Deletion of IL-4 receptor alpha on dendritic cells renders BALB/c mice hypersusceptible to *Leishmania major* infection

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I dedicate this thesis to all those that shared in my journey towards a Ph.D,

Most especially my parents, Shamilla and Rick, for bearing my absence with smiling faces on many special occasions, and my brother Lazarus, for understanding me in both the moments of joy and despair.

DECLARATION

I, Ramona Hurdayal, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part thereof is being, has been, or is to be submitted for another degree in this or any other University.

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Ramona Hurdayal
16th May 2013

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LIST OF PUBLICATIONS

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

1. Deletion of IL-4 receptor alpha on dendritic cells renders BALB/c mice hypersusceptible to *Leishmania major* infection.
   Hurdayal, R. and Nieuwenhuizen., N., Revaz-Breton M., Smith, L., Hoving, J.C., Parihar, S.P., Reizis B., and Brombacher., F.

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2. Simvastatin-induced autophagy results in protection against cutaneous Leishmaniasis in mice.
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   (In preparation)

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   Revaz-Breton, M., Hurdayal, R., Einhorn, A., Nieuwenhuizen, N and Brombacher, F.

   (In preparation)

4. Dendritic Cell-Mediated Vaccination Relies on Interleukin-4 Receptor Signaling to Avoid Tissue Damage after *Leishmania major* Infection of BALB/c Mice.
   Masic, A., Hurdayal, R., Nieuwenhuizen, N., Brombacher., F and Moll, H. 2012.
   PLoS Negl Trop Dis. 6(7): e1721.

5. Allergic airway disease is unaffected by the absence of IL-4Rα-dependent alternatively activated macrophages.
   Nieuwenhuizen, N., Frank, K., Jayakumar, J., Emedi, B., Hurdayal, R., Horsnell, W., Lopata AL and Brombacher, F. 2012.
   Journal of Allergy and Clinical Immunology. 130(3):743-750

6. Anti-peptide antibodies differentiate between plasmodial lactate dehydrogenases.
   Hurdayal, R., Achilonu, I., Choveaux, D., Coetzer, TH., and Goldring, JPD. 2010.
   Peptides. 31(4):525-532

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ABBREVIATIONS

Abs	-	Antibodies
APCs	-	Antigen-presenting cells
AP	-	Alkaline phosphatase
AP-1	-	Activator protein-1
APC	-	Allophycocyanin
Arg I	-	Arginase I
β2-ME	-	Beta-Mercaptoethanol
BCA	-	Bicinchoninic Acid Protein Estimation
Bio	-	Biotinylated
Baso	-	Basophils
BCR	-	B cell receptor
BM	-	Bone marrow
BMDCs	-	Bone marrow-derived dendritic cells
CD	-	Cluster of differentiation
cDCs	-	Conventional dendritic cells
CNS	-	Central nervous system
CpG	-	Cytosine phosphorylated guanidine
Cre	-	Cyclization recombinase
DCs	-	Dendritic cells
DMEM	-	Delbucco's Minimal Eagle's Medium
DTH	-	Delayed type hypersensitivity
ELISA	-	Enzyme-linked Immunosorbent Assay
Eos	-	Eosinophils
EPO	-	Eosinophil protease
FACS	-	Fluorescence-activated cell sorter
FCS	-	Fetal calf serum
FDCs	-	Follicular dendritic cells
FITC	-	Fluorescein isothiocyanate
FP	-	Footpad
γδ	-	Gamma delta T cells

GFP	-	Green fluorescent protein
GM-CSF	-	Granulocyte macrophage-colony stimulating factor
H&E	-	Haematoxylin and eosin
HRP	-	Horseradish peroxidase
IFN-γ	-	Interferon gamma
Ig	-	Immunoglobulin
IL	-	Interleukin
IL-4Rα	-	Interleukin-4 receptor-alpha
IL-12β2	-	Interleukin-12 receptor-beta2
IL-13Rα1	-	Interleukin-13 receptor-alpha1
IL-13Rα2	-	Interleukin-13 receptor-alpha2
iNOS	-	Inducible nitric oxide synthase
IRS	-	Insulin receptor substrate
JAK	-	Janus tyrosine kinases
LCs	-	Langerhans cells
LPG	-	Lipophosphoglycan
L. brasiliensis	-	*Leishmania brasiliensis*
L. donovani	-	*Leishmania donovani*
L. major	-	*Leishmania major*
L. mexicana	-	*Leishmania mexicana*
L. monocytogenes	-	*Listeria monocytogenes*
LN	-	Lymph Node
LPS	-	Lipopolysaccharide
mAbs	-	Monoclonal antibodies
MACS	-	Magnetic labeled bead cell separation
MHC I	-	Major histocompatibility complex class I
MHC II	-	Major histocompatibility complex class II
Mph	-	Macrophage
M. tuberculosis	-	*Mycobacterium tuberculosis*
N. brasiliensis	-	*Nippostrongylus brasiliensis*
ND	-	Not detected

Neut	-	Neutrophils
NK	-	Natural killer
NKT	-	Natural killer T cell
NO	-	Nitric oxide
OD	-	Optical density
PAMPS	-	Pathogen-associated molecular patterns
pDCs	-	Plasmacytoid dendritic cells
PRRs	-	Pattern recognition receptors
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PE	-	Phycoerythrin
PNP	-	4-Nitrophenylphosphate
RPMI	-	Roswell Park Memorial Institute
RT	-	Room temperature
SEM	-	Standard error of the mean
S. Mansoni	-	*Schistosoma mansoni*
STAT	-	Signal transducer and activator of transcription
T-bet	-	T-box expressed in T cells
TCR	-	T cell receptor
Tc	-	T cytotoxic
TCR	-	T cell receptor
Th	-	T helper
Th1	-	T helper 1 cells
Th2	-	T helper 2 cells
TGF	-	Transforming growth factor
TipDCs	-	TNFα-inducible nitric oxide synthase-producing dendritic cells
TNF	-	Tumor necrosis factor
TLR	-	Toll-like receptors
Treg	-	T regulatory
WK	-	Week

ABSTRACT

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In BALB/c mice, susceptibility to infection with the intracellular parasite *Leishmania major* is driven largely by the development of T helper 2 (Th2) responses and the production of interleukin (IL)-4 and IL-13, which share a common receptor subunit, the IL-4 receptor alpha chain (IL-4Rα). While IL-4 is the main inducer of Th2 responses, paradoxically it has been shown that exogenously administered IL-4 can promote dendritic cell IL-12 production and enhance Th1 development if given early during infection. To investigate the relevance of this theory during *in vivo* infection, a novel BALB/c mouse model lacking IL-4Rα expression specifically on dendritic cells and alveolar macrophages ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice) was generated by gene targeting and site-specific recombination using the cre/*lox*P system under control of the CD11c locus. Functional characterization showed selective impairment of IL-4Rα function on dendritic cells from $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice. Following infection with *L. major*, $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice became hypersusceptible to disease, presenting earlier and increased footpad swelling, necrosis and parasite burdens, upregulated Th2 cytokine responses and increased type 2 antibody production as well as impaired classical activation of macrophages. Hypersusceptibility in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice was accompanied by a striking increase in parasite burdens in peripheral organs such as the spleen, liver and even brain. Inflammatory $CD11b^{+}$ dendritic cells were the major infected cell population in infected $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice and showed deficiencies in iNOS production resulting in increased parasite loads. IL-4Rα-deficient DCs produced reduced IL-12 but increased IL-10 due to impaired DC instruction, with increased mRNA expression of IL-23p19 and activin A, cytokines previously implicated in susceptibility to cutaneous Leishmaniasis. Together, these data demonstrate that abrogation of IL-4Rα signaling on DCs is severely detrimental to the host, leading to rapid disease progression and increased survival of parasites in infected $CD11b^{+}$ dendritic cells.

CHAPTER 1:
INTRODUCTION

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INTRODUCTION

1.1 Innate and adaptive immunity

The mammalian immune system is a highly versatile defence system that has evolved to recognise, differentiate and eliminate pathogens or other foreign antigens from the body's 'self' components (Kuby *et al.*, 2006). Functionally, two main branches of the immune system exist, termed innate and adaptive immunity. The innate immune response provides the first line of defence, occurring rapidly on recognition of antigens. However, innate immunity is not specific and does not improve on repeated encounters with the same pathogen (Kenneth *et al.*, 2008).

Innate immunity relies on pattern recognition receptors (PRRs) to recognise common pathogen-associated molecular patterns (PAMPs) (Dempsey *et al.*, 2003). Of note, Toll-like receptors (TLRs), well known signaling PRRs that recognise PAMPs, are essential for antibacterial immune responses (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999a), immunity against protozoan parasites (Kropf *et al.*, 2004; Abou Fakher *et al.*, 2009) and innate antiviral responses (Doyle *et al.*, 2002). PRRs can be found intracellularly and extracellularly of phagocytic cells and work in conjunction with other immune cells to provide immunity (Kenneth *et al.*, 2008).

Central to the innate response are macrophages and neutrophils that recognise, engulf and kill invading pathogens. Eosinophils release destructive enzymes, like eosinophil protease (EPO), which target extracellular parasites that are too large to be phagocytosed. Basophils and mast cells also release a variety of destructive enzymes, cytokines and inflammatory molecules. Eosinophils, mast cells and basophils are also important during allergic inflammatory reactions. Natural Killer (NK) cells are large granular lymphocytes that kill tumour cells or cells infected by intracellular pathogens. The dendritic cells (DCs) internalise and degrade foreign pathogens. However, their main role is the presentation of pathogen antigens on their cell surface to naïve T lymphocytes to initiate adaptive immune responses (Kropf *et al.*, 2004; Kuby *et al.*, 2006; Kenneth *et al.*, 2008).

In contrast to innate immunity, adaptive immune responses are slower, being acquired over time, mount defences that are highly specific and retain a "memory" of the invading pathogen which confers life-long immunity to a repeated pathogen. Adaptive immunity relies on specific recognition functions of T and B lymphocytes. Both cells express a

diverse repertoire of antigen receptors on their cell surface, encoded by gene rearrangement. This enables the immune system to recognise and respond to virtually any antigen an individual may be exposed to, and build a strong antigen-specific response against it. The antigen-receptors of B cells, the B-cell receptors (BCRs) are immunoglobulin (Ig) molecules. Upon activation, B cells may differentiate into effector plasma cells secreting large quantities of the BCR in the form of antibodies or memory B cells providing long-lasting immunity. Cytotoxic T cells (Tc), characterised by expression of the CD8 co-receptor, and helper T cells (Th), characterised by expression of the CD4 co-receptor, are the two main types of T cells. $CD8^+$ cytotoxic T cells kill cells infected with viruses or other intracellular pathogens whilst $CD4^+$ T helper cells release cytokines that stimulate other immune cells, which act more directly in the protection against infectious pathogens. The antigen-receptors of T cells, the T cell receptors (TCRs) are membrane-bound proteins whose function is to signal naïve T cells for activation (Dempsey *et al.*, 2003; Kenneth *et al.*, 2008). In addition to TCR signaling, T cell activation requires co-stimulatory signaling to promote antigen-specific T cell expansion and differentiation and to upregulate cytokine expression. CD28 is the major T cell costimulatory receptor and interacts with the B7 ligand family (CD80/CD86), receptors that are expressed on antigen-presenting cells (APCs) (Chen and Flies, 2013). Critical to an effective immune response is the influence of soluble cytokines in the microenvironment of the cells as well as the recognition and presentation of foreign antigens by the APCs.

1.1.1 Cytokines

Cytokines are low molecular weight (~ 25 kDa) regulatory proteins secreted by a range of immune cells to a variety of stimuli (Brombacher, 2000). The production of cytokines is transient and has a short half-life, being constitutively expressed when needed or remaining at baseline levels in steady-state. Their mode of action is either autocrine, affecting the cells that produce them, paracrine, affecting surrounding cells, or endocrine, affecting distant cells (Kenneth *et al.*, 2008). Cytokines play an important role in stimulating or inhibiting the differentiation, proliferation or function of immune cells as well as modulating the type of immune responses that should be elicited towards a particular pathogen (Dempsey *et al.*, 2003). For instance, interferon-γ (IFN-γ) secreted by the T helper 1 (Th1) subset of $CD4^+$ T cells and cytotoxic $CD8^+$ T cells induce the cellular immune response during infection with intracellular pathogens. In contrast, cytokines interleukin (IL)-4, IL-5, IL-9, IL-10 and IL-13 produced by the Th2 subset of $CD4^+$ T cells

induce the humoral immune response in response to extracellular pathogens and allergens. In addition, cytokines can also direct the differentiation of IL-17-producing Th17 cells (Harrington *et al.*, 2005; Weaver *et al.*, 2007) or IL-10 producing regulatory T cells.

1.1.2 Dendritic cells

Antigen-presenting cells include dendritic cells, macrophages and B cells. Whilst B cells are the most abundant APC population (Moulin *et al.*, 2000), DCs are sparsely distributed but are the most efficient in uptake, processing and presentation of antigens to T cells. These cells therefore form a crucial link between innate and adaptive immunity. DCs are also defined as migratory cells characterised by numerous branched projections, the dendrites, which gives the cell its name and aids in uptake, processing and presentation of antigens to T cells. DC progenitors in the bone-marrow differentiate into precursor cells that home to tissues, where they reside as immature cells with high phagocytic ability, sampling their surrounding environment for foreign antigens (Figure 1.1). Following infection or tissue damage, the intrinsic properties of immature DCs (Table 1.1) allows them internalise antigen, either by receptor-mediated phagocytosis/endocytosis or macropinocytosis, mature and migrate to lymphoid organs where they participate in antigen-presentation to T cells (Banchereau *et al.*, 2000). Several factors, such as pathogen products, bacterial DNA/RNA, viral dsRNA and cytokines, can trigger DC transition from immature antigen-capturing cells to mature antigen-presenting cells (Banchereau *et al.*, 2000). DCs utilise different receptors to recognise antigens for internalization, such as Fc receptors for antigen-antibody complexes, C-type lectin receptors for glycoproteins and PRRs such as TLRs for pathogen specific molecules (Brandonisio *et al.*, 2004). This allows DCs to recognise and respond to a wide range of pathogenic stimuli.

Mature DCs undergo several phenotypic changes subsequent to antigen-presentation as listed in Table 1.1. Morphological changes are also observed, such as increased migratory capacity, loss of adhesion molecules and reorganization of the cytoskeleton (Banchereau *et al.*, 2000). DCs will express Major Histocompatibility Complex (MHC) molecules at their cell surface which bear fragments of pathogen antigens for presentation to naïve T cells. DCs carrying MHC Class II molecules present antigen to naïve $CD4^+$ Th cells whilst MHC Class I molecules present antigen to $CD8^+$ cytotoxic T cells. Recognition of MHC-antigen complexes by naïve T cells is followed by interaction between co-stimulatory molecules expressed by DCs (CD40) and their ligands expressed by T cells (CD40L) (Banchereau *et al.*, 2000; Brandonisio *et al.*, 2004).

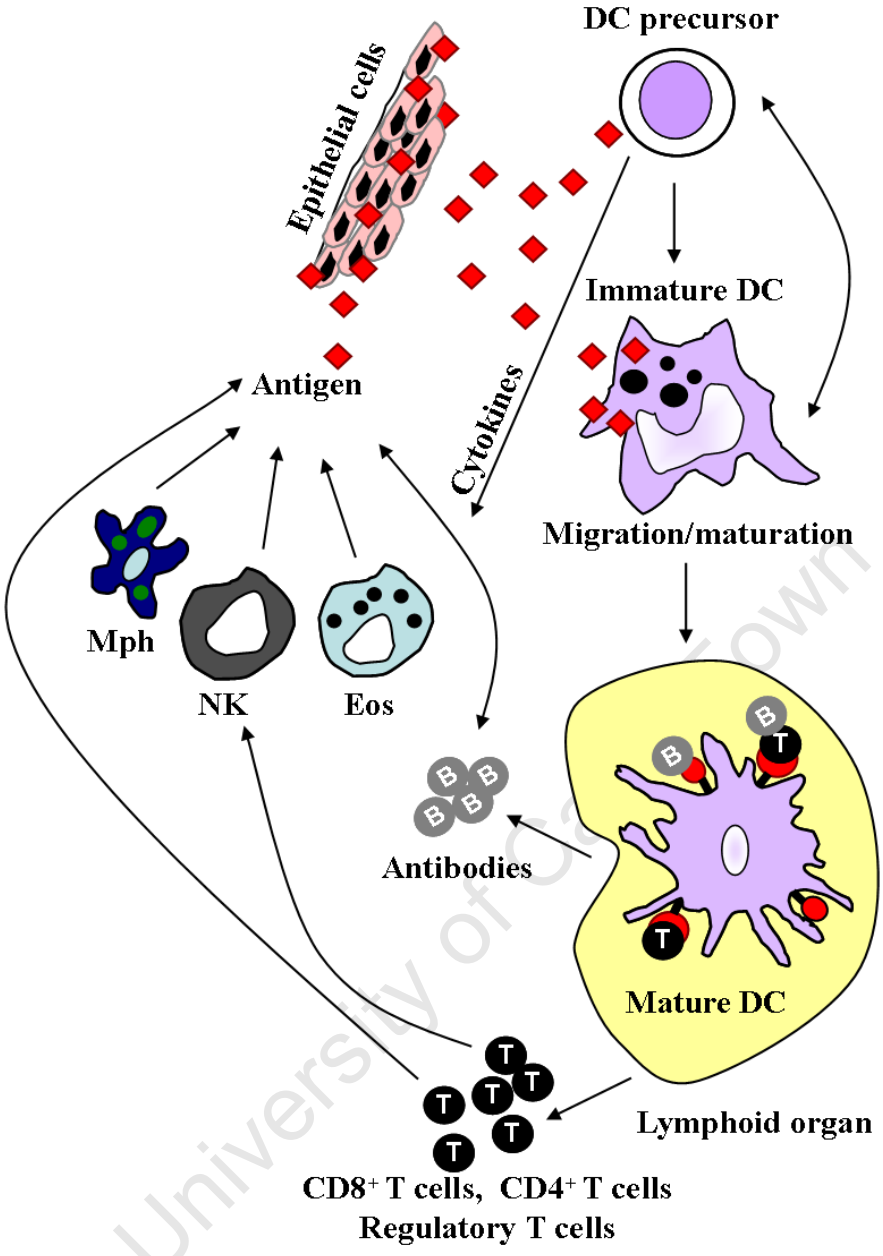

Figure 1.1: The life cycle of dendritic cells at different stages of development.
DC precursors enter tissue as immature DCs. Upon encounter with pathogens, the intrinsic properties of immature DCs, allows them to capture antigen and release cytokines which activate eosinophils (Eos), macrophages (Mph) and natural killer (NK) cells to the site of infection. After antigen capture, immature DCs migrate to lymphoid organs and acquire a mature phenotype. DCs process the captured antigens and present peptide-MHC on their cell surface to antigen-specific T lymphocytes. Antigen-presentation by DCs is characterised by different DC properties, which helps to induce T cell expansion and differentiation. Activated helper T and cytotoxic T cells migrate to the site of infection. The helper T cells secrete cytokines which further activates eosinophils, macrophages and NK cells whilst cytotoxic T cells destroy the infected cells. B cell activation occurs after contact with T cells and DCs. B cells mature into antibody-secreting plasma cells to neutralise the infecting pathogen or opsonise them for improved uptake by phagocytes. Illustration adapted and redrawn from previous publications (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000).

Table 1.1: Properties of immature and mature dendritic cells during development

Immature DC-Antigen capture	Mature DC-Antigen Presentation
High intracellular MHC II	High surface MHC II
High endocytosis	Low endocytosis
High phagocytosis	Low phagocytosis
High CCR1, CCR5, CCR6	Low CCR1, CCR5, CCR6
Low CCR7	High CCR7, CD58
Low CD80, CD86	High CD54, CD86
Low CD54, CD58	High CD40, CD83, CD80
Low CD40, CD83, CD68	Low CD68
Actin cables present	Actin cables absent

Once DCs present antigen, a variety of immune effector cells are activated and regulated. These include induction of antigen-specific $CD4^+$ Th cells and $CD8^+$ cytotoxic T cells, B cells to secrete antigen-specific antibodies as well as stimulation of mononuclear phagocytes, eosinophils and NK cells (Figure 1.1). Expression of chemokines/chemokine receptors and cytokines by DCs is also upregulated, the former to guide effector cells and recruit circulating DC precursors to the site of injury and draining lymph node and the latter, to guide further differentiation of effector T cells most appropriate to combat the invading pathogen (Table 1.1) (Banchereau *et al.*, 2000). Given their role in immune responses, DCs are flexible cells and provide the necessary information that determines the type of immune response that should be elicited. For example, Th1 or Th2 phenotypes are dependent on DC production of various cytokines. DCs secrete IL-12 and IL-18, which regulate Th1 responses and IFN-γ production which in turn, increases the microbicidal action of activated macrophages. DC production of anti-inflammatory cytokines, like IL-10, suppresses the induction of uncontrolled immune responses (Colino and Snapper, 2003) whilst production of pro-inflammatory cytokines such as, tumor necrosis factor (TNF-α), IL-1 and IL-6, on antigen capture are known to induce DC maturation and modulate DC movement (Banchereau and Steinman, 1998).

1.1.2.1 Heterogeneity of dendritic cell subsets in humans

In humans, two pathways of DC development have been identified, myeloid and lymphoid DC development. Myeloid DCs were shown to differentiate from human monocytes in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 *in vitro* (Romani *et al.*, 1994; Wu and Liu, 2007). Lymphoid DCs were recognised based on the fact that lymphoid tissue-resident DCs expressed markers, such as CD8α, CD4 and CD25, that are associated with lymphoid cells (Wu and Liu, 2007). DC subsets in humans include $CD11c^+$ myeloid conventional DCs (cDCs) found circulating in the blood, $CD11c^+$ lymphoid cDCs resident in lymphoid tissue, $CD11c^-$ plasmacytoid DCs (pDCs) found in lymphoid organs and epidermal Langerhans cells (LC) along with dermal DCs in the skin (Brandonisio *et al.*, 2004; Segura *et al.*, 2012). Conventional DCs are directly involved in antigen-presentation and activation of naïve T cells whilst pDCs generate large amounts of type I interferons, particularly important during viral infections (Gilliet *et al.*, 2008; Cervantes-Barragan *et al.*, 2012).

1.1.2.2 Heterogeneity of dendritic cell subsets in mice

Studies in mice have provided more detailed insight into the development and differentiation of DC populations. Both myeloid and lymphoid DC development has been identified and can be further sub-divided into skin DCs, plasmacytoid DCs, follicular DCs and monocyte-derived DCs (Figure 1.2). Differentiation of all DC subsets, with the exception of follicular DCs, arises from hematopoietic stem cells (HSC) in the bone-marrow that give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) as illustrated in Figure 1.2. Careful studies on murine precursor cells transferred into irradiated mice have shown that all DC subsets can be generated from both myeloid and lymphoid precursors (Manz *et al.*, 2001), which give rise to common macrophage-DC progenitors (MDP) that further differentiate into common DC-progenitors (CDP). CDPs exclusively give rise to myeloid and lymphoid DCs through a precursor DC population, the pre-DCs (Figure 1.2) (Shortman and Naik, 2007; Wu and Liu, 2007).

Myeloid and lymphoid DCs differ in phenotype, function and localization. Myeloid DCs are known for their migratory properties and migrate from peripheral tissues to lymphoid organs whilst lymphoid DCs lack migratory functions and reside in lymphoid tissue. Myeloid DCs include DC subsets found in the skin, lung, intestinal tract, liver, kidneys, LN, spleen and thymus whilst lymphoid DCs are resident only in lymphoid organs (LN, spleen and thymus) (Figure 1.2). Myeloid DCs, and not lymphoid DCs, expand in murine

bone-marrow cultures stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF) (Scheicher *et al.*, 1992). In contrast, Flt3 ligand (tyrosine kinase 3 ligand) has been reported to induce the differentiation of both myeloid and lymphoid DCs from mouse bone-marrow progenitors (Lyman and Jacobsen, 1998) and upon *in vivo* injection (Pulendran *et al.*, 1997; Shurin *et al.*, 1997).

