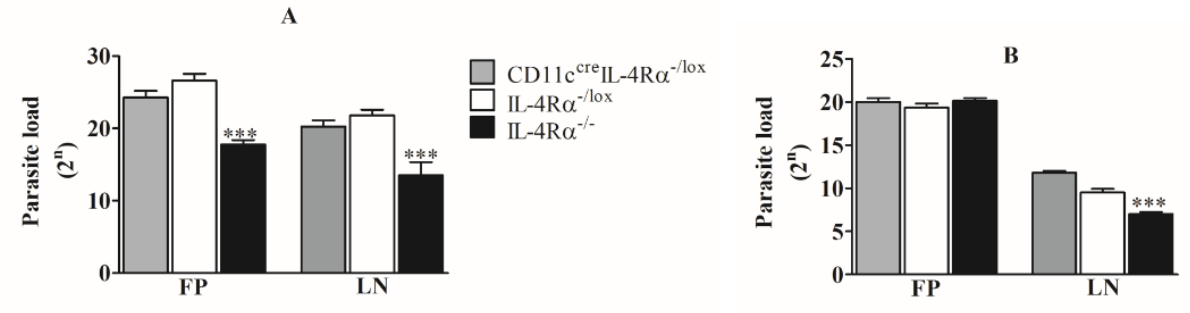

Figure 3.2: Enumeration of parasite burden in CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice following *L. mexicana* infection. Both female and male mice were subcutaneously infected with 2x10^6 *L. mexicana* promastigotes into the hind footpad and monitored for lesion development for 8 weeks. Mice were sacrificed and the footpad and lymph nodes (LN) were isolated. Using limiting dilution assays parasite burden was enumerated in single-cell suspensions of the footpad and lymph node of female (**A**) and male (**B**) mice and the mean parasite burden in CD11c^{cre}IL-4Rα$^{-/lox}$ were compared to IL-4Rα$^{-/lox}$ mice. A representative of two individual experiments is shown with mean± SEM of 4-6 mice. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test), defining differences to IL-4Rα$^{-/lox}$ mice as significant ***p≤0.001).

Next, we investigated whether deficiency of IL-4Rα signaling on both macrophage and neutrophil cells contributed to resistance to *L. mexicana* infection in global IL-4Rα$^{-/-}$ mice (Alexander et al., 2002). To compare the CL disease caused by *L. mexicana* in LysM^{cre}IL-4Rα$^{-/lox}$ with IL-4Rα$^{-/lox}$ littermate control, animals were subcutaneously infected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad, lesion progression was measured, and parasite load was quantified using LDA in both footpad and lymph node. Like CD11c^{cre}IL-4Rα$^{-/lox}$ findings, LysM^{cre}IL-4Rα$^{-/lox}$ mice developed rapid non-healing footpad lesions similar to their littermate controls, in both females (**Figure 3.3A**) and males (**Figure 3.3B**). Besides, when male and female mice were compared, no sex differences were observed in lesion size progression during the 8-week infection period (**Figure 3.3C**), hence suggesting that no significant effect of sex hormones on macrophage and neutrophil cell function in the control of *L. mexicana* infection in IL-4Rα$^{-/-}$ BALB/c mice (Alexander et al., 2002). Considering LDA, our data revealed a similar parasite burden in the footpad, between LysM^{cre}IL-4Rα$^{-/lox}$ and their littermate controls both in female (**Figure 3.3D**) and male (**Figure 3.3E**) mice. A similar trend was observed regarding parasite dissemination into the LN of both female (**Figure 3.3D**) and male (**Figure 3.3E**) mice.

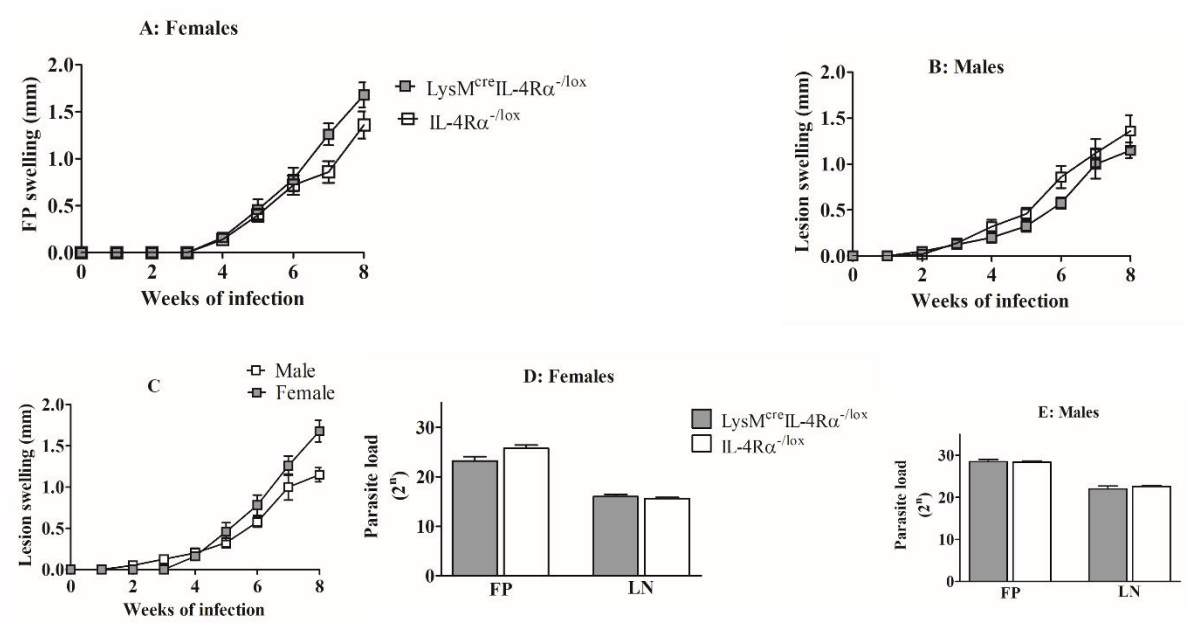

Figure 3.3: Lesions and parasite burdens in LysM^{cre}IL-4Rα$^{-/lox}$ mice with *L. mexicana*. LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice were subcutaneously injected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad. The footpad swellings for females (**A**) and males (**B**) were measured weekly for 8 weeks. FP swelling between male and female LysM^{cre}IL-4Rα$^{-/lox}$ mice was compared during 8 weeks of *L. mexicana* infection (**C**). Mice were sacrificed and parasite burden was determined by limiting dilution assay in the footpad and LN of female (**D)** and male (**E**) mice. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice. A P value of ≤0.05 was considered significant (n=4-6).

Overall, these findings suggest that the expression of IL-4Rα on DCs and macrophage/neutrophil cells is dispensable in the control of lesion development, parasite growth, and dissemination in both males and females BALB/c mice following *L. mexicana* infection.

3.1.2 The absence of IL-4Rα signaling on DCs and macrophages/neutrophils does not change the humoral responses in *L. mexicana* infected BALB/c mice.

Antibody production by activated B cells has been shown to enhance the pathogenesis of leishmaniasis (Al-Qadhi et al., 2015; Wanasena, 2008). In contrast, other studies have strongly demonstrated the protective roles of B cells and antibodies during *Leishmania* infection (Woelbing et al., 2006).

Accordingly, we analyzed type 1 and type 2 antibody responses, characterized by the production of IgG2a/IgG2b and IgG1/total IgE antibodies, respectively (Gibson-Corley et al.,

2014). Type 1 antibody responses comprising of IgG2a/b have been shown to confer protection to *Leishmania* infection (Gibson-Corley et al., 2014; Hurdayal et al., 2017; Hurdayal et al., 2013) whereas type 2 antibodies (IgE and IgG1) have been implicated in susceptibility (Al-Qadhi et al., 2015; de Lima et al., 2021; Sousa-Atta et al., 2002). Since both, the CD11c^{cre}IL-4Rα$^{-/lox}$, LysM^{cre}IL-4Rα$^{-/lox}$ compared to their littermate IL-4Rα$^{-/lox}$ control mice displayed a non-healing lesion progression, the levels of serum total IgE and IgG1 subclass of antibodies were assayed by ELISA to determine whether the infected mice had a skewed type 2 response. Similarly, we compared the antibody titres for IgG2a/b by ELISA between the DCs and macrophages/neutrophils-IL-4Rα deficient mice with their respective littermate controls. Findings from this study demonstrate that there were no differences in the levels of total IgE (**Figure 3.4A**) and IgG1(**Figure 3.4B**) antibody titers in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c female mice when compared to their littermate controls. Similarly, there were no observed significant differences in the titers of IgG2b (**Figure 3.4C**) and IgG2a (**Figure 3.4C**) antibodies produced in the serum of *L. mexicana*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c female mice when compared to their littermate control.

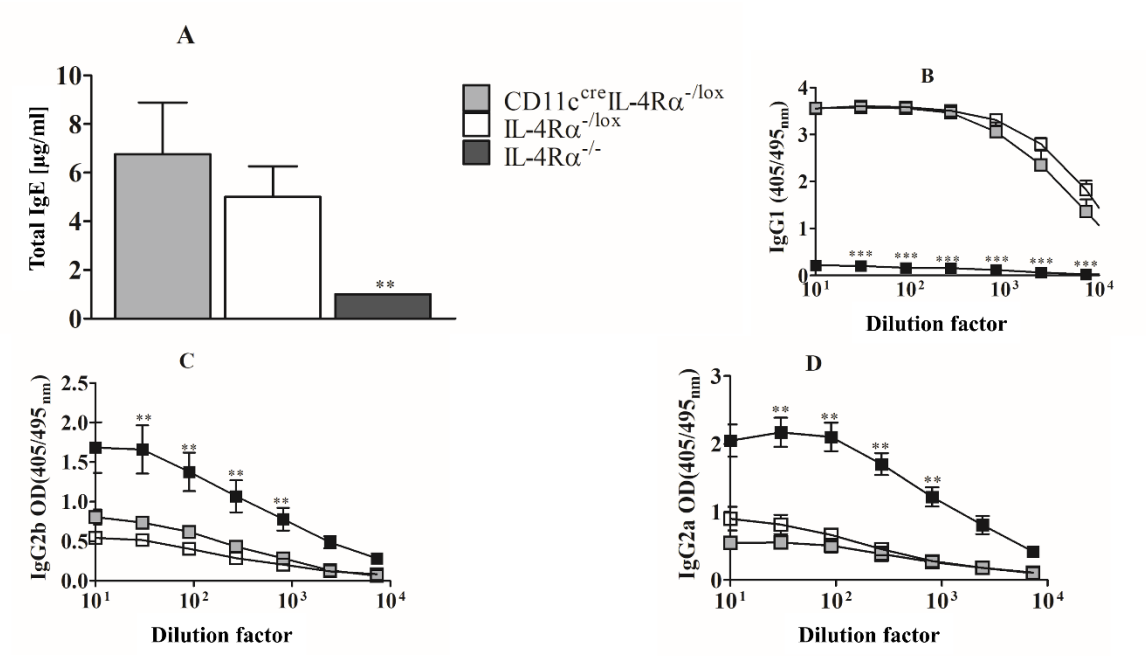

Figure 3.4: Serum levels of antibody responses following infection of female CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice with *L. mexicana*. Mice were subcutaneously injected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad. Sera from CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate control mice, infected with *L. mexicana* for 8 weeks were assayed for total IgE in the serum of (**A)** using ELISA. Similarly, *L. mexicana*-specific IgG1 (**B**), IgG2b (**C**), and IgG2a (**D**) were determined in the serum using ELISA. Data represent means±SEM from duplicate experiments (n=4-6). Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice. A P value of ≤.05 was considered significant (***P ≤ 0.001 and **P≤ 0.01).

In CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c male mice, when compared to their littermate controls, we found no significant differences in the levels of total IgE (**Figure 3.5A**), IgG1 (**Figure 3.5B**), IgG2a (**Figure 3.5C**), and IgG2b (**Figure 3.5D**) between the two mouse strains. However, the global IL-4Rα$^{-/-}$ mice had significantly decreased total IgE (**Figure 3.5A**), IgG1 (**Figure 3.5B**) but significantly increased levels of IgG2a (**Figure 3.5C**) and IgG2b (**Figure 3.5C**) antibody responses, emphasizing their Type 1 response (Gibson-Corley et al., 2014).

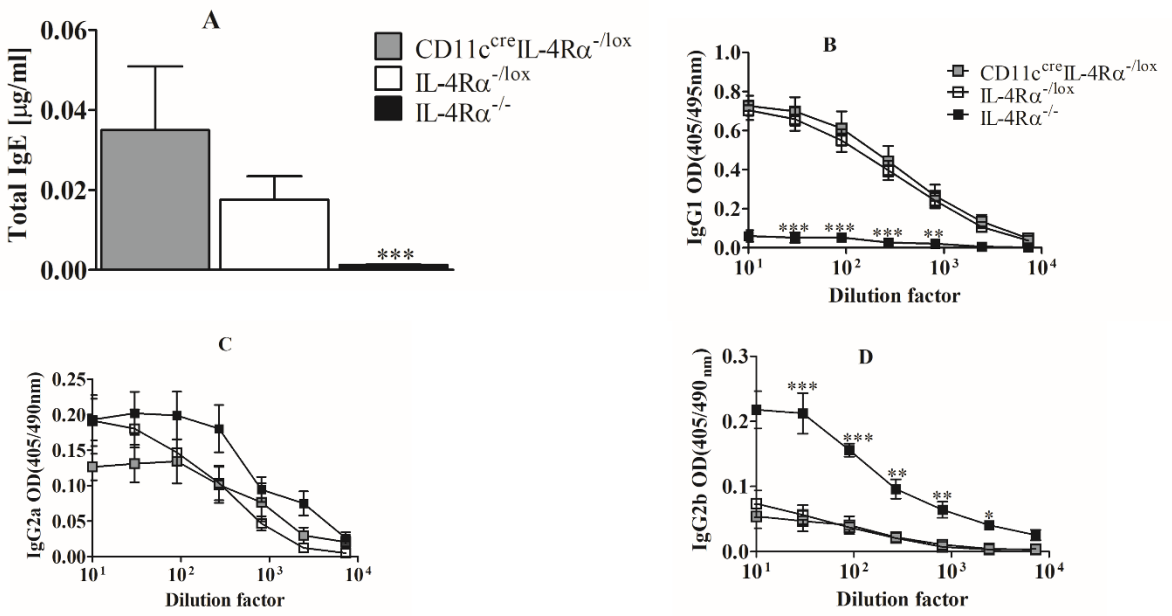

Figure 3.5: Serum levels of antibody responses following infection of male CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ BALB/c mice with *L. mexicana*. Mice were subcutaneously injected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad. Sera from CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate control mice, infected with *L. mexicana* for 8 weeks were assayed for total IgE in the serum of (**A)** using ELISA. Similarly, *L. mexicana*-specific IgG1 (**B**), IgG2a (**C**), and IgG2b (**D**) were determined in the serum using ELISA. Data represent means±SEM from duplicate experiments. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice. A P values of *P ≤ 0.05; **P ≤ 0.01 and ***P≤ 0.001 were considered significant (n=4-6).

In line with the non-healing progressive disease phenotype displayed by macrophage/neutrophil IL-4Rα$^{-/lox}$-deficient female mice, both type 2; total IgE (**Figure 3.6A**), IgG1(**Figure 3.6B**) and type 1; IgG2a (**Figure 3. 3.6C**), IgG2b (**Figure 3.6D**) antibody responses production was comparable to IL-4Rα$^{-/lox}$ mice. A similar trend was observed in total IgE (**Figure 3.7A**), IgG1(**Figure 3.7B**), IgG2a (**Figure 3.7C**), and IgG2b (**Figure 3.7C**) antibody levels in serum of male mice.

Overall, these findings indicate that the loss of IL-4Rα signaling on DC and macrophage/ neutrophil cells does not influence the production of IgE or *Leishmania* antigen-specific IgG antibody isotypes in BALB/c mice during *L. mexicana* infection.

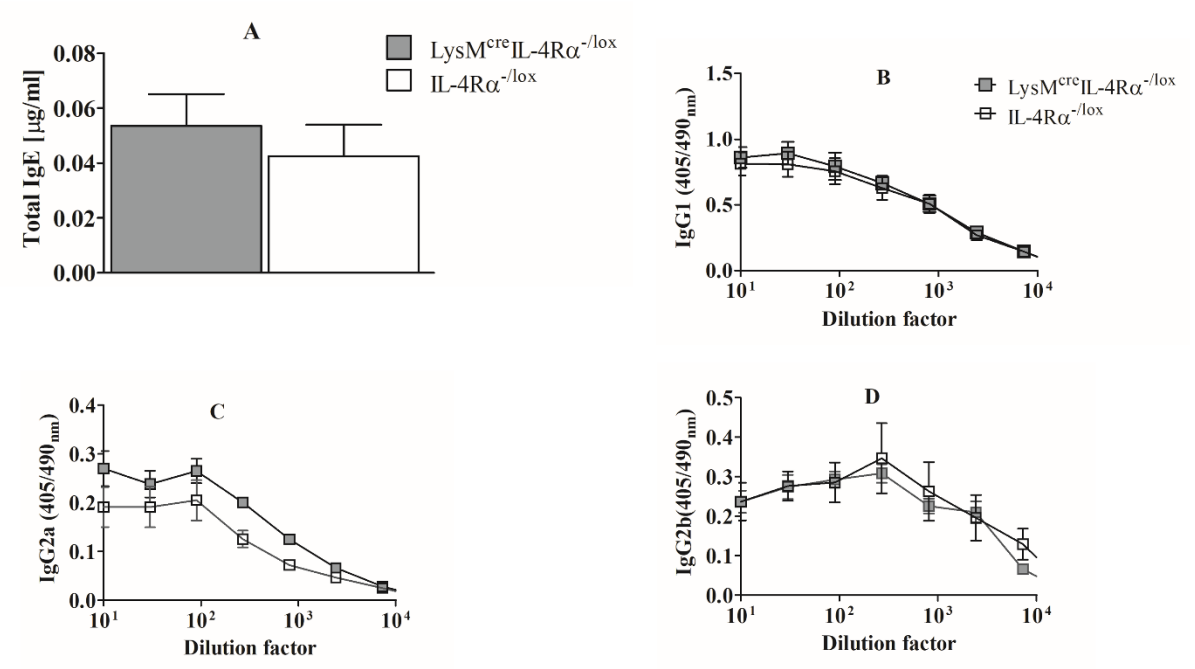

Figure 3.6: Measurement of antibody response in LysM^{cre}IL-4Rα$^{-/lox}$ female mice at week 8 p.i with *L. mexicana*. Mice were subcutaneously injected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad. Sera from LysM^{cre}IL-4Rα$^{-/lox}$ and littermate control mice, infected with *L. mexicana* for 8 weeks were assayed for total IgE in the serum of (**A)** using ELISA. Similarly, *L. mexicana*-specific IgG1 (**B**), IgG2a (**C**), and IgG2b (**D**) were determined in the serum using ELISA. The levels of the antibodies in LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared with their littermate control using the unpaired student t-test. A value of $p \leq 0.05$ was considered statistically significant (n=4-6).