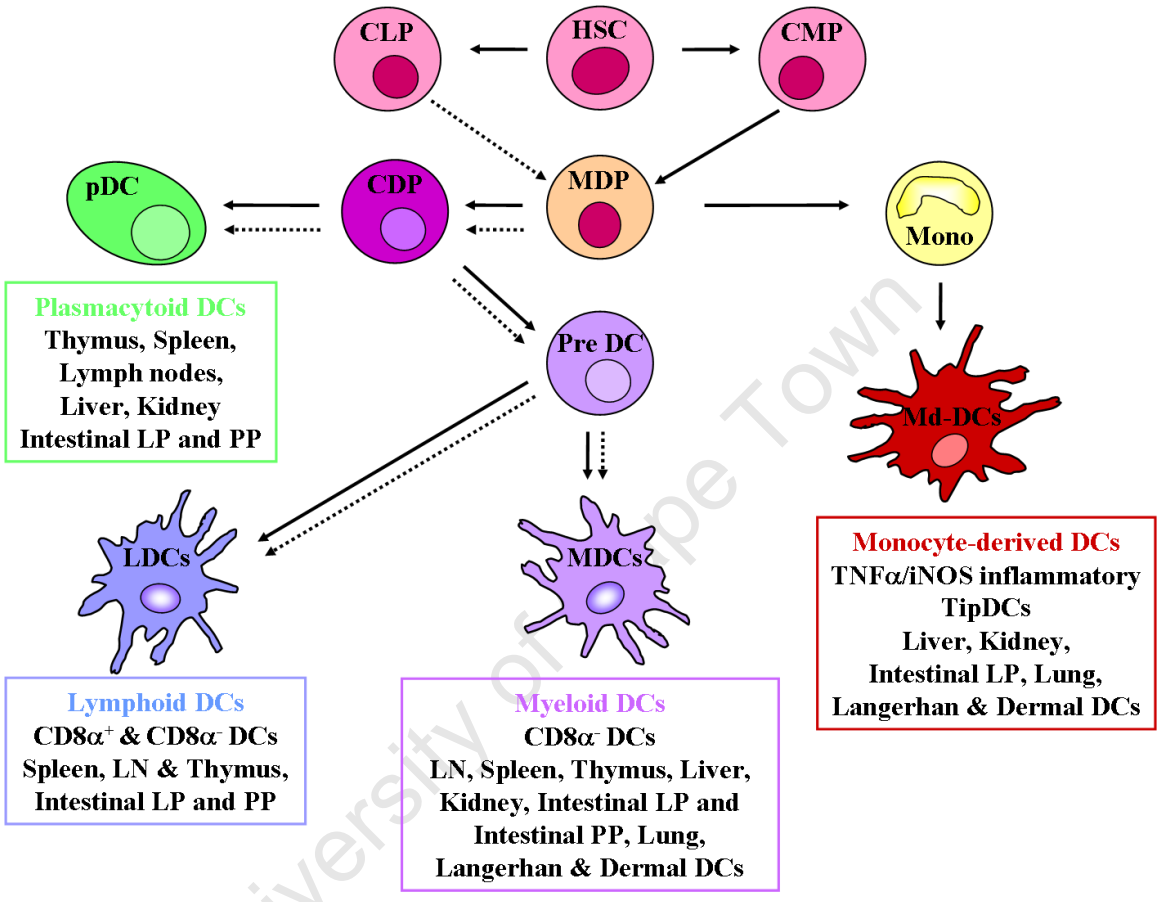

Figure 1.2: Differentiation of dendritic cells from haematopoietic stem cells.
Haematopoietic stem cells (HSCs) differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) which can differentiate into macrophage-dendritic cells (DC) progenitors and give rise to common DC progenitors (CDP) and monocytes. CDPs specifically give rise to DCs through pre-DC precursor cells (pre-DC). CDPs can also give rise to plasmacytoid DCs (pDCs). pre-DCs migrate to lymphoid and peripheral tissues where they differentiate into either lymphoid or myeloid DCs. Monocytes give rise to monocyte-derived DC subsets in peripheral tissues. Myeloid DC differentiation is denoted by the solid line (⟵) and lymphoid DC differentiation is denoted by the broken line (◂····). Illustration drawn from previous publications (Shortman and Liu, 2002; Shortman and Naik, 2007; Wu and Liu, 2007; Kushwah and Hu, 2011).

Myeloid and lymphoid DC subsets are differentiated by the CD8α marker, which is expressed as a homodimer by lymphoid DCs, but absent from myeloid DCs (Pulendran *et al.*, 1997; Vremec and Shortman, 1997). Along with the $CD8\alpha^+$ lymphoid DCs, there are the $CD8\alpha^-$ lymphoid DCs that lack expression of the $CD8\alpha^+$ homodimer. The $CD8\alpha^-$

lymphoid DCs in turn are further divided by the expression of $CD4^+$ and give rise to $CD8^-$ $CD4^+$ and $CD8^-CD4^-$ DCs (Shortman and Naik, 2007). Myeloid and lymphoid DCs do share some common traits such as the expression of high levels of the integrin α chain CD11c, MHC molecules and co-stimulatory or adhesions molecules. In addition, both subsets have been shown to efficiently prime antigen-specific $CD4^+$ T cells (Maldonado-Lopez *et al.*, 1999).

Lymphoid DCs reside in the T cell-rich areas of the spleen and lymph nodes whereas myeloid DCs localise to the marginal zone bridging channels of the spleen, but can be induced to home to the T cell-rich areas upon stimulation by pathogen products or pro-inflammatory signals (Banchereau *et al.*, 2000). The myeloid $CD8\alpha^-$ DCs are more phagocytic than $CD8\alpha^+$ lymphoid DCs, whilst the latter produces higher levels of IL-12. IL-12 induces potent production of IFN-γ in $CD8\alpha^+$ lymphoid DCs and $CD4^+/CD8^+$ T cells and plays a role in cytotoxicity of NK cells (Pulendran *et al.*, 1997). Thus, $CD8\alpha^+$ DCs tend to induce a biased Th1 response whereas $CD8\alpha^-$ DCs tend to induce a biased Th2 response owing to their production of Th1 inhibitory cytokines, IL-10 and transforming growth factor (TGF-β). A unique property of $CD8\alpha^+$ lymphoid DCs is their ability to engulf and cross-present exogenous antigens through MHC Class I (Shortman and Liu, 2002; Kushwah and Hu, 2011).

Myeloid skin DCs include Langerhans cells and dermal DCs, which represent the model migratory DCs. LCs express higher levels of the Birbeck-granule-associated molecule, Langerin, than dermal DCs. Dermal DCs can be subdivided into $Langerin^+$ and $Langerin^-$ dermal DCs with different roles in initiating immune responses in the skin (Brewig *et al.*, 2009; Henri *et al.*, 2010; King *et al.*, 2010). These DCs have a slow turnover and initiate immune responses to pathogens that gain access to epidermal or dermal skin layers. Development of LC DCs appears to be dependent on TGF-β since TGF-β-deficient mice are devoid of LC DCs (Borkowski *et al.*, 1996). LC DCs migrate from the skin carrying antigenic information to the draining LN. However, controversy remains as to whether LC DCs themselves present the antigen to T cells or whether they pass the antigen on to $CD8\alpha^+$ lymphoid DCs for final presentation (Moll *et al.*, 1993; Ritter *et al.*, 2004; Allan *et al.*, 2006). On the other hand, dermal DCs have been shown to not only carry parasite antigen from the skin to the draining LN but also to present it to antigen-specific naïve T cells (Ritter *et al.*, 2004; Brewig *et al.*, 2009).

Plasmacytoid DCs can be differentiated from both lymphoid and myeloid precursors and are relatively long-lived cells found circulating in both lymphoid and non-lymphoid organs. pDCs lack normal dendritic form and instead, morphologically resemble antibody-producing B-lineage plasma cells. pDCs secrete type I interferons, which are key cytokines involved in viral immunity and contributes to the activation of NK, B and T cells as well as myeloid DCs (Kushwah and Hu, 2011). pDCs are differentiated based on low/intermediate expression of CD11c and MHC II markers and selective expression of Siglec-H and PDCA-1 (Randolph *et al.*, 2008). pDCs are generally poor activators of T cell proliferation because these cells are incompetent at antigen capture, processing and presentation (Colonna *et al.*, 2004). However, upon activation with IL-3 or pathogen products, murine pDCs differentiate into conventional DC morphology, acquire a mature phenotype and instruct adaptive immunity through production of IL-12 (Shortman and Liu, 2002; Randolph *et al.*, 2008).

Follicular DCs (FDCs) are a non-migratory population located in B cell-rich areas and germinal centres of lymphoid organs. FDCs sustain the viability, growth and differentiation of activated B cells. Interestingly, FDCs develop from mesenchymal precursors unlike other DC subsets that are derived from bone-marrow haematopoietic stem cells. FDCs express a unique set of surface markers, including all complement receptors, which allows them to capture and display antigen-antibody complexes on their cell surface for activation of B cells. B cells that recognise the antigen-antibody complexes on FDCs process the antigen and present it as peptide-MHC complexes to antigen-specific T cells (Banchereau and Steinman, 1998).

Monocytes serve as precursor cells in peripheral tissues and can give rise to monocyte-derived DCs (or macrophages) under inflammatory or steady-state conditions in addition to epidermal LC DCs after skin inflammation (Randolph *et al.*, 1999; Wu and Liu, 2007; Kushwah and Hu, 2011). Monocyte-derived DCs take up antigen and migrate to the draining LN for presentation to antigen-specific T cells. Among the monocyte-derived DCs, the inflammatory DCs, also known as TNF-α/inducible nitric oxide synthase (iNOS)-producing "TipDCs", produce TNF-α, nitric oxide, IL-12 and stimulate Th1 cells. These monocyte-derived inflammatory DCs are derived from the $Ly6C^{+}$ monocytes and can be distinguished from normal DC subsets by their high level expression of CD11b along with CD11c and the distinct absence of CD4 or CD8 expression (Shortman and Naik, 2007). The TipDCs have been implicated in protection against infections caused by intracellular

bacteria and protozoan parasites (Serbina *et al.*, 2003; Leon *et al.*, 2007; De Trez *et al.*, 2009).

1.1.3 CD4$^+$ T helper cell differentiation

CD4$^+$ Th cells are important in orchestrating adaptive immunity and immunological memory to a variety of stimuli. They initiate, regulate or suppress adaptive immune responses in response to infection. Hence, when their numbers are low or functions are lost, an individual becomes susceptible to a host of infectious disorders. During TCR activation of naïve Th cells via stimulation of their cognate antigen by DCs, CD4$^+$ T cells may differentiate into four distinct lineages. Each lineage has a distinct set of cytokines, signalling transducer and activator of transcription (STAT) proteins and transcription factors that promote its differentiation as illustrated in Figure 1.3. The ability of Th cells to differentiate into any of these lineages during an immune response depends on the nature and quantity of the pathogen, the type of APC and co-stimulatory molecules, their effector function and location, their pattern of cytokine secretion and their expression of specific master transcription factors (Onah and Nawa, 2000; Zhu et al., 2010).

IL-12 and IFN-γ act in collaboration with STAT1 and transcription factor T-bet to drive the differentiation of CD4$^+$ Th1 cells that secrete IFN-γ and IL-2 (Figure 1.3). Th1 cells are important for the activation of macrophages and cytotoxic CD8$^+$ T cells, production of protective IgG antibodies and delayed-type hypersensitivity (DTH) reactions in clearance of intracellular pathogens (Zhu et al., 2010). T-bet acts by upregulating expression of the IL-12 receptor (IL-12Rβ2) which in turn stimulates further IL-12 and IFN-γ secretion. IFN-γ produced by the differentiated cell creates a positive feedback loop in promoting further cell differentiation (Zhu et al., 2010).

Th2 cells secrete IL-4, IL-13 and IL-10, which are important for the clearance of extracellular parasites (Zhu et al., 2010) and mediate asthma and allergic responses to ubiquitous antigens which can lead to anaphylactic shock and death (Holgate, 1999). Th2 cell differentiation is driven by IL-4 and signaling via the IL-4 receptor alpha chain (IL-4Rα), which together signal the STAT6 pathway and the Th2 master regulator, GATA3 (Figure 1.3) (Zhu et al., 2010). Both Th1 and Th2 responses counter-regulate each other. T-bet and STAT4 promote IFN-γ secretion and increased IL-12Rβ2 expression in Th1 cells, but expression of Th2 associated GATA3 is suppressed. Similarly, in Th2 cells, GATA3/STAT6 increase IL-4 production but expression of Th1 associated STAT4 is

downregulated. Moreover, IL-10 inhibits Th1 responses whilst IL-12 suppresses Th2 responses (Zhu et al., 2010).

Differentiation of $CD4^+$ IL-17-producing cells (Th17) cells is induced by IL-23, IL-6 and TGF-β1 which together signal RORγT and STAT3 for defence against extracellular bacteria, fungi and autoimmune diseases (Figure 1.3) (Weaver et al., 2007; Zhu et al., 2010). Differentiation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) relies on stimulation with TGF-β and IL-2, whilst IL-6 plays an inhibitory role in the commitment of this cell lineage. Tregs produce IL-10 and TGF-β which are cytokines that tend to inhibit rather than activate T cell responses. Thus, Tregs and their inhibitory cytokines exert a regulatory role which is important in maintaining self-tolerance and immune modulation during exaggerated immune responses.

Although the Th1/Th2/Th17/Treg paradigm is widely accepted, other Th lineages have also been proposed, including Th3 cells (TGF-β-producing $CD4^+$ T cells) (Weiner, 2001), Tr1 cells (IL-10-producing $CD4^+$ T cells) (Groux *et al.*, 1997), Th9 cells (IL-9-producing $CD4^+$ T cells) (Veldhoen *et al.*, 2008), Th5 cells (IL-5-producing $CD4^+$ T cells) (Kurowska-Stolarska *et al.*, 2008) and T follicular helper (Tfh) cells (King et al., 2008; Zhu and Paul, 2010). However, controversy still remains as to whether these cells represent distinct lineages separate from the four known lineages since the cytokines they produce and transcription factors they express are also shared among the four known cell lineages and therefore not unique (Zhu et al., 2010). In addition, T cells are more plastic than initially thought and can shift between either of the Th lineages. For these reasons, further investigations are required to uncover the relationships between Th1/Th2/Th17/Treg and Th3/Tr1/Th9/Th5 and Tfh cells in response to infection.

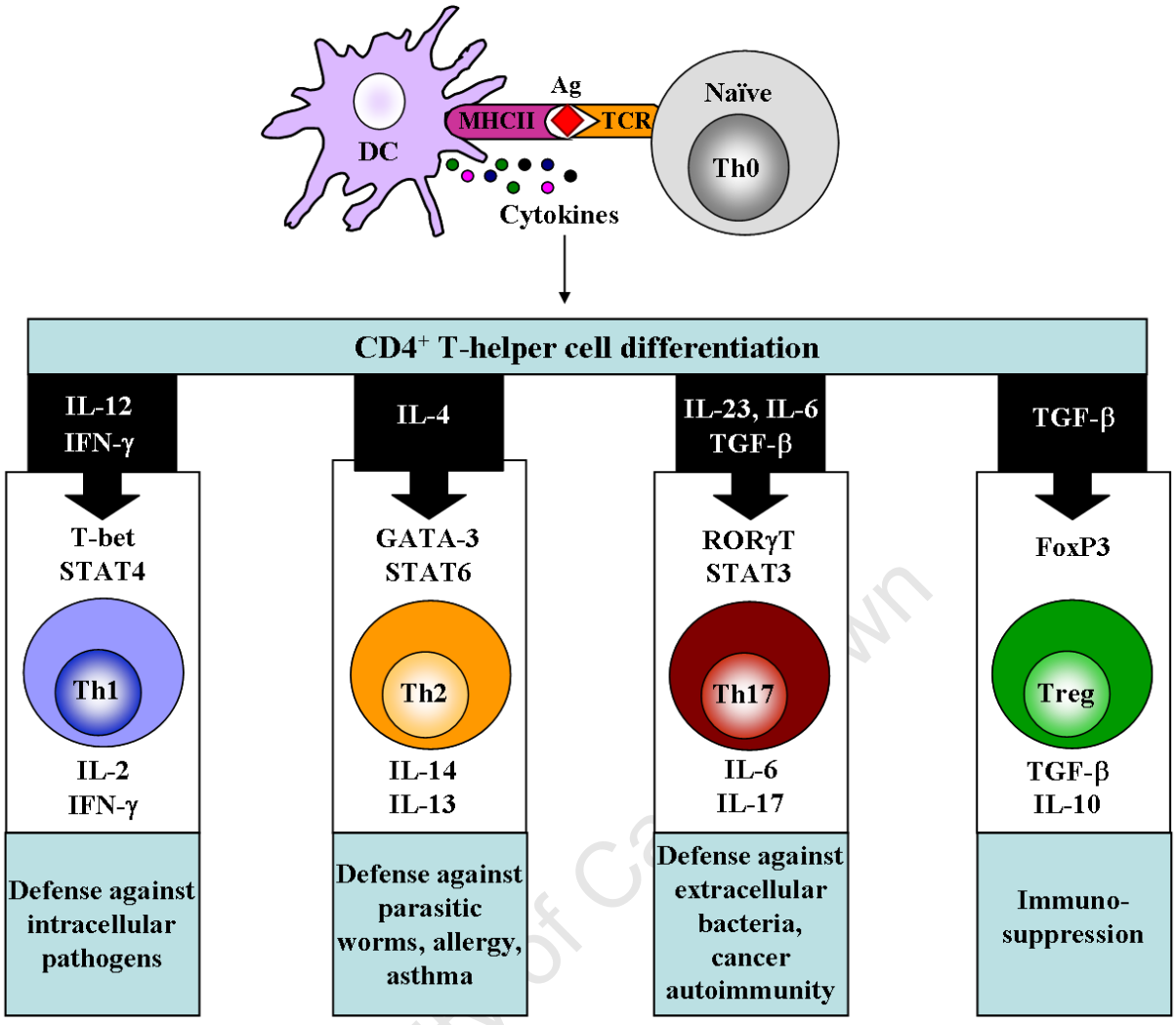

Figure 1.3: CD4$^+$ T helper cell differentiation.
Dendritic cells present MHC Class II-loaded antigens to naïve CD4$^+$ Th0 cells. Naïve CD4$^+$ Th0 cells recognise the loaded antigen by their T cell receptor (TCR) and differentiate into an effector T cell lineage depending on the cytokine milieu in the local environment. Th1 differentiation is influenced by IL-12, IL-18 and IL-2 along with T-bet/STAT4 whilst Th2 differentiation is influenced by IL-4 together with GATA-3/STAT6. Inflammatory cytokines IL-23, IL-6, TGF-β and RORγT/STAT3 drive the differentiation of Th17 cells. TGF-β along with FoxP3 induces the differentiation of immunosuppressive regulatory T cells (Treg). Illustration adapted and redrawn from previous publications (Brombacher, 2000; Kenneth *et al.*, 2008; Zhu *et al.*, 2010; Craft, 2012).

1.2 Interleukin-4 and Interleukin-13

1.2.1 Interleukin-4

Interleukin-4 (IL-4) plays a critical role in initiating and regulating Th2-type immune responses (Himmelrich *et al.*, 2000). Murine IL-4 is 14-19 kDa glycoprotein co-localized on chromosome 11 together with IL-5 and IL-13 genes. It is produced by CD4$^+$ Th2 cells (Launois *et al.*, 1995) (Figure 1.3) in addition to NK T cells (Yoshimoto and Paul, 1994), γ/δ T cells (Ferrick *et al.*, 1995), basophils (Min *et al.*, 2004), mast cells (Plaut *et al.*, 1989)

and eosinophils (Sabin *et al.*, 1996). Apart from regulating the differentiation of Th2 cells, IL-4 also controls immunoglobulin class switching by activated B cells. IL-4 specifies human B cells to switch to the expression of IgE and IgG4 (Gascan *et al.*, 1991) whilst in mice, to IgE and IgG1, with the concomitant suppression of IgM, IgG2a and IgG2b (Coffman *et al.*, 1986; Snapper *et al.*, 1988). IL-4 suppresses the expression of IFN-γ (Nelms *et al.*, 1999) and increases the expression of MHC II molecules (Noelle *et al.*, 1984), Fc epsilon receptors (CD23) (Defrance *et al.*, 1987), co-stimulatory molecules CD80 and CD86 (Stack *et al.*, 1994), IL-4 receptor (Ohara and Paul, 1987) and together with TNF-α, enhances endothelial cell adhesiveness of T cells (Thornhill *et al.*, 1991). Although IL-4 has been shown to be the primary inducer of Th2 responses, studies have reported IL-4-independent Th2 differentiation (Noben-Trauth *et al.*, 1997; Brombacher, 2000; Mohrs *et al.*, 2000; Ritz *et al.*, 2002; Cunningham *et al.*, 2004). Furthermore, Th2 differentiation and production of Th2 cytokines was also found to be independent of IL-4, IL-4Rα signaling and STAT6 regulation during infection (Noben-Trauth *et al.*, 1997; Finkelman *et al.*, 2000; Jankovic *et al.*, 2000).

1.2.2 Interleukin 13

Murine interleukin-13 (IL-13) is an immunoregulatory cytokine with a molecular weight of 10-14 kDa (Hershey, 2003) and co-localizes on chromosome 11 together with the genes for IL-4, IL-5 and IL-3. Like IL-4, murine IL-13 also promotes upregulation of MHC II antigens, co-stimulatory (CD80/CD86) molecules and adhesion molecules. However, unlike IL-4, murine IL-13 has been shown not to affect Th2 differentiation, B cell switching or upregulation of the low-affinity IgE receptor (CD23), probably due to the absence of a functional IL-13 receptor on those cells in mice. On the other hand, human B lymphocytes do respond to IL-13 (Brombacher, 2000). Activated Th2 cells, NK T cells, mast cells, basophils, dendritic cells and NK cells produce IL-13 (McKenzie *et al.*, 1993; de Saint-Vis *et al.*, 1998; Hoshino *et al.*, 1999b; Brombacher, 2000). IL-13 also has immunosuppressive and anti-inflammatory effects on macrophages and monocytes, including suppression of pro-inflammatory cytokines and chemokines. Nitric oxide (NO) production along with antibody-mediated cytoxicity is also inhibited by IL-13. IL-13 also exerts an effect on innate immune cells and is responsible for activating mast cells and modulating eosinophil function (Hershey, 2003). IL-13 production is also crucial for the expulsion of intestinal parasites during *Nippostrongylus brasiliensis* infection (McKenzie *et al.*, 1998a; Urban *et al.*, 1998), regulates immune responses during infection with

Schistosoma mansoni (Chiaramonte *et al.*, 1999) and is a mediator of allergic asthma (Grunig *et al.*, 1998) and anaphylaxis (Fallon *et al.*, 2001).

1.2.3 The IL-4 and IL-13 receptor complexes

The common functions between IL-4 and IL-13 can be attributed to the shared IL-4 receptor alpha (IL-4Rα) chain (Brombacher, 2000). This was demonstrated in competitive studies wherein mice treated with IL-4 antagonists or anti-IL-4Rα antibodies inhibited both IL-4 and IL-13 responses (Aversa *et al.*, 1993; Zurawski *et al.*, 1993; Hilton *et al.*, 1996). The IL-4Rα is a 140 kDa chain and serves as a common monomer in both the type I and type II IL-4 receptor complexes (Figure 1.4). It is expressed in fairly low numbers on haematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocytes and brain tissue (Nelms *et al.*, 1999). IL-4Rα associates with the gamma common (γc) to form the type I IL-4 receptor and with the 65-70 kDa IL-13-binding receptor chain (IL-13Rα1) to form the type II IL-4/IL-13 receptor (Figure 1.4) (Nelms *et al.*, 1999). IL-4 binds the IL-4Rα chain with high affinity but IL-13 binds the IL-13Rα1 chain with low affinity. However, when paired with the IL-4Rα chain, IL-13 binds the IL-13Rα1 chain with high affinity forming an active signaling unit (LaPorte *et al.*, 2008). Expression of IL-13Rα1 is absent on human or murine T cells but constitutively expressed on B cells, epithelial cells and monocytes in both mice and humans (Gauchat *et al.*, 1997; Graber *et al.*, 1998). IL-13 shows a higher binding affinity for the α2 chain of the IL-13 receptor (IL-13Rα2) which is a 55-60 kDa protein (Figure 1.4). Up until recently, IL-13Rα2 was considered a decoy receptor for IL-13 devoid of signal transduction since its short cytoplasmic domain does not contain any binding motifs for signaling molecules (MacDonald, 2006). However, recent reports have demonstrated a signaling pathway for IL-13 through the IL-13Rα2 chain, which induces production of TGF-β1 and mediates fibrosis (Fichtner-Feigl *et al.*, 2006). In human and mouse models, soluble forms of both IL-4Rα and IL-13Rα2 exist (Figure 1.4), capable of binding IL-4 and IL-13 with high affinity. In doing so, the soluble receptors can act as competitive inhibitors of both IL-4 and IL-13 and modulate their effector responses (Zhang *et al.*, 1997; Jung *et al.*, 1999).

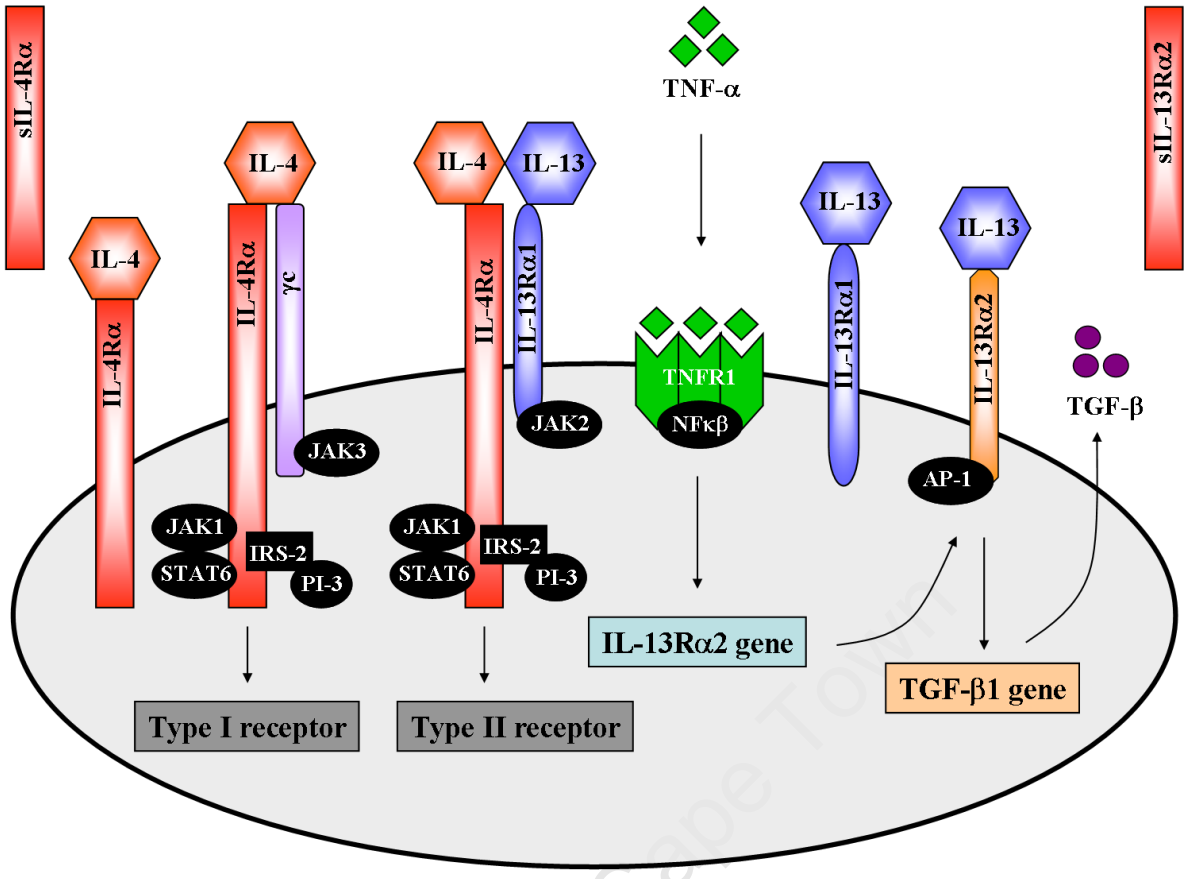

Figure 1.4: The IL-4 and IL-13 receptor complexes.
IL-4 interacts with the IL-4Rα chain in combination with either the γc to form the Type I receptor or with the IL-13Rα1 to form the Type II receptor complex. The IL-4Rα chain signals via the JAK1/STAT6 pathway whilst JAK3 associates with the γc chain and JAK2 with the IL-13Rα1. IL-13 interacts with the type II receptor complex (through IL-13Rα1) or with the IL-13Rα2. A signaling pathway for IL-13 via IL-13Rα2 has recently been identified. TNF-α induces upregulation of IL-13Rα2 expression. IL-13 then binds to the IL-13Rα2 which activates AP-1 to induce gene expression and secretion of soluble TGF-β. Illustration adapted and redrawn from previous publications (Nelms *et al.*, 1999; Brombacher, 2000; Hershey, 2003; MacDonald, 2006; Hoving, 2010).