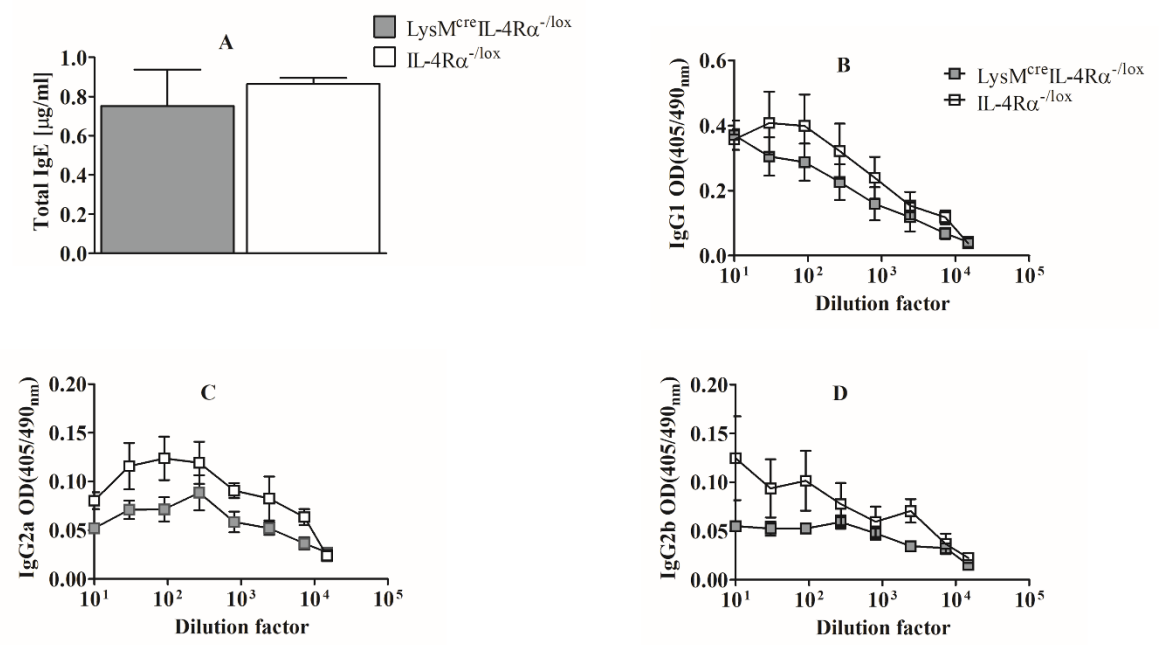

Figure 3.7: Measurement of antibody response in LysM^{cre}IL-4Rα$^{-/lox}$ male mice at week 8 p.i with *L. mexicana*. Mice were subcutaneously injected with 2×10^6 metacyclic promastigotes of *L. mexicana* in the footpad. Sera from LysM^{cre}IL-4Rα$^{-/lox}$ and littermate control mice, infected with *L. mexicana* for 8 weeks were assayed for total IgE in the serum of (**A)** using ELISA. similarly, *L. mexicana*-specific IgG1 (**B**), IgG2a (**C**), and IgG2b (**D**) were determined in the serum using ELISA. The levels of the antibodies in LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared with their littermate control using the unpaired student t-test. A value of $p \leq 0.05$ was considered statistically significant (n=4-6).

3.1.3 Type 2 immune responses were increased in CD11c^{cre}IL-4Rα$^{-/lox}$ mice but did not tilt hyper-susceptibility over littermate IL-4Rα$^{-/lox}$ mice.

Susceptibility and resistance to CL caused by *Leishmania* infection are modulated by Th1/type 1 and Th2/type-2-derived cytokine responses (Alexander and Brombacher, 2012) therefore, we investigated cytokine responses. Following *L. mexicana* infection, popliteal LN cells from CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice were re-stimulated with αCD3 and heat-killed *L. mexicana* promastigotes after which, cell supernatants were assayed for IFN-γ, IL-4, IL-13, IL-10, and IL-12p70.

At week 8 p.i the amount of IFN-γ secreted by LN cells of female mice was similar between CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (**Figure 3.8A**). In contrast, global IL-4Rα deficient mice had significantly increased IFN-γ compared to the littermate controls (**Figures 3.8A).** Investigation on IL-4 revealed significantly increased levels in CD11c^{cre}IL-4Rα$^{-/lox}$ compared to their littermate controls (**Figure 3.8B**). There were observed increased levels of IL-13 production by LN cells of CD11c^{cre}IL-4Rα$^{-/lox}$ mice even though not significant when

compared to the littermate controls (**Figure 3.8C**). Similarly, no differences were observed in levels of IFN-γ (**Figure 3.8D**) production by CD11c^{cre}IL-4Rα$^{-/lox}$ male mice when compared to their littermate controls. However, comparable to female mice, CD11c^{cre}IL-4Rα$^{-/lox}$ male mice had significantly increased levels of IL-4 (**Figure 3.8E**) with the increased trend for IL-13 (**Figure 3.8F**), even though not statistically significant.

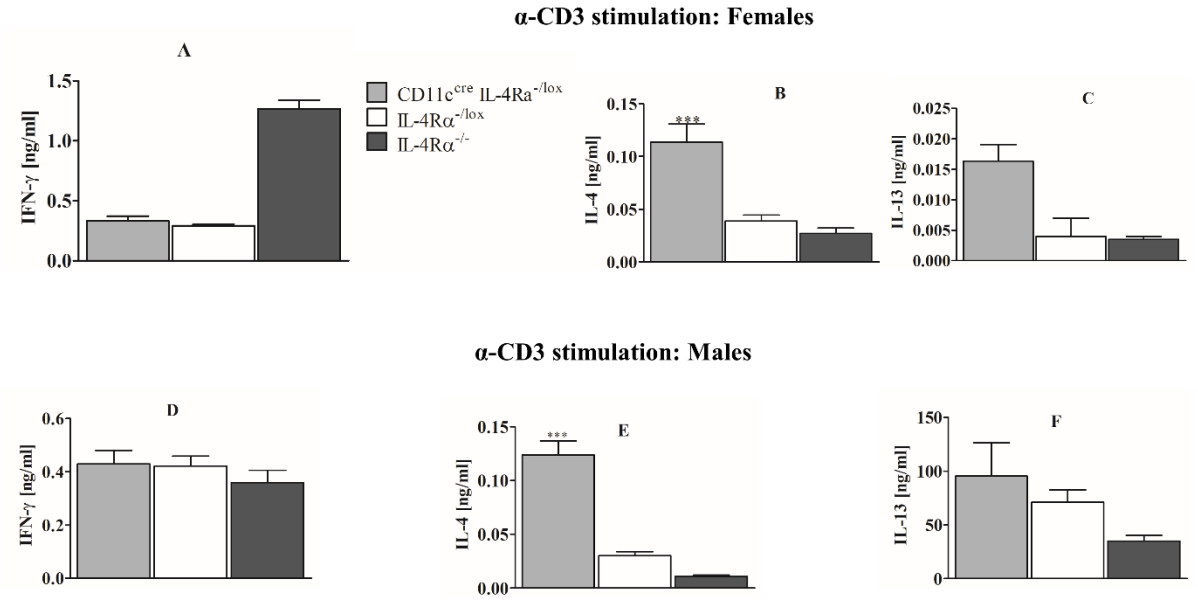

Figure 3.8: Cytokine production by pLN cells of *L. mexicana*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice female and male and littermate IL-4Rα$^{-/lox}$ controls. Cells from popliteal lymph node (pLNs) of experimental female mice infected subcutaneously with *L. mexicana* promastigotes at week 8 p.i were stimulated with α-CD3. IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**) were measured in female mice and IFN-γ (**D**), IL-4 (**E**), IL-13 (**F**) were measured in male mice using ELISA. Data represent means and SEM of duplicate experimental assays. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (***p ≤0.001) (n=4-6).

To supplement general non-specific T cell responses (αCD3 stimulation), we investigated antigen-specific immune responses by measuring the levels of IFN-γ, IL-4, IL-13, IL-10 and IL-12p70 cytokine production by LN cells of CD11c^{cre}IL-4Rα$^{-/lox}$ mice stimulated with heat-killed *L. mexicana* promastigotes using ELISA and compared to their littermate controls. Our results revealed that LN cells from CD11c^{cre}IL-4Rα$^{-/lox}$ female mice had similar levels of IFN-γ production when compared to the littermate IL-4Rα$^{-/lox}$ mice (**Figure 3.9A**). However, the type 2 cytokines, IL-4 (**Figure 3.9B**) and IL-13 (**Figure 3.9C**) were significantly increased in CD11c^{cre}IL-4Rα$^{-/lox}$ when compared to IL-4Rα$^{-/lox}$ mice. These findings, therefore, suggest that IL-4 and IL-13, responsible for type 2 immune responses and host

susceptibility to CL, although substantially increased in CD11c^{cre}IL-4Rα$^{-/lox}$ mice did not promote hyper susceptibility compared to littermate controls as previously reported (Hurdayal et al., 2013). IL-10, an immunoregulatory cytokine (Ng et al., 2013) was similar between CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ female mice (**Figure 3.9D**). Studies have demonstrated that to suppress parasites proliferation in cells during *Leishmania* infection, the balance between inflammatory and regulatory networks is crucial (Gomes-Silva et al., 2007). In this current study, the ability of pLN cells was assessed for inflammatory cytokine IL-12p70 secretion following *L. mexicana* infection. Notably, no significant differences in IL-12p70 secretion were observed between female CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls (**Figure 3.9E**).

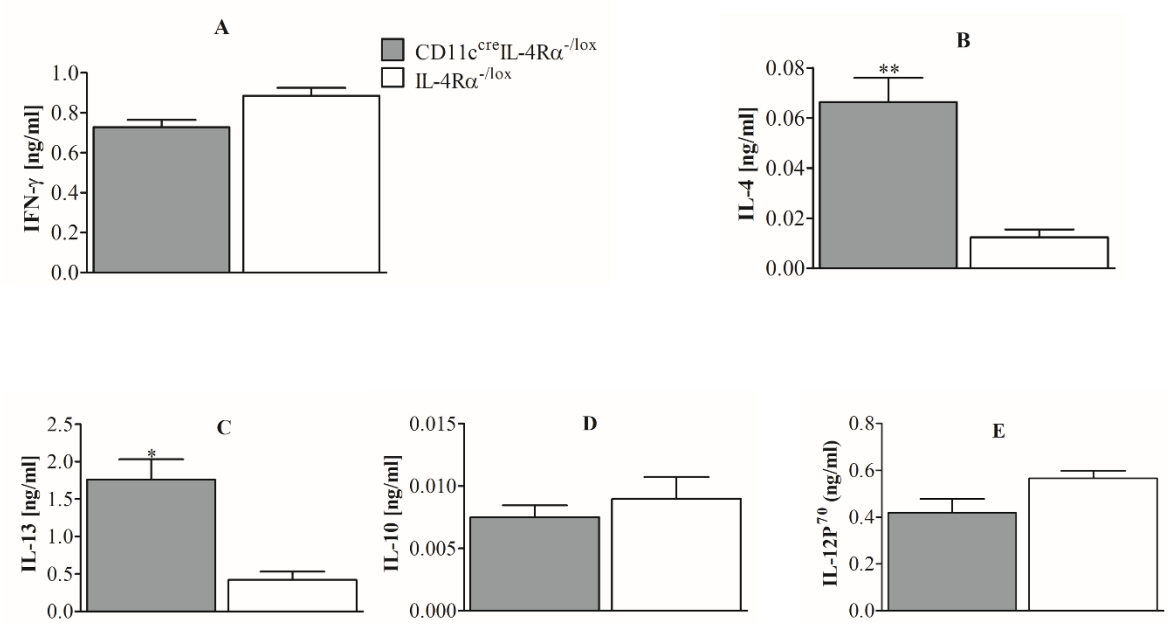

Figure 3.9: Cytokine production by pLN cells of *L. mexicana*-infected female CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate IL-4Rα$^{-/lox}$ controls. Cells from pLNs of experimental female mice infected subcutaneously with *L. mexicana* promastigotes at week 8 p.i were stimulated with heat-killed *L. mexicana* promastigotes for 72hrs. IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**), IL-10 (**D**), IL-12p70 (**E**) were measured using ELISA. Data represent means and SEM of one of the duplicate experimental. Statistical analysis was performed using an unpaired student t-test, comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (*p ≤0.05; **p ≤0.01) (n=4-6).

Comparable to females, in males, heat-killed stimulated pLN cells produced similar amounts of IFN-γ (**Figure 3.10F**) between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls but significantly increased IL-4 (**Figure 3.10G**). The IL-13 levels (**Figure 3.10H**), were similar between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and their littermate controls. However, we also found

significantly increased levels of IL-10 in male CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to their littermate controls (**Figure 3.10I**). IL-12P^{70} production by pLN cells stimulated with heat-killed *L. mexicana* promastigotes was unchanged between male CD11c^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ BALB/c mice (**Figure 3.10J**). Despite elevated levels of IL-10 in male mice deficient with IL-4Rα signaling on DCs, implying that some level IL-4-mediated DC-IL-12 instruction was altered between the two mouse strains, however, they did not display hyper susceptibility compared to their littermate controls, possibly tempered by equivalent IL-12p70 production.

These findings suggest that the IL-4/IL-13 signaling through IL-4Rα on DCs is essential in controlling an excessive type 2 cytokine response during *L. mexicana* challenge in mice. However, this does not break the balance between susceptibility to hyper-susceptibility. Furthermore, IL-4-mediated DC instruction appears to be species-specific.

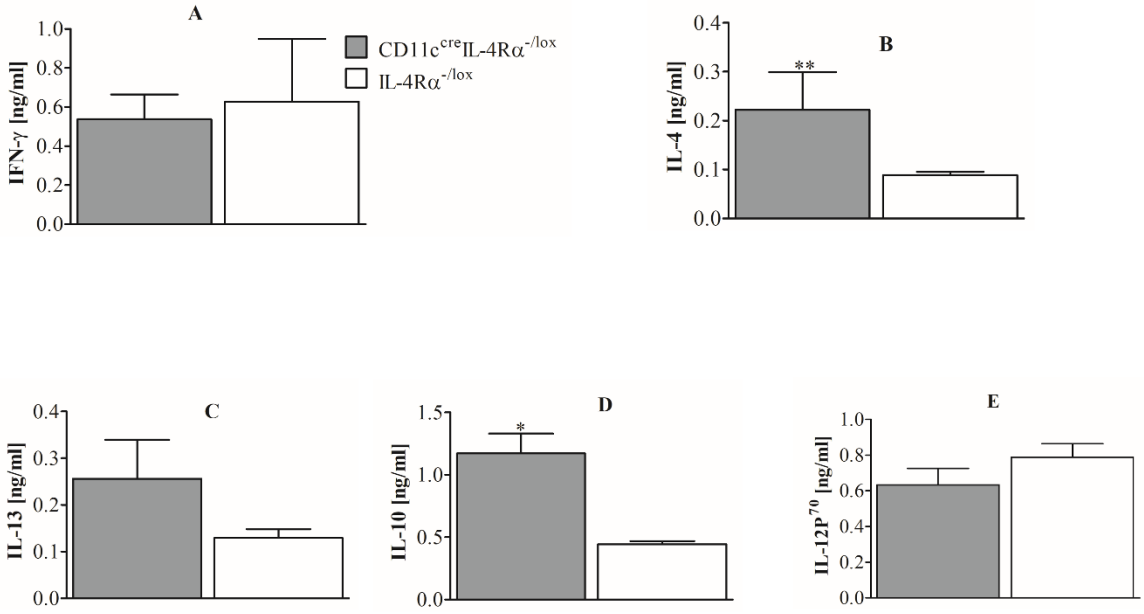

Figure 3.10: Cytokine production by pLN cells of *L. mexicana*-infected male CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ BALB/c mice. Cells from popliteal lymph nodes (pLNs) of experimental male mice infected subcutaneously with *L. mexicana* promastigotes at week 8 p.i were stimulated with heat-killed *L. mexicana* promastigotes for 72hrs. IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**), IL-10 (**D**), IL-12p70 (**E**) were measured using ELISA. Data represent means±SEM of one of the duplicate experiments. Statistical analysis was performed using an unpaired student t-test, comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (*p ≤0.05; **p ≤0.01) (n=4-6).

3.1.4: IL-4Rα deficiency in macrophage and neutrophil cells results in the production of immunoregulatory cytokines differentially in a sex-dependent manner following infection with *L. mexicana*.

Following *L. mexicana* infection in LysM^{cre}IL-4Rα$^{-/lox}$, we sought to investigate the effects of abrogation of IL-4Rα signaling on macrophages/neutrophils on IL-10 and TGF-β production. Both these cytokines have been implicated in cutaneous leishmaniasis (Schwarz et al., 2013; Hejazi et al., 2012). In addition, levels of IFN-γ and archetypal Th2 cytokines, IL-4 and IL-13, were measured in LN cells from mice infected subcutaneously with 2×10^6 metacyclic promastigotes of *L. mexicana* parasites stimulated with α-CD3 for 72 hrs.

Lymph node cells from female LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice stimulated with α-CD3 secreted similar levels of IFN-γ (**Figure 3.11A**), IL-4 (**Figure 3.11B**), and IL-10 (**Figure 3.11C**) except with significantly increased TGF-β levels in female LysM^{cre}IL-4Rα$^{-/lox}$ mice (**Figure 3.11D**). Surprisingly there was a significantly increased IFN-γ in LysM^{cre}IL-4Rα$^{-/lox}$ male mice when compared to their littermate controls (**Figure 3.11A**), however, this did not overall change the CL outcome. In contrast, the levels of IL-4 (**Figure 3.11B**), IL-10 (**Figure 3.11C**), and TGF-β (**Figure 3.11D**) production in LysM^{cre}IL-4Rα$^{-/lox}$ male mice were comparable to their littermate controls.