1.2.4 Mechanisms of IL-4 and IL-13 signaling through the IL-4Rα chain

Both IL-4 and IL-13 signaling via the IL-4Rα chain involves activation of the Janus-family kinases (JAK) for signal transduction. JAK1 associates with the IL-4Rα chain while JAK3 associates with the γc chain and JAK2 with the IL-13Rα1 (Figure 1.4) (Yin *et al.*, 1994; Murata *et al.*, 1996; Nelms *et al.*, 1999). IL-4 engagement with the IL-4Rα chain results in tyrosine phosphorylation of the IL-4Rα chain itself as well as phosphorylation of STAT6 and IRS-2 (Insulin receptor substrate 2) by JAKs, which then associates with the phosphoinositol-3 kinase (PI-3) (Figure 1.4) (Nelms *et al.*, 1999; Brombacher, 2000). Macrophages in STAT6-deficient mice had impaired IL-13-mediated functions which confirmed that IL-13 also uses the JAK/STAT6 pathway for signal transduction (Figure

1.4) (Takeda *et al.*, 1996; Brombacher, 2000). IL-13 signaling through the IL-13Rα2 first requires engagement of the IL-13Rα1/IL-4Rα complexes in conjunction with TNF-α signaling which increases surface expression of IL-13Rα2. IL-13 then binds to the IL-13Rα2 and, through activation of the transcription factor, AP-1, drives secretion of TGF-β (Figure 1.4) (Fichtner-Feigl *et al.*, 2006; MacDonald, 2006).

1.2.5 Macrophage activation

Macrophages exhibit significant plasticity, which allows them to respond to various environmental signals and change their activation phenotype (Mosser and Edwards, 2008). Tissue macrophages have been classified into three distinct subsets, which together regulate inflammation and wound healing (Figure 1.5) (Gordon, 2003). Classically activated macrophages occur during Th1 responses and mediate cellular immunity against intracellular pathogens via the production of pro-inflammatory cytokines (IL-1 and TNF), reactive oxygen species (ROS) and nitric oxide. Activation of classical macrophages is mediated by IFN-γ and TNF signaling (Mosser and Edwards, 2008; Classen *et al.*, 2009). Conversely, alternatively activated macrophages, also known as wound-healing macrophages, are activated by Th2 cytokines, IL-4 and IL-13, signaling through the IL-4Rα chain (Classen *et al.*, 2009). Studies have shown that alternative macrophages have important roles not only in regulating wound healing (Mosser and Edwards, 2008) but also in defense against helminths (Herbert *et al.*, 2004) and fungal infections (Muller *et al.*, 2007) whilst others have demonstrated a detrimental role during infection with protozoan parasites (Holscher *et al.*, 2006). Alternatively activated macrophages upregulate the expression of specific markers such as, arginase I (Arg I), mannose receptor CD206 (Mrc1) and resistin-like alpha (RELMα) (Figure 1.5). Importantly, IL-4 inhibits iNOS expression thereby inhibiting classically activated macrophages. The third macrophage subset, termed 'regulatory' macrophages, secrete large amounts of IL-10 in response to Fc receptor-γ ligation (Sutterwala *et al.*, 1998; Mosser and Edwards, 2008).

Recently it has been shown that murine DCs can also respond to IL-4 *in vivo* and *in vitro* and become alternatively activated in a manner similar to that described for alternatively activated macrophages, by upregulating multiple alternative activation markers such as Ym-1, Dectin-1, mannose receptor and RELM-α (Cook *et al.*, 2012). Moreover, expression of RELM-α on these alternative DCs was found to play a role in priming optimal Th2 responses. In separate studies, classical activation of DCs was has also been

reported. These DCs were found to secrete iNOS, similar to classically activated macrophages, and contributed to resistance during infection with *L. monocytogenes* (Serbina *et al.*, 2003) and *Brucella sp* (Copin *et al.*, 2007). These data highlight that classical and alternative activation of DCs is also possible although the mechanisms through which these occur remain unidentified and require further investigation.

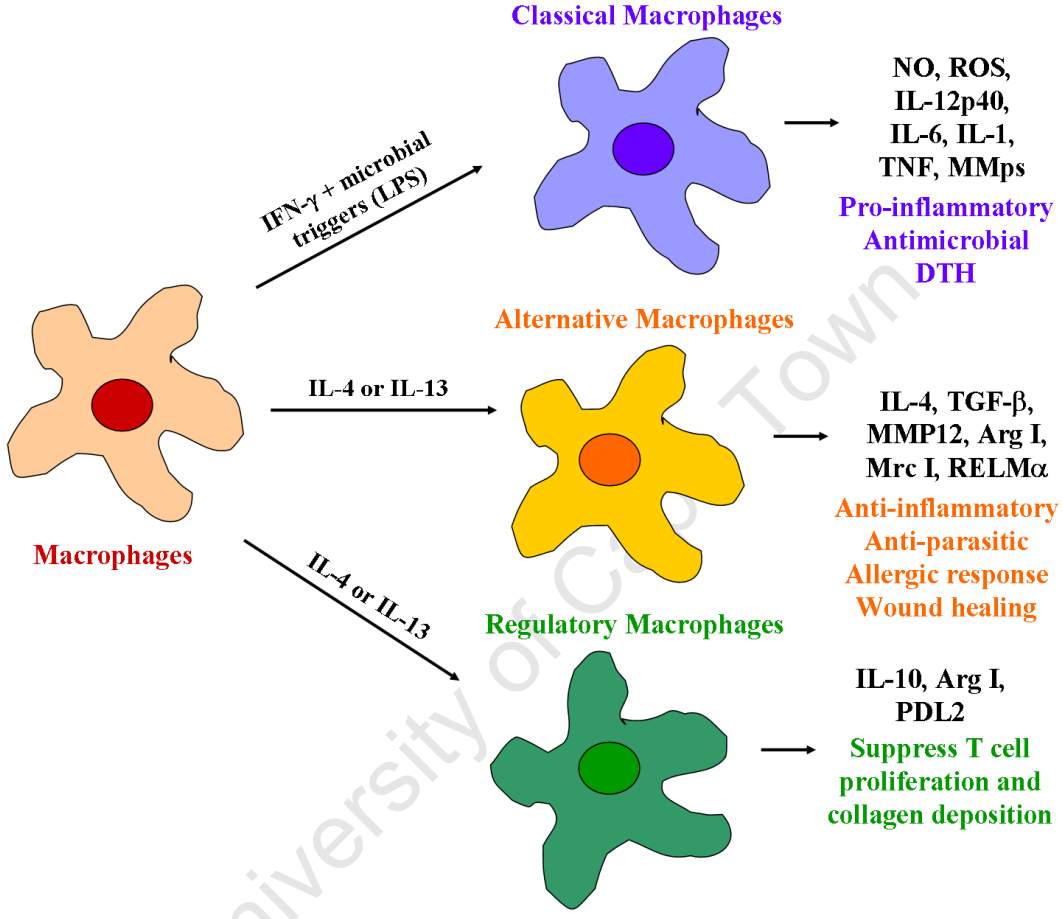

Figure 1.5: Activation of classical, alternative and regulatory macrophages.
Classical activation of macrophages is induced by Th1 cytokines, such as IFN-γ, in combination with microbial stimuli (e.g. LPS) and secrete NO and pro-inflammatory cytokines. Alternative and regulatory macrophages are activated by Th2 cytokines (IL-4/IL-13). Alternatively activated macrophages secrete IL-4 and Arg I that favor parasite growth have an anti-inflammatory responses. Regulatory macrophages produce high amounts of IL-10 that can suppress immune responses. Adapted and redrawn from previous publications (Gordon, 2003; Murray and Wynn, 2011).

1.3 Leishmaniasis

1.3.1 Background

Leishmania spp. are obligate, intracellular protozoan parasites that are transmitted by female sand flies of the *Phlebotomus* spp. and cause the disease, Leishmaniasis (Alexander *et al.*, 1999; Herwaldt, 1999). Leishmaniasis is characterised by different clinical manifestations which depend on both the genetic background of the host and the *Leishmania* spp. initiating infection. These include cutaneous Leishmaniasis, caused by *L. major*, *L. tropica* and *L. mexicana*; mucocutaneous Leishmaniasis, caused by *L. braziliensis*; and visceral Leishmaniasis, caused by *L. donovani*, *L. infantum*, *L. chagasi* or *L. amazonensis* (Figure 1.6) (Alexander *et al.*, 1999). Cutaneous Leishmaniasis is localized to dermal tissue surrounding the site of inoculation, but parasites may migrate to draining lymph nodes and visceralise during chronic infection. Mucocutaneous Leishmaniasis (also known as "Espundia") results in total or partial deconstruction of the mucous membranes of the nose, mouth and genitalia. Visceral Leishmaniasis (also known as "Kala-azar") is usually fatal if left untreated and results from the metastatic spread of parasites to internal organs such as the spleen and liver (Alexander *et al.*, 1999). A fourth clinical symptom, post Kala-azar dermal Leishmaniasis (PKDL) has been identified as a squeal to "kala-azar" following treatment and appears in a dermatotropic form of *L. donovani* infection, serving as a reservoir of visceral parasites (Figure 1.6) (Zijlstra *et al.*, 2003; Sharma and Singh, 2009).

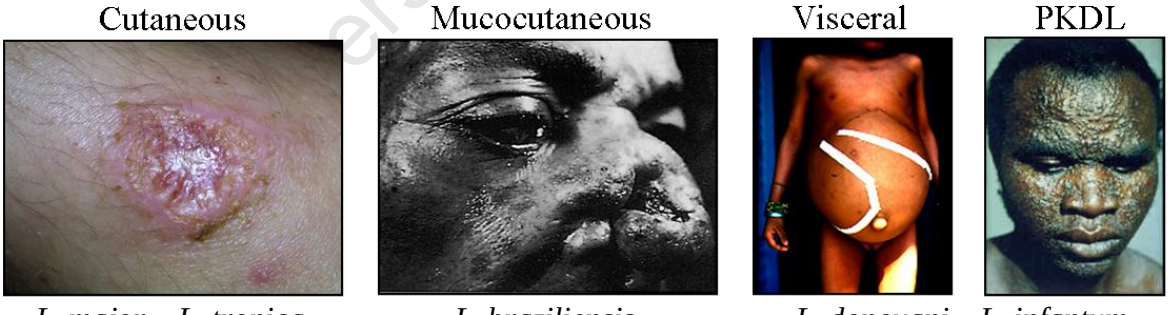

Figure 1.6: Clinical manifestations of Leishmaniasis in humans.
Infection with *L. major*, *L. tropica* or *L. mexicana* causes localized cutaneous Leishmaniasis and presents as ulcers on the skin. Mucocutaneous Leishmaniasis results from infection with *L. braziliensis* and leads to progressively destructive ulcerations of the mucosa. Visceral Leishmaniasis caused by *L. donovani* or *L. infantum* is fatal if left untreated and leads to enlargement of the spleen and liver due to the systemic spread of parasites. Post-kala-azar dermal Leishmaniasis (PKDL) is a complication of visceral Leishmaniasis following treatment and presents as lesions on the skin.

Currently there is no effective vaccine for Leishmaniasis and control depends on early detection and prompt treatment to prevent death from visceral Leishmaniasis and morbidity from cutaneous and mucosal Leishmaniasis (Herwaldt, 1999).

1.3.2 Epidemiology of Leishmaniasis

It is estimated that Leishmaniasis is endemic in 88 countries, with 12 million people currently infected and a population of 350 million at risk of contracting the disease (Sharma and Singh, 2009). The incidence of disease is approximately 1.5 million per annum for cutaneous Leishmaniasis and 500 000 per annum for visceral Leishmaniasis (Desjeux, 2004). Disability-adjusted life years (DALYs) lost due to Leishmaniasis is close to 2.4 million (Desjeux, 2004). Greater than 90% of all cutaneous Leishmaniasis cases occur in Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru whilst the tropical and sub-tropical regions of Africa, Asia, Southern Europe as well as South and Central America are highly endemic for visceral Leishmaniasis (Sharma and Singh, 2009). Thus far, over 21 *Leishmania* species are known to infect humans and 30 *Phlebotomus* sandfly species are identified as vectors (Desjeux, 2004).

There has been a steady, worrying increase in the incidence of Leishmaniasis. Increases have been mainly attributed to behavioural and environmental changes, including the development of new settlements, building of new dams, deforestation and migration from rural to urban areas. The rising incidence of Leishmaniasis is evident in the recent outbreaks that have been reported. The incidence of CL in Brazil increased from 21,800 cases in 1998 to 60,000 cases in 2003 (Desjeux, 2004). In 2009, the deadliest outbreak of visceral Leishmaniasis began in Sudan, with more than 6,363 new cases of infection and 303 fatalities (WHO, 2010). Moreover, individual risk factors such as poverty, malnutrition, mounting anti-leishmanial drug resistance and immunosuppression owing to HIV co-infection has contributed to the rising incidence of disease. During HIV co-infection, the loss of $CD4^+$ T cells implies a loss of T cells capable of recognizing *Leishmania* antigens and stimulating a response against it (Olivier *et al.*, 2003). Moreover, the *Leishmania* parasite accelerates the replication of the HIV virus promoting the development of AIDs and in doing so, reduces the life expectancy of HIV^+ patients (Cacopardo *et al.*, 1996; Cruz *et al.*, 2006). Thus, although it is clear that *Leishmania* is an established disease, these complicating factors enable its classification as a re-emerging threat (Desjeux, 2004; Goto and Lindoso, 2010).

1.4 Cutaneous Leishmaniasis

Like all other *Leishmania* parasites, cutaneous *Leishmania* parasites have a digenetic life cycle and exist in two distinct morphologies. The flagellated promastigote forms multiply and develop extracellularly within the digestive tract of the female sandfly vectors, and are transmitted to the mammalian host during a blood meal. Once inside the mammalian host, promastigote parasites infect phagocytic macrophages, neutrophils and even dermal DCs (Figure 1.7) (Ng *et al.*, 2008; Peters *et al.*, 2008; Ribeiro-Gomes *et al.*, 2012). Within the phagolysosomes of macrophages, the promastigote parasites transform into non-motile amastigotes and continue to replicate in this form through complex host-parasite interactions.

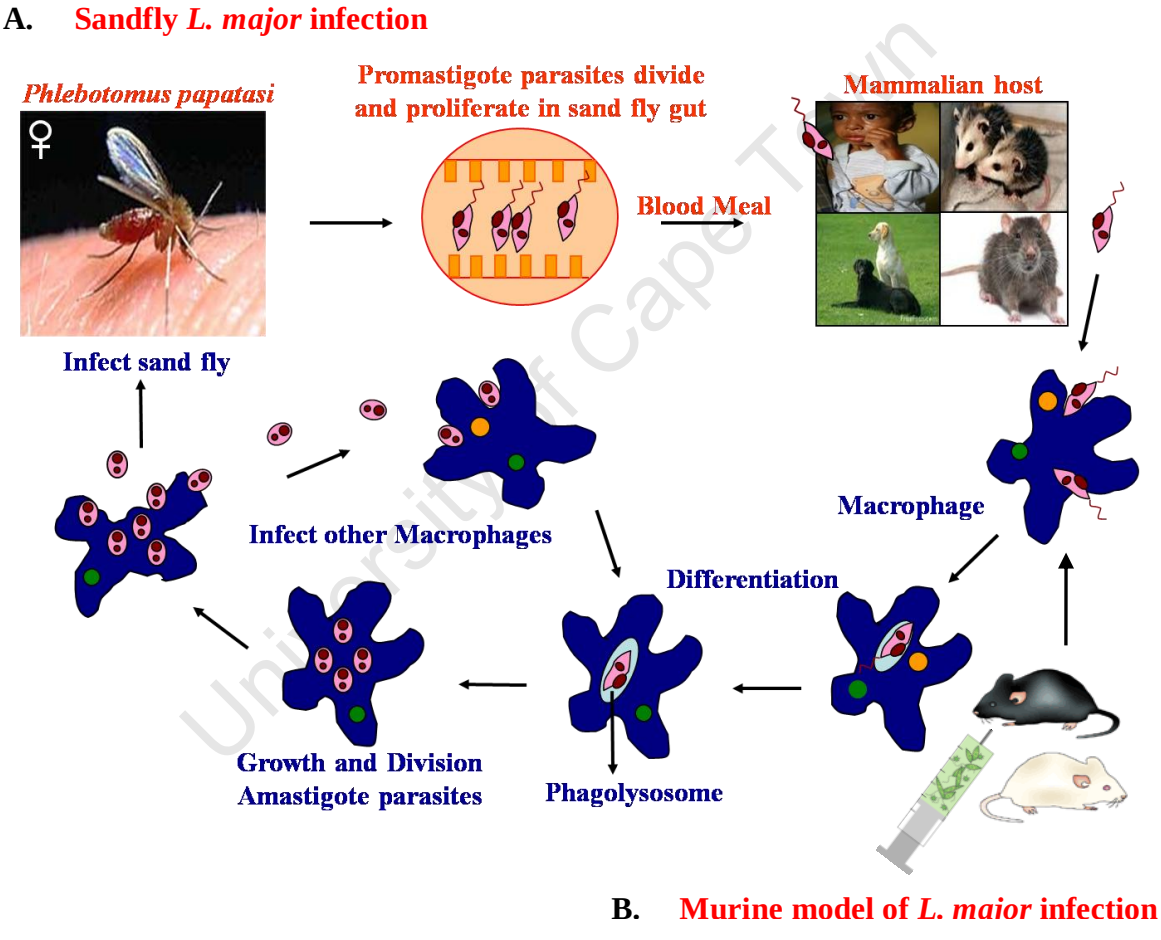

Figure 1.7: Life cycle of *Leishmania major* parasites.
A. Metacyclic promastigote *L. major* parasites are regurgitated into the skin of a mammalian host by the bite of an infected female sandfly. The parasites are phagocytosed by macrophages, where they survive in phagolysosomes, and transform into replicating amastigotes. When infected macrophages burst, amastigotes infect other macrophages, or infected macrophages are ingested by the sandfly once again to repeat the cycle. Amastigote parasites then transform into flagellate promastigotes in the gut of the sandfly and wait to be transmitted when the sandfly takes another blood meal. **B**. In a murine model of *L. major* infection, mice are infected with promastigote parasites via subcutaneous, intravenous, intradermal or intranasal routes and follow the same life cycle as described for the natural route of infection by sandflies. Illustration drawn from previous publications (Bogdan *et al.*, 1996; Sacks and Noben-Trauth, 2002; Lipoldova and Demant, 2006).

Infected macrophages may then burst releasing amastigote parasites that spread the infection by infecting other cells, or they can be ingested by a feeding sandfly to repeat the cycle (Figure 1.7) (Alexander *et al.*, 1999; Murray *et al.*, 2005).

To identify correlates of immune protection, which may aid in vaccine design and therapeutic strategies, experimental models of cutaneous Leishmaniasis have been established in which disease is induced by infecting mice subcutaneously with *L. major*. The *L. major* mouse model provides an excellent model system for investigating the mechanisms underlying Th1/Th2 cell differentiation relating to resistance/susceptibility to intracellular infection. Most laboratory strains (such as C57BL/6) are able to control *L. major* disease progression and heal lesions, raising a polarised Th1 response (Figure 1.8). In contrast, due to a genetic predisposition, susceptible BALB/c mice show progressive lesion development with dissemination of parasites to visceral organs which is associated with a polarized Th2 response following infection (Sacks and Noben-Trauth, 2002) (Figure 1.8). However, evidence also suggests that a low dose of *L. major* is capable of inducing a protective Th1 response in susceptible BALB/c mice whilst only a transient Th2 response is observed in resistant C57BL/6 mice due to the expansion of IFN-γ-producing $CD8^+$ T cells (Bretscher *et al.*, 1992; Menon and Bretscher, 1998; Uzonna *et al.*, 2004).

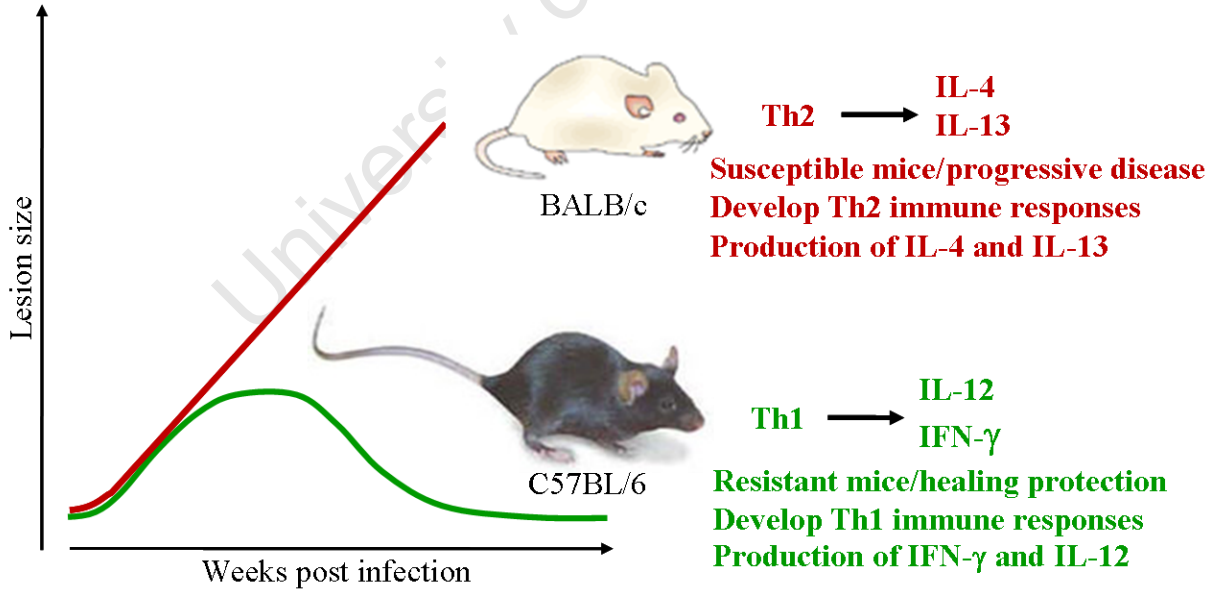

Figure 1.8: Disease progression in a murine model of *Leishmania major* infection.
Laboratory strains of mice are infected by needle inoculation of a large number of promastigote parasites (10^4-10^7) into subcutaneous, intravenous, intradermal or intranasal sites. The susceptible BALB/c mice develop an IL-4 driven Th2 response with production of IL-4 and IL-13. There is no elimination of parasites leading to progressive disease. In contrast, the resistant C57BL/6 mice develop an IL-12 driven Th1 response characterized by the production of IFN-γ. Parasites are eliminated leading to a healing response. Illustration drawn from previous publications (Bogdan et al., 1996; Brombacher, 2000; Sacks and Noben-Trauth, 2002).

1.4.1 Evasion of L. major parasites from primary host immune responses during infection

The *Leishmania* parasite expresses specific virulence factors which aid in establishing intracellular parasitism by: (a) evading humoral responses, (b) attachment and phagocytosis of parasites, (c) intracellular survival of endocytosed parasites, (d) transformation from promastigote to amastigote form and (e) replication of amastigotes (Chang and McGwire, 2002). Among the virulence factors are two important molecules, the metalloprotease gp63 and lipophosphoglycan (LPG). *Leishmania* Gp63 interacts with the complement protein C3b and converts it to an inactive state, C3bi. Since C3b is a potent immune opsonin critical in activating complement-mediated cell lysis for clearance of pathogens, its inactivation to C3bi shifts complement function toward phagocytosis and promotes uptake of *Leishmania* by means of C3b receptors (CR1 and CR3) on innate phagocytic cells such as macrophages, neutrophils and dendritic cells. Gp63 has also been associated with suppression of oxidative burst (Sorensen *et al.*, 1994; Bogdan *et al.*, 1996; Alexander *et al.*, 1999). Aside from protecting the *Leishmania* parasite against complement and oxidative burst, Gp63 also promotes inactivation of protective host cell signaling pathways thereby favouring *Leishmania* survival and progression within its host (Olivier *et al.*, 2012).

In the case of *L. major*, LPG facilitates attachment and entry of promastigote and amastigote parasites into macrophages (Bogdan *et al.*, 1996) and like Gp63, protects the parasites against complement-mediated lysis. In addition, LPG transiently inhibits phagosome maturation which gives the promastigotes sufficient time to differentiate into the more oxidative-resistant amastigotes (Desjardins and Descoteaux, 1997; Peters and Sacks, 2006). LPG also acts as a protective shield, physically blocking oxygen radicals from reaching the parasite surface (Alexander *et al.*, 1999). *Leishmania* is also reported to interfere with protein kinase C activity (PKC) (Guler *et al.*, 2011) and suppress macrophage iNOS expression and NO production (Proudfoot *et al.*, 1996). Once engulfed, *Leishmania* parasites must evade the oxidative burst in the phagosome and acidic enzymes in the phagolysosome released by macrophages to degrade the parasite. Consequently, the parasites produce acidic phosphatases to reduce the intensity of the oxidative burst and resist the attack of acidic enzymes by maintaining a neutral intracellular pH through a proton pump on its surface (Sharma and Singh, 2009). More recently, *Leishmania* proteinases as important virulence factors have been implicated in tissue invasion, survival

in macrophages and immune modulation by parasites (Silva-Almeida *et al.*, 2012). Current literature further indicates that *Leishmania* exosomes are immunomodulatory virulence factors responsible for delivery of immunosuppressive *Leishmania* molecules (such as Gp63) to host target cells (Silverman *et al.*, 2010; Olivier *et al.*, 2012).

1.4.2 Cell mediated host immune responses against L. major

Experimental infection with *L. major* in mice defined the Th1/Th2 paradigm of resistance/susceptibility to intracellular infection. Resistant C57BL/6 mice develop healing Th1 responses with production of IL-12 and IFN-γ, which upregulate the expression of inducible nitric oxide synthase leading to classical activation of macrophages and subsequent killing of intracellular parasites by effector NO production (Figure 1.9) (Heinzel *et al.*, 1993; Stenger *et al.*, 1994; Guler *et al.*, 1996; Park *et al.*, 2002; Holscher *et al.*, 2006). In contrast, lack of healing in susceptible BALB/c mice is associated with a Th2 response characterized by secretion of IL-4, IL-5, IL-9 and IL-13, high type 2 anti-*Leishmania* antibody titres and alternative activation of macrophages (Figure 1.9) (Locksley and Scott, 1991; Kopf *et al.*, 1996; Matthews *et al.*, 2000; Iniesta *et al.*, 2002; Sacks and Noben-Trauth, 2002; Arendse *et al.*, 2005). Earlier studies demonstrated that IFN-γ deficient mice developed Th2 responses and increased susceptibility to *L. major* infection (Wang *et al.*, 1994). Similarly, genetically resistant C57BL/6 mice lacking IL-12 displayed increased IL-4 expression and progressive disease to *L. major* (Mattner *et al.*, 1996). On the other hand, disruption of the IL-4 gene or treatment with anti-IL-4 antibodies in susceptible BALB/c mice caused these animals to become highly resistant to *L. major* infection (Sadick *et al.*, 1990; Kopf *et al.*, 1996). Thus, it is now widely accepted that IL-4 induces Th2 and inhibits Th1 whereas IL-12/IFN-γ induces Th1 and inhibits Th2 differentiation during cutaneous Leishmaniasis.

The early IL-4 response to *L. major* is confined to a population of $CD4^+$ T cells expressing a Vβ4Vα8 T cell receptor that recognize the *Leishmania* antigen LACK (*Leishmania* homologue of receptors for activated C kinase) (Launois *et al.*, 1997). Evidence for this comes from the observation that infected Vβ4-deficient BALB/c mice mount strong Th1 responses and heal cutaneous lesions (Himmelrich *et al.*, 2000). Both BALB/c and C57BL/6 mice secrete IL-4 early after infection, however, production of IL-4 is sustained in susceptible BALB/c mice and transient in resistant C57BL/6 mice (Morris *et al.*, 1992; Belkaid *et al.*, 2000).

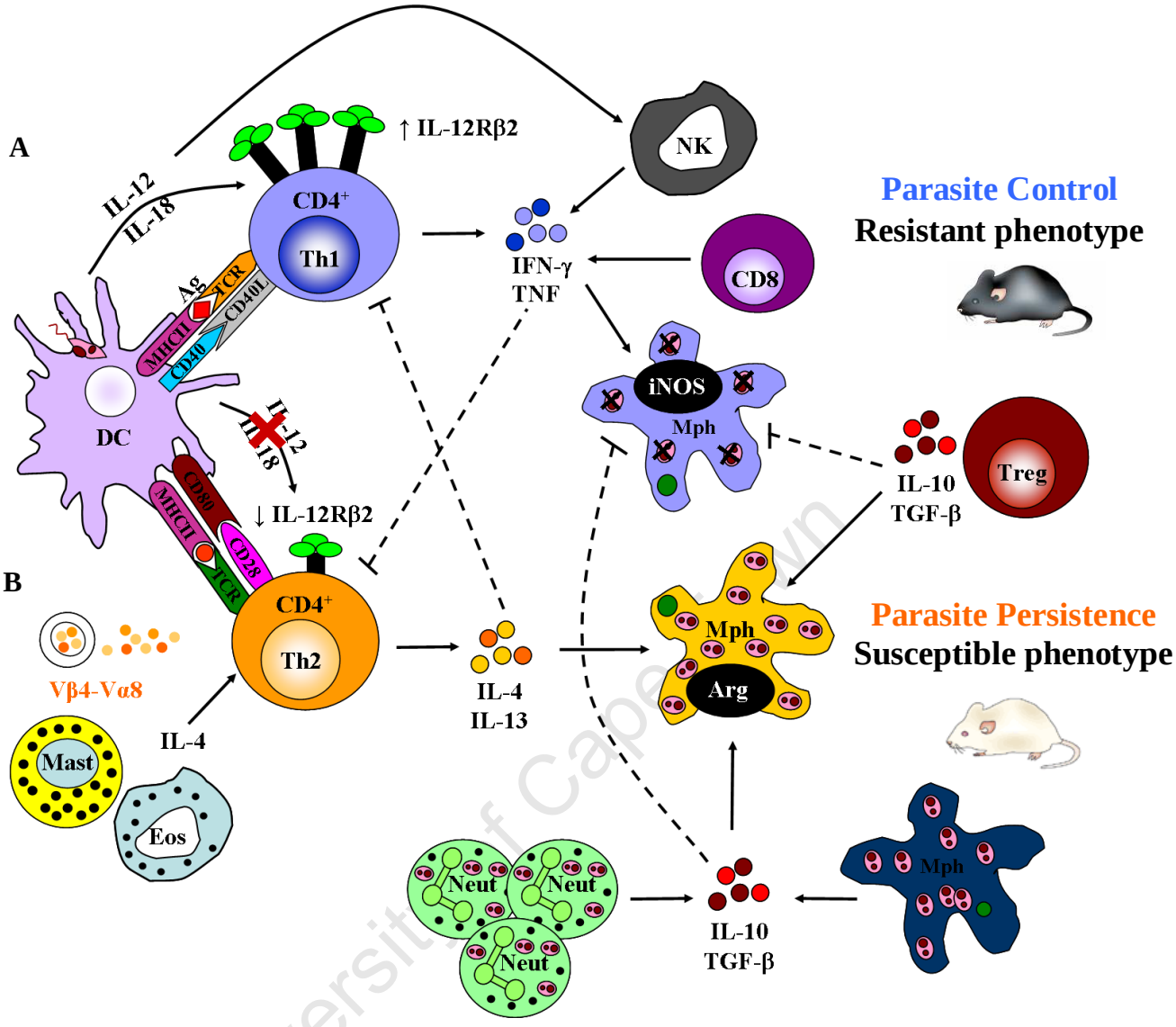

Figure 1.9: Immune responses to *L. major* in resistant and susceptible mice.
A. Upon interaction with *L. major* parasites, activated dendritic cells (DCs) upregulate expression of CD40 and produce IL-12 and IL-18. These cytokines interact with the IL-12 receptor β2-chain (IL-12Rβ2) whose expression is increased in activated Th1 cells. Together, a dominant Th1 response is generated by the production of IFN-γ and TNF-α which upregulate the expression of inducible nitric oxide synthase (iNOS) and activate infected macrophages for intracellular killing. $CD8^+$ T cells and natural killer (NK) cells control infection by producing IFN-γ and effector killing by macrophages leading to parasite control. **B**. In contrast, parasite persistence in BALB/c mice is due to the failure of an IL-12-dependent redirection of the early Th2 response. This could be due to continued expression of early IL-4 from Vβ4Vα8 $CD4^+$ T cells, mast cells and eosinophils at the inoculation site and draining lymph nodes, delayed secretion of IL-12 or loss of responsiveness to IL-12 due to reduced expression of the IL-12Rβ2 chain on activated Th2 cells. IL-4 and IL-13 contribute to susceptibility by suppressing Th1 responses and inhibiting intracellular killing by macrophages. This leads to arginase production by macrophages which favor parasite growth. Sustained neutrophil recruitment to the site of infection and regulatory T cells promote susceptibility and inhibit Th1 responses by the production of IL-10 and TGF-β, that inhibit activation of macrophages. Solid lines (⟵) represent activation and broken lines (◂⋯⋯) represent inhibition. Illustration drawn from previous publications (Bogdan *et al.*, 1996; Sacks and Noben-Trauth, 2002; Sharma and Singh, 2009; Liu and Uzonna, 2012).

It appears that resistant mouse strains redirect the early Th2 response in an IL-12-dependent mechanism towards a protective Th1 response while in susceptible mice the Th2 response persists and dominates the disease outcome by suppressing effector mechanisms needed for parasite killing (Sypek *et al.*, 1993; Sacks and Noben-Trauth, 2002). This was confirmed by the early neutralisation of IL-12, which abrogated resistance in C57BL/6 mice and led to the production of Th2 cytokines, which selectively inhibited the production of nitric oxide by IFN-γ-activated macrophages (Heinzel *et al.*, 1993; Scharton-Kersten *et al.*, 1995). Since IL-12 has been shown to redirect the early Th2 response to *L. major* and promote resistance (Heinzel *et al.*, 1993; Sypek *et al.*, 1993), it suggests that the IL-12 response is either dysfunctional or inhibited in susceptible BALB/c mice. A loss of IL-12 signaling due to reduced expression of the IL-12 receptor β2-chain (IL-12Rβ2) on activated Th2 cells has been proposed to explain the defective IL-12 response in BALB/c mice (Figure 1.9) (Hondowicz *et al.*, 2000).

Furthermore, arginase I production in BALB/c induced by Th2 cytokines in alternatively activated macrophages has been shown to be essential for *Leishmania* survival and growth (Figure 1.9) (Iniesta *et al.*, 2002). In fact, impairment of alternative macrophage activation delays *L. major* disease progression in susceptible BALB/c mice (Holscher *et al.*, 2006). IL-13 (Matthews *et al.*, 2000), IL-9 (Arendse *et al.*, 2005), IL-17 (Lopez Kostka *et al.*, 2009), IL-10 (Kane and Mosser, 2001), TGF-β (Li *et al.*, 1999), co-stimulatory molecule interactions (OX40-OX40L; CD28-B7) (Brown *et al.*, 1996; Akiba *et al.*, 2000), and maintenance of inflammatory neutrophils at the site of parasite inoculation (Tacchini-Cottier *et al.*, 2000) are additional susceptibility factors inducing a persistent Th2 response (Figure 1.9). IL-10 suppresses Th1 responses and deactivates macrophages (Figure 1.9) (Belkaid *et al.*, 2001; Noben-Trauth *et al.*, 2003) and IL-10-deficient BALB/c mice display increased resistance to *L. major* infection (Kane and Mosser, 2001). Studies have indicated that regulatory T cells (Tregs) ($CD4^+CD25^+$) constitute a source of IL-10 that favours the persistence of parasites in leishmanial skin lesions (Figure 1.9) (Belkaid *et al.*, 2002a). Furthermore, IL-10-producing Tregs also suppress protective immunity and induce uncontrolled parasite growth in *L. major*-infected BALB/c mice (Xu *et al.*, 2003). Thus, IL-10 and Treg cells are important regulators of resistance/susceptibility to cutaneous Leishmaniasis.

IFN-γ-induced NO production by classically activated macrophages is crucial for the killing of *L. major* parasites. Indeed, iNOS-deficient mice are highly susceptible to

infection (Wei *et al.*, 1995). In addition to IFN-γ and IL-12, other protective mediators assist in the regulation of Th1 development and parasite clearance during *L. major* infection. Specifically, C57BL/6 mice genetically deficient for Th1 transcription factor T-bet (Szabo *et al.*, 2002), STAT4 (Stamm *et al.*, 1999), TNF (Wilhelm *et al.*, 2001) or CD40-CD40L interactions (Campbell *et al.*, 1996; Kamanaka *et al.*, 1996) show an increased susceptibility to infection. IL-18 augments IL-12 activity (Yoshimoto *et al.*, 1998) and protects against *L. major* infections (Monteforte *et al.*, 2000). IL-12 activates innate natural killer cells and $CD8^+$ T cells to produce IFN-γ, which in turn contributes to Th1 responses by increasing expression of the IL-12R by activated Th1 cells and activating macrophages for intracellular killing (Figure 1.9) (Scharton-Kersten *et al.*, 1995; Belkaid *et al.*, 2002b). Collectively, it appears that IL-12 and the other Th1-promoting factors redirect the early IL-4/Th2 responses to *L. major* infection in resistant mice. However, IL-12/IL-12R signaling remains crucial to a healing Th1 response and IL-4 remains central to a non-healing Th2 response.

1.5 Dendritic cells and macrophages in *L. major* infection

IL-12 is critical for driving IFN-γ production from NK cells and $CD4^+$/$CD8^+$ T cells to induce protective immunity against *L. major* infection (Reiner and Locksley, 1995). Since *Leishmania* infect macrophages, early studies focused on macrophages as the source of IL-12. However, it was found that macrophages from both susceptible and resistant strains of mice made very little IL-12 in response to *Leishmania* promastigotes and the parasite was in fact able to inhibit IL-12 secretion by macrophages (Reiner *et al.*, 1994; Carrera *et al.*, 1996; Belkaid *et al.*, 1998). In contrast to macrophages, DCs play an important role in secreting IL-12 and subsequently orchestrating Th1 cell differentiation and protective immunity to *Leishmania* infection (Liu and Uzonna, 2012). *Leishmania*-infected macrophages also secrete immuno-regulatory cytokines such as IL-10 and TGF-β which inhibit their killing capacity and promote susceptibility (Alexander *et al.*, 1999; Belkaid *et al.*, 2001) whilst IFN-γ, IL-1, TNF-α and type I interferons activate macrophages and enhance its leishmanicidal activity (Wang *et al.*, 1994; Diefenbach *et al.*, 1998; Von Stebut *et al.*, 2003).

Macrophages can phagocytose *L. major* via the CR3 receptor (Mosser and Edelson, 1985) or through Fc receptors with IgG-coated parasites (Liu and Uzonna, 2012). CR3-mediated ingestion of *L. major* amastigotes by Langerhans DCs has been reported (Blank *et al.*,

1993) whilst C-type lectin, Fcγ and Toll-like receptors have been implicated in the ingestion of promastigotes by DCs (Colmenares *et al.*, 2004; Woelbing *et al.*, 2006; Tuon *et al.*, 2008). Whilst both macrophages and DCs are considered major APCs and present antigen via the MHC Class II pathway, only DCs can prime and stimulate *L. major* specific $CD8^+$ T cells via the MHC Class I pathway (Belkaid *et al.*, 2002b; Woelbing *et al.*, 2006). Moreover, *L. major* suppresses MHC Class II presentation of parasite and non-parasite antigen to $CD4^+$ T cells in infected macrophages, possibly as a ploy to evade detection by the adaptive immune system (Fruth *et al.*, 1993). Importantly, DCs and not macrophages, can be used to vaccinate against Leishmaniasis *in vivo* (Flohe *et al.*, 1998; Ahuja *et al.*, 1999; von Stebut *et al.*, 2000) whilst macrophages and not DCs are the major effector cells responsible for destruction of *Leishmania* parasites. Thus, although DCs and macrophages are ontogenetically related, they play distinctly different roles in the initiation and propagation of immune responses against *Leishmania* parasites.

1.5.1 Interaction of dendritic cell subsets with L. major parasites

Various studies have indicated that different DC subsets respond differently to infection by *Leishmania* parasites. Murine epidermal Langerhans cells were found to be the first subset of dendritic cells to phagocytose *L. major* amastigotes, but not promastigotes, in the skin and secrete IL-12 (Moll, 1993; von Stebut *et al.*, 1998). Parasite uptake was associated with an increase in DC activation as shown by the upregulation of surface expression of MHC Class I and Class II and co-stimulatory molecules (Liu and Uzonna, 2012). Langerhans DCs were initially thought to transport parasite antigen from the infected site to the draining lymph node for presentation to antigen-specific T cells (Blank *et al.*, 1993; Moll *et al.*, 1993). However, new evidence suggests that although dermal $Langerin^-$ DCs and LC DCs may transport *L. major* antigens to the draining lymph node, only dermal DCs activate $CD4^+$ T cells (Ritter *et al.*, 2004). Follow-up studies demonstrated that mice lacking MHC Class II antigens exclusively on LCs, and not dermal DCs, were able to control *L. major* infection confirming that LCs are dispensable for triggering T cell responses during *Leishmania* infection (Lemos *et al.*, 2004). Moreover, a recent study has indicated that LCs may even play a pathogenic role during low-dose *L. major* infection due to their induction and expansion of Tregs (Kautz-Neu *et al.*, 2011b).

Plasmacytoid DCs are also detected in the infected skin and are activated by *Leishmania* DNA to secrete IL-12 and increased levels of IFN-α/β, which have been shown to induce protection against *L. major* infection in BALB/c mice (Remer *et al.*, 2007). Monocyte-

derived dendritic cells (mDCs) are also recruited to the site of infection. mDCs can efficiently engulf parasites and secrete early IL-12 and pro-inflammatory cytokines (Konecny *et al.*, 1999; Soong, 2008). Activated mDCs have been implicated in stimulating NK cells and inducing a more powerful parasite-specific IFN-γ-mediated Th1 response (Leon *et al.*, 2007). CD40-CD40L interactions and exogenous stimuli such as IL-1, IFN-γ, IFN-γ/LPS were also shown to enhance DC-derived IL-12 production during infection (Campbell *et al.*, 1996; Kamanaka *et al.*, 1996; von Stebut *et al.*, 2000). TLR9-dependant activation of DCs by *Leishmania* DNA is also reported to be involved in protection to *L. major* infection (Abou Fakher *et al.*, 2009). Murine lymphoid DCs in the spleen also differ in their ability to phagocytose amastigote parasites with $CD8\alpha^+$ DCs being the least permissive to amastigotes compared to $CD8\alpha^-$ DCs. However, $CD8\alpha^+$ DCs are the best producers of IL-12p70 in response to infection (Henri *et al.*, 2002).

It has also been reported that antibody opsonisation of *Leishmania* enhances uptake of the parasites by Langerhans cells and monocyte-derived DCs, which is associated with increased IL-10 release. DCs that have taken up opsonised *Leishmania* preferentially prime IL-10-producting $CD4^+$ T cells, thus contributing to increased susceptibility to infection (Soong, 2008). In contrast, other studies have demonstrated that uptake of antibody-opsonized parasites activates mDCs to secrete IL-12 and induce protective immunity. These different findings indicate that antibodies do play a regulatory role in cutaneous Leishmaniasis to mediate different effector responses (Soong, 2008).

Some studies have speculated that the different DC responses to *Leishmania* parasites may be attributed to genetic resistance or susceptibility of mice to infection. Whilst some studies have shown that DCs from both susceptible and resistant mice exhibit comparable upregulation of MHC, co-stimulatory molecules and release of IL-12p70 (von Stebut *et al.*, 1998), other studies have demonstrated increased IL-4 receptor and down-regulation of CD40 and IL-12p40 expression on DCs from BALB/c but not resistant C57BL/6 mice (Heinzel *et al.*, 1998; Mbow *et al.*, 2001; Moll *et al.*, 2002). Overall, it may seem that *Leishmania* parasites have developed strategies to inhibit DC-mediated Th1 responses in susceptible mice. However, more studies need to be conducted to provide clear evidence if the observed differences in DCs between susceptible and resistant mice contribute to pathogenesis and/or protection to disease.

1.5.2 Evasion of L. major parasites from dendritic cell presentation

Leishmania parasites also modulate and impair DC functions to escape the host immune system and establish infection. Products secreted by *L. major* promastigotes have been shown to inhibit the motility of murine splenic DCs (Jebbari *et al.*, 2002) whilst migration of *L. major* infected murine Langerhans cells is inhibited by LPG (Ponte-Sucre *et al.*, 2001). *Leishmania* parasites can modulate chemokine receptor expression, such as CCR2 and CCR5 on DCs, which may contribute to this inhibition of DC motility (Steigerwald and Moll, 2005). Moreover, absence of CCR2 leads to increased susceptibility to *L. major* infection, dominated by Th2 cytokines (Sato *et al.*, 2000). Overall, this suggests that *Leishmania* parasites have the ability to interfere with DC transportation of antigen to or within lymphoid tissue and thus, further delay the onset of acquired immunity.

Further studies have demonstrated that *Leishmania* parasites hinder the onset of adaptive immunity by delaying DC maturation/activation in order to allow establishment of infection (Moll *et al.*, 1995). Moreover, *L. major* infected murine $CD11c^+$ lymph node DCs maintain high parasite loads owing to reduced MHC Class II antigens and no detectable CD86 expression (Muraille *et al.*, 2003). LPGs of *L. major* promastigotes impair the induction of Th1 responses by down-modulating DC-derived IL-12 production (Liu *et al.*, 2009). Epidermal Langerhans DCs from susceptible BALB/c mice also down-regulate IL-12p40 expression in response to *L. major* infection (Moll *et al.*, 2002). DCs are also modulated by IL-10 production during *Leishmania*, infection which generally tends to favor Th2 responses by inhibiting Th1 responses.

1.5.3 Immunobiology of human cutaneous Leishmaniasis and dendritic cell interactions

L. major infection in resistant strains of mice resembles self-limiting cutaneous Leishmaniasis in humans (Sharma and Singh, 2009). However, in human *L. major* infection, a distinct dichotomy in the T cell response against *Leishmania* parasites is not clearly evident as demonstrated in murine models. Generally, a mixed Th1/Th2 cytokine response is seen in patients with cutaneous Leishmaniasis (Ajdary *et al.*, 2000), however, IL-13, IL-4, IL-10 and TGF-β have been detected in the skin after initial infection suggesting that Th2 cytokines play an immunoregulatory role in human cutaneous Leishmaniasis (Melby *et al.*, 1994; Ajdary *et al.*, 2000). Nonetheless, clearance of infection still depends on IL-12 induced IFN-γ production (Kolde *et al.*, 1996). Myeloid-derived human dendritic cells secrete IL-12p70 in conjunction with CD40-CD40L interactions following *L. major* infection (Marovich *et al.*, 2000). Uptake of *Leishmania*

promastigotes results in DC maturation and upregulation of HLA-DR and co-stimulatory molecules. Human DCs also respond well to antibody-opsonised parasites *in vitro* and secrete IL-12 upon activation mimicking the *in vivo* infection (Sharma and Singh, 2009).

1.6 IL-4Rα signaling in cutaneous Leishmaniasis

Recently, IL-4Rα signaling has been investigated for its intriguing role in affecting the outcome of various parasitic infectious diseases such as cutaneous Leishmaniasis (Mohrs *et al.*, 1999), acute Schistosomiasis (Mountford *et al.*, 2001; Herbert *et al.*, 2004; Dewals *et al.*, 2009) and nematode infections (Urban *et al.*, 1998; Horsnell *et al.*, 2007; Schmidt *et al.*, 2012). Both IL-4 and IL-13 can signal through the IL-4Rα chain therefore deletion of the IL-4Rα impairs downstream signaling of both IL-4 and IL-13 responses via trancription factor STAT6. This has been established in animal studies during *L. major* infection in which susceptible BALB/c mice deficient for IL-4 (Kopf *et al.*, 1996), IL-13 (Matthews *et al.*, 2000) or STAT6 (Stamm *et al.*, 1998) were able to control disease progression.

Initial control of *L. major* during the acute phase of infection in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ deficient BALB/c mice is equivalent, with both strains of mice showing reduced footpad swelling, parasite loads and type 1 antibody responses (Mohrs *et al.*, 1999). However, in contrast to IL-$4^{-/-}$ mice, IL-4R$\alpha^{-/-}$ mice developed progressive disease and necrotic footpad lesions during the chronic phase of infection. In contrast to IL-$4^{-/-}$ mice, the absence of IL-13-mediated functions in IL-4R$\alpha^{-/-}$ mice implicated IL-13 as a susceptibility factor in chronic *L. major* infection (Mohrs *et al*., 1999). Furthermore, IL-13 transgenic C57BL/6 mice were shown to develop a susceptible phenotype to acute Leishmaniasis with impaired IL-12 and IFN-γ production, while IL-13-deficient BALB/c mice remained comparatively resistant (McKenzie *et al.*, 1998b; Matthews *et al.*, 2000). IL-13 promotes susceptibility by activating IL-4/IL-13 alternative macrophages and suppressing secretion of NO, IL-12 and/or IL-18 (McKenzie *et al*., 1998b; Matthews *et al*., 2000).