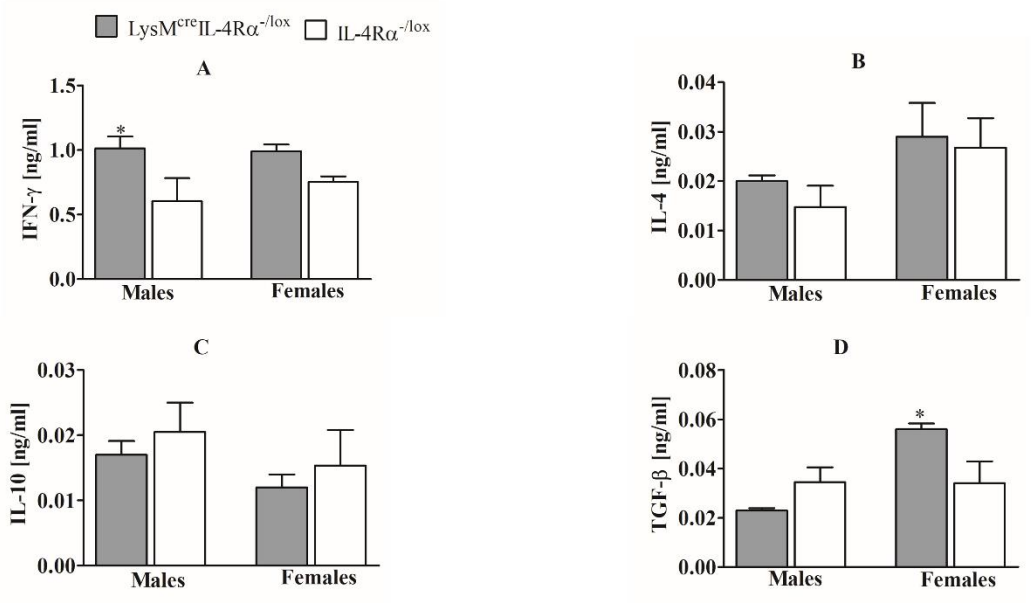

Figure 3.11: Cytokine measurements in LysM^{cre}IL-4Rα$^{-/lox}$ mice and their littermate controls infected with *L. mexicana* promastigotes. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* metacyclic promastigotes and at 8 weeks post-infection the cells were isolated from LNs and stimulated with αCD3 for 72hrs. Supernatants were collected and assayed for IFN-γ (**A, E**), IL-4 (**B, F**), IL-10 (**C, G**), and TGF-β (**D, H**) in females and males mice respectively using ELISA. Means and SEM are presented. Data presented are from two separate experiments. Levels of cytokine in LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared to their littermate controls. Samples size n= 4-6 mice. Differences were compared using one-way ANOVA (Bonferroni post-test) (*P ≤0.05).

In order to investigate antigen-specific responses to supplement the general T cell response by α-CD3 stimulation, we analyzed the above cytokines in supernatants from pLN cells of LysM^{cre}IL-4Rα$^{-/lox}$ mice stimulated with heat-killed *L. mexicana* promastigotes using ELISA. Analysis revealed similar levels of IFN-γ (**Figure 3.12A**), IL-4 (**Figure 3.12B**), IL-13 (**Figure 3.12C**), and TGF-β (**Figure 3.12D**) production by LN cells of both male and female LysM^{cre}IL-4Rα$^{-/lox}$ mice when compared to their littermate controls. This data on the immune response is indicative of the similar non-healing lesion progression between LysM^{cre}IL-4Rα$^{-/lox}$ mice and their littermate controls (**Figure 3.3 A, B**).

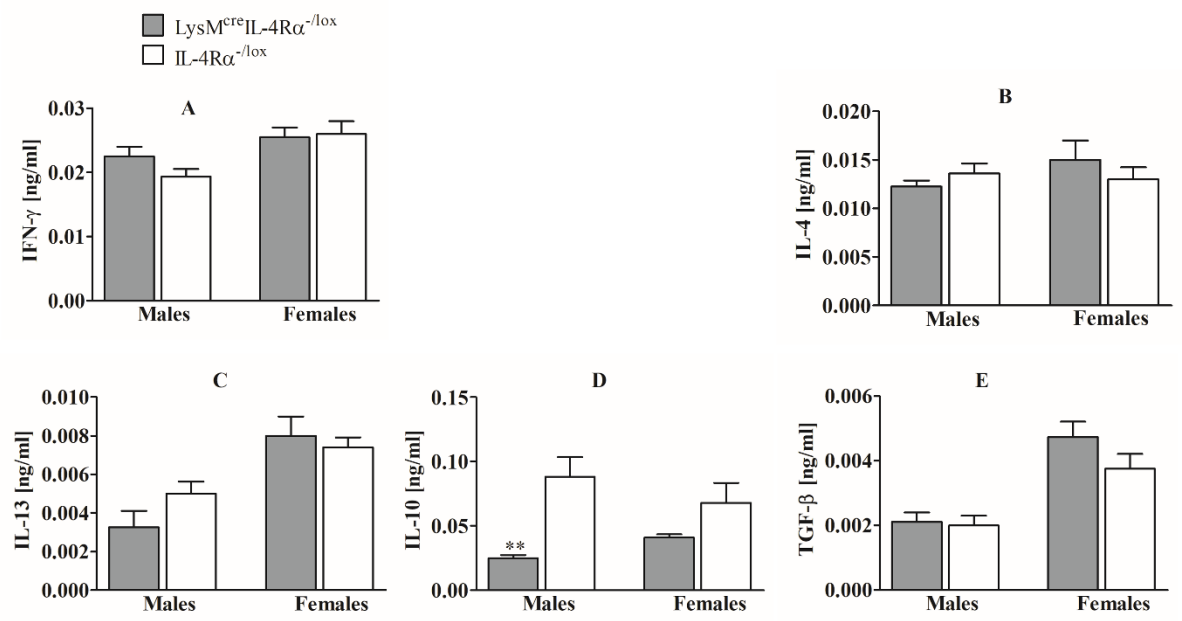

Figure 3.12: Cytokine measurements in LysM^{cre}IL-4Rα$^{-/lox}$ mice and their littermate controls infected with *L. mexicana* promastigotes. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* metacyclic promastigotes and at 8 weeks post-infection the cells were isolated from LNs and stimulated with heat-killed *L. mexicana* promastigotes for 72hrs. Supernatants were collected and assayed for IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**), IL-10 (**D**), and TGF-β (**E**) in male and female mice using ELISA. Means ±SEM are presented. Data presented are from two separate experiments. Levels of cytokine in LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared to their littermate controls. Samples size n= 4-6 mice. Differences were compared using one-way ANOVA (Bonferroni post-test). (**P ≤0.01).

3.1.5 The loss of IL-4Rα signaling on DC and macrophages/neutrophils does not either alter the expansion of lymphocyte populations or their activation in the pLN compared to littermate IL-4Rα$^{-/lox}$ controls.

Evidence has shown that CD4$^+$ and CD8$^+$ T lymphocytes play different roles in the outcome of leishmaniasis (da Silva Santos and Brodskyn, 2014). CD4$^+$ T cell memory phenotypes can modulate immune responses by secreting specific cytokines hence capable of influencing disease outcome (Luckheeram et al., 2012).

Accordingly, the expansion of CD4$^+$, and CD8$^+$ T lymphocytes together with activation and memory phenotypes of CD4$^+$ T cells were analyzed in both CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mouse strains infected with *L. mexicana* promastigotes using flow cytometry and compared to their littermate IL-4Rα$^{-/lox}$ controls.

$CD4^+$ and $CD8^+$T cells were gated as $CD19^-CD3^+CD4^+CD8^-$, $CD19^-CD3^+CD4^-CD8^+$ respectively whereas their activated T cells were determined by CD44 expression. The central and memory phenotypes of T cells were gated as $CD44^+CD62L^+$ and $CD44^+CD62L^-$ respectively (Hurdayal et al., 2013) (**Appendix 2**).

Percentage $CD4^+$ and $CD8^+$ T cells present in LNs of $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ females were comparable to their IL-4R$\alpha^{-/lox}$ littermate controls (**Figures 3.13A, B**). However, $CD4^+$ and $CD8^+$ T cells were significantly increased and decreased in global IL-4Rα deficient BALB/c mice respectively, when compared to littermate controls (**Figures 3.13A, B**). The percentage of activated $CD4^+$/$CD8^+$ T cells, their central and effector memory, and naïve phenotypes were similar between $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ and their IL-4R$\alpha^{-/lox}$ littermate controls (**Figures 3.13A, B**). In contrast, global IL-4Rα deficient female mice had significantly increased $CD4^+$ T cells, activated and effector memory $CD4^+$ T cells. In contrast, activated, central and effector memory $CD8^+$ T cells were significantly decreased in IL-4R$\alpha^{-/-}$ mice (**Figures 3.13A, B**).

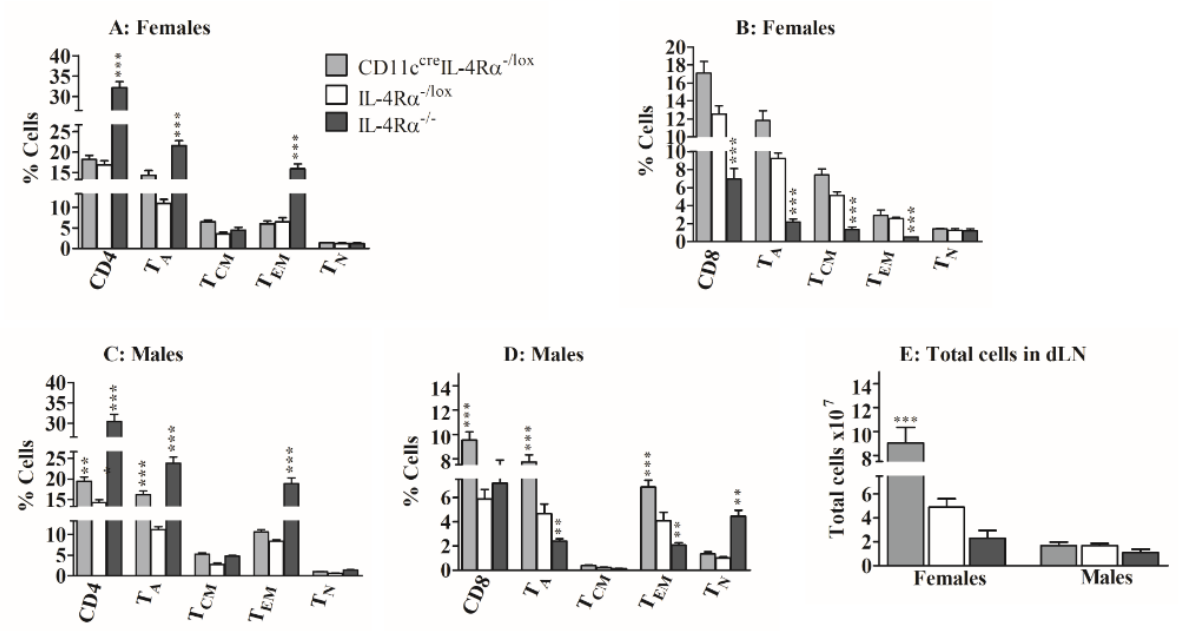

Figure 3.13: Frequency of lymphocytes expansion and activation during *L. mexicana* infection in CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice. Mice were infected subcutaneously with 2×10^6 *L. mexicana* promastigotes into the hind footpad. At week 8 after infection, frequency of $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells with their activated (TA), central memory (T_{CM}), effector memory (T_{EM}), and Naïve T cells (T_N) were enumerated in the LNs of female mice by flow cytometry. Similarly, the frequency of $CD4^+$ (**C**) and $CD8^+$ (**D**) T cells with their T_A, T_{CM}, T_{EM}, and T_N were enumerated in the LNs of male mice. Total cell numbers infiltration into the draining LN were also shown (**E**). Cells were differentiated based on following markers; $CD4^+$ T cells ($CD4^+CD3^+CD19^-$), $CD8^+$ T cells ($CD8^+CD3^+CD19^-$), T_A ($CD4^+CD8^+CD44^+$), T_{CM} ($CD4^+/CD8^+CD44^+CD62L^+$), T_{EM} T cells ($CD4^+/CD8^+CD44^+CD62L^-$) and T_N ($CD4^+/CD8^+CD44^-CD62L^+$). Data from one experiment representative of two performed are shown. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (**p≤0.01; ***p≤0.001) (n=4-6).

Examination of pLN cells of male CD11c^{cre}IL-4Rα$^{-/lox}$ mice surprisingly revealed a significantly increased percentage of $CD4^+$ and $CD8^+$ T cells when compared to their littermate controls (**Figures 3.13C, D**). This trend was observed in the frequency of activated and effector memory of both $CD4^+$ and $CD8^+$ T cells (**Figures 3.13C, D**). However, no significant differences were observed in the frequency of central memory and naïve phenotypes of both $CD4^+$ and $CD8^+$ T cells in the LN of CD11c^{cre}IL-4Rα$^{-/lox}$ when compared to their littermate controls (**Figures 3.13C, D**). Notably, male IL-4Rα$^{-/-}$ mice had differentially significantly increased frequency of naïve $CD8^+$ T cell populations compared to their $CD4^+$ T cells counterparts (**Figures 3.13C, D**). More importantly, the CD11c^{cre}IL-4Rα$^{-/lox}$ female mice had significantly increased total pLN cell expansion when compared to their littermate controls (**Figure 3.13E**), explaining the differences in absolute cell numbers observed between the two-mouse strain.

Considering the absolute cell numbers which is a product of percentages and total cell numbers derived from the pLN, CD11c^{cre}IL-4Rα$^{-/lox}$ female mice had increased CD4^{+} and CD8^{+} T cells with increased expression of CD44 surface markers, hence exhibiting increased activation (**Figures 3.14 A, B**). No differences were observed in memory CD4^{+} T cell phenotypes or their naïve status in female CD11c^{cre}IL-4Rα$^{-/lox}$ mice (**Figure 3.14A**) when compared to their littermate controls. Comparatively, only the central memory CD8^{+} T cells were significantly increased in CD11c^{cre}IL-4Rα$^{-/lox}$ female mice when compared to the littermate control (**Figure 3.14 B**).

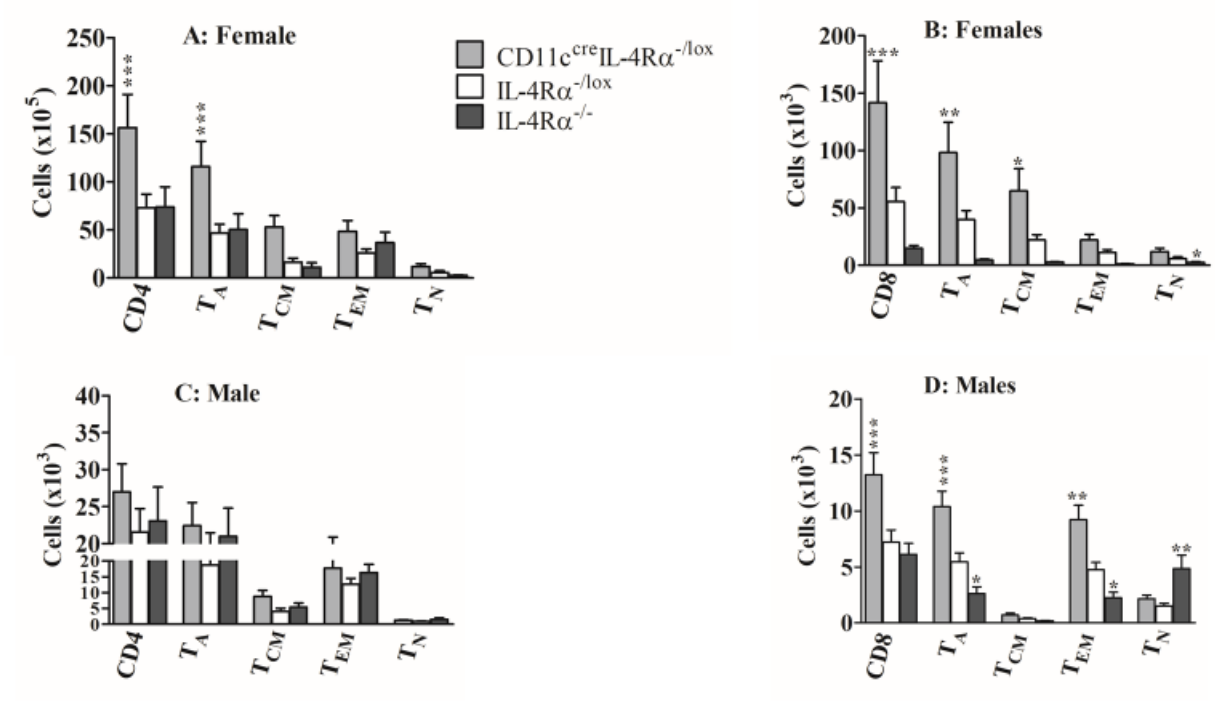

Figure 3.14: Calculated absolute numbers of lymphocytes expansion and activation during *L. mexicana* infection in female CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice. Mice were infected subcutaneously with 2×10^6 *L. mexicana* promastigotes into the hind footpad. Eight weeks post-infection, mice were sacrificed and the absolute numbers of CD4^{+} (**A**) and CD8^{+} (**B**) T cells in the LNs of female mice with their respective activated (T_A), central memory (T_{CM}), effector memory (T_{EM}), and Naïve (T_N) T cells were quantified. Similarly, the absolute numbers of CD4^{+} (**C**) and CD8^{+} (**D**) T cells with their activated (T_A), central memory (T_{CM}), effector memory (T_{EM}), and Naïve T cells (Werner) T cells were enumerated in the LNs of male mice. Values of one experiment representative of two performed are shown. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (*P≤0.05, **p ≤0.01; ***p≤0.001) (n=6).