The role of IL-4 and IL-4Rα in *Leishmania* infection mice is controversial since conflicting reports have suggested that although IL-4 is important, it is not the sole mediator of susceptibility in BALB/c mice. Whilst Kopf *et al.* (1996) and Mohrs *et al*. (1999) demonstrated that IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice were able to control acute Leishmaniasis, Noben-Trauth *et al.* (1996) showed that IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ deficient BALB/c mice remained susceptible to disease and could not contain parasites. This

discrepancy between the two studies could be attributed to the use of parasite substrains of different virulence which may have affected the outcome of *L. major* infection in IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ BALB/c mice. Important to note from either of the studies mentioned above is that even in the absence of IL-4 or IL-4Rα, Th2-cell development and Th2-related cytokines were still present, albeit in different Th1/Th2 ratios (Kopf *et al.*, 1996; Noben-Trauth *et al.*, 1996; Noben-Trauth *et al.*, 1999). To address the conflicting issues on the role of IL-4 in Th2-cell development and Th2-cytokines during *L. major* infection, subsequent studies followed IL-4 expression and T helper cell development *in vivo* in *L. major*-infected IL-4R$\alpha^{-/-}$ deficient BALB/c mice. This study confirmed that IL-4Rα-independent mechanisms underlie the default Th2 pathway in *L. major*-infected BALB/c mice because despite a clear absence of IL-4/IL-13-mediated functions in IL-4R$\alpha^{-/-}$ deficient mice, unimpaired Th2 polarization and IL-4-producing CD4$^+$ T cells as well as other Th2-related cytokines were still present (Mohrs *et al.*, 2000) Similar findings were also reported for IL-4$^{-/-}$ deficient BALB/c mice (Noben-Trauth *et al.*, 1997; Kropf *et al.*, 1999). Together, these findings contradict the idea that IL-4 is the sole regulator of susceptibility to *L. major* infection. The accumulating reports instead suggest that both IL-4-dependent and IL-4-independent factors contribute to the susceptibility phenotype in *L. major*-infected BALB/c mice (Brombacher, 2000).

Given the conflicting data on IL-4Rα signaling in the outcome of *L. major* infection, several studies have aimed to clarify the role of IL-4Rα-signaling in specific cell populations in resistance or susceptibility to infection. Given the central role of T cells in *L. major* infection (Scott *et al.*, 1988) and controversial roles of IL-4 (Kopf *et al.*, 1996; Noben-Trauth *et al.*, 1996), specific abrogation of IL-4Rα on CD4$^+$ T cells (with variable deletion on CD8$^+$ T cells) in Lck^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice was investigated during *L. major* infection. Lack of IL-4Rα signaling on CD4$^+$ T cells led to a healing phenotype in BALB/c mice (Radwanska *et al.*, 2007). Resistance to *L. major* in Lck^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice was higher than in global IL-4R$\alpha^{-/-}$ mice and was associated with early IL-12 mRNA induction which led to increased IFN-γ production, elevated iNOS expression and increased DTH responses, similar to genetically resistant C57BL/6 mice. This demonstrated that impairment of IL-4Rα-dependent CD4$^+$ Th2 cells in the presence of IL-4/IL-13-responsive non-CD4$^+$ T cells transforms non-healing BALB/c mice to a healer

phenotype, indicating a beneficial role for IL-4Rα signaling on other cell types in cutaneous Leishmaniasis.

In another study, Holscher and colleagues analysed IL-4Rα signaling on macrophages/neutrophils in *L. major*-infected LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice (Holscher *et al.*, 2006). Their study revealed that the absence of IL-4Rα on macrophages (and neutrophils) significantly delayed disease progression, although Th2 and type 2 antibody immune responses were similar to littermate controls. These data revealed that IL-4Rα-mediated effects on macrophages are involved in the development of early disease progression after *L. major* infection. Delayed disease progression in *L. major*-infected LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice was attributed to inhibition of alternative activation of macrophages, leading to classical activation of macrophages, with enhanced NO production and decreased arginase activity. These data reinforced that alternatively activated macrophages enhance susceptibility to cutaneous Leishmaniasis. Since it is known that IL-4Rα engagement suppresses IL-12 production in macrophages (Herbert *et al.*, 2004), the authors postulated that IL-4Rα deficiency on macrophages and neutrophils could have led to IL-4Rα-dependant protective mechanisms involving other phagocytic cells, such as dendritic cells, thereby resulting in the early protection to *L. major* in LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. This proposal is especially reasonable given the fact that dendritic cells have been implicated in Th1 cell development owing to the newly coined "IL-4-mediated DC instruction" theory.

1.6.1 IL-4-mediated dendritic cell instruction in cutaneous Leishmaniasis

The fact that IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ BALB/c mice remained susceptible to *L. major* (Noben-Trauth *et al.*, 1999) challenged the prevailing view that IL-4 and IL-4-producing Th2 cells are antagonists of Th1 immune responses. Although some studies have clearly shown IL-4 to be the primary inducer of Th2 responses (Sadick *et al.*, 1990; Himmelrich *et al.*, 2000), paradoxically other studies have also shown that IL-4 can promote Th1 development and the initiation of protective immunity. IL-4 was shown to promote IL-12 production by bone-marrow derived dendritic cells (BMDCs) stimulated with CpG or lipopolysaccharide (LPS) (Hochrein *et al.*, 2000; Biedermann *et al.*, 2001; Lutz *et al.*, 2002). Furthermore, administration of 1 μg of recombinant IL-4 at 0 and 8 hours after infection in BALB/c mice with *L. major* led to increased IL-12 mRNA expression by dendritic cells (DCs) *in vivo*, promoted Th1 responses and rendered mice resistant to infection. In contrast, two

additional injections of recombinant IL-4 during the period of T cell priming, which occurs 12–16 h after parasite inoculation, abrogated the resistance induced by IL-4 during the first 8 h and induced Th2 differentiation and progressive disease (Biedermann *et al.*, 2001). This study provided a new paradigm for the mode of action of IL-4 and gave rise to the "IL-4-mediated dendritic cell instruction" theory.

The IL-4 instruction theory suggests that IL-4 acting on DCs during DC activation instructs them to produce IL-12, resulting in the polarisation of naïve T cells into Th1 cells, and enhancing control of *Leishmania* infection. On the other hand, IL-4 present during the period of T cell priming polarises naïve T cells towards a Th2 phenotype, resulting in continued production of Th2 cytokines and parasite persistence (Figure 1.10). Early sources of IL-4 include Vβ4Vα8 $CD4^+$ T cells, mast cells, eosinophils and basophils. Therefore, it appears that the outcome of disease in *L. major* infected BALB/c mice depends on the timing of IL-4 interacting with different cell types. Naturally, IL-4Rα expression on DCs and naïve T cells is central to this instruction for signaling the IL-4 to exert its downstream effects on the immune response.

A follow-up study demonstrated that IL-4-mediated DC instruction is dependent on IL-10 since IL-4 treatment of DCs stimulated with LPS or CpG suppresses IL-10 production resulting in enhanced IL-12 secretion (Figure 1.10). Furthermore, the authors found that IL-4 inhibited IL-10 production only in DCs but not other APCs, such as B cells, and failed to up-regulate IL-12 production in IL-10-deficient DCs (Yao *et al.*, 2005). IL-4 could potentially exert its effects on dendritic cells using either or both of the Type I and Type II IL-4Rα receptor complexes (Lutz *et al.*, 2002). However, experiments in BMDCs demonstrated that enhancement of IL-12 production depends exclusively on the Type I IL-4R complex and IL-4 *in vitro*. IL-4 and IL-13-mediated maturation of DCs occurs via the Type II IL-4R complex (Lutz *et al.*, 2002). IL-13 is unable to signal via the Type I IL-4R complex and has been shown not to enhance BMDC-derived IL-12 production (Lutz *et al.*, 2002; Masic *et al.*, 2012). However it was found that both IL-4 and IL-13 enhanced maturation of BMDCs, via the Type II IL-4R complex. The ability of IL-4 to instruct IL-12 production by dendritic cells, enhancing Th1 responses, suggests that IL-4Rα signaling on DCs could play a role in resistance to *L.major* infection.

Although IL-4-mediated DC instruction of Th1 immunity is the most investigated role of IL-4 signaling on DCs, studies have also considered the possibility that IL-4 signals on

DCs could promote Th2 responses. Since there is little evidence to suggest that DCs themselves secrete IL-4 to favor Th2 differentiation, other DC-derived factors have been proposed to aid the process, for instance, secretion of Th1 inhibiting cytokines, IL-10 and TGF-β, increased secretion of IL-6 (Rincon *et al.*, 1997) or upregulation B7-1 and B7-2 costimulatory molecules (Moser and Murphy, 2000). Recently, a study demonstrated a direct role for IL-4Rα-signaling of IL-4 on DCs in the development of alternatively activated DCs that prime optimal Th2 responses to *S. mansoni* antigens (Cook *et al.*, 2012). Altogether, the general hypothesis on IL-4 signals on DCs contributing to Th2 development is that the Th2 pathway may occur as the default pathway in the absence of IL-12 production by DCs.

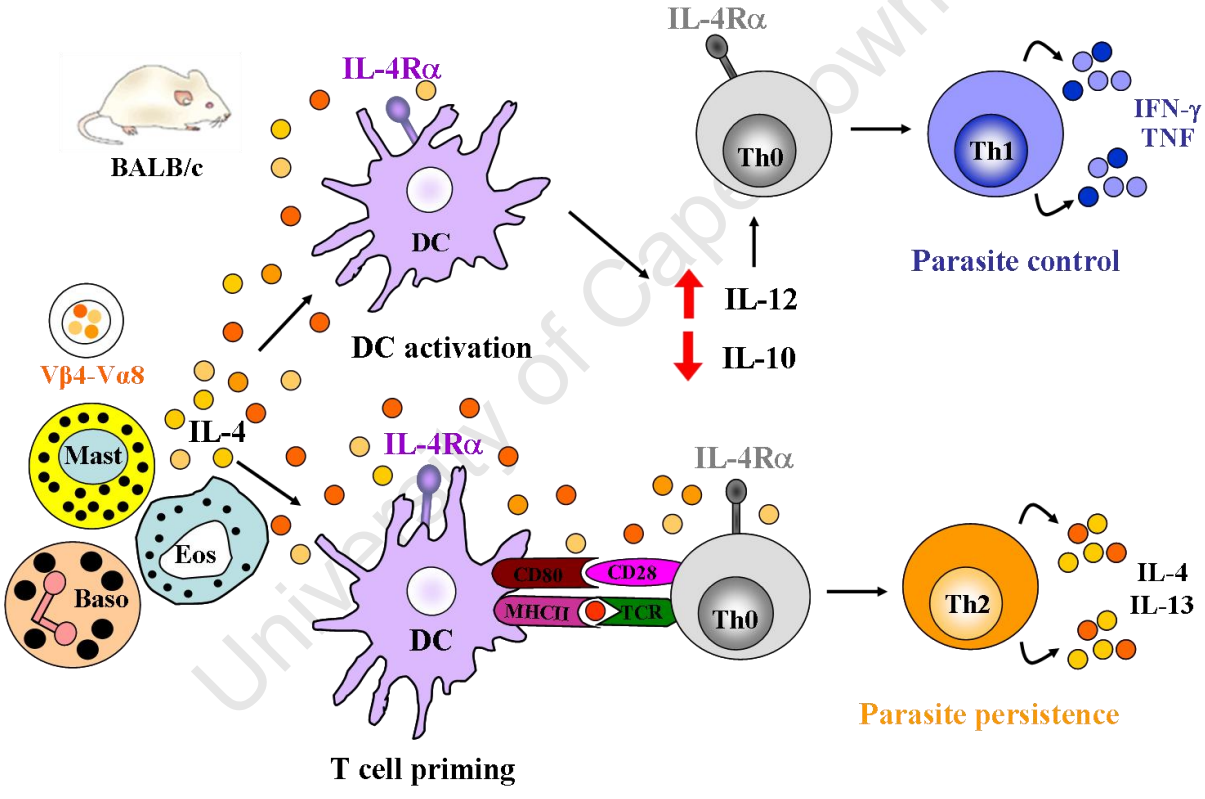

Figure 1.10: The "IL-4-mediated dendritic cell instruction" theory
Biedermann and colleagues reported a paradox wherein IL-4 can promote both Th1 and Th2 responses in BALB/c mice. If early IL-4 is administered early during the period of dendritic cell (DC) activation, it instructs DCs to produce IL-12 which leads to a polarised Th1 response and parasite control. Increased IL-12 production was shown to be due to a decrease in immunosuppressive IL-10 production, mediated by IL-4Rα signaling. However, if IL-4 is present during the period of T cell priming, IL-4 in the microenvironment of cells induces naïve T cells to differentiate into Th2 cells resulting in an environment that favours parasite persistence. Early sources of IL-4 could include Vβ4Vα8 $CD4^+$ T cells, mast cells, eosinophils and/or basophils. IL-4Rα expression on either DCs or naïve T cells is central to this instruction for signaling the IL-4.

1.7 Generation of dendritic cell-specific IL-4Rα deficient mice using the Cre/*lox*P system

In order to determine the effects of IL-4Rα signaling through dendritic cells in *L. major* infection, we generated $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice, in which dendritic-cell specific deletion of the *il4ra* gene was achieved by the bacteriophage derived Cre/*lox*P recombination system under control of the *cd11c* locus (Figure 1.11) (Caton *et al.*, 2007). In this system, cyclization recombinase (Cre) inserted downstream of the cell-specific promoter recognizes a pair of *lox*P sequences flanking the gene of interest (Exon 7 to Exon 9 of IL-4Rα). Cre-recombinase removes the intervening DNA by bringing the two *lox*P sites together (Nagy, 2000). CD11c encodes the integrin alpha X chain which is a transmembrane protein typically expressed on DCs and alveolar macrophages (Banchereau *et al.*, 2000). CD11c is mainly involved in phagocytosis but other diverse roles in antigen-presentation, inflammation, innate immunity and tissue homeostasis have also been described (Sadhu *et al.*, 2007). Generation of $CD11c^{cre}$IL-$4R\alpha^{-/-}$ BALB/c mice is illustrated in Figure 1.11. Transgenic $CD11c^{cre}$ mice were backcrossed to BALB/c for 9 generations, then intercrossed with global IL-4Rα (IL-$4R\alpha^{-/-}$) BALB/c mice to generate $CD11c^{cre}$IL-$4R\alpha^{-/-}$ BALB/c mice. Littermate mice were then subsequently intercrossed with floxed IL-4Rα (IL-$4R\alpha^{lox/lox}$) BALB/c mice (Exon 6 to Exon 8 flanked by *lox*P) (Herbert *et al.*, 2004) to yield $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice (Hurdayal, and Nieuwenhuizen, *et al.* Submitted manuscript). A similar approach using the Cre/*lox*P system has been used previously to generate mice with a conditional deletion of IL-4Rα on macrophages/neutrophils ($LysM^{cre}$IL-$4R\alpha^{-/lox}$), $CD4^+$ T cells (Lck^{cre}IL-$4R\alpha^{-/lox}$) and smooth muscle cells (SM-MHC^{cre}IL-$4R\alpha^{-/lox}$) (Herbert *et al.*, 2004; Horsnell *et al.*, 2007; Radwanska *et al.*, 2007).

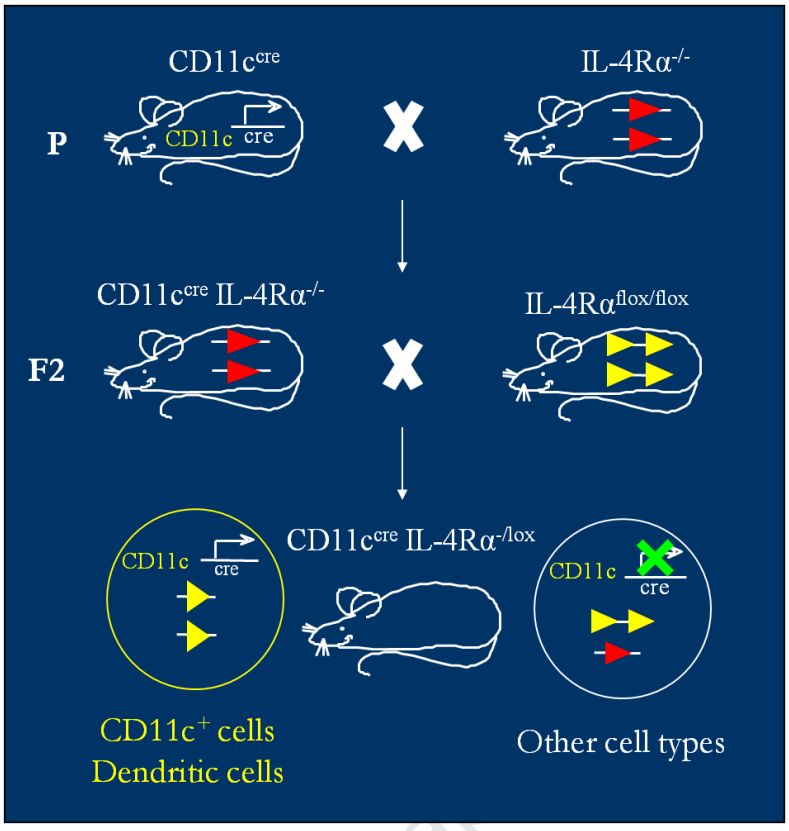

Figure 1.11: Generation CD11c^{cre}IL-4Rα$^{-/-}$ BALB/c mice.
Global IL-4Rα$^{-/-}$ BALB/c mice were intercrossed with transgenic BALB/c mice expressing Cre-recombinase under control of the *CD11c* locus. Littermate mice (F2) were then crossed with IL-4Rα$^{lox/lox}$ mice to generate CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. The "floxed" IL-4Rα allele (yellow arrows) and deleted allele (red arrows) are shown. In cells where the CD11c promoter is recognised, IL-4Rα is deleted and the functions of IL-4/IL-13 through the receptor are impaired. In all other cell types, IL-4Rα is expressed normally with unimpaired IL-4/IL-13-mediated functions.

1.8 Hypothesis and research objectives

In this study, we investigate the role of IL-4Rα signaling on dendritic cells during *L. major* infection *in vivo*. Most studies on the effects of IL-4 and IL-13 on DCs were conducted using *in vitro* BMDC cultures and thus the *in vivo* effects of IL-4Rα signaling on DCs during acute cutaneous Leishmaniasis are unknown. Therefore, we created dendritic cell specific IL-4Rα-deficient BALB/c mice ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$) by cell-specific gene targeting using the Cre-*lox*P system (Figure 1.11). We hypothesized that IL-4Rα signaling of IL-4 on DCs is important in instructing optimal Th1 responses and subsequent control of acute cutaneous *L.major* infection. Our objectives were as follows:

(A) To investigate whether IL-4-mediated dendritic cell instruction occurs in cutaneous Leishmaniasis *in vivo* using $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice.

(B) To establish whether dendritic cell-specific deletion of IL-4Rα affects the outcome of acute *L. major* infection in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice by determining parasite burdens, cellular and humoral immune responses and macrophage effector functions in *L. major*-infected

CHAPTER 2:

MATERIALS AND METHODS

CHAPTER 2

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2.1 Generation and genotyping of CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice.

Dendritic cell-specific IL-4Rα-deficient (CD11c^{cre}IL4Rα$^{-/lox}$) BALB/c mice were generated and characterized by our laboratory (Hurdayal and Nieuwenhuizen *et al.* Submitted manuscript). Briefly, CD11c^{cre} mice (Caton *et al*., 2007) were crossed with IL-4Rα$^{lox/lox}$ BALB/c mice (Herbert *et al*., 2004) and complete IL-4Rα$^{-/-}$ BALB/c mice (Mohrs *et al.*, 1999) to generate hemizygous CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. Hemizygous littermate controls (IL-4Rα$^{-/lox}$) were used as controls in cutaneous Leishmaniasis experiments together with wild type (WT) BALB/c mice and global IL-4Rα$^{-/-}$ BALB/c mice. C57BL/6 mice were used as resistant control mice in the *L. major* infection model. Specific primers shown in Table 2.1 were used to for genotyping CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. Mouse genotyping was performed by Wendy Green and Rayaana Fredericks at the Division of Immunology.

Table 2.1: Primers used in genotyping of CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice

Gene Target	Primer
WT IL-4Rα – Forward	5' TGACCTACAAGGAACCCAGGC 3'
WT IL-4Rα – Reverse	5' CTCGGCGCACTGACCCATCT 3'
Deletion IL-4Rα – Forward	5' GGCTGCTGACCTGGAATAACC 3'
Deletion IL-4Rα – Reverse	5' CCTTTGAGAACTGCGGGCT 3'
LoxP – Forward	5' GGCTGCTGACCTGGAATAACC 3'
LoxP – Reverse	5' GTTTCCTCCTACCGCTGATT 3'
Cre – Forward	5' ATGCCCAAGAAGAAGAGGAAGGT 3'
Cre – Reverse	5' GAAATCAGTGCGTTCGAACGCTAGA 3'

All mice were housed in specific-pathogen free barrier conditions in individually ventilated cages at the University of Cape Town, South Africa. Mice were age and sex matched and used between 8-12 wks of age. This study was performed in strict accordance with the recommendations of the South African National Guidelines and University of Cape Town of practice for laboratory animal procedures. All mouse experiments were performed according to protocols approved by the Animal Research Ethics Committee of the Health

Sciences Faculty, University of Cape Town (Permit Number: 009/042). All efforts were made to minimize suffering of the animals.

2.2 IL-4Rα responsiveness in bone marrow-derived dendritic cells (BMDCs)

Bone marrow-derived dendritic cells **(**BMDCs) were generated from bone marrow progenitors using mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma-Aldrich) as previously described (Lutz *et al.*, 1999). Briefly, bone marrow was harvested from the femurs of 8-10 weeks old $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and littermate control mice. Cells (2×10^6) were cultured for 10 days in sterile Petri dishes with complete Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich) medium containing 10% fetal calf serum (FCS) (Gibco Life Technologies, Paisly, UK), 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen Life Technologies), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% β-mercaptoethanol (Sigma-Aldrich) 200 U/ml GM-CSF or 50% GM-CSF-rich supernatant generated by NIH3T3 cells. On Day 10, non-adherent cells were harvested and 5×10^5 BMDCs were stimulated with LPS (Sigma-Aldrich; 1 μg/ml) or *Leishmania major* IL-81 promastigotes (MOI: 10 parasites/cell) in the presence or absence of 1000 U/ml recombinant mouse IL-4 or IL-13 (rIL-4/rIL-13, BD Biosciences) for 48 hrs. Following incubation, IL-12p40, IL-12p70 and IL-10 cytokines were measured in culture supernatants by ELISA.

2.3 *Leishmania major* infections

L. major LV39 (MRHO/SV/59/P) (Mattner *et al.*, 1996), *L. major* IL81 (MHOM/IL/81/FEBNI) and GFP-expressing *L. major* IL81 (MHOM/IL/81/FEBNI) (kind gift from Prof. Heidrun Moll, University of Würzburg, Germany) strains were maintained by continuous passage in susceptible BALB/c mice. Parasites were harvested from footpad skin lesions and popliteal lymph nodes of infected animals and cultured *in vitro* in 10 ml Schneiders medium (Sigma-Aldrich) supplemented with 20% FCS in a tissue culture flask (Corning). After 7 days in a humidified chamber at RT (stationary growth phase), the parasites were fixed in 2% gluteraldehyde, enumerated on a Neubauer cytometer slide and prepared for infection studies. Prior to infection, experimental mice were randomly selected but were age and sex matched and used between 8-12 weeks of age. Anaesthetised mice were inoculated subcutaneously with 2×10^6 or 2×10^5 stationary phase metacyclic promastigote parasites into the left hind footpad in a volume of 50 μl of Hank's Balanced Salt solution (HBSS) (Invitrogen). Swelling of infected footpads was monitored weekly,

unblinded, using a Mitutoyo micrometer caliper (Brütsch, Zürich, Switzerland). Mice infected with *L. major* LV39 were sacrificed at either 3 or 8 weeks after infection whilst mice infected with the virulent IL81 strains were sacrificed at 4 weeks after infection (see Appendix A for additional reagents for all experiments).

2.4 Quantification of viable *L. major* parasite burden

Quantification of viable parasite burden was determined by the limiting dilution method (Mohrs *et al.*, 1999). The infected footpad was collected in 2 ml Schneiders medium supplemented with 20% FCS whilst popliteal lymph nodes, spleens, livers and brains were collected in 2 ml complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol and 1% HEPES (Invitrogen Life Technologies). Spleens were weighed after being removed from infected animals. Footpads, spleens, livers and brains were homogenised and lymph nodes were pressed through a 70 μM strainer to obtain single cell suspensions in a final volume of 6.4 ml (100 μl = 2^6 parasites). Two-fold serial dilutions were prepared in 96 well flat-bottom plates using Schneider's media with 20% FCS in a final volume of 100 μl. After 7 days incubation at RT, each individual well was examined and scored microscopically for the presence of parasite growth. Prior to removal of mouse brain tissue for detection of parasite burden, animals were perfused with 20 ml sterile saline solution. Detection of viable parasite burden was estimated as previously described (Mohrs *et al.*, 1999).

2.5 Isolation and stimulation of lymph node cells

Lymph nodes from infected mice were collected in complete DMEM containing 10% FCS (Gibco Life Technologies, Paisly, UK), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Deisenhofen, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). Single cell suspensions were prepared by pressing draining lymph nodes through a 70 μM cell strainer (Falcon, New Jersey, USA) followed by centrifugation at 1300 rpm for 5 min at 4°C. Pelleted cells were resuspended in 5 ml complete DMEM and viable cells enumerated by Trypan Blue exclusion (Sigma-Aldrich). Cell were then cultured in triplicates at 1×10^6/ml in 48-well plates with anti–CD3 (100 μg/ml) or soluble *L. major*-antigen (SLA, 50 μg/ml) and incubated at 37°C and 5% CO_2. Supernatants were removed after 48 hrs, and cytokines (IL-4, IL-13, IFN-γ, IL-12 and IL-10) were measured by ELISA.