Considering male mice, contrary to findings on percentages, no differences were exhibited in the absolute cell numbers of $CD4^+$ T cells in pLN of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to their littermate controls (**Figure 3.14C**). The activation status of these $CD4^+$ T cells remained unchanged between the strains. In a similar trend, the central, effector memory and naïve phenotypes of $CD4^+$ T cells were comparable between the $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and their littermate controls (**Figure 3.14C**). In contrast, $CD8^+$ T cells, their activation, and effector memory were significantly increased in male $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice when compared to their littermate controls, whereas the central memory and naïve $CD8^+$ T cells were comparable between the two mouse strains (**Figure 3.14D)**. Unlike in female mice, global male $IL\text{-}4R\alpha^{-/-}$, mice had significantly increased naïve $CD8^+$ T cells when compared to the littermate controls (**Figure 3.14D)**.

Analysis of pLN of mice with deficiency of IL-4Rα signaling on macrophage and neutrophil cells revealed that the frequency of $CD4^+$ T cells activated $CD4^+$ T cells, their central and effector memory phenotypes were unchanged between $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ of both female (**Figure 3.15A)** and male (**Figure 3.15B**) mice. A similar trend was observed in B cell populations (**Figure 3.15 C**). Similarly, the calculated absolute cell numbers were similar between the $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and their littermate $IL\text{-}4R\alpha^{-/lox}$ mice of both female (**Figure 3.15 D)** and male (**Figure 3.15 E)** mice, evident on the similar total number of cells in the pLN of $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and their littermate control mice (**Figure 3.15 F)**.

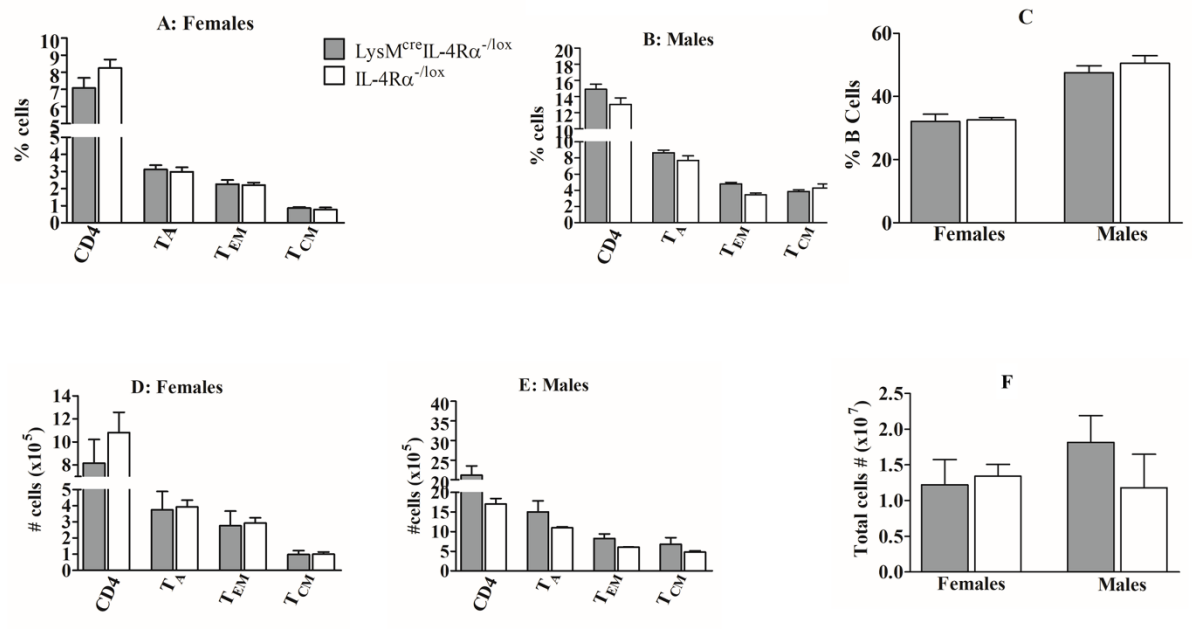

Figure 3.15: Expansion of CD4$^+$ T cells, activation, and memory phenotypes in pLN of LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. Mice were infected subcutaneously with 2×10^6 *L. mexicana* promastigotes into the hind footpad. Eight weeks post-infection, mice sacrificed, single-cell suspension from pLNs was stained for surface markers. Using flow cytometry, the % of CD4$^+$ T cells, their activation state, effector, and central memory were determined in both females (**A**) and males (**B**). % B cells **(C)** were also shown. The absolute cell numbers were calculated in females (**D**) and males (**E**) based on the frequencies and the total number of cells (**F**). Both % and absolute cell numbers in pLN of LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared to littermate IL-4Rα$^{-/lox}$ controls. A P value of ≤0.05 was considered significant (n=6).

3.1.6 IL-4 and IL-13 production by CD4 and CD8 is differentially regulated in the absence of IL-4Rα on DCs and macrophages/neutrophils.

T cells are important in conferring susceptibility or protection of host to CL disease (Santos and Brodskyn, 2014), and CD4$^+$ and CD8$^+$ T cells are responsible for the production of cytokines that mediate either protection or promote CL disease. To this end, this study investigated the ability of lymphocytes from the pLNs of CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ to secrete both Th1 and Th2 cytokines, to determine the immune mechanisms behind a similar disease phenotype in both strains compared to littermate IL-4Rα$^{-/lox}$ controls.

3.1.6.1 Increase of IL-4 and IL-13 production by $CD4^+$ and $CD8^+$ T cells in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice influence the outcome of *L. mexicana*-induced CL in a sex-dependent manner.

In line with cytokine production by LN cells stimulated with both αCD3 and *L. mexicana* heat-killed promastigotes, analysis of pLN cells of female $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice revealed a similar percentage of $CD4^+$ and $CD8^+$ T cells producing IFN-γ whereas a slight trend for increased percent of IL-4, and IL-13 was observed when compared to IL-$4R\alpha^{-/lox}$ mice as opposed to increased IFN-γ and decreased IL-4 and IL-13 in resistant global IL-$4R\alpha^{-/-}$ mice (**Figures 3.16A, B**). Accordingly, there was a significant increase in the absolute number of $CD4^+$ and $CD8^+$ T cells producing IL-4 and IL-13 in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ compared to IL-$4R\alpha^{-/lox}$ (**Figures 3.16C, D**), indicative of enhanced Th2 responses however not tilting to hyper-susceptibility as reported previously (Hurdayal et al., 2013).

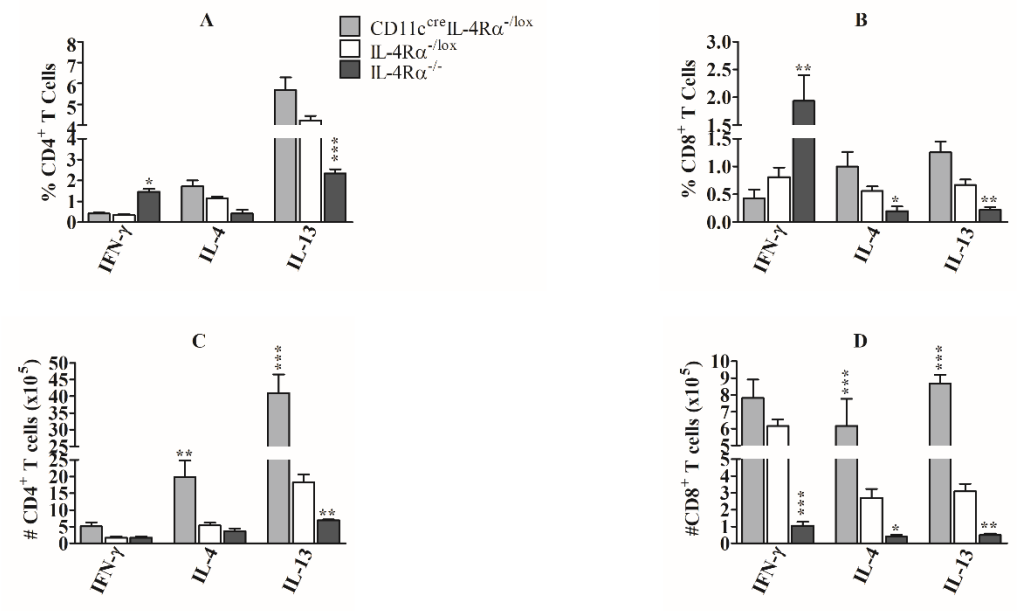

Figure 3.16: Cytokine-producing T cells from $CD11c^{cre}$IL-$4R\alpha^{-/lox}$, IL-$4R\alpha^{-/lox}$, and IL-$4R\alpha^{-/-}$ female mice infected with *L. mexicana*. Female mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* and 8 weeks post-infection. 2×10^6 cells were isolated from popliteal lymph node cells were stimulated with PMA and ionomycin for 2 hours followed by 4hrs incubation with monensin. Cells were seeded in 96 well plates, stained with fluorochromes conjugated antibodies, and analyzed using a flow cytometer. Percentage $CD4^+$ (**A**), $CD8^+$ (**B**), absolute cell numbers $CD4^+$ (**C**), $CD8^+$ (**D**) cytokine-producing T cells were determined. Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-$4R\alpha^{-/lox}$ BALB/c mice as significant (*$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$) (n=6).

Dendritic cell-specific IL-4Rα-deficient male mice had a similar percentage of IFN-γ (**Figure 3.17A**), IL-4 (**Figure 3.17A**), and IL-13 (**Figure 3.17A**) $CD4^+$ cytokine producers in their pLN. Unlike female mice, the male $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had a similar trend in the absolute numbers of IFN-γ (**Figure 3.17D**), IL-4 (**Figure 3.17E**), and IL-13 (**Figure 3.17F**) $CD4^+$ cytokine-producing cells when compared to their littermate controls. Similarly, the percentage of IFN-γ, IL-4, and IL-13 (**Figure 3.18A**) $CD8^+$ cytokine-producing cells were comparable between male $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and the littermate controls. A similar trend was observed in absolute cell numbers of IFN-γ, IL-4, and IL-13 $CD8^+$ T cell producers (**Figure 3.18B**). These findings, therefore, demonstrate a sex-dependent T cell cytokine production in mice with loss of IL-4Rα signaling on DCs.

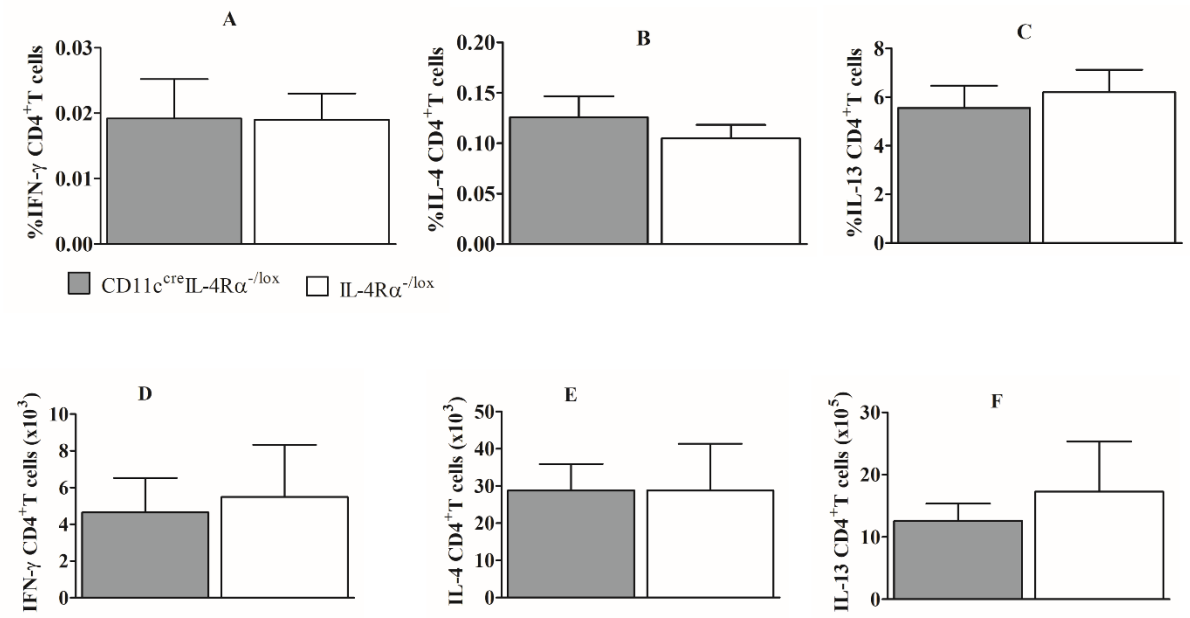

Figure 3.17: Cytokine producing $CD4^+$ T cells from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ male mice infected with *L. mexicana*. Male mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* and 8 weeks post-infection. 2×10^6 cells were isolated from popliteal lymph node cells were stimulated with PMA and ionomycin for 2 hours followed by 4hrs incubation with monensin. Cells were seeded in 96 well plates, stained with fluorochromes conjugated antibodies, and analyzed using a flow cytometer. Percentage IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**) producing $CD4^+$ T cells. In addition, absolute cell numbers of IFN-γ (**D**), IL-4 (**E**), IL-13 (**F**) producing $CD4^+$ T cells were calculated. Statistical analysis was performed using an unpaired student t-test comparing the differences to $IL\text{-}4R\alpha^{-/lox}$ BALB/c mice. A P value of ≤ 0.05 was significant. The data presented was one of the two independent experiments (n=6).

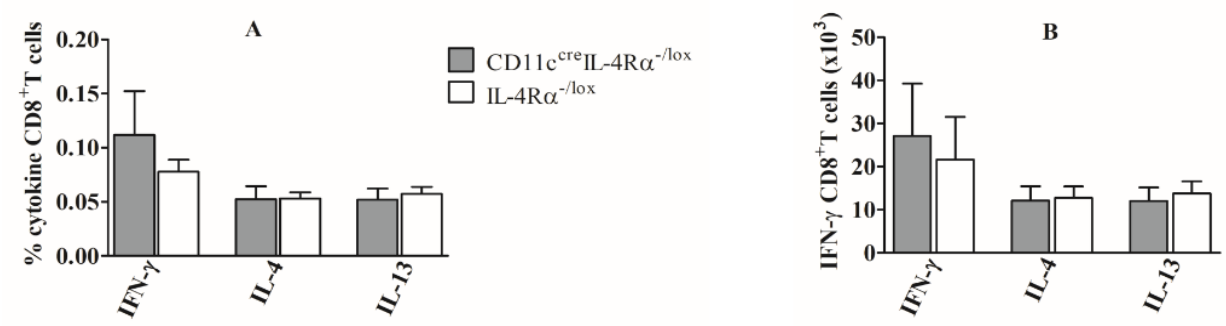

Figure 3.18: Cytokine producing CD8$^+$ T cells from CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ male mice infected with *L. mexicana*. Male mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* and 8 weeks post-infection. 2×10^6 cells were isolated from popliteal lymph node cells were stimulated with PMA and ionomycin for 2 hours followed by 4hrs incubation with monensin. Cells were seeded in 96 well plates, stained with fluorochromes conjugated antibodies, and analyzed using a flow cytometer. Percentage IFN-γ, IL-4, and IL-13 producing CD8$^+$ T cells (**A**). In addition, absolute cell numbers of IFN-γ, IL-4, IL-13 producing CD8$^+$ T cells (**B**). Statistical analysis was performed using an unpaired student t-test comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice. A P value of ≤ 0.05 was significant. The data presented was one of the two independent experiments (n=6).

3.1.6.2 Infected macrophage/neutrophil cell-specific IL-4Rα$^{-/-}$ mice have similar induction of Th1/Th2 cytokines by CD4$^+$ T cells following *L. mexicana* infection.

Flow cytometry analysis revealed that the percentage of IFN-γ CD4$^+$ T producers defining the quality of Th1 responses were not statistically significant between LysM^{cre}IL-4Rα$^{-/lox}$ and littermate IL-4Rα$^{-/lox}$ in both female and males (**Figures 3.19A, B**). A similar trend was observed in IL-4 and IL-13 CD4$^+$ producing T cells (**Figures 3.19A, B**).

Similarly, the absolute numbers of IFN-γ, IL-4, and IL-13-producing CD4$^+$ T cells were unchanged between LysM^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls in both female and male mice (**Figures 3.19C, D**). However, analysis of pLN cells demonstrated that IL-4Rα signaling via macrophages/neutrophils promotes the induction of IL-10 producing CD4$^+$ T cells in males following infection with *L. mexicana*, indicating a sex-dependent function (**Figure 3.19D**).

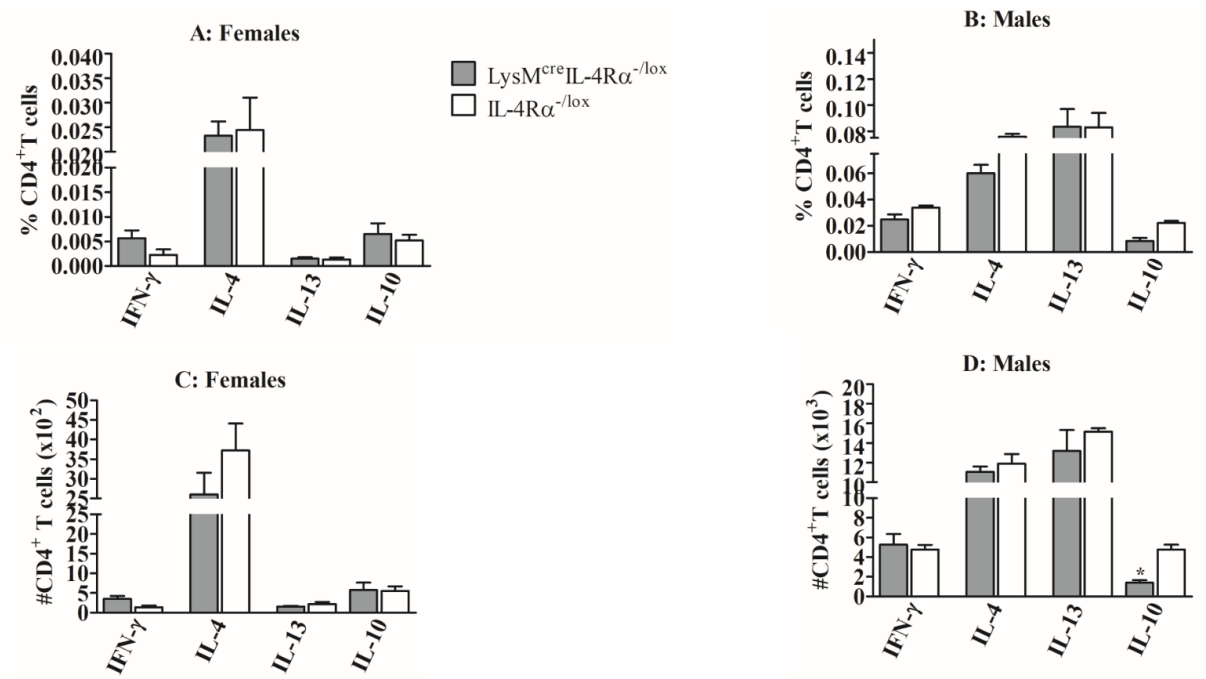

Figure 3.19: Cytokine producing CD4$^+$ T cells in LysM^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate controls. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* and 8 weeks post-infection. 2×10^6 cells were isolated from popliteal lymph node cells were stimulated with PMA and ionomycin for 2 hours followed by 4hrs incubation with monensin. Cells were seeded in 96 well plates, stained with fluorochromes conjugated antibodies, and analyzed using a flow cytometer. Percentage cells producing IFN-γ, IL-4, IL-13, and IL-10 were determined in females (**A**) and Male (**B**) using a flow cytometer. Absolute numbers of cytokine producing CD4$^+$ T cells were calculated in both females (**C**) and males (**D**). Percentage and the absolute number of cytokine-producing CD4$^+$ T cells by LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared to littermate IL-4Rα$^{-/lox}$ controls. A P value of ≤0.05 was considered significant (n=6).