2.6 Isolation and antigen-specific restimulation of $CD4^+$ T cells

$CD4^+$ T cells from total lymph node cell suspensions (described above) were positively selected using anti-CD4 microbeads (MACS, Miltenyi Biotec), according to the manufacturer's instructions. Briefly, total lymph node cells were incubated with anti-CD4 microbeads and passed through an LS separation column. The magnetically labelled CD4 cells remain bound to the column whilst unlabelled cells flow through. Following washing 3×, the columns were removed from the magnetic field and viable $CD4^+$ cells were counted using Trypan Blue exclusion (Sigma-Aldrich) to a purity of >95%, as confirmed by FACS analysis. Individual spleens were collected from either WT BALB/c or C57BL/6 mice into 5 ml complete DMEM. Single cell suspensions were prepared by pressing spleens through a 70 µm cell strainer (Falcon, New Jersey, USA) followed by centrifugation at 1300 rpm for 5 min at 4°C. Splenocytes were depleted of RBCs by hypotonic lysis in Red Cell Lysis Buffer (see Appendix A) followed by centrifugation at 1300 rpm, 5 min at 4°C. Pelleted cells were incubated with anti-Thy1.2 for 30 min at 4°C. After washing, splenocytes were incubated with rabbit serum (1:5) for 30 min at 37°C to allow T cell depletion of Thy1.2-labeled splenocytes by complement-mediated lysis thereby enriching antigen-presenting cells (APCs) in suspension. APCs were then treated with 50 µg/ml mitomycin C (Sigma-Aldrich) for 20 min at 37°C and washed extensively in complete DMEM. Cells were resuspended in 2 ml complete DMEM and stained with Trypan Blue (Sigma-Aldrich) to count viable cells. A total of 2×10^5 purified $CD4^+$ T cells and 1×10^5 APCs were cultured with soluble *Leishmania* antigen (50 µg/ml). After 72 hrs incubation at 37°C and 5% CO_2, supernatants were collected and cytokine production was analysed by ELISA (see Appendix A for additional reagents for all experiments).

2.7 Isolation of footpad and spleen cells

Footpads and spleens of infected mice were collected in DMEM containing 2% Penicillin/Streptomycin. Muscle tissue was separated from infected footpads and digested in DMEM medium containing 2% Penicillin/Streptomycin supplemented with Collagenase IV (Sigma-Aldrich; 1 mg/ml) and DNase I (Sigma-Aldrich; 1 mg/ml) at 37°C and 5% CO_2 for 60 min. Following incubation, Collagenase IV activity was stopped by adding an equal volume of DMEM supplemented with 10% FCS. Single cell suspensions were isolated by pressing footpad tissue through 40 µM cell-strainers into 50 ml Falcon tubes (Corning). Spleen cells were isolated by pressing through 70 µM cell-strainers and depleted of RBCs by hypotonic lysis in Red Cell Lysis Buffer followed by centrifugation at 1300 rpm for 5

min at 4°C. Pelleted cells were washed, resuspended in 10 ml complete DMEM and stained with Trypan Blue (Sigma-Aldrich) to count viable cells.

2.8 Flow cytometry

Total lymphocytes, splenocytes and footpad cells were isolated as described above, resuspended in FACS buffer and transferred into FACS tubes for staining. Cells were centrifuged at 1500 rpm for 5 min at 4°C. Cell pellets were stained in 50 µl antibody mix containing 1% heat-inactivated rat serum and 10 ug/ml anti-FcR. The anti-FcR (clone: 2.4G2) was used in order to block non-antigen-specific binding of immunoglobulins to the FcγII and FcγIII receptors. Cells were resuspended in antibody mix and incubated for 20 min at 4°C. Unbound antibody was removed by washing and centrifugation at 1500 rpm for 5 min at 4°C. Finally, stained cells were resuspended in 300 µl FACS buffer for acquisition. Expression of surface markers on cell populations were identified using fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotinylated mAbs against: SiglecF-PE (clone E50-2440), CD11c-PE or APC (clone HL3), MHCII-biotin (clone M5/114), F4/80-PE (clone: A3-1), anti-CD124-PE, CD3-FITC/PerCP (clone 145-2C11), CD19-PE (clone 1D3), CD11b-FITC or PE (clone M1/70), CD4-PerCP (clone GK1.5), CD103-Biotin (clone M290), CD8-PE (clone: 53.6.72), GR-1-PE (clone RB68C5), rat anti-mouse IgG2a-PE, CD11c-biotin (clone HL3) and rat-anti mouse IgG2a biotin (all BD Bioscience, Erembodegem, Belgium), PDCA-APC (clone JF05-IC2.4.1) and SiglecH-PE (clone 551.3D3) (R&D Systems). Biotin-labelled antibodies were detected by staining with either streptavidin-allophycocyanin (APC) or streptavidin-peridinin chlorophyll protein (PerCP). For intracellular cytokine staining, popliteal lymph node cells from *L. major* infected mice were isolated as described above and seeded at 2×10^6 cells/well in 96 well V-bottom plates (Nunc). Cells were stimulated at 37°C for 4 hours in complete DMEM with phorbal myristate acetate (Sigma-Aldrich) (50ng/ml) and ionomycin (Sigma-Aldrich) (250 ng/ml) as well as monensin (200 µM) (Sigma-Aldrich), which was added to accumulate expressed cytokins. Dendritic cells were identified by staining with CD11c PE-Cy7 (BD Bioscience, clone N418) and MHCII APC (BD Bioscience, clone M5/114). Following staining, cells were washed and fixed in 2% paraformaldehyde for 20 min at 4°C. Following washing, staining and fixation, cells were permeabilized with Permeabilization buffer for 30 min at 4°C. Intracellular cytokines were stained with anti-IL-10 (clone JES5-16E3), anti-IL-12 (clone C15.6) and isotype controls (BD Bioscience) all conjugated to PE. Cells were acquired using a FACS Calibur machine (BD

Immunocytometry systems, San Jose, CA, USA) and data were analyzed using Flowjo software (Treestar, Ashland, OR, USA) (see Appendix A for additional reagents for all experiments).

2.9 Antibody detection by ELISA

Blood samples were taken from tail-vein bleeds and collected into serum separator tubes (BD Bioscience, San Diego, CA). Samples were centrifuged at 8000 rpm for 10 min at 4°C. *L. major*-specific IgG1, IgG2a and IgG2b were measured by indirect ELISA using 5 µg/ml soluble *L. major* antigen (SLA) for coating (overnight at 4°C) on Nunc MaxiSorp flat-bottom 96 well ELISA plates (Nunc, Reskilde Denmark). SLA was prepared from stationary phase metacyclic promastigotes by sonication 3 times for 30 sec at 4°C in the presence of protease inhibitors (Sigma-Aldrich). Soluble cell supernatant was collected by centrifugation at 8000 rpm for 10 min at 4°C and frozen at -80°C until needed. Protein concentration was measured using the BCA protein kit (Pierce, Rockford, IL) according to manufactures instructions. Following overnight incubation, the plates were blocked with 2% milk powder for 3hrs at 37°C. Plates were then washed 3× and serum was added in 3-fold serial dilutions followed by overnight incubation at 4°C. After washing 3×, alkaline phosphatase (AP)-labelled goat-anti-mouse isotype-specific antibodies (Southern Biotechnology, Birmingham, USA) were added for 1hr at 37°C. Total IgE was measured by sandwich ELISA using clone 84.1C to coat ELISA plates and anti-mouse IgE (Southern Biotechnology, USA) for detection (Nieuwenhuizen *et al.*, 2007). 4-Nitrophenylphosphate (PNP, Fluka, Switzerland) at 1 mg/ml dissolved in substrate buffer was added to all samples and the absorbance measured at 405 nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany) (see Appendix A for additional reagents for all experiments).

2.10 Cytokine detection by ELISA

The concentration of cytokines IFN-γ, IL-4, IL-13, IL-12p40, IL-12p70 and IL-10 were determined by sandwich ELISA. Nunc MaxiSorp flat-bottom 96 well ELISA plates were coated with purified anti-IL-4 (clone 11B11, 2 µg/ml), anti-IFN-γ (clone An18KL6, 1 µg/ml), anti-IL-13 (clone 38213, 1 µg/ml), anti-IL-10 (clone JES052A5, 1 µg/ml) or anti-IL-12p40/p70 (p40 clone C15.6, p70 clone 9A5, 1 µg/ml) (all BD Pharminogen) antibodies, diluted in carbonate coating buffer, pH 9.5 and incubated overnight at 4°C. Plates were then blocked with 2% milk powder for 3hrs at 37°C, washed 3× with ELISA wash buffer, after which purified recombinant cytokine standards IFN-γ, IL-4, IL-13, IL-

10 or IL-12p40/p70 (BD Pharmingen) or cell supernatants were added in 3-fold serial dilutions and incubated overnight at 4°C. Following washing 3×, biotinylated anti-mouse secondary antibodies for IL-4 (clone: BVD6-24G2), IFN-γ (clone: XMG1.2), IL-13 (goat anti-mouse, 0.5 µg/ml), IL-10 (goat anti-mouse, 0.5 µg/ml) or IL-12p40/p70 (clone C17.8) (all BD Pharmingen) were added for 3hrs at 37°C. Plates were then washed 4× and incubated with Horseradish peroxidase (HRP)-labeled streptavidin for 1hr at 37°C followed by incubation with TMB Peroxidase Substrate (KPL, USA) for detection of antigen-antibody complexes. The reaction was stopped with 1 M H_2SO_4 and absorption measured at 450 nm (see Appendix A for additional reagents for all experiments).

2.11 Nitric oxide synthase and arginase measurements

Lymph node and footpad cells collected at week 4 after *L. major* IL81 infection were restimulated with LPS (Sigma-Aldrich; 10 ng/ml) for 48 hrs at 37°C in 5% CO_2. Following incubation, supernatants were collected for quantification of iNOS activity as previously described (Modolell *et al.*, 1995). Briefly, a nitric oxide standard (Na_2NO_3, 2mM) was serially diluted in DMEM in a 96−well plate and supernatants were diluted 1/3 in triplicate. 25µl of Griess reagent A = Sulfanilamide (1% sulfanilamide in 3% phosphoric acid) was then added to the plate followed by an equal volume of Griess reagent B = NED solution (N−1−napthyl ethylenediamine dihydrochloride). The plate was then gently rocked to mix the solutions and incubated for 5 - 10 minutes until a pink dye developed across standard wells. Absorption was then measured at 540 nm. Arginase activity was measured in cell lysates as previously described (Corraliza *et al.*, 1994; Modolell *et al.*, 1995). Briefly, cells were lysed with 50 µl of 0.1% Triton X-100 (Sigma-Aldrich). After lysis, 50 µl of 10 mM $MnCl_2$ (Merck) and 50 mM Tris-HCl (Merck) were added, and the enzyme was activated by heating for 10 min at 55°C. Arginine hydrolysis was performed by incubating 25 µl of the activated lysate with 25 µl of 0.5 M L-arginine (Merck; pH 9.7) at 37°C for 60 min. The reaction was stopped with 400 µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). As a measure of arginase activity, the urea concentration was quantified at 540 nm after addition of 25 µl of α-iso-nitrosopropiophenone (Sigma-Aldrich; dissolved in 100% ethanol) followed by heating at 95°C for 45 min. One unit of arginase activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol urea/min. Expression of intracellular iNOS and arginase was further analyzed in $CD11b^+CD11c^-MHCII^+$ macrophages and $CD11b^+CD11c^+MHCII^+$ DCs by flow cytometry using rabbit anti-mouse

iNOS (Abcam) with goat anti-rabbit PE (Abcam) and goat anti-mouse arginase (Santa Cruz Biotechnology) with donkey anti-goat PE (Abcam). Purified goat IgG and rabbit IgG were used as controls.

2.12 Histological analysis

Footpads, spleens and livers were removed from infected mice, fixed in ice-cold 4% formaldehyde in phosphate buffered saline and embedded in wax. Wax-embedded tissues were cut into 2 µm sections and stained with either haematoxylin and eosin (H&E) or Giemsa for detection of inflammatory cells and *L. major* amastigote parasites. The processing and staining of histology sections was performed by Lizette Fick at the Department of Surgery, Groote Schuur Hospital.

2.13 Immunohistochemistry

OCT (Tissue-Tek; Sakura, Zoeterwoude, Netherlands)-embedded spleen and brain tissue from mice infected with GFP-expressing *L. major* IL81 parasites for 4 weeks was cut into 10 µm frozen sections and mounted on 3-aminopropyltriethoxysilane (AEPES)-coated slides. Following acetone fixation of spleen sections, nuclei were stained with Hoechst whilst dendritic cells and macrophages were stained using biotinylated anti-CD11c mAb (BD Biosciences) and biotinylated anti-CD68 (RayBiotech), respectively, followed by a streptavidin-Cy3 conjugate (Sigma-Aldrich). Cryosections of mouse brain tissue were acetone-fixed and then stained with nuclear stain Hoechst. Coverslips were then mounted on sections using Mowiol 4-88 mounting medium (Calbiochem) with anti-fade (Sigma-Aldrich). Images were acquired and analyzed by Ziess LSM 510 confocal microscope (Jena, Germany).

2.14 Confocal microscopy of sorted cells

Footpad macrophages ($CD11b^+MHCII^+CD11c^-$) and lymph node B cells ($CD19^+CD3^-CD11c^-$) were first isolated as described above. Cells were seeded into chamber slides and localization of GFP^+ parasites were viewed and analysed by LSM 510 confocal microscopy.

2.14 Quantitative RT-PCR

$CD11c^+$ cells from total lymph node cell suspensions (described above) were positively selected using anti-CD11c microbeads (MACS, Miltenyi Biotec), according to the manufacturer's instructions (described above). Eluted $CD11c^+$ cells were further sorted for

$CD11c^{+}MHCII^{+}$ dendritic cells on a FACS vantage flow cytometer (BD Biosciences, Ferndale, South Africa) to a purity of > 99% as determined by FACS. Total RNA was extracted from sorted dendritic cells using Tri reagent (Applied Biosystems, Carlsbad, Calif) and mini-elute columns (Quiagen, http://www.quiagen.com) according to the manufacturer's protocol. cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche), and real-time PCR of dendritic cell-specific genes (Table 2.2) was performed by using Lightcycler FastStart DNA Master PLUS SYBR Green I reaction mix (Roche) on a Lightcycler 480 II (Roche). Values were normalized according to the expression of the housekeeping genes *HPRT* or *rS12*.

Table 2.2: Primers for quantitative RT-PCR of dendritic cell-specific genes

Gene Target	Primer
IL-12p40 – Forward	5' CTGGCCAGTACACCTGCCAC 3'
IL-12p40 – Reverse	5' GTGCTTCCAACGCCAGTTC 3'
IL-18 – Forward	5' TGGTTCCATGCTTTCTGG 3'
IL-18 – Reverse	5' TCCGTATTACTGCGGTTGT 3'
IL-10 – Forward	5' AGCCGGGAAGACAATAACTG 3'
IL-10 – Reverse	5' CATTTCCGATAAGGCTTGG 3'
IL-23p19 – Forward	5' CAGCTTAAGGATGCCCAGGTT 3'
IL-23p19 – Reverse	5' TCTCACAGTTTCTCGATGCCA 3'
βA subunit (Activin A) – Forward	5' GAGAGGAGTGAACTGTTGCT 3'
βA subunit (Activin A) – Reverse	5' TACAGCATGGACATGGGTCT 3'
HPRT – Forward	5' GGCCATGAGGCTGGATCTC 3'
HPRT – Reverse	5' AACATTTGAATCCTGCAGCCA 3'

2.15 Statistics

Data is represented as mean ± SD. Statistical analysis was performed using the unpaired Student's *t* test or 1-way Anova with Bonferroni's post test, defining differences to IL-$4R\alpha^{-/lox}$ mice as significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) unless otherwise stated (Prism software; http://www.prism-software.com).

CHAPTER 3:

RESULTS

CHAPTER 3

RESULTS

3.1 Generation and characterization of CD11c^{cre}IL-4Rα$^{-/lox}$ mice

Mice expressing Cre recombinase (Cre) under control of the CD11c locus (Caton *et al.*, 2007) were backcrossed to BALB/c for 9 generations, then intercrossed with global IL-4Rα$^{-/-}$ BALB/c mice (Mohrs *et al.*, 1999) to generate CD11c^{cre}IL-4Rα$^{-/-}$ mice on a pure BALB/c genetic background. These mice were subsequently intercrossed with floxed IL-4Rα$^{lox/lox}$ BALB/c mice (exon 6 to 8 flanked by *loxP*) (Herbert *et al.*, 2004) to generate CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. Characterization of CD11c^{cre}IL-4Rα$^{-/lox}$ mice is described in Hurdayal and Nieuwenhuizen *et al.*, (Submitted manuscript). Analysis of IL-4Rα surface expression on different cell types by flow cytometry demonstrated that IL-4Rα was efficiently depleted in DCs of the lymph nodes, spleen, skin and lungs, when compared with IL-4Rα$^{-/lox}$ littermate controls and IL-4Rα$^{-/-}$ mice (Figure 3.1).

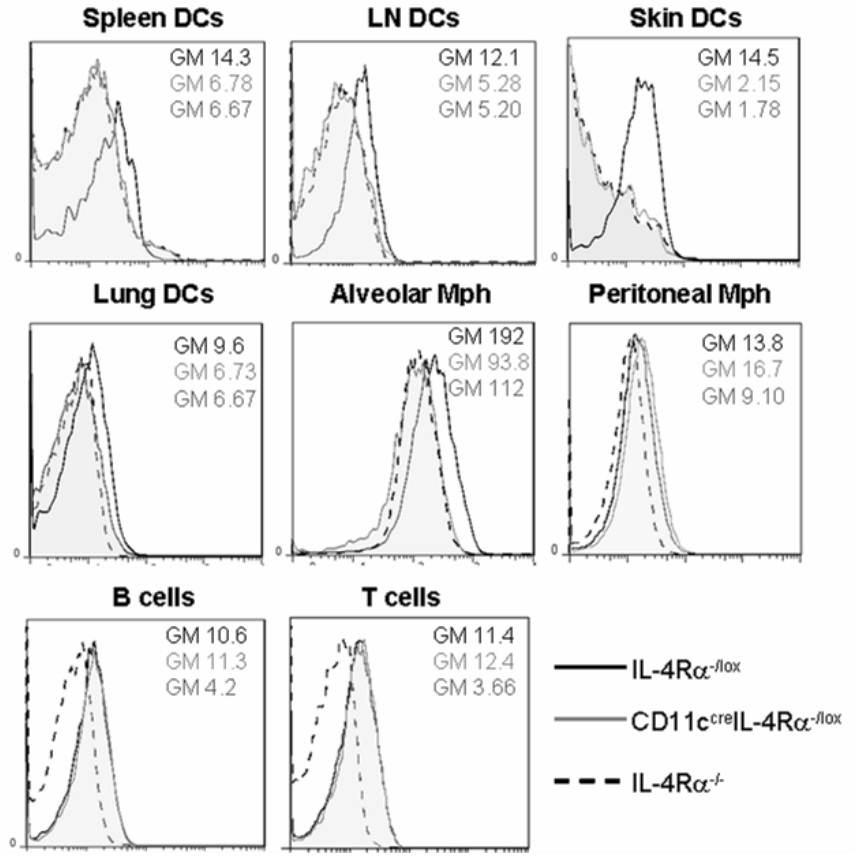

Figure 3.1: Characterization of CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice
IL-4Rα surface expression was analyzed by flow cytometry from naïve IL-4Rα$^{-/lox}$ (solid line), IL-4Rα$^{-/-}$ (dashed line) and CD11c^{cre}IL-4Rα$^{-/lox}$ (grey tinted) mice. Dendritic cells (DCs) were CD11c^{+}MHCII^{+} (SiglecF^{-} in lungs), alveolar macrophages (Mph) were CD11c^{+}SiglecF^{+}, peritoneal macrophages were F480^{+}CD11b^{+}, B cells were CD19^{+}CD3^{-} and T cells were CD3^{+}CD19^{-}. GM = geometric mean. Experiments and analysis performed by Dr. N.E. Nieuwenhuizen.

As expected $CD11c^{+}$ alveolar macrophages also had abrogated IL-4Rα surface expression. Other cell types such as T cells, B cells and macrophages had comparable IL-4Rα expression to IL-4$R\alpha^{-/lox}$ littermate controls.

3.2 Selective impairment of IL-4Rα function on dendritic cells from $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice

To assess functional impairment of DCs in $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice, we generated bone marrow-derived dendritic cells (BMDCs) and stimulated them with LPS in the presence or absence of recombinant IL-4 or IL-13. Total yield of BMDCs from precursor cells seeded was similar in $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice and littermate controls and viability after maturation was also comparable. IL-4 has been shown to promote Th1 immune responses by enhancing DC production of IL-12 in an IL-4Rα dependent manner, better known as "IL-4-mediated DC instruction theory" (Biedermann *et al.*, 2001; Lutz *et al.*, 2002). As previously reported, BMDCs derived from mice with a functional IL-4Rα, the IL-4$R\alpha^{-/lox}$ mice and BALB/c wildtype controls, had significantly increased IL-12p40 production after the addition of IL-4. In contrast, LPS/IL-4 stimulated BMDCs derived from $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice or from global IL-4$R\alpha^{-/-}$ mice, with a functional deletion of the IL-4Rα chain, showed similar levels of IL-12 to those stimulated with LPS alone, with the addition of IL-4 having no effect (Figure 3.2A). These results demonstrate functional impairment of IL-4Rα signaling on DCs from $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice. Interestingly, after the addition of LPS alone, BMDCs with a functional IL-4Rα already showed a trend towards increased IL-12p40 levels, suggesting that endogenous levels of IL-4 released from small numbers of contaminating granulocytes in culture could influence these BMDCs. In contrast, stimulation of BMDCs with exogenous IL-13 did not increase levels of IL-12p40 (Figure 3.2A), which has been reported in previous DC stimulation studies (Lutz *et al.*, 2002).

Furthermore, addition of recombinant IL-4 and IL-13 to BMDC cultures had no significant effect on BMDC maturation in WT or $CD11c^{cre}$IL-4$R\alpha^{-/lox}$ mice, as shown by similar expression levels of DC activation markers such as MHCII, CD80 and CD86 (Figure 3.2B). These results are consistent with a very recent publication (Cook *et al.*, 2012), which also found that IL-4 stimulation of DCs did not significantly alter their activation phenotype.

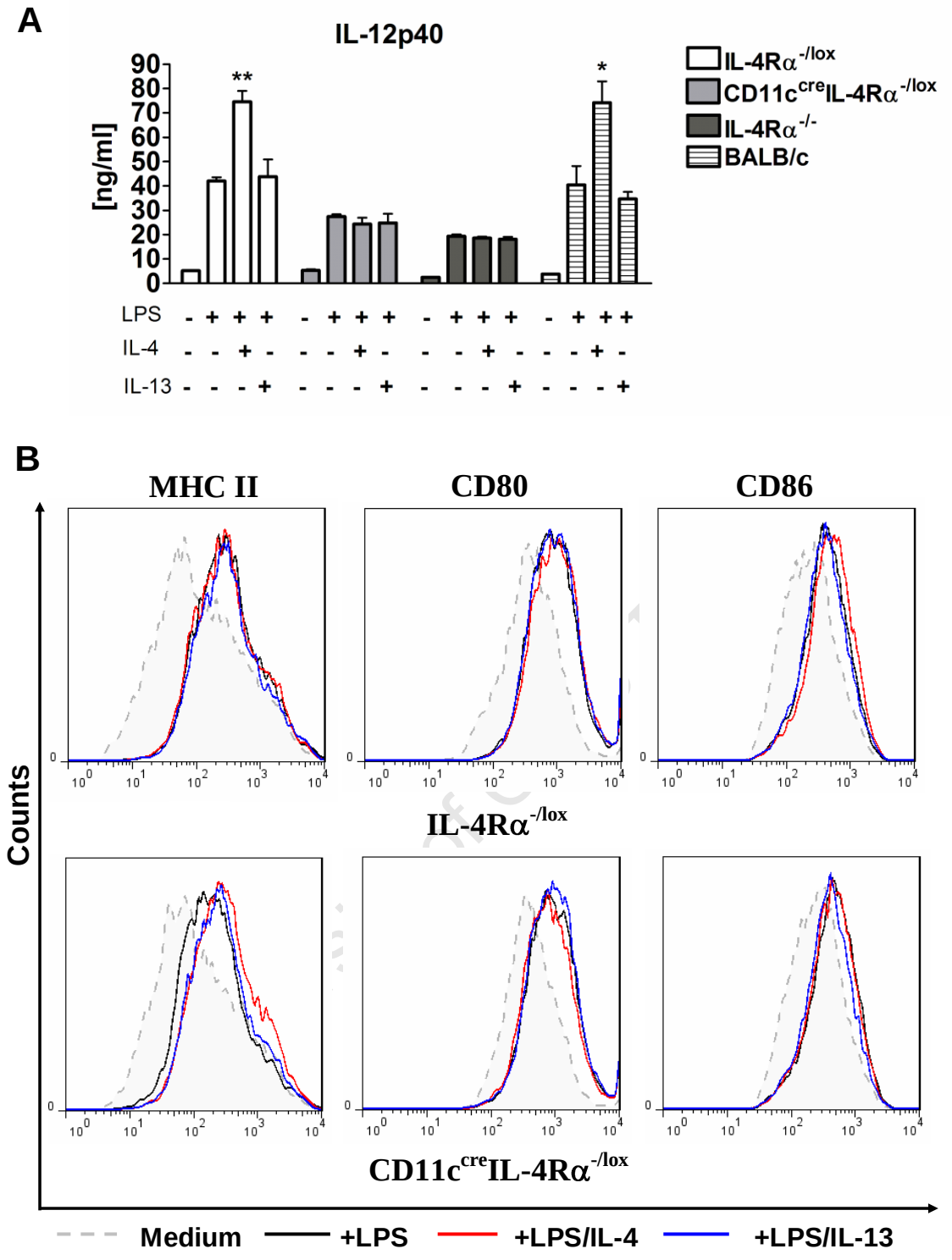

Figure 3.2: Functional impairment of dendritic cells in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice.
Bone marrow derived DCs (BMDCs) were generated from bone marrow progenitors in CD11c^{cre}IL-4Rα$^{-/lox}$ and control mice.
(A) BMDCs were stimulated with LPS in the presence or absence of IL-4 or IL-13 and IL-12p40 was measured in the supernatants 48 hours later. *, p <0.05, **, $p \leq 0.01$.
(B) BMDCs were stimulated with LPS (black line) in the presence or absence of IL-4 (red line) or IL-13 (blue line) plus medium control (grey tinted) and expression of DC activation markers was analysed by flow cytometry.
A representative of one of two individual experiments is shown with values ± SEM. Statistical analysis was performed defining differences to LPS treatment as significant (*, $p \leq 0.05$; **, $p \leq 0.01$).

3.3 CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice are hypersusceptible to acute *Leishmania major* infection

3.3.1 Acute infection with L. major LV39 and L. major IL81

In order to investigate the role of IL-4Rα signaling on DCs during cutaneous Leishmaniasis, CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice were infected subcutaneously with 2 × 10^6 stationary phase metacyclic promastigotes of *L. major* LV39 (MRHO/SV/59/P; Figure 3.3, A-C) with a more virulent *L. major* IL81 (MHOM/IL/81/FEBNI; Figure 3.4, A-C) or with GFP-expressing IL81 (MHOM/IL/81/FEBNI; Figure 3.4, D-F) strains into the hind footpad.

GFP-expressing IL81 parasites were used in order to facilitate *in vivo* tracking of *Leishmania* parasites in some experiments. Infection studies with *L. major* IL81 and GFP-expressing IL81 parasites were compared to first ascertain whether insertion of the GFP cassette in the *L. major* genome hampered its ability to infect experimental animals. As depicted in Figure 3.4, A-C and D-F, infection kinetics between *L. major* IL81 and GFP-expressing IL81 *Leishmania* parasites were similar, confirming that the GFP insert did not have an inhibitory effect on *Leishmania* infection.

As previously shown (Mohrs *et al.*, 1999; Radwanska *et al.*, 2007), C57BL/6 mice and IL-4Rα$^{-/-}$ deficient BALB/c mice controlled lesion development during acute infection with all indicated *L. major* strains (Figure 3.3A, Figure 3.4A and 3.4D), which correlated with low parasite numbers in infected footpads (Figure 3.3B, Figure 3.4B and 3.4E) and draining lymph nodes (LN) (Figure 3.3C, Figure 3.4C and 3.4F). Susceptible WT BALB/c and IL-4Rα$^{-/lox}$ littermate control mice developed progressive footpad swelling after infection with all indicated strains (Figure 3.3A, Figure 3.4A and 3.4D), with increased parasite burdens in the infected footpads (Figure 3.3B, Figure 3.4B and 3.4E) and draining LN (Figure 3.3C, Figure 3.4C and 3.4F). Hemizygous (IL-4Rα$^{-/lox}$ mice) had slightly reduced footpad swelling compared to BALB/c mice in IL81 infection. The greater virulence of the *L. major* IL81 strain is reflected in more rapid disease progression, with footpad swelling and parasite burden reaching similar levels by 4 weeks (Figure 3.4) to those obtained with *L. major* LV39 in 8 weeks (Figure 3.3).

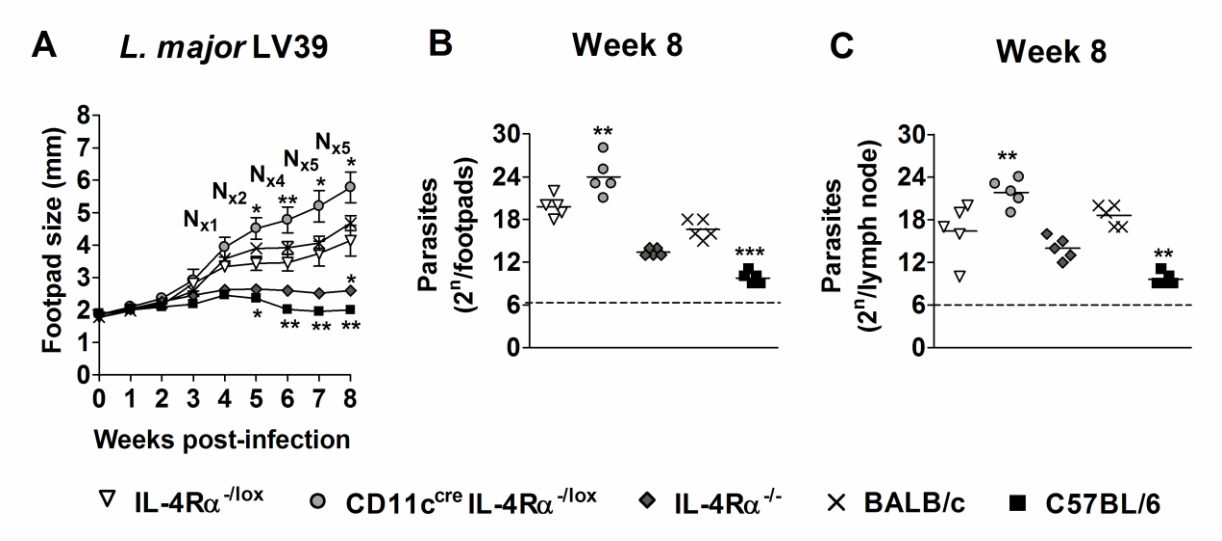

Figure 3.3: CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice are hypersusceptible to acute *L. major* LV39 infection.

Mice were infected with *L. major* LV39 (MRHO/SV/59/P) strain.

(A-C) Footpad swelling was measured at weekly intervals (5 mice per group) infected subcutaneously with 2×10^6 stationary phase metacyclic *L. major* promastigotes into the hind footpad. Parasite burden was determined by limiting dilution of homogenized footpads **(B)** and single-cell suspensions of lymph nodes **(C)** at 8 weeks after infection.

"N" indicates necrosis or ulceration/mouse. A representative of one of two individual experiments is shown with values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Of importance, CD11c^{cre}IL-4Rα$^{-/lox}$ mice were hypersusceptible to acute *L. major* infection compared to heterozygous littermate controls and BALB/c mice, showing significantly worsened disease progression when infected with all three strains (Figure 3.3 and Figure 3.4), with earlier and dramatically larger footpad lesions, and development of early necrosis (Figure 3.3A, Figure 3.4A and 3.4D). Increased disease progression was accompanied by significantly higher parasite numbers in the footpads (Figure 3.3B, Figure 3.4B and 3.4E) and LN (Figure 3.3C, Figure 3.4C and 3.4F) of infected animals.

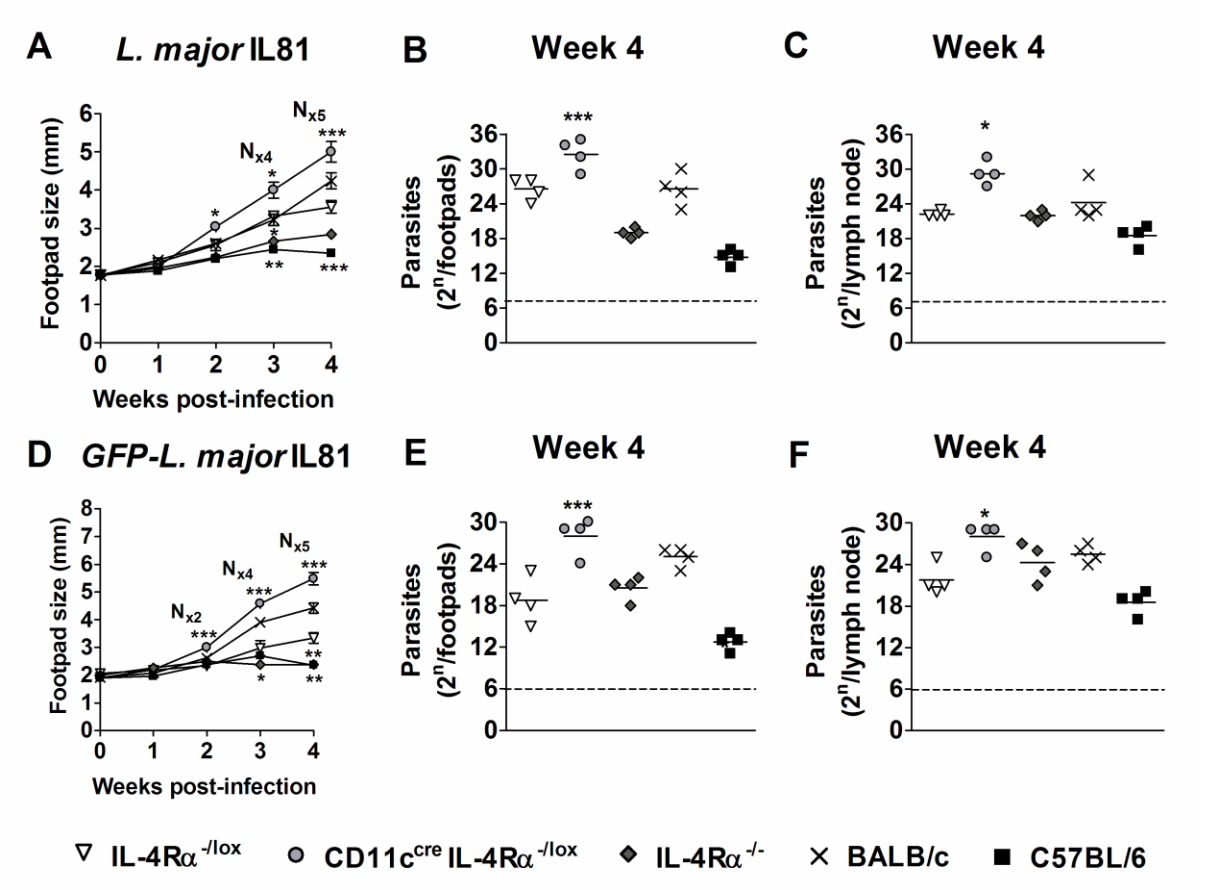

Figure 3.4: CD11c^{cre}IL-4R$\alpha^{-/lox}$ BALB/c mice are hypersusceptible to acute *L. major* IL81 infection.

(A-C) Mice were infected with the virulent *L. major* IL81 (MHOM/IL/81/FEBNI) strain. Footpad swelling was measured at weekly intervals in mice (4 per group) infected subcutaneously with 2×10^6 stationary phase metacyclic *L. major* promastigotes into the hind footpad **(A)**. Parasite burden was measured by limiting dilution of homogenized footpads **(B)** and single-cell suspensions of lymph nodes **(C)** at 4 weeks after infection.

(D-F) Mice were infected with a GFP-expressing *L. major* IL81 (MHOM/IL/81/FEBNI) strain. Footpad swelling was measured at weekly intervals (5 mice per group) infected subcutaneously with 2×10^6 stationary phase metacyclic *L. major* promastigotes into the hind footpad **(D)**. Parasite burden was measured by limiting dilution of homogenized footpads **(E)** and single-cell suspensions of lymph nodes **(F)** at 4 weeks after infection

"N" indicates necrosis or ulceration/mouse A representative of one of two individual experiments is shown with values ± SEM. Statistical analysis was performed defining differences to IL-4R$\alpha^{-/lox}$ mice as significant (*, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$).

3.3.2 Histopathological analysis in infected footpads of CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice

Macroscopic examination of infected CD11c^{cre}IL-4R$\alpha^{-/lox}$ footpads at week 4 after infection with the virulent IL81 showed severe, non-healing inflammatory lesions (Figure 3.5A). Histopathological analysis in footpads of CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice revealed severe destruction of epidermis, connective tissue and bone as a result of footpad necrosis, accompanied by increased inflammatory cells and a high load of extracellular *L. major* amastigotes (Figure 3.5B). In contrast, infected footpads of IL-4R$\alpha^{-/lox}$ revealed moderate

dermal inflammatory infiltrates with mostly intact epidermis, connective tissue and bone (Figure 3.5B). Together, these data reveal that IL-4Rα signaling on DCs play an important role in host protection against acute *L. major* infection.

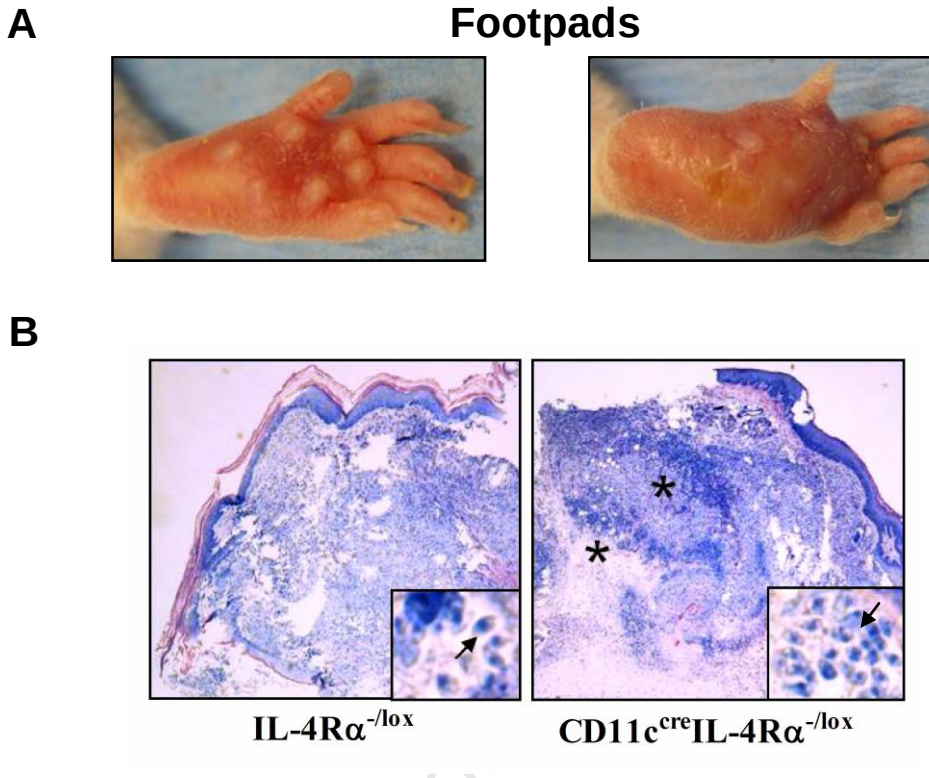

Figure 3.5: Dermal inflammation and amastigote parasites in footpads of CD11c^{cre}IL-4Rα$^{-/lox}$ infected mice.
Mice were infected with the virulent *L. major* IL81 (MHOM/IL/81/FEBNI) strain. Infected footpads were collected at week 4 after infection.
(A) Representative images of *L. major* IL81-infected footpads at week 4.
(B) Formalin-fixed footpads were stained with Giemsa for histopathology (original magnification ×40; asterisks indicate inflammatory foci and insets, arrows indicate amastigote parasites ×800).
Representative images of one of two individual experiments is shown.

3.4 A shift towards Th2-type immune responses in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice during *L. major* infection

In order to determine if the hypersusceptibility observed in CD11c^{cre}IL-4Rα$^{-/lox}$ mice during acute Leishmaniasis (IL81) was accompanied by an altered immune response, we examined cellular and humoral Th1/Th2-type immune responses during acute Leishmaniasis. Antigen-specific restimulation of CD4^{+} T cells sorted from the LN of infected mice and co-cultured with fixed antigen-presenting cells and soluble *Leishmania* antigen (SLA) revealed a significantly reduced IFN-γ response in CD11c^{cre}IL-4Rα$^{-/lox}$ mice in comparison to the resistant C57BL/6 or IL-4Rα$^{-/-}$ strains as well as to the susceptible IL-4Rα$^{-/lox}$ littermate controls (Figure 3.6A). Conversely, the levels of IL-4, IL-13 and IL-10

were significantly higher in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and C57BL/6 mice (Figure 3.6B, 3.6C and 3.6D).

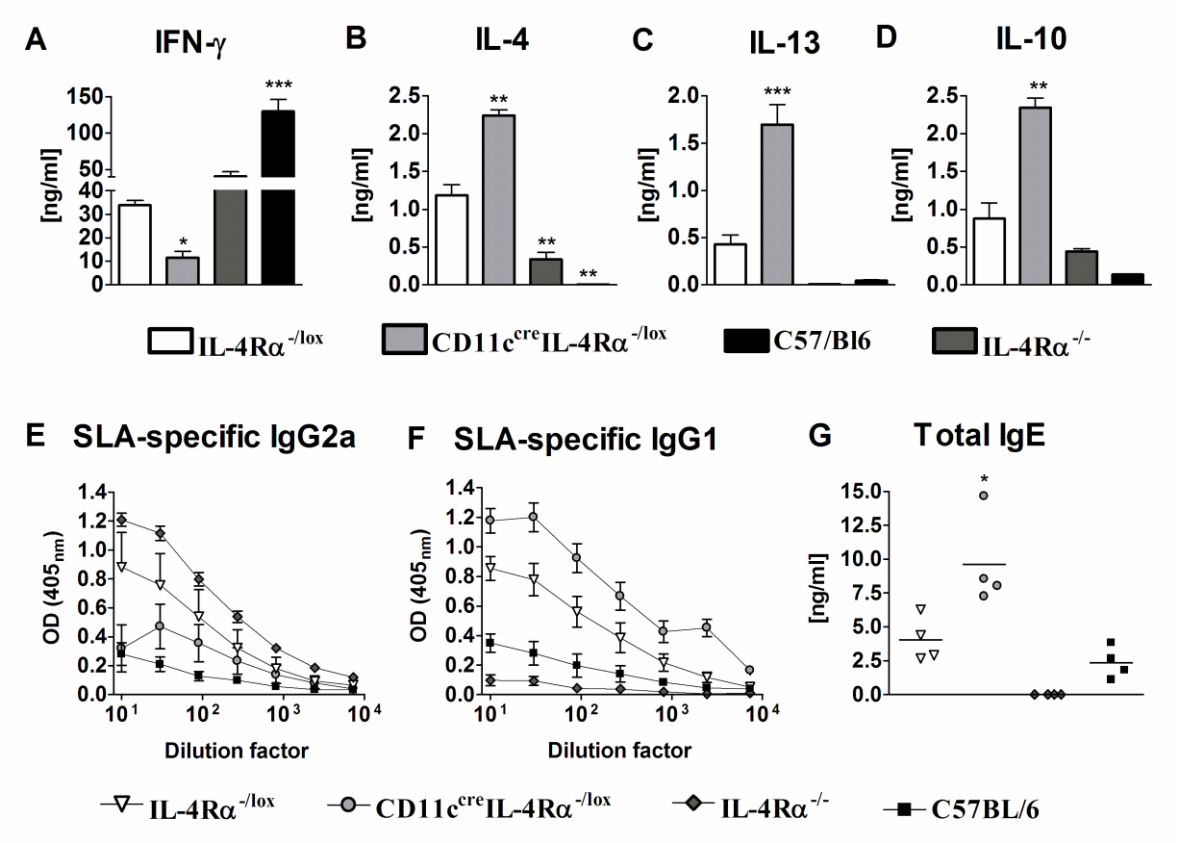

Figure 3.6: T helper 2 immunity is enhanced in hypersusceptible CD11c^{cre}IL-4Rα$^{-/lox}$ mice during acute *L. major* IL81 infection.
Experimental mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 promastigotes into the hind footpad.
(A-D) At week 4 post infection, total CD4$^+$ T cells from the draining lymph node were restimulated for 72 hrs with APCs and soluble *Leishmania* antigen (SLA). The production of IFN-γ **(A)**, IL-4 **(B)**, IL-13 **(C)** and IL-10 **(D)** was determined by ELISA.
(E-G) Also at week 4 post infection, antigen-specific IgG2a **(E),** IgG1 **(F)** and total IgE **(G)** antibody production was quantified from infected sera by ELISA.
A representative of one of two individual experiments is shown with values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (*, $p \leq 0.05$, **, $p \leq 0.01$ ***, $p \leq 0.001$).

Similar results were also obtained with T cell mitogenic (anti-CD3) and antigen-specific restimulation of total lymph node cells at week 4 after infection (data not shown). The observed shift towards Th2-type responses was further confirmed systemically in the quality of *Leishmania*-specific antibody immune responses. Sera of week 4 infected mice revealed a predominant type 1 antibody response in IL-4Rα$^{-/-}$ mice, as shown by elevated levels of *Leishmania*-specific IgG2a (Figure 3.6E). In contrast, CD11c^{cre}IL-4Rα$^{-/lox}$ mice displayed a predominant type 2 antibody response characterized by marked production of

IgG1 and total IgE which was significantly higher than that observed in littermate IL-4Rα$^{-/lox}$ mice (Figure 3.6F and 3.6G). Similar results were obtained from infections with *L. major* LV39 (data not shown). Together, these data indicate that CD11c^{cre}IL-4Rα^{-lox} mice have a shift towards a dominant CD4^{+} Th2-type immune response, which is likely to contribute to the hypersusceptible disease phenotype.

3.5 The effect of a lower *L. major* LV39 parasite dose in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice

3.5.1 CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice are hypersusceptible to 10-fold lower dose of L. major LV39 parasites

We next sought to examine the influence of parasite dose on disease progression and Th1/Th2 cell differentiation *in vivo* in CD11c^{cre}IL-4Rα$^{-/lox}$ mice and control groups (Figure 3.7 and 3.8). Infection using a 10-fold lower dose (2×10^5) of *L. major* LV39 parasites also resulted in a hypersusceptible disease phenotype in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.7A) accompanied by significantly higher parasite numbers in the footpads (Figure 3.7B) and LN (Figure 3.7C) of infected animals. IL-4Rα$^{-/-}$ and C57BL/6 mice developed a healing response to 2×10^5 *L. major* infection, characterized by lower numbers of parasites in the footpad (Figure 3.7B) and LN (Figure 3.7C).

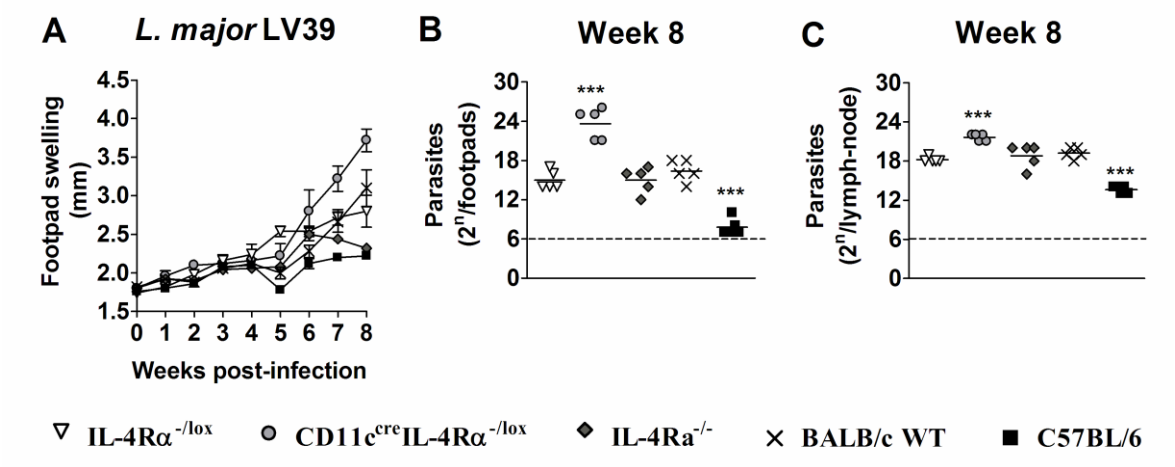

Figure 3.7: CD11c^{cre}IL-4Rα$^{-/lox}$ mice are hypersusceptible to 10-fold lower dose cutaneous *L. major* infection.

Experimental mice were infected subcutaneously with 2×10^5 stationary phase metacyclic *L. major* LV39 promastigotes into the hind footpad.

(A-C) Footpad swelling was measured at weekly intervals in mice (5 per group) infected subcutaneously with 2×10^5 metacyclic *L. major* promastigotes into the hind footpad **(A)**. "N" indicates necrosis or ulceration/mouse. Parasite burden was determined by limiting dilution of single-cell suspensions from homogenized footpads **(B)** and lymph nodes **(C)** at week 8.

A representative of one of two individual experiments is shown with values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (***, $p \leq 0.001$).

3.5.2 Cytokine and antibody responses in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice during 2×10^5 L. major LV39 infection

Infection with a 10-fold lower dose of *L. major* LV39 did not promote a switch to a protective Th1 response in either CD11c^{cre}IL-4Rα$^{-/lox}$ or in BALB/c control mice (Figure 3.8), and showed reduced IFN-γ and increased IL-4 secretion (Figure 3.8A and 3.8B). CD11c^{cre}IL-4Rα$^{-/lox}$ mice also had a predominant type 2 antibody response characterized by reduced IgG2a (Figure 3.8C) and marked production of IgG1 and total IgE (Figure 3.8D and 3.8E), which was significantly higher than that observed in littermate IL-4Rα$^{-/lox}$ mice. C57BL/6 maintained a strong IFN-γ healing response to 2×10^5 *L. major* parasites up to 8 weeks (Figure 3.8). Taken together, infection with a 10-fold lower dose of *L. major* confirmed the results obtained with the high dose infection.