3.1.7 The deficiency of IL-4Rα signaling on DCs and macrophages/neutrophils does influence the infiltration of myeloid cells into the LN of mice infected with *L. mexicana*.

Myeloid cells are important for the replication and clearance of *Leishmania* (Rossi and Fasel, 2018). More importantly, myeloid cells are critical in the development of the immune response against *Leishmania* parasites through antigen presentation T cells leading to the secretion of cytokines, chemokines, and microbicidal products (Polari et al., 2019).

Since CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice had uncontrolled footpad swelling and parasite growth like their littermate controls, this study investigated whether IL-4Rα deficiency on DCs and macrophage/neutrophil cells may have modulated myeloid cell recruitment into the LN and their maturation by evaluating the frequency of macrophages, DCs, and neutrophils using flow cytometry. Activated macrophages were identified as CD11b$^+$Ly6G$^-$F4/80$^+$CD11c$^-$MHCII$^+$ whereas neutrophils were CD11b$^+$Ly6G$^+$ (Sheng et al.,

2017). The conventional DCs (cDCs) were gated as $CD11b^{+}Ly6G^{-}F4/80^{-}CD11c^{+}MHCII^{+}$ and monocyte-derived DCs (MoDCs) were identified as $CD11c^{+}F4/80^{+}$ cells (Cao et al., 2015) (**Appendix 3**).

The deletion of IL-4Rα on DCs did not alter the percent infiltration and expansion of activated macrophages, cDCs, neutrophils, or Mo-DCs populations in the LN of female $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ compared to their littermate controls. The frequency of activated macrophages (**Figure 3.20A**), cDCs (**Figure 3.20B**), neutrophils (**Figure 3.20C**), and Mo-DCs (**Figure 3.20D**) was similar between both $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and littermate controls; however, there was observed markedly significantly lower frequency of activated macrophages (**Figure 3.20A**), cDCs (**Figure 3.20B**), and Mo-DCs (**Figure 3.20D**) in female $IL\text{-}4R\alpha^{-/-}$ mice compared to $IL\text{-}4R\alpha^{-/lox}$ mice. A similar trend was shown when considering absolute cell numbers of activated macrophages (**Figure 3.20E**), cDCs (**Figure 3.20F**), neutrophils (**Figure 3.20G**), and Mo-DCs (**Figure 3.20H**).

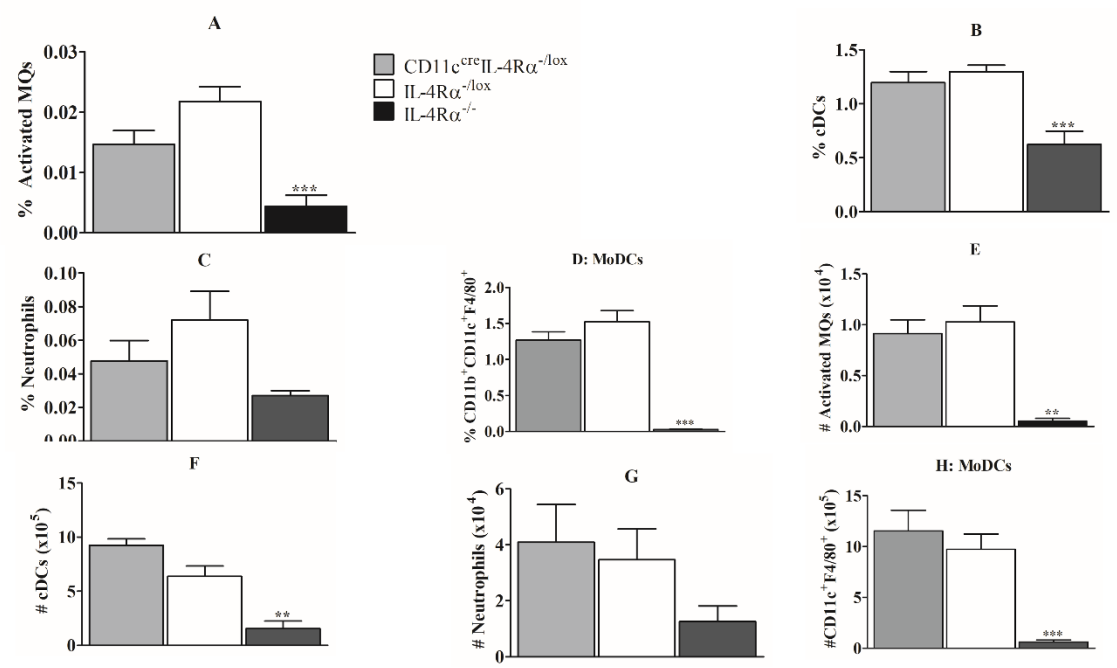

Figure 3.20: Myeloid cell populations in LNs from CD11c^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ female mice infected with *L. mexicana*. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* metacyclic promastigotes and at 8 weeks post-infection the cells were isolated from LNs and characterized by flow cytometry. The frequency of activated macrophage (**A**), conventional DC (cDCs) (**B**), neutrophils (**C**), and monocyte-derived DCs (Mo-DCs) (**D**) cells infiltration into LNs were determined. Absolute cell numbers for activated macrophages (**E**), cDCs (**F**), neutrophils (**G**), and Mo-DCs (**H**) were calculated by multiplying the percentages and total cell numbers. Activated macrophages (MQs) were identified as CD11b$^+$CD11c$^-$F4/80$^+$MHCII$^+$), cDCs (CD11b$^+$CD11c$^+$MHCII$^+$), Neutrophils (CD11b$^+$L6G$^+$), and Mo-DCs (CD11c$^+$F4/80$^+$) (**Appendix 3**). Statistical analysis was performed using one-way ANOVA (Bonferroni post-test) comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice as significant (**P≤0.01; ***p ≤0.001). Results are representative of one of two independent experiments (n=6).

By sex-matching, using flow cytometry, we also analyzed cells in pLN of male CD11c^{cre}IL-4Rα$^{-/lox}$ mice in order to investigate whether the lack of IL-4Rα signaling on DCs influence the infiltration of myeloid cells. The deficiency of IL-4Rα expression on DCs did not alter the frequency of activated macrophages (**Figure 3.21A**), cDCs (**Figure 3.21B**), and neutrophils (**Figure 3.21C**) in pLN when compared to littermate controls. Like the frequencies, the absolute cell numbers of activated macrophages (**Figure 3.21D**), cDCs (**Figure 3.21E**), and neutrophils (**Figure 3.21F**) were comparable between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and the littermate IL-4Rα$^{-/lox}$ mice.

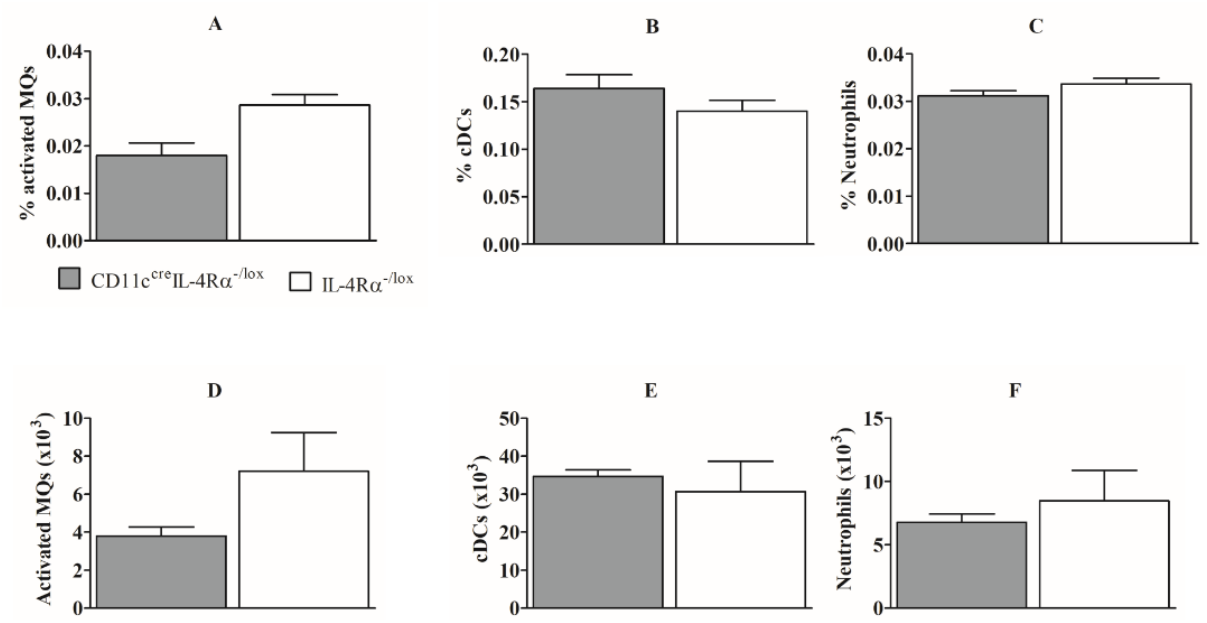

Figure 3.21: Myeloid cell populations in LNs from CD11c^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ male mice infected with *L. mexicana*. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* metacyclic promastigotes and at 8 weeks post-infection the cells were isolated from pLNs and characterized by flow cytometry (**Appendix 3**). The frequency of activated macrophage (MQs) (**A**), Dendritic cells (DCs) (**B**), and neutrophils (**C**) cells infiltration into LNs were determined. Absolute cell numbers of activated macrophage (MQs) (**D**), Dendritic cells (DCs) (**E**), and neutrophils (**F**) were calculated by multiplying the percentages and total cell numbers in LNs. Statistical analyses were performed using an unpaired student t-test comparing the differences to IL-4R$\alpha^{-/lox}$ BALB/c mice. A p-value of $\leq$ 0.05 was significant. Results are representative of one of the two experiments (n=6).

Comparatively, macrophages/neutrophils cell-specific IL-4R$\alpha^{-/-}$ female mice had an unchanged frequency of mature macrophages (**Figure 3.21A**), cDCs (**Figure 3.21B**), and neutrophils (**Figure 3.21C**) populations infiltration and expansion in the pLN when compared to littermate controls. Notably, there was no significant difference in the frequency of activated macrophage (**Figure 3.21D**), cDC (**Figure 3.21E**), and neutrophil (**Figure 3.21F**) cell populations between male LysM^{cre}IL-4R$\alpha^{-/lox}$ mice compared to their littermate controls.

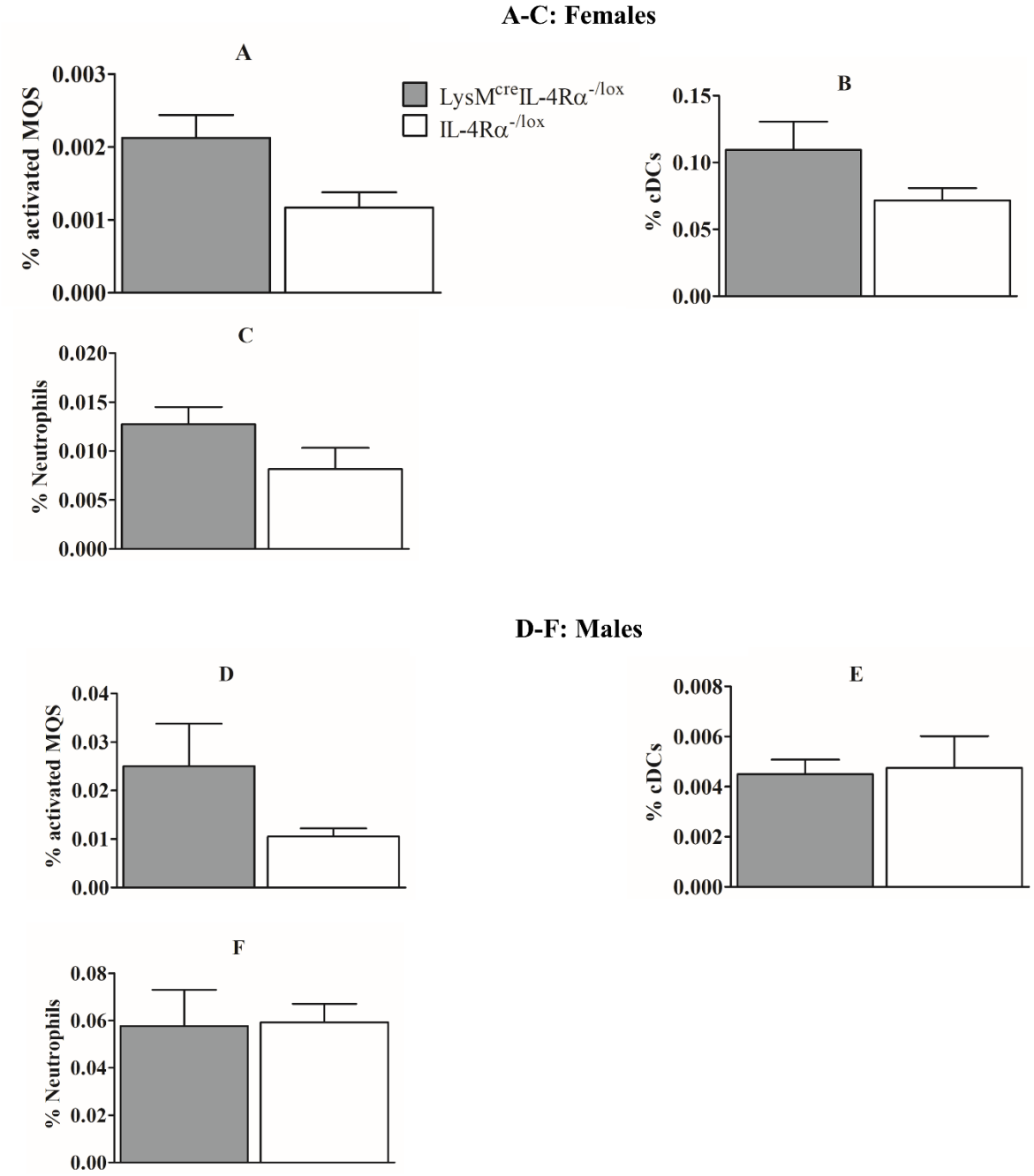

Figure 3.22: Myeloid infiltration into pLN of LysM^{cre}IL-4Rα$^{-/lox}$ mice. Mice were injected subcutaneously in the footpad with 2×10^6 *L. mexicana* metacyclic promastigotes and at 8 weeks post-infection the cells were isolated from LNs and characterized by flow cytometry. The frequency of activated macrophages (**A, D**), cDCs (**B, E**), and neutrophils (**C, F**) in both female and male mice, respectively, were enumerated in pLN using flow cytometry. The percentage of cells in LysM^{cre}IL-4Rα$^{-/lox}$ mice were compared to littermate IL-4Rα$^{-/lox}$ controls using an unpaired student t-test. A P value of ≤0.05 was considered significant (n=6).

Overall, this study demonstrates that deficiency of IL-4Rα signaling on DCs, and macrophages/neutrophils does not influence both infiltrations of activated macrophages,

cDCs, and neutrophils into pLN or their expansion when compared to IL-4Rα$^{-/lox}$ littermates. In addition, the infiltration and expansion of myeloid cells in pLN of LysM^{cre}IL-4Rα$^{-/lox}$ mice were not dependent on sex.

3.1.8 Frequency and absolute cell numbers of IL-12 DC producing cells in DC-IL-4Rα cell-specific IL-4Rα$^{-/-}$ mice following *L. mexicana* infection.

IL-4/IL-13 signaling through IL-4Rα regulates DC maturation and functions consequently affecting adaptive immune responses (Webb et al., 2007; Ahn et al., 2005). During the early phase of *Leishmania* infection, Il-4 signaling on DCs is important in initiating type 1 responses by secreting IL-12 that drives expansion of Th1 immune responses (Hurdayal et al., 2013). Having observed similar nonhealing progressive lesion sizes (**Figures 3.1 A, B**), we aimed to investigate whether the absence or presence of IL-4Rα signaling on DCs affects their IL-12 production following *L. mexicana* infection. Cells from pLN of *L. mexicana* infected CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice were stained with fluorochromes conjugated antibodies for surface markers for DCs and IL-12 cytokine. In corroboration with similar disease phenotype, the percentage of the expression of IL-12 by DCs from CD11c^{cre}IL-4Rα$^{-/lox}$ infected *L. mexicana* parasites, were similar to the IL-4Rα$^{-/lox}$ in both female (**Figures 3.23 A**) and male (**Figures 3.23 B**) mice. The absolute cell numbers of DCs expressing IL-12 did not differ between CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ littermates in female (**Figures 3.23 C**) and male (**Figures 3.23 D**) either. This suggests that IL-4-DC IL-12 instruction in female and male CD11c^{cre}IL-4Rα$^{-/lox}$ mice was not affected despite increased secretion of IL-4 and IL-13 cytokines by pLN cells as shown in figures **3.8B/E, 3.9B, 3.10B**.

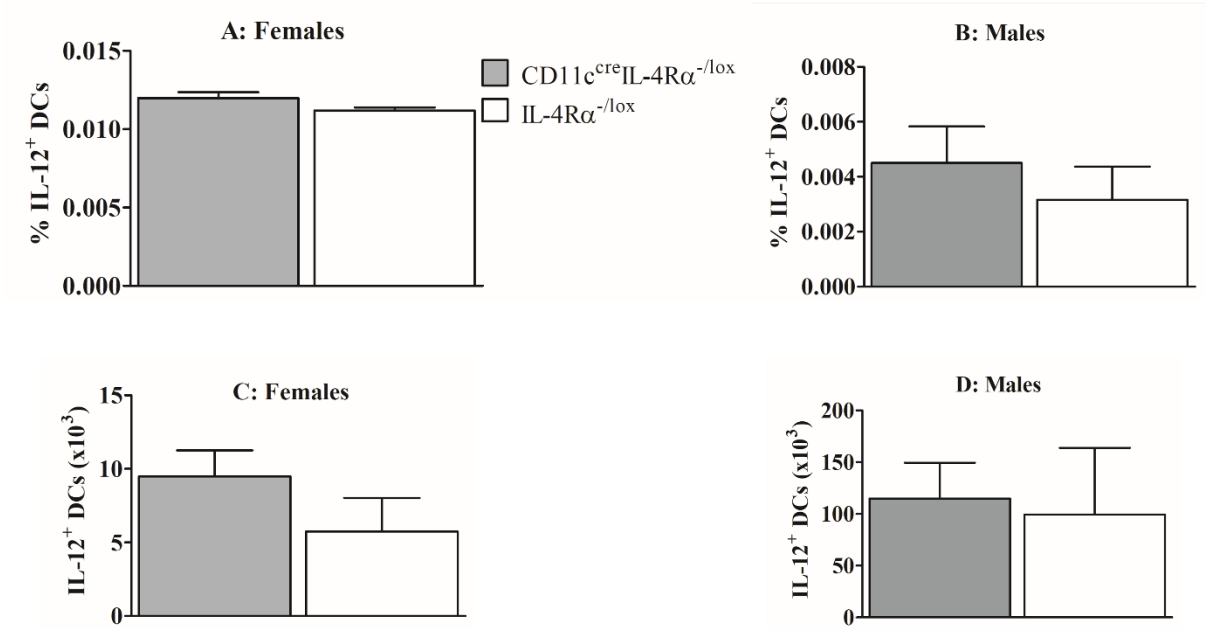

Figure 3.23: IL-12 DCs producers from pLN of *L. mexicana*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ BALB/c mice. Cells from pLNs of experimental female mice infected subcutaneously with *L. mexicana* promastigotes at week 8 p.i were stimulated with PMA/ionomycin and monensin for 6 hours at 37ºC, 5% CO_2. The percentage of DCs producing IL-12 in females (**A**) and males (**B**) was determined by flow cytometry. The absolute cell numbers of IL-12 producing DCs were calculated in female (**C**) and male (**D**) mice as the product of frequency and total cell numbers. Statistical analysis was performed using an unpaired student t-test comparing the differences to IL-4Rα$^{-/lox}$ BALB/c mice. A value of $p \leq 0.05$ was considered significant (n=6).

3.1.9 Deficiency of IL-4Rα signaling on DCs and macrophages/neutrophils did not alter nitrite concentration and arginase 1 derived metabolite in pLN cells following *L. mexicana* infection

Il-4Rα signaling on MQ drives an M2 macrophage phenotype (Weng et al., 2018; Wang et al., 2014). Accordingly, we investigated macrophage effector functions in CD11c^{cre}IL-4Rα$^{-/lox}$ **and** LysM^{cre}IL-4Rα$^{-/lox}$ mice subcutaneously infected in a footpad with *L. mexicana* promastigotes.

CD11c^{cre}IL-4Rα$^{-/lox}$ mice were hyper-susceptible during *L. major* infection when compared to littermate controls, with significantly decreased levels of iNOS indicative of the pivotal role of NO in protection against CL (Hurdayal et al., 2020; Hurdayal et al., 2013).

Since there was observed increased levels of IL-4 and IL-13 in the pLN cells of CD11c^{cre}IL-4Rα$^{-/lox}$ compared to their littermate controls, the study sought to investigate if the pLN cells

exhibited any alteration in their effector functions in nitrite and urea secretion by virtue of intrinsic iNOS and arginase I activity respectively (Rivera-Fernández et al., 2019; Contreras et al., 2014). Cells from pLN stimulated with LPS for 72hrs in 37°C, 5% incubation produced comparable levels of nitrite and urea between CD11c^{cre}IL-4Rα$^{-/lox}$ and their littermate controls in both males and females (**Figures 3.24A, B**). This data suggests that following *L. mexicana* challenge, BALB/c mice with loss of IL-4Rα expression on DCs does not modulate the activity of iNOS and arginase-1 enzymes.

Similarly, after LPS restimulation of pLN cells, urea and nitrite production was unchanged between LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, compared with littermate controls (**Figures 3.24C, D**). Although this current study did not directly evaluate the secretion of iNOS and arginase I, the data is indicative of the inability to polarize cells to a classically activated phenotype that efficiently enhances leishmanicidal activity hence failure to control the infection.

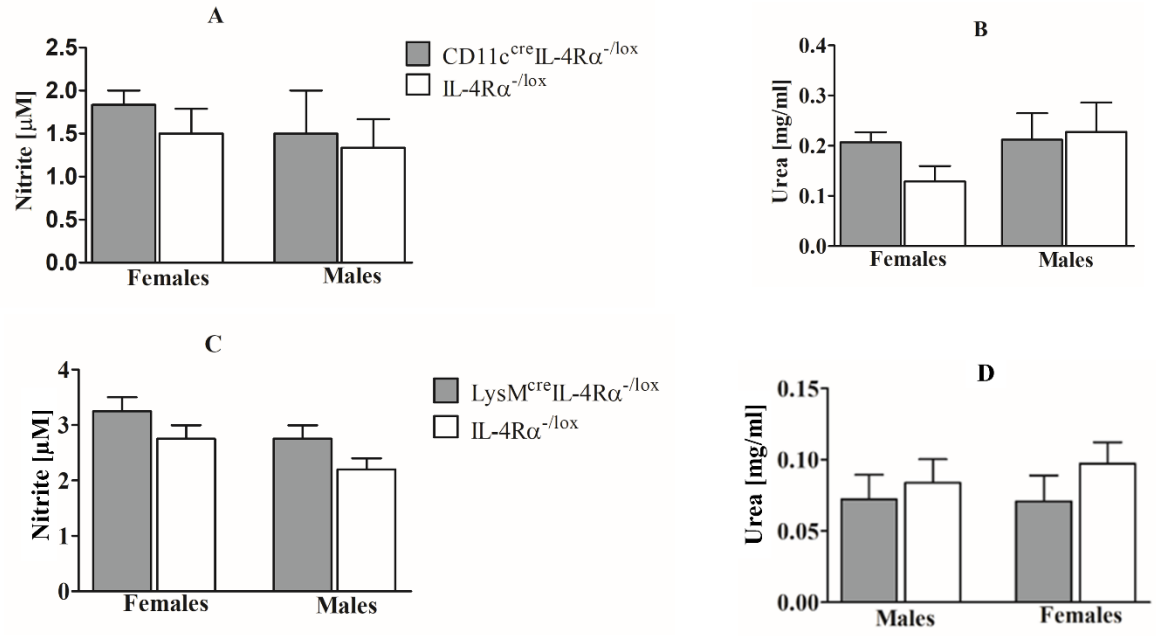

Figure 3.24: Measurement of nitrite and urea production by LN cells of CD11c^{cre}IL-4Rα$^{-/lox}$, LysM^{cre}IL-4Rα$^{-/lox}$ mice and their littermate IL-4Rα$^{-/lox}$. LN cells from *L. mexicana* infected male and female CD11c^{cre}IL-4Rα$^{-/lox}$, LysM^{cre}IL-4Rα$^{-/lox}$ mice and their respective littermate IL-4Rα$^{-/lox}$ were stimulated with 1µg/ml LPS for 72 hrs at 37°C, 5% CO_2. Supernatants were collected for quantification of nitrite and pelleted cells were used for urea analysis. Levels of nitrite in pLN cells of male and female CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice (**A, C**) were quantified. In addition, the quantities of arginase 1 derived metabolites, urea was investigated in both male and female CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice (**B, D**). Data were analyzed using one-way ANOVA (Bonferroni post-test) defining significant differences to IL-4Rα$^{-/lox}$ BALB/c mice were defined as $p \leq 0.05$.

3.1.10 IL-4 mediated DC instruction *in vitro* is unaltered in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to their littermate controls during *L. mexicana* infection.

In this study, CD11c^{cre}IL-4Rα$^{-/lox}$ mice had increased IL-4 and IL-13 compared to their littermate controls. IL-4 is known to modulate DC instruction by the production of IL-12 via IL-4Rα signaling and impairment of IL-10 (Biedermann et al., 2001; Yao et al., 2005). To investigate IL-4-mediated DC instruction during *L. mexicana* infection in absence of IL-4Rα-responsive DCs, BMDCs were generated from CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. BMDCs were infected and treated with either IL-4 and/or lipopolysaccharide (LPS) for 48 hours. The frequency and maturation status of BMDCs stimulated with IL-4 and /or LPS in the presence or absence of *L. mexicana* promastigotes were evaluated *in vitro* by the expressions of MHCII molecules on CD11c^{+} cells. The addition of IL-4 and LPS in presence of *L. mexicana* promastigotes cultures did not alter the frequency of the MHCII molecule on BMDCs (**Figures 3.25A, B**).

The mean fluorescence intensity and frequency of activation marker CD80 on BMDCs after *in vitro* addition of IL-4/LPS in cultures stimulated with *L. mexicana* promastigotes was also comparable between IL-4Rα -deficient and IL-4Rα sufficient BMDCs (**Figures 3.25C, D**). To investigate IL-4-mediated IL-12 instruction of DC, the levels of Il-12 and IL-10 of BMDCs induced by the addition of IL-4 and/or LPS co-cultured with *L. mexicana* promastigotes were evaluated. IL-4Rα-deficient BMDCs producing IL-12 did not differ among all cultures stimulated with IL-4 and/or LPS when compared to IL-4Rα sufficient BMDCs **(Figure 3.26A**). In addition, the levels of IL-10 of infected BMDCs stimulated with IL-4 and/or LPS were not significantly different between IL-4Rα-deficient and IL-4Rα sufficient BMDCs **(Figure 3.26B**). Similarly, the BMDCs treated with only LPS produced both IL-12p70 and IL-10 cytokines that were comparable between IL-4Rα-deficient and IL-4Rα functional BMDCs (**Figures 3.26A, B**).

Inducible NO synthase (iNOS) enzyme shares L-arginase as a substrate with arginase I enzyme, and the depletion of this substrate by either enzyme regulates macrophage phenotype hence important in regulating macrophage effector functions (Hölscher et al., 2006). Altogether, we evaluated the levels of both iNOS and arginase 1 derived metabolites including nitrite, and urea respectively. Infected IL-4Rα deficient BMDCs stimulated with IL-4 and/or LPS produced similar levels of nitrite **(Figure 3.26C**) and urea **(Figure 3.26D**) in cultures when compared to IL-4Rα functional BMDCs, suggesting that the presence of IL-4

did not influence either a classical or alternative activation of BMDCs. The IL-4Rα-deficient BMDCs treated with only LPS did not produce significantly different amounts of nitrite and urea when compared to IL-4Rα sufficient BMDCs (**Figures 3.26C, D**).

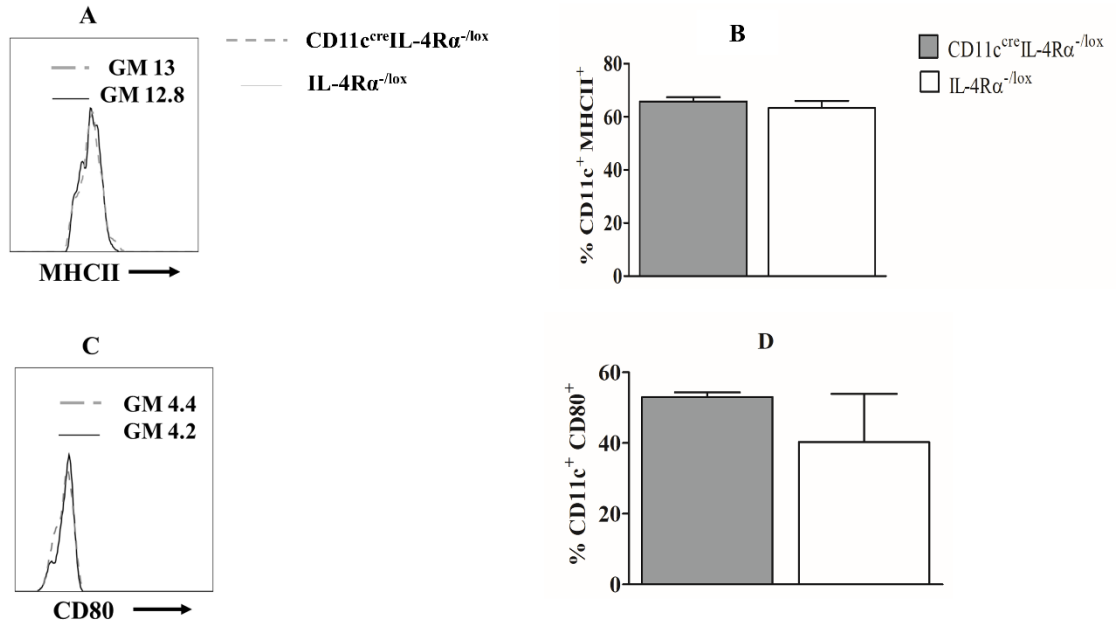

Figure 3.25: Abrogation of IL-4Rα expression in BMDCs does not affect their maturation and activation *in vitro*. Bone marrow cells harvested from the female mice were differentiated in RPMI 1640 containing 50% GMCSF supernatant incubated at 37ºC, 5 % CO_2 for 10 days. $5x10^5$ cells were treated with IL-4 in presence of 1µg/ml LPS or *L. mexicana* parasites (MOI 10:1). Cells stimulated with LPS only were used as controls. After 48hrs of incubation cells, supernatants were collected for analysis of the geometrical mean and % expression of MHCII (**A & B**), and CD80 (**C & D**) using flow cytometry. Data were analyzed using an unpaired student t-test defining differences to IL-4Rα functional BMDCs. A value of $P \leq 0.05$ was considered significant.

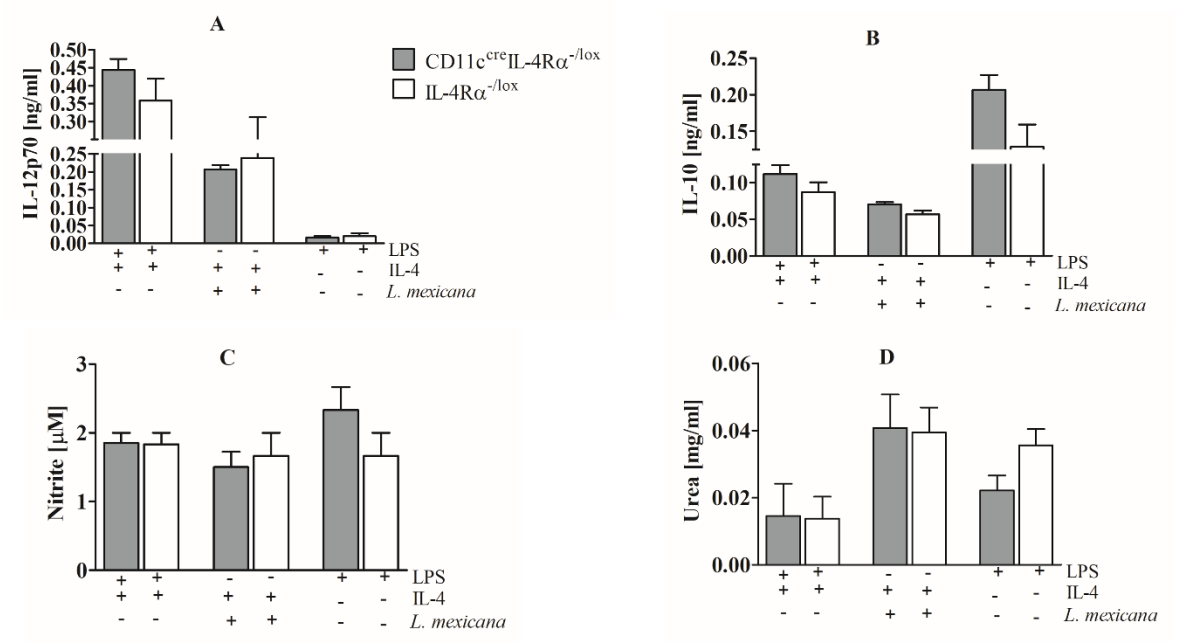

Figure 3.26: IL-4Rα deficient BMDCs have unchanged IL-4-DC-IL-12 and IL-10 instruction following *L. mexicana* infection *in vitro*. Bone marrow cells harvested from the female mice were differentiated in RPMI 1640 containing 50% GMCSF supernatant incubated at 37^{o}C, 5 % CO_2 for 10 days. 5x10^5 cells were treated with IL-4 in presence of 1μg/ml LPS or *L. mexicana* parasites (MOI 10:1). Cells stimulated with LPS only were used as controls. After 48hrs incubation of cells, supernatants were collected for analysis of IL-12p70 (**A**) and IL-10 (**B**) using ELISA. Nitrite was also assayed in supernatants (**C**) and pelleted cells were used for arginase activity by measuring levels of urea (**D**). Data were analyzed using one-way ANOVA (Bonferroni post-test) defining differences to IL-4Rα sufficient BMDCs. A value of P≤0.05 was considered significant.

3.1.11 BMDMs from LysM^{cre}IL-4Rα$^{-/lox}$ produce IL-12p70 and regulatory IL-10/ TGF-β similar to their littermates during *L. mexicana* infection.

As in CD11c^{cre}IL-4Rα$^{-/lox}$, we analyzed levels of IL-12p70, IL-10, and TGF to investigate the effects of IL-4Rα signaling on macrophage polarization in LysM^{cre}IL-4Rα$^{-/lox}$ mice. BMDMs were generated from LysM^{cre}IL-4Rα$^{-/lox}$ male mice and their littermate IL-4Rα$^{-/lox}$ controls. BMDMs were treated with IFN-γ or IL-4 for M1 and M2 differentiation respectively. Macrophages also produce NO in presence of LPS (Han et al., 2017) hence, BMDMs were thereafter treated with LPS for 48 hours post-infection to initiate NO production. The infected BMDMs from LysM^{cre}IL-4Rα$^{-/lox}$ mice, stimulated/unstimulated with IL-4 did not induce a significant increase in IL-10 production when compared with their littermate control (**Figure 3.26A**), however, infected BMDMs stimulated with IFN-γ had significantly increased IL-10 by BMDMs from LysM^{cre}IL-4Rα$^{-/lox}$ male mice compared to their littermate controls (**Figure**

3.26A). Infected BMDMs from IL-4Rα$^{-/lox}$ male mice stimulated /unstimulated with IL-4 did not induce a significantly increased IL-12p70 compared to LysM^{cre}IL-4Rα$^{-/lox}$ BMDMs (**Figure 3.26B**). Similarly, infected IL-4Rα deficient BMDMs stimulated /unstimulated with IFN-γ did not induce a significantly increased IL-12p70 compared to BMDMs from IL-4Rα$^{-/lox}$ mice (**Figure 3.26B**). Increased levels of TGF-β have been associated with active CL disease (Montoya et al., 2018), hence we investigated whether the absence of IL-4Rα signaling on macrophages/neutrophils affects their production by BMDMs. Infected BMDMs from LysM^{cre}IL-4Rα$^{-/lox}$ mice stimulated /unstimulated with either IL-4 or IFN-γ did not trigger any changes in TGF-β production compared to those from littermate controls (**Figure 3.26C**). These findings demonstrate that IL-4Rα signaling on macrophages does not alter capacity in secreting IL-12p70, IL-10, and TGF-β. Similarly, no differences were observed in the ability of IL-4 to induce the BMDMs with a functional IL-4Rα to produce nitrite when compared to IL-4Rα deficient BMDMs (**Figure 3.26D**), suggesting that IL-4 had no additive effect on classical activation of macrophages.

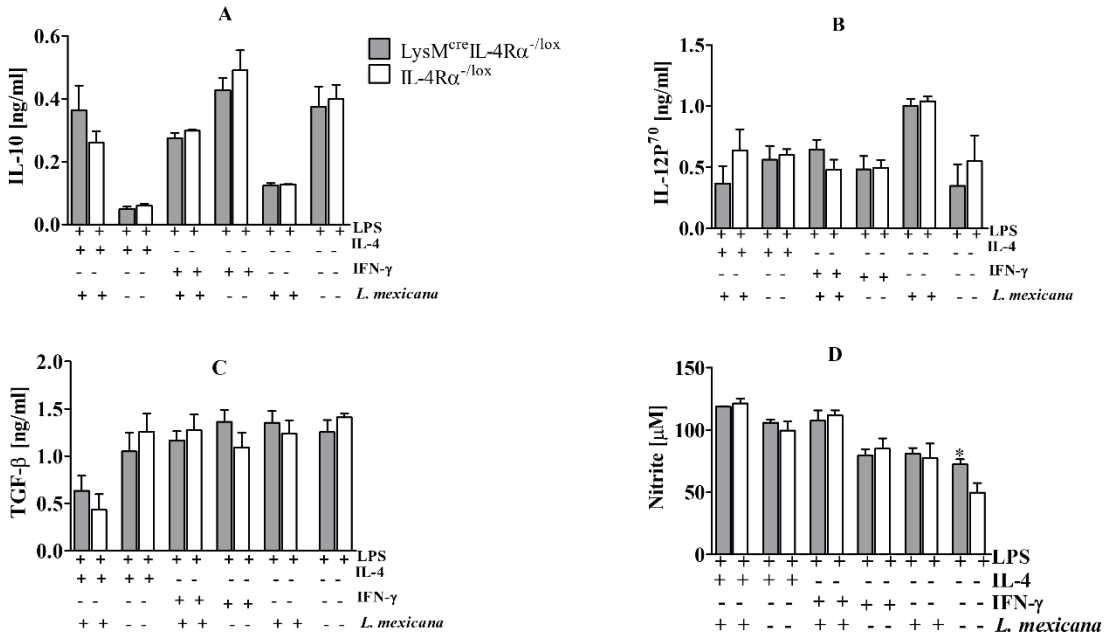

Figure 3.26: Measurement of cytokine production by BMDMs from male LysM^{cre}IL-4Rα$^{-/lox}$ mice and their littermate IL-4Rα$^{-/lox}$. BMDMs cells were generated from BM cells from two mouse strains. Infected BMDMs were stimulated with IL-4 or IFN-γ for 16hrs before being incubated with LPS/ IFN-γ for 48hrs. The supernatant was gently collected after centrifugation at 1500 rpm 4ºC for 5min. levels of IL-10 (**A**), IL-12p70 (**B**), and TGF-β (**C**) cytokines were determined using ELISA. The concentration of nitrite was also determined from supernatants using Griess reaction (**D**). Data were analyzed using one-way ANOVA (Bonferroni post-test), defining differences to BMDMs from IL-4Rα$^{-/lox}$ BALB/c mice as significant (*p≤0.05; **p≤0.01).

CHAPTER 4:

DISCUSSIONS AND CONCLUSIONS

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4.1 Discussion

Studies have shown that gene knockout and Cre/lox conditional knockout mouse models are invaluable tools in elucidating the functions of genes in specific cells and tissues (Ray et al., 2000). Hence, investigation of the gene knockout on specific cells during *Leishmania* infection will reveal how host cell-parasite interaction contributes to either susceptibility or protection. The IL-4Rα pathway, a common receptor for IL-4 and IL-13 signaling, is essential in mediating susceptibility to CL. In global abrogation of IL-4Rα, the progression of cutaneous leishmaniasis, caused by *L. mexicana* is severely impaired resulting in protection from infection and eventually a healing outcome (Alexander et al., 2002). Hence the need for studies on the abrogation of IL-4Rα on cell-specific to investigate the cell responsible for resistance to CL infection.

Interleukin-4/IL-13 signaling has been implicated in the maturation and activation of myeloid cells leading to the production of pro-inflammatory or anti-inflammatory cytokines important for eliminating some parasites (Bosurgi et al., 2017; Heeb et al., 2020; Hurdayal et al., 2013). Myeloid cells as a whole comprise of DCs, macrophages, and neutrophils (Devlin et al., 2020). In terms of DCs, IL-4 has been shown to instruct IL-12-derived DC production by virtue of reduced IL-10 (Yao et al., 2005) hence driving Th1 immune responses (Biedermann et al., 2001). Accordingly, the abrogation of IL-4Rα on DCs in BALB/c mice ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$) led to the host's hypersusceptibility to *L. major* infection, hence indicating a protective role for IL-4Rα signaling on DCs in mice (Hurdayal et al., 2013). In addition, IL-4Rα signaling regulates M2, alternatively activated, macrophage polarization. Indeed, M2 macrophage polarization is induced by IL-4 cytokine milieu and has opposing disease-regulating functions to those of the M1 phenotype (Zhou et al., 2017). M2 macrophages are permissive for the growth of CL-causing *Leishmania* parasites leading to disease severity, primarily through the metabolism of polyamine to produce urea that favors the survival of *Leishmania parasites* (Lee et al., 2018; Muxel et al., 2017). In contrast, the M1 macrophages phagocyte *Leishmania* pathogens, produce proinflammatory cytokines and trigger oxidative burst that effectively leads to the elimination of the parasites (Tomiotto-Pellissier et al., 2018; Zanluqui et al., 2015).

On the other hand, neutrophils migrate to the site of infection following a gradient of chemo-attractants like CXCR2-binding chemokines and become the first innate cells that respond to *Leishmania* infections (Phillipson and Kubes, 2011). At the site of infection, neutrophils employ mechanisms like phagocytosis, degranulation, the release of reactive oxygen species (ROS), and the formation of neutrophil extracellular traps (NETs) to kill or immobilize the pathogens (Bonne-Année et al., 2014; Mayadas et al., 2014). Importantly, IL-4/IL-13 mediated IL-4Rα signaling in neutrophils inhibits their recruitment and effector functions (Impellizzieri et al., 2019). Thus, loss of IL-4Rα signaling in neutrophils may perhaps enhance their massive recruitment to the site of infection and activate their effector function suggesting that they may lead to the control of *Leishmania* infections.

LysM^{cre}IL-4Rα$^{-/lox}$ are mice on susceptible BALB/c genetic background with deficiency of genes that are controlled by lysosome (*lysm*) locus, thus they have a deficiency of IL-4Rα signaling in both macrophages and neutrophils (Hölscher et al., 2006). These mice infected in the footpad with *L. major* promastigotes were able to control the infection in the acute phase, however a similar disease phenotype to littermate control in the chronic phase. More importantly, no sex differences were observed (Hölscher et al., 2006). In contrast, IL-4Rα deficiency in macrophages/neutrophils exhibited minimal effects on the control of CL in BALB/c mice infected in the rump with *L. mexicana* amastigotes (Bryson et al., 2011). Together, these studies, therefore, suggest that *Leishmania* parasite spp, their developmental stage, and/or site of infection may influence the CL disease outcome. Considering the effector functions of myeloid cells and their functional regulation by IL-4/IL-13, this study aimed to investigate whether infection of CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice with *L. mexicana* could impact host resistance to *L. mexicana*-induced CL owing to the absence of IL-4Rα on DCs and macrophages/neutrophils. To achieve this, the mice were infected with promastigotes of *L. mexicana*, LV4, subcutaneously in the footpad, and at 8 weeks p.i, disease progression, humoral and cellular immune responses were evaluated.

CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice exhibited non-healing footpad swelling and parasite loads similar to their littermate IL-4Rα$^{-/lox}$ mice suggesting that IL-4Rα responsive DCs and macrophages/neutrophils do not play role in modulating CL disease caused by *L. mexicana*. These studies, therefore, demonstrate that IL-4Rα signaling on DCs and macrophages/neutrophils is not the reason why IL-4Rα$^{-/-}$ mice are protected from *L. mexicana* infection (Alexander et al., 2002). Moreover, sex hormones have been shown to

impact the immune responses against leishmaniasis (Lockard et al., 2019; Snider et al., 2009). Since sexes have been found to differ in the intensity, prevalence, and pathogenesis in various infections including CL (Bryson et al., 2011; Fathi et al., 2020; vom Steeg and Klein, 2016), disease phenotypes in mice deficient of IL-4Rα signaling on DCs and macrophage/neutrophil cells between male and female mice were compared. By and large, this study found no sex-related difference associated with *L. mexicana* infection in the presence or absence of IL-4Rα expression on DCs and macrophage/neutrophil cells. This is similar to other *L. major* studies using IL-4Rα cell-specific deficient mice (Hurdayal et al., 2013; Hölscher et al., 2006). However, it contrasts with studies using IL-4Rα-$CD4^+$ T cell-deficient mice infected with *L. mexicana* in the rump that demonstrated sex-dependent CL disease outcome (Bryson et al., 2011). In comparison with studies by Bryson et al 2011, the close similarity in the disease phenotype of $LysM^{cre}$IL-4$R\alpha^{-/lox}$ and IL-4$R\alpha^{-/lox}$ mice was independent of the site of infection and developmental stage of *L. mexicana* parasite. Comparatively, it is now clear that the outcome of CL infection is influenced by the parasite species as highlighted in DC-IL-4Rα deficient mice infected with *L. major* which were hypersusceptible (Hurdayal et al., 2013).

IL-4/IL-13 signaling drives type 2 inflammation due to the expansion of Th2 cells, which are a source of IL-4 and IL-13 in both autocrine and paracrine signaling. One hallmark function of these cytokines is the downstream pathways that result in isotype switching of antigen-primed B cells to IgG1 and IgE production (Gandhi et al., 2016). Humoral immune response influences both susceptibility and disease promotion during *Leishmania* infection. In particular, the type 2 antibody responses consisting of IgE and IgG1 are associated with disease pathology whereas type 1 antibody responses (IgG2a/b) are associated with protection (Chu et al., 2010; de Lima et al., 2021). Accordingly, total IgE and *Leishmania*-specific IgG1 and IgG2a/b antibodies were measured. No differences in IgE and IgG1 were found between $CD11c^{cre}$IL-4$R\alpha^{-/lox}$/ $LysM^{cre}$IL-4$R\alpha^{-/lox}$ mice and littermate controls but the systemic high titres of IgG1 exhibited by $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ and littermate control compared to resistant IL-4$R\alpha^{-/-}$ mice may account for the non-healing response in these animals. Indeed, higher titers of IgG1 have been corroborated with active CL disease in humans (Fagundes-Silva et al., 2012). In support, during *L. mexicana* infection IgG1 binds with affinity to FcγRIII on macrophages leading to secretion of IL-10 cytokine that is known to suppress NO production and inhibits Th1 immune responses, like IFN-γ production (Thomas and Buxbaum, 2008). In addition, $IgG1^{-/-}$ and B cell-deficient mice are protected from CL caused by *L. major* and *L. amanzonensis* infection (Wanasen et al., 2008; Ronet et al., 2008; Miles,

2005; Kima et al., 2000). In line with the above studies, this present study validates that host susceptibility to *L. mexicana* infection is mediated by the high titers of IgG1 antibodies (de Lima et al., 2021; Magalhães et al., 2021). Overall, the findings from this study demonstrate that the presence or absence of IL-4Rα signaling on DCs and macrophages/neutrophils does not lead to a cascade of events that alter B cell antibody class switching and the production of IgG1.

Studies have shown that IL-13 also activates B cells to induce IgG1 (and IgE) class switching (Junttila, 2018). CD11c^{cre}IL-4Rα$^{-/lox}$ mice exhibited an increased level of IL-4 and IL-13 by LN cells stimulated with αCD3 and heat-killed *L. mexicana* promastigotes. Unexpectedly, however, the increased IL-4 and IL-13 cytokines in CD11c^{cre}IL-4Rα$^{-/lox}$ mice did not increase IgG1 production when compared to littermate controls. Alternatively, these cytokines could have delayed class switching to IgG2a/b in CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice when compare to IL-4Rα$^{-/-}$ mice (Collins and Jackson, 2013) corroborating the non-healing response. Comparatively, similar levels of IL-13 between LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ BALB/c mice may suggest similar levels of IgG1 production by B cells.

Total IgE, another type 2 antibody, was significantly lower in IL-4Rα$^{-/-}$ mice but increased in CD11c^{cre}IL-4Rα$^{-/lox}$ mice and their littermate control, again validating that IgE plays a role in susceptibility to *L. mexicana* infections. Similarly, the comparable levels of IgE between LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ are indicative of the lack of CL disease control. In line with these findings, a clinical study on patients from Brazil and Iraq revealed increased titers of IgE antibodies hence associating it with disease severity during CL (Al-Qadhi et al., 2015; Sousa-Atta et al., 2002). The low levels of type 1 antibodies, namely, IgG2a and IgG2b, exhibited in CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/-}$ mice also demonstrate further the systemic inability of these mice to control the infection as type 1 antibodies have been associated with parasite clearance (Gibson-Corley et al., 2014; Hurdayal et al., 2013). In this context, a previous study showed that interaction of mouse IgG1-amastigote immune complexes with FcγRIII on macrophages is detrimental in *L. mexicana* infections, but not with interactions involving IgG2a/c (Chu et al., 2010). Moreover, IgG2a facilitates the killing of *L. amanzonesis* in macrophages (Gibson-Corley et al., 2014). Hence, in future studies, it will be interesting to determine the clinical phenotype during *L. mexicana* infection in B cell specific IL-4Rα$^{-/-}$ BALB/c mice.

The quality and magnitude of T cell-producing cells determine the disease outcome (Macedo et al., 2012). Studies on CL have shown that the control of infection is dependent on the production of Th1 cytokines (Govender et al., 2018; Hurdayal et al., 2013). Indeed, increased $CD4^+$ and $CD8^+$ T cells producing IFN-γ are known to mediate protection in *Leishmania* infections in mice (Kumar et al., 2014; Macedo et al., 2012). However, in this study, neither the frequency nor the absolute cell numbers of IFN-γ $CD4^+$ T cell producers were significantly different between mice with DC and macrophage/neutrophil cell-specific IL-4Rα deletion and their littermate IL-4Rα$^{-/lox}$ mice, an observation that was not sex-dependent.

Lymphocytes from CL patients produce IFN-γ upon stimulation with *Leishmania* antigens and previous studies have shown evidence in the stimulation of macrophages and DCs in LN, fuelling a rapid escalation of NO production that induces parasite killing (De Trez, 2009; Sacks, 2002). In general, there is an agreeable trend in similar IFN-γ production by pLN cells stimulated by αCD3 and the percentage of CD4 T cell IFN-γ producers in mice with presence or absence of IL-4Rα signaling on DCs. This corroborates the unchanged differences in footpad lesion swellings. Notably, αCD3 stimulated pLN cells to produce significantly increased IFN-γ in mice with IL-4Rα deficiency on macrophages/neutrophils. However, increased levels of IL-4 and IL-13 in CD11c^{cre}IL-4Rα$^{-/lox}$ over IL-4Rα$^{-/lox}$ mice determined using ELISA is the same as the percentage and absolute numbers of T cell producers, however this did not render them hypersusceptible. This may be due to the role of Treg cells in limiting and suppression of immune response. Hence Tregs may be involved in regulating exaggerated Th2 immune responses through secretion of TGF-β, however requires further investigations to ascertain this suggestion. Interestingly, presence of IL-2 has been shown to favor the differentiation of effector T cells (Kalia et al., 2010), and Treg can interfere with differentiation of effector Th2 cells by perhaps modulating the availability of IL-2, hence suggesting further studies on this mechanistic action of immune regulation. More importantly, although Th1 immune responses mediated by IFN-γ production are vital in protection against *L. mexicana* infection, it is more likely that this is not the case with BALB/c mice devoid of IL-4Rα signaling on macrophages/neutrophils.

Interleukin-10 or TGF-β cytokines are involved in the immune modulation and wound healing during CL (Abdoli et al., 2017; Franca-Costa et al., 2015; Hejazi, 2012; Rodrigues et al., 2014). More importantly, TGF-β and IL-10 cytokines inhibit Th1 responses through inhibition of NO production, hence leading to failure in the control of parasite survival

(Kedzierski and Evans, 2014). However, no differences were observed in these cytokines, between female LysM^{cre}IL-4Rα$^{-/lox}$ and their littermate IL-4Rα$^{-/lox}$ mice. Hence, the loss of IL-4Rα signaling on macrophage and neutrophil cells in females did not alter immunoregulatory cytokines. Even though IL-10 and TGF-β cytokines were decreased and increased in male LysM^{cre}IL-4Rα$^{-/lox}$ mice compared to their littermate controls respectively, no differences in clinical phenotype were observed, owing to their association with CL disease severity (Hejazi et al., 2012; Miles et al., 2005).

The presence of CD4$^+$ and CD8$^+$ T cells is important in the control of CL as shown in *L. branziliensis* and *L. major* infections (Siewe et al., 2016: Santos Cda et al., 2013). Besides, DCs stimulate the naive T cells to become effector cells. Similarly, macrophages can stimulate CD4$^+$T cells to fully functional effector cells (Pozzi et al., 2005). Consequently, the maturation, activation, and cytokines production from the DCs and macrophages, that is mediated by IL-4 signaling via IL-4Rα, will greatly influence the priming and activation of T cells (Vento-Tormo et al., 2016; Cook et al., 2012). Hence, we hypothesized that the loss of IL-4Rα signaling on DCs, and macrophages may affect their maturation and activation, consequently affecting the activation and proliferation of CD4$^+$ and CD8$^+$ T cells. In this study, it was demonstrated that IL-4Rα signaling on DC and macrophage/neutrophil cells does not modulate CD4$^+$ and CD8$^+$ T cells expansion as no significant differences were observed between IL-4Rα cell-specific deficient mice and their littermate controls. Analysis of pLN cells using flow cytometry revealed the role of IL-4Rα signaling on DC in activating CD8$^+$ T cells during *L. mexicana* infections are sex-dependent, as male CD11c^{cre}IL-4Rα$^{-/lox}$ mice had significantly increased frequency of activated CD8$^+$ T cells compared to their littermate controls. However, female CD11c^{cre}IL-4Rα$^{-/lox}$ mice had comparable activated CD8$^+$ T cells. Surprisingly, considering the total cell numbers in the pLN, both male and female CD11c^{cre}IL-4Rα$^{-/lox}$ mice had significantly increased activated CD8$^+$ T cells compared to their littermate controls. This discrepancy makes it difficult to corroborate IL-4Rα signaling on DCs in priming and activation of cytotoxic CD8$^+$ T cells during *L. mexicana*.

As expected these activated CD4$^+$ T cells did not translate to differences in effector memory CD4$^+$ T cells which are the main IFN-γ secreting cells during *L. major* infections (Carvalho et al., 2012b). Even though this study did not evaluate the ability of effector memory T cells in the production of IFN-γ, clinical studies involving individuals with a history of *L. major* or *L. tropica* infections have highlighted that CD4$^+$ T cells are responsible for IFN-γ secretion

(Keshavarz-Valian et al., 2013). Following this observation, the increased frequency of effector memory $CD4^+$ T cell phenotypes in female $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice may be indicative of increased IFN-γ-$CD4^+$ T cell producers in *L. mexicana* resistant global $IL\text{-}4R\alpha^{-/-}$ mice.

Central memory T cells (T_{CM}) can survive longer and upon encountering antigen are poised to proliferate into effector memory cells that secrete cytokines (Zaph et al., 2004). The expansion of T_{CM} was not affected by DC and macrophage/neutrophil cell-specific IL-4Rα deletion suggesting that upon encounter with *L. mexicana* antigens, these mice may not be better equipped to galvanize enhanced protection following *L. mexicana* infection when compared to wild-type mice. T_{CM} cells primarily homed in pLN unlike the T_{EM} (that are mostly housed in peripheral organs) are majorly specialized to solve systemic infections (Martin and Badovinac, 2018), indicative of close similarity in the disease phenotype during 8week *L. mexicana* infection between mouse strains with DC and macrophage/neutrophil cell-specific IL-4Rα deletion and their littermate controls. Small proportions of naïve cells were found in pLN of mice with IL-4Rα deficiency on DC cells which was similar to littermate controls indicating the magnitude of naïve T lymphocytes to scan the APC for the presence of immunogenic peptides presented on MHC molecules during immune surveillance is not affected by the loss of IL-4Rα signaling on DCs (Veigar-Fernandes et al., 2000). Overall, these findings demonstrate that the IL-4 responsive DC and macrophage/neutrophil cells do not alter the expansion and proliferation of the frequencies of central memory and naïve T cells.

Innate cells such as macrophages, neutrophils, and DCs form the first line of defense against CL causing *Leishmania* parasites before the onset of adaptive immunity. Surprisingly, these cells may also favor parasite survival and proliferation hence promoting CL disease (Bogdan, 2020; Rossi and Fasel, 2018). The presence or absence of IL-4Rα expression on DCs and macrophages/neutrophils did not affect the infiltration of the cDCs, activated macrophages, and neutrophils into pLN. Studies have shown sex-dependent differences in neutrophil infiltration and hormonal regulation of the population of macrophages in females (Kay et al., 2015; Scotland et al., 2011). However, this study suggests that sex hormones did not influence the infiltration of these myeloid cells into pLN of mice with deficiency of IL-4Rα expression on DCs, macrophages/neutrophils. Mature DCs with a similar expression of MHCII molecules between $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and the littermate controls did

correspond to the unaltered capacity of DCs to modulate the differentiation of naïve $CD4^+$ T cells into memory phenotypes, in contrast to *L. major* infections (Hurdayal et al., 2020). Studies involving $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice infected with *L. major* demonstrated reduced expression of IL-12 by DCs (Hurdayal et al., 2020), surprisingly, in *L. mexicana* infection, DCs exhibit unchanged IL-12 expression, suggesting that inflammatory responses by DCs are dependent on *Leishmania* species.

Conventional DCs (cDCs) are specialized in the uptake of *Leishmania* parasites, process, and present parasite-derived antigens to T cells. More importantly, the cDC1 identified by their CD8α expression as opposed to cDC2, cross-present antigens to $CD8^+$ T cells (Feijó et al., 2016; den Haan et al., 2000). In addition, cDC1 are specialized in the production of IL-12p70 hence they are less permissive to *Leishmania* infection, unlike cDC2 cells that skew $CD4^+$ T cells differentiation to Th2 cells (Martínez-López et al., 2015; Henri et al., 2002). Therefore, in this study, it is more likely that the cDCs may have majorly consisted of cDC2 subsets since the marked increased IL-4 and IL-13 production by $CD4^+$ T cells in the pLN of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice during *L. mexicana* infection may indicate skewed Th2 cells.

Inflammatory DCs, also known as monocyte-derived DCs (moDCs) mostly expand during infections as highlighted in viral infection, in an IFN-γ-dependent manner (Shin et al., 2019). Analysis of the MoDCs revealed unchanged frequencies regardless of the presence or absence of IL-4Rα signaling on DCs. But the $IL\text{-}4R\alpha^{-/-}$ mice that controlled *L. mexicana* infection had decreased frequencies of these cells, demonstrating the vital role MoDCs play in susceptibility to *L. mexicana* infections.

The maturation of DCs is important making them efficient in presenting antigens to T cells, consequently increasing their potential in cytokine production. Briefly, BMDCs were generated from the bone marrow of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ mice. Stimulation of IL-4Rα deficient BMDCs with IL-4 and/or LPS in presence of *L. mexicana* promastigotes does not alter the expressions of MHCII molecules when compared to littermate controls. Investigation on whether exogenous IL-4 treated BMDCs become activated during *L. mexicana* infection, expression of CD80 was not altered when compared to IL-4Rα sufficient BMDCs, as highlighted in *L. major* infections (Feijó et al., 2016, Hurdayal et al., 2013).

Interleukin-12 is important in mounting the Th1 immune responses involved in the control of *Leishmania* infection (Osero et al., 2020). IL-4 enhances the production of IL-12 by DC

through the IL-4Rα signaling pathway and by virtue of reduced IL-10 (Biedermann et al., 2001; Yao et al., 2005). Investigation on IL-12 production mediated by BMDCs stimulated with IL-4 during *L. mexicana* infection revealed unchanged levels between BMDCs from CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. The regulatory IL-10 cytokine is a pointer to CL disease promotion (Dayakar et al., 2019), as their levels were comparable between IL-4Rα deficient BMDCs and IL-4Rα sufficient BMDCs.

Nitric oxide generated from both macrophages and DCs is important in the control of the growth and survival of *Leishmania* parasites (Muxel et al., 2017). To evaluate the activity of iNOS during *L. mexicana* infection, the concentration of nitrite in the pLN cells of both CD11c^{cre}IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice was measured using Griess reaction (Sun et al., 2003), and compared to littermate controls. The production of nitrogen intermediates (nitrite) was comparable between the mouse strains indicative of poor control of the infection, hence the close similarity in disease phenotype. These data suggest that classically activation of DCs during *L. mexicana* infection is not IL-4Rα-dependent. More importantly, the similar levels of urea and nitrite metabolites produced by infected IL-4Rα deficient BMDCs treated with exogenous rIL-4/LPS are indicative of unimpaired DC effector function highlighting the presence or absence of IL-4Rα signaling on DCs does not alter the effector functions of BMDCs during *L. mexicana* infection (Contreras et al., 2014).

A similar non-healing progressive lesion development between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and their littermate controls may have been due to equal parasitization of macrophages by neutrophils (Pereira et al., 2019; Laskay et al., 2003). IL-4 affects neutrophil counts and migration into the LN during bacterial infections (Woytschak et al., 2016), however, IL-4/IL-13 signaling via IL-4Rα on DCs did influence the infiltration of neutrophils into pLN. Similarly, mice lacking IL-4Rα signaling on macrophages/neutrophils do not experience a differential influx of neutrophils into the pLN compared to littermate controls. Notably, neutrophil depleted mice are protected from *L. mexicana* infection as highlighted in resistant IL-4Rα$^{-/-}$ mice (Hurrell et al., 2015).

Studies have demonstrated that IL-4Rα-deficient macrophages when stimulated with LPS are classically activated (La Flamme et al., 2012). In contrast, LysM^{cre}IL-4Rα$^{-/lox}$ mice infected with *L. major* exhibited an alternative activation of macrophages, hence a better parasite control (Hölscher et al., 2006). Next, in line with equivalent the numbers of activated macrophages infiltrating into the pLN in both macrophage/neutrophil-IL-4Rα deficient mice

and littermate controls during *L. mexicana* infection, the IL-4Rα deficient BMDMs stimulated with IL-4 and LPS did not equally produce significantly different levels of inflammatory (IL-12p70) and regulatory (IL-10 and TGF-β) cytokines when compared to IL-4Rα sufficient BMDMs. The equivalent concentration of nitrite between $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice and littermate controls demonstrate the unchanged shift in macrophage effector function. In general, these findings, suggest that IL-4Rα signaling on macrophages and neutrophils is inconsequential in modulating their effector functions.

Finally, IL-13 also binds to IL-13Rα2 (Lupardus et al., 2010). IL-13/ IL-13Rα2 signaling axis on DCs, macrophage, and neutrophils may be a compensatory signaling mechanism in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice (Rahaman et al., 2002). Transient expression of IL-13Rα2 on DCs, macrophage, and neutrophils may have inhibited IL-4 mediated signaling in littermate controls resulting in a similar expansion of frequency of T cells and infiltration of myeloid cells consequently leading to the similar disease phenotype.

4.2 Conclusions

This study aimed to investigate the specific cell in IL-4Rα$^{-/-}$ mice that contribute to control against *L. mexicana* infection. Earlier studies on the IL-4Rα deficient $CD4^+$ T cell population demonstrate that protection against *L. mexicana* infection in IL-4Rα$^{-/-}$ mice is mediated in part by non-$CD4^+$ T cells (Bryson et al., 2011). In efforts to investigate which of these non-$CD4^+$ T cells mediate disease protection, mouse strains with IL-4Rα deficiency on DCs and macrophages/neutrophils infected with *L. mexicana* parasites in the footpad revealed a similar disease outcome to their 'wild-type' littermate controls, that is not sex-dependent suggesting a minimal effect of sex hormones in IL-4Rα signaling on DCs and macrophage/neutrophil cells function.

This study highlights that during subcutaneous infection in the footpad with *L. mexicana* promastigotes, IL-4Rα deficiency on DCs and macrophages/neutrophils in BALB/c mice does not drive CL disease protection in IL-4Rα$^{-/-}$ mice. The findings rule out the important role of IL-4Rα signaling on DCs, macrophages, and neutrophils in the immune responses characterized by antibody production, cytokine secretion by pLN cells, cellular responses, and iNOS and arginase-1 enzyme activities in BALB/c mice during *L. mexicana* infection.

Importantly, this study demonstrates that the lack of IL-4Rα signaling on DCs leads to heightened IL-4 and IL-13 production in BALB/c mice infected with *L. mexicana*. However, additional mechanistic studies will be necessary to better investigate why these mice were not hyper-susceptible to *L. mexicana* infection when compared to littermate controls.

The decreased IL-10 and heightened TGF-β levels observed in BALB/c mice with deficiency of IL-4Rα expression on macrophages and neutrophils were inconsistent with close similar non-healing disease phenotype in comparison to littermate controls reinforcing the idea that Th2 immune responses mediated by IL-4/IL-13 cytokines are associated with active CL disease caused by *L. mexicana* infection.

Overall, the studies on $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice demonstrate protection against CL caused by *L. mexicana* infection is independent of IL-4Rα signaling on DCs, macrophages, and neutrophils. It is possible that IL-4Rα signaling on other cells, besides CD4T cells, may be involved in the protection.

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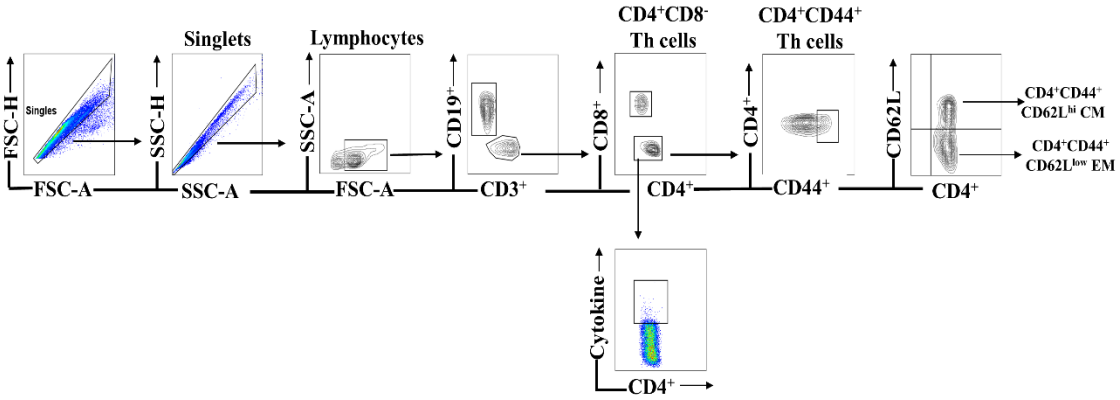
APPENDICES

Appendix 1: Preparation of Plutznik media

PLUTZNIK Media	Stock Conc.	Final Conc.	500ml
Inactivated fetal calf serum #	100%	10%	50ml
Inactivated horse serum #	100%	5%	25ml
L929 conditioned medium* # NARJIS7/7/11	100%	30%	150ml
L-glutamine	200mM	2mM	5ml
Na-Pyruvate	stock		10 ml
Pen-Strep	200X	1X	2.5 ml
2-β-Mercaptoethanol	1000X (50mM)	1X (50 uM)	0.5 ml
*DMEM medium	100%	50%	267 ml

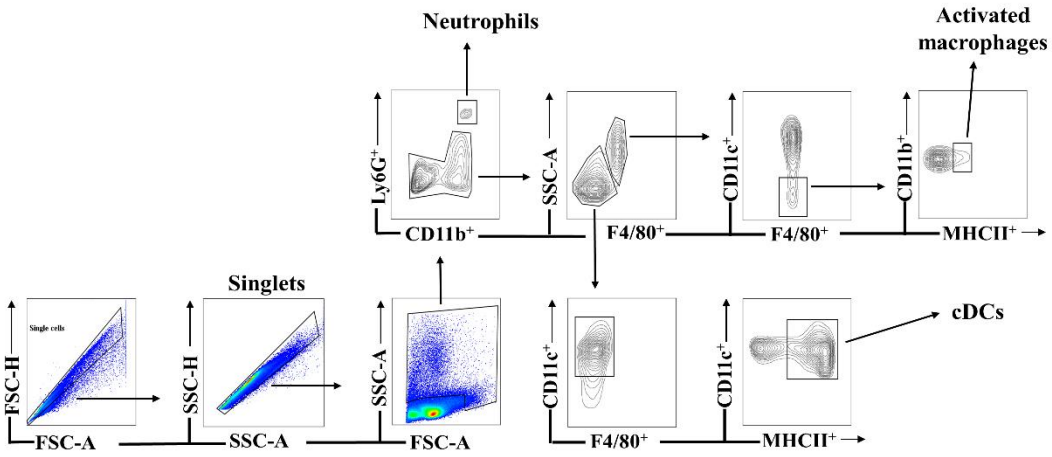
Appendix 2: Lymphoid gating



Appendix 2: Flow cytometry gating for lymphocytes. The lymphocyte cells were enumerated by analysing CD19, CD3, CD4, CD8, CD44, CD62L surface marker expressions. $CD19^{-}CD3^{+}$ cells were described as T cells. $CD4^{+}$ T cells were gated as $CD19^{-}CD3^{+}CD4^{+}CD8^{-}$, $CD8^{+}$ T cells gated as $CD19^{-}CD3^{+}CD4^{-}CD8^{+}$, Activated $CD4^{+}$ and $CD8^{+}$ T cells were identified as $CD44^{+}$ cells, whereas their central (T_{CM}) and effector memory (T_{EM})

phenotypes identified as $CD44^{+}CD62L^{+}$, $CD44^{+}CD62L^{-}$ cells respectively. Finally, naïve phenotypes (T_N) were identified as $CD44^{-}CD62L^{+}$.

Appendix 3: Myeloid gating



Appendix 3: Gating strategy for myeloid cells. $CD11b^{+}CD11c^{+}F4/80^{-}MHCII^{+}$ cells were described as conventional DCs (cDCs). Activated macrophages were gated as $CD11b^{+}CD11c^{-}F4/80^{+}MHCII^{+}$, neutrophils gated as $SSC^{+}CD11b^{+}Ly6G^{+}$, and monocyte-derived DCs (Mo-DCs) were identified as $CD11b^{+}CD11c^{+}F4/80^{+}$ cells.