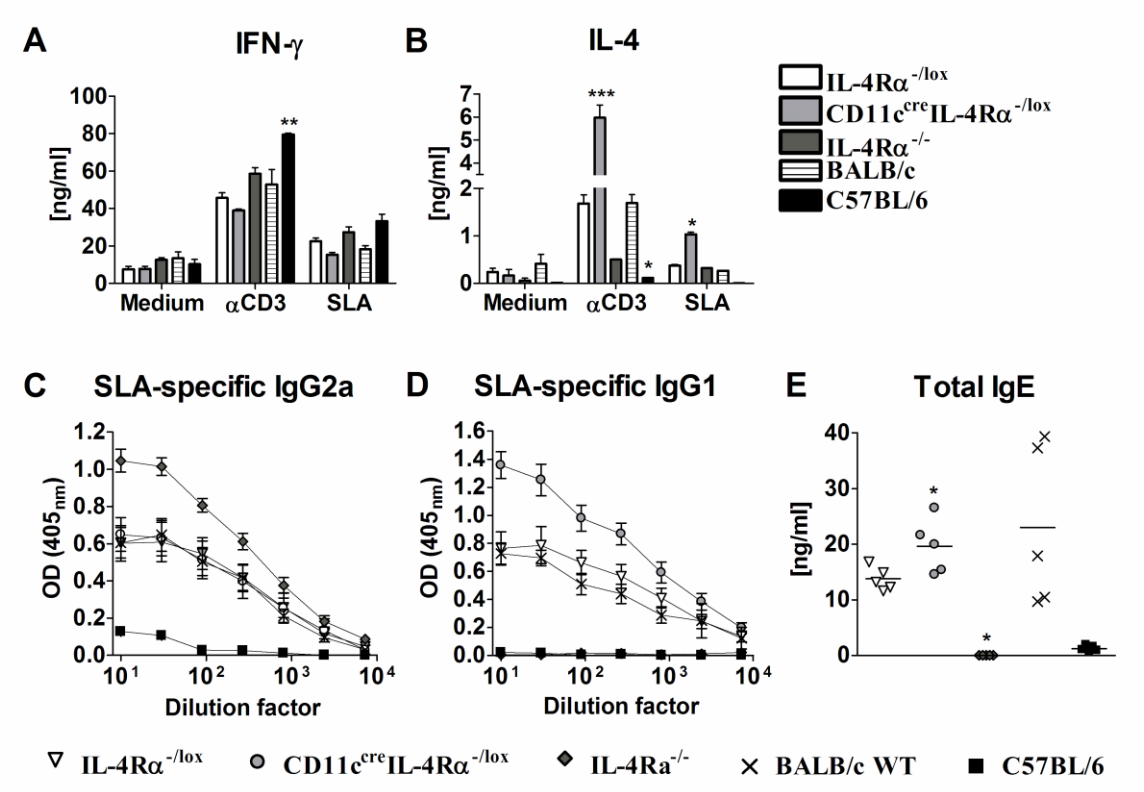

Figure 3.8: T helper 2 immunity is enhanced in hypersusceptible CD11c^{cre}IL-4Rα$^{-/lox}$ mice during 10-fold lower dose infection with *L. major* LV39.
Mice were infected subcutaneously with 2×10^5 *L. major* LV39 promastigotes into the hind footpad. **(A-B)** At week 8 after infection, total cells from the draining lymph node were restimulated for 72 hrs with medium, αCD3 and soluble *Leishmania* antigen (SLA). The production of IFN-γ **(A)** and IL-4 **(B)** was determined by ELISA.
(C-F) At week 8 after infection, antigen-specific IgG2a **(C)**, IgG1 **(D)** and total IgE **(E)** antibody production was quantified from infected sera by ELISA.
A representative of one of two individual experiments is shown with mean values ±SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (*, $p \leq 0.05$, **, $p \leq 0.01$; ***, $p \leq 0.001$).

3.6 Absence of IL-4Rα signaling on dendritic cells leads to alternative activation of macrophages during *L. major* infection

As IFN-γ-induced nitric oxide synthase (iNOS) production by classically activated macrophages is a key control mechanism in *L. major* infection (Stenger *et al.*, 1994), the activation state of macrophages was determined in the infected footpad at week 4 after infection. Inflammatory macrophages (FSC^{hi}, $CD11b^{+}MHCII^{+}CD11c^{-}$) from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had significantly reduced iNOS expression compared to those of littermate $IL\text{-}4R\alpha^{-/lox}$ control mice (Figure 3.9A).

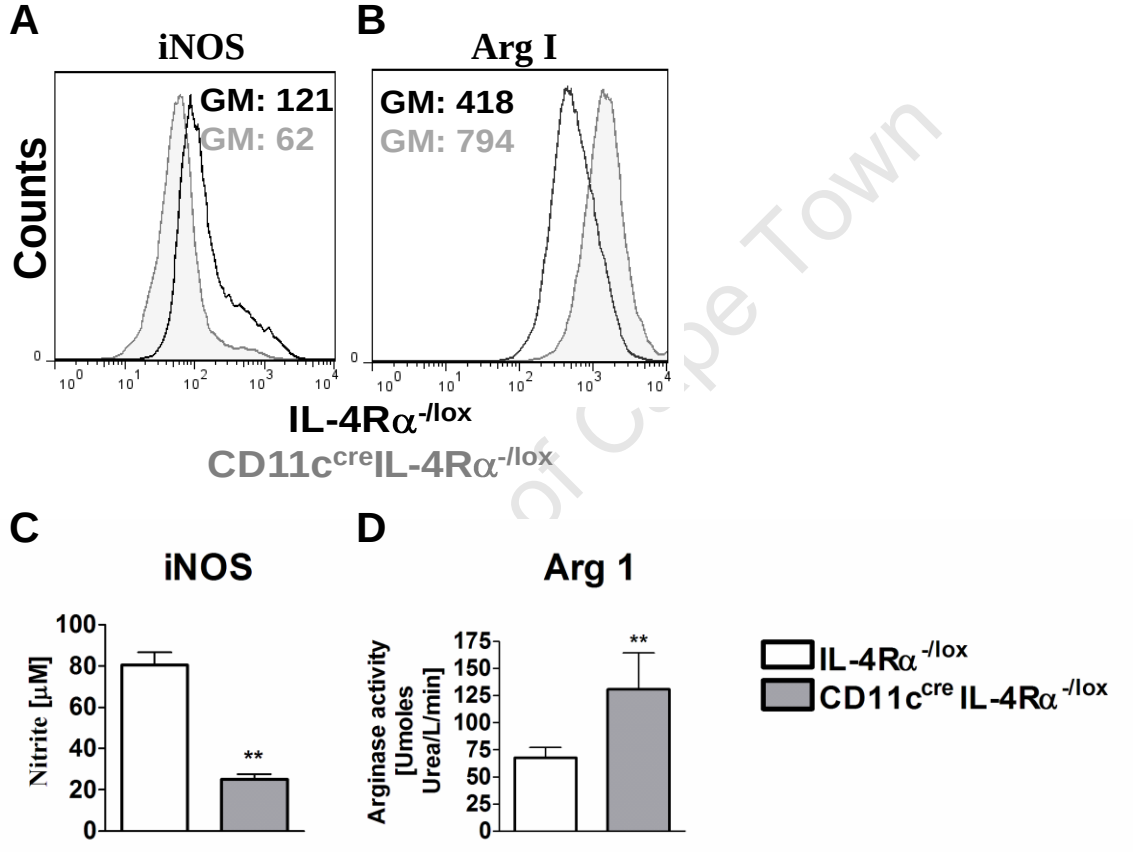

Figure 3.9: Macrophages in footpads of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice have reduced iNOS and increased Arg I during *L. major* IL81 infection.
Experimental mice were infected with *L. major* IL81 and at week 4 after infection, the activation phenotypes of inflammatory macrophages at the site of infection were analysed.
(A-B) Total cells were isolated from infected footpads and surface-stained for FSC^{hi} $CD11b^{+}MHCII^{+}CD11c^{-}$ macrophages followed by intracellular staining for iNOS **(A)** and arginase 1 **(B)**. GM = Geometric Mean.
(C-D) Total cells were isolated from infected footpads and stimulated with 10 ng/ml LPS for 72 h. Production of NO was determined in cell supernatants **(C)** and cell lysates were assayed for arginase I production **(D)**. A representative of two individual experiments is shown with mean values ± SEM. Statistical analysis was performed defining differences to $IL\text{-}4R\alpha^{-/lox}$ mice as significant (**, $p \leq 0.01$).

Conversely, expression of arginase I, a marker of alternatively activated macrophages, was higher in macrophages of CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.9B). This altered phenotype in macrophage activation was confirmed in iNOS and arginase activity assays performed on total footpad cells stimulated with LPS. Macrophages from CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice produced significantly reduced amounts of NO and significantly increased arginase activity in response to LPS stimulation, in comparison to control mice (Figure 3.9C and 3.9D). Together, these results demonstrate reduced macrophage killing effector functions in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, which could account for the uncontrolled parasite replication observed in these animals during acute Leishmaniasis.

3.7 CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice have increased *L. major* loads in peripheral organs

3.7.1 GFP^{+} L. major in immune cell populations in spleens of CD11c^{cre}IL-4Rα$^{-/lox}$ mice

At week 8 after infection with *L. major* LV39 and GFP-IL81 strains, CD11c^{cre}IL-4Rα$^{-/lox}$ mice displayed noticeable splenomegaly (Figure 3.10), which prompted us to evaluate parasite dissemination in peripheral organs using two-fold limiting dilution assays. As studies in BALB/c mice have suggested that the presence of parasites in peripheral organs could occur as a result of parasites disseminating to these sites within 24 hours from the onset of infection, but beginning to replicate at a later time-point (Laskay *et al.*, 1995; Schilling and Glaichenhaus, 2001), we decided to evaluate parasite dissemination to peripheral organs at both an early and late time-points.

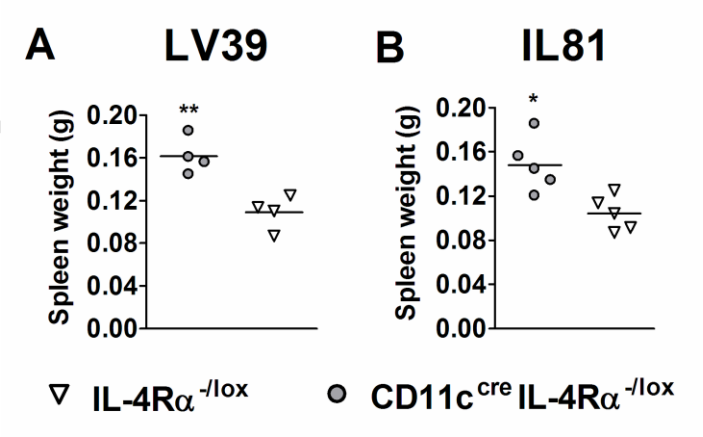

Figure 3.10: Spleen weights in CD11c^{cre}IL-4Rα$^{-/lox}$ mice during *L. major* infection
(A-B) Mice were infected subcutaneously with 2 × 10^{6} stationary phase metacyclic *L. major* LV39 **(A)** and GFP-expressing *L. major* IL81 (MRHO/SV/59/P) strain **(B)** into the hind footpad and spleens were weighed at week 8 and week 4 after infection, respectively.
A representative of one of two individual experiments is shown with mean values ±SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (*, $p \leq 0.05$, **, $p \leq 0.01$).

In GFP$^+$ *L. major* IL81 infected mice, an increase in GFP$^+$ parasites was not detectable in the spleen at 1 or 3 days post infection using flow cytometry, whereas at week 4, GFP$^+$ DCs was significantly increased compared to day 0 (Figure 3.11), suggesting that parasite dissemination in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice occurred at a later time-point. Moreover, both at early time-points and at week 4, DCs contained significantly higher GFP$^+$ loads than macrophages and neutrophils.

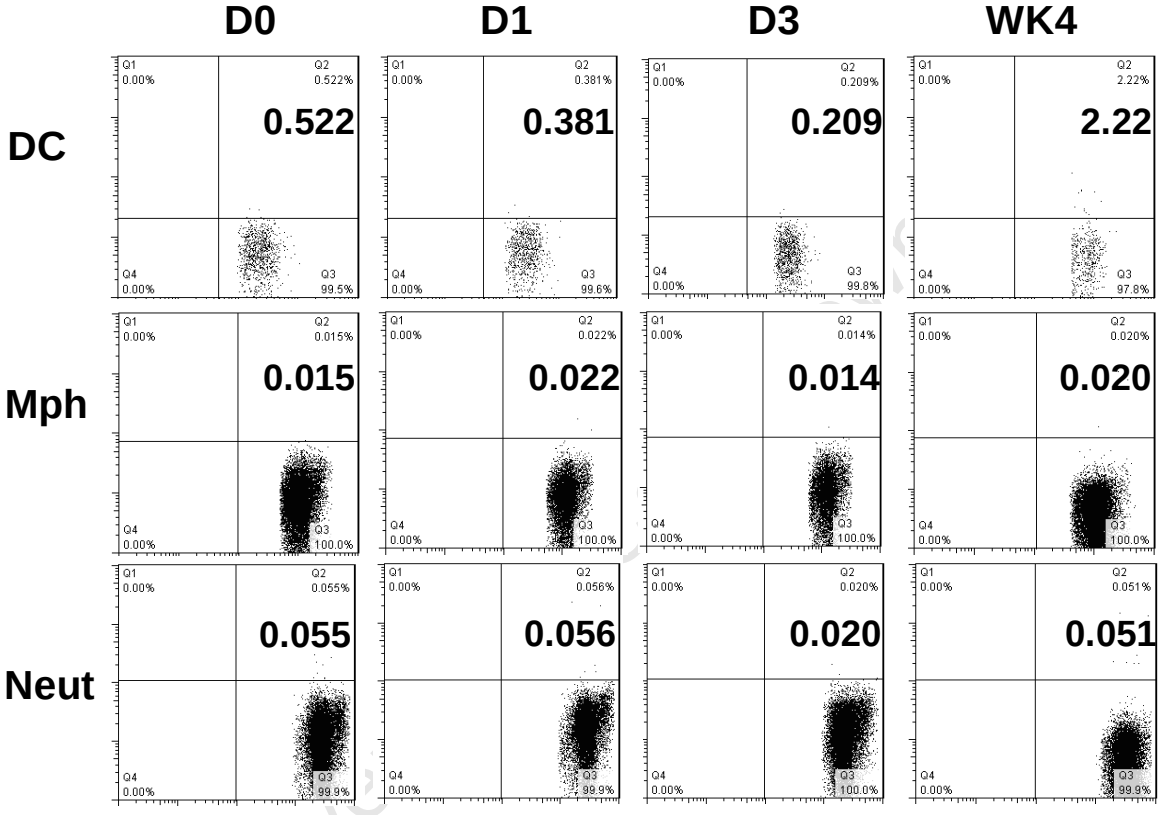

Figure 3.11: GFP$^+$-*L. major* IL81 parasites in immune cell populations in spleen in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice.
Mice were infected subcutaneously with 2 × 10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 (MRHO/SV/59/P) strain into the hind footpad. At Day 0 (D0), Day 1 (D1), Day 3 (D3) and Week 4 (WK4) after infection, total spleen cells were surface stained for DCs (CD11c$^+$MHCII$^+$), Mph (CD11b$^+$MHCII$^+$CD11c$^-$) and neutrophils (GR1^{hi}CD11c$^-$). The percentage of infiltrating GFP$^+$-infected cells were determined by flow cytometry.
A representative of one of two individual experiments is shown.

3.7.2 Parasite burdens in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice during L. major infection

We next evaluated parasite burdens in CD11c^{cre}IL-4R$\alpha^{-/lox}$ and littermate control mice during the acute phase of *L. major* infection. In *L. major* LV39 infected CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice, parasites were only found in footpads and the draining lymph nodes at week 3, whereas by week 8 parasites were detected in the spleen and liver in both CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice and littermate controls (Figure 3.12A and 3.12B). Parasite burdens were much

higher in the organs of infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice, compared to littermate control mice. Moreover, depending on the degree of hypersusceptibility, it was surprising to find that *L. major* parasites had disseminated as far as the brain by week 8 after infection in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, but not in control mice (Figure 3.12C).

Similar disease progression was also observed after infection with *L. major* IL81 (Figure 3.12C), where CD11c^{cre}IL-4Rα$^{-/lox}$ mice already displayed noticeable splenomegaly at 4 weeks post infection (Figure 3.10B) and had strikingly increased parasite burdens in all organs analyzed, including the brain (Figure 3.12C).

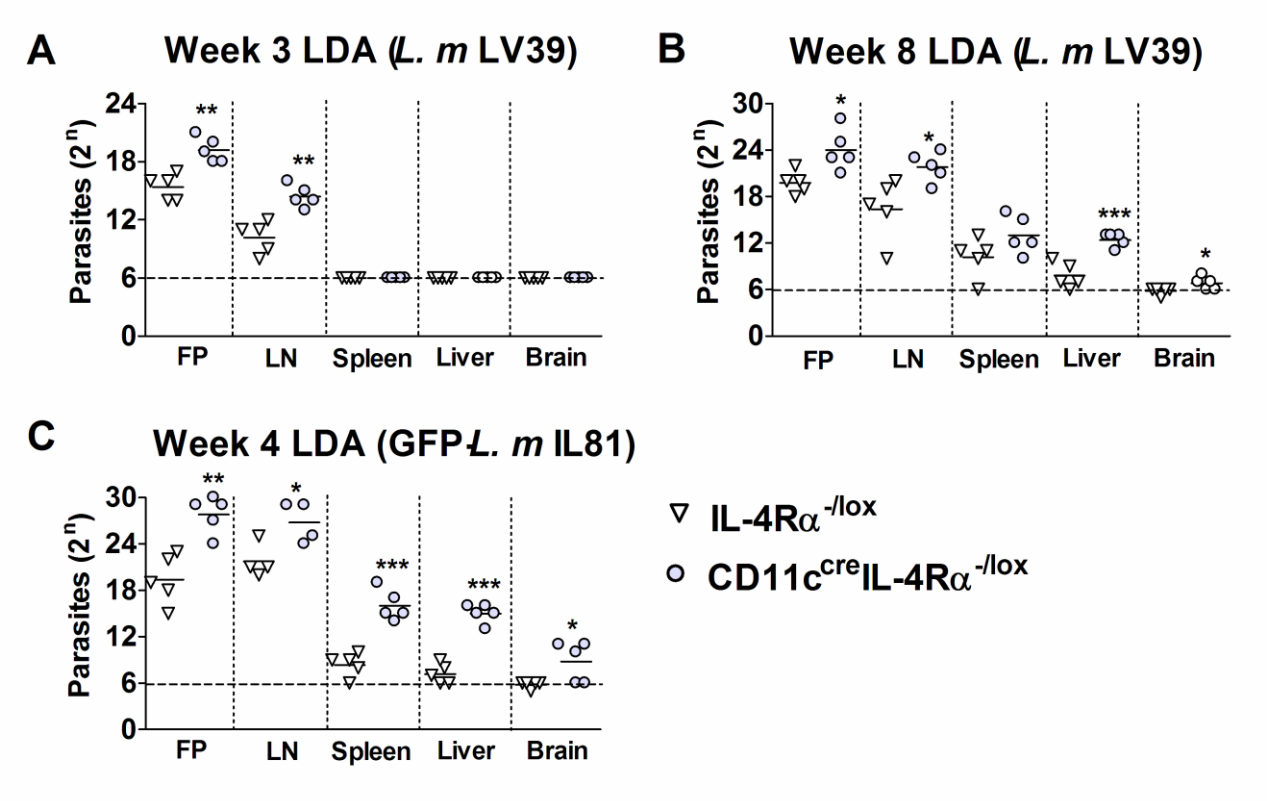

Figure 3.12: Impaired IL-4-mediated dendritic cell instruction results in increased *L. major* parasite loads.
CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice were infected subcutaneously with 2×10^6 stationary phase metacyclic *L. major* (*L. m* LV39 or GFP-*L. m* IL81 promastigotes into the hind footpad.
(A-B) Parasite load was determined by limiting dilution assay (LDA) of homogenized footpads and single-cell suspensions from lymph nodes, spleens, livers and brains at week 3 **(A)** and week 8 **(B)** after infection with *L. major* (*L. m* LV39).
(C) Similarly, parasite load was determined by limiting dilution assay of homogenized footpads and single-cell suspensions from lymph nodes, spleens, livers and brains at week 4 after infection with GFP-expressing *L. major* (*L. m* IL81).
A representative of one of two individual experiments is shown with mean values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$). FP = Footpad and LN = Lymph node

3.7.3 Histological analysis in peripheral tissues of CD11c^{cre}IL-4Rα$^{-/lox}$ mice during L. major infection

Histological analysis confirmed the increased presence of disseminated parasites in the spleen and liver of CD11c^{cre}IL-4Rα$^{-/lox}$ mice (IL81, week 4), as shown by the high load of extracellular *L. major* amastigotes (spleen) and the prevalence of inflammatory foci and leishmanial bodies in mononuclear cells (liver) (Figure 3.13A).

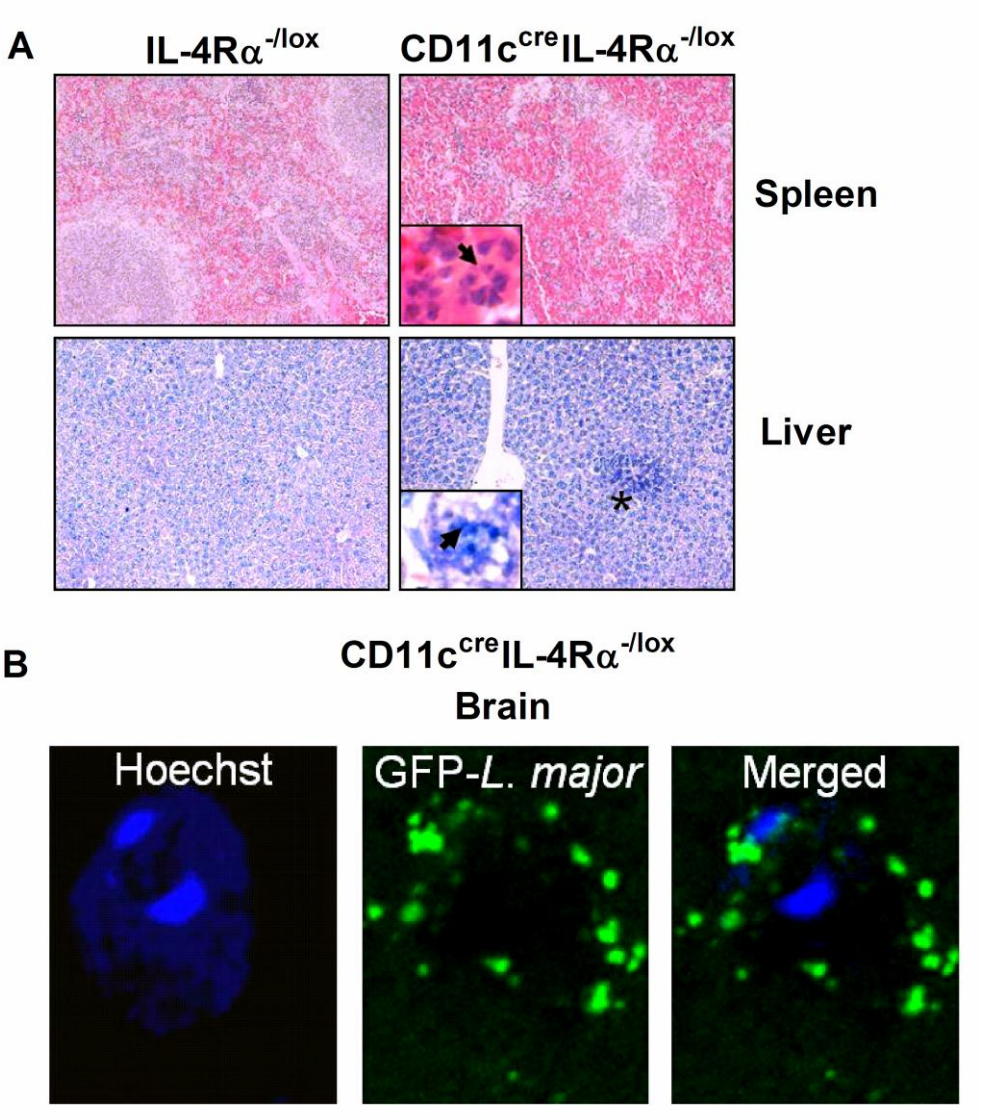

Figure 3.13: Dissemination of *L. major* parasites in peripheral organs of infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice.
CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate control mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-*L. major* IL81 parasites in the hind footpad.
(A) At week 4 after infection, formalin-fixed spleen and liver were stained with H&E and Giemsa respectively, for histopathology (original magnification ×100; asterisks indicate inflammatory foci and insets, arrows indicate amastigote parasites ×800).
(B) Cryosections of brain tissue were stained with Hoechst nuclear stain (Blue) and visualized by confocal microscopy for GFP$^+$-*L. major* amastigote parasites (original magnification ×400). A representative of one of two individual experiments is shown.

To rule out the possibility that the parasite burdens observed in homogenised brain tissue of infected CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice was a consequence of parasites present in the blood brain barrier or the meninges, the localization of GFP$^+$ *L. major* parasites in brain tissue of perfused CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice at week 4 after infection was examined by confocal microscopy. GFP$^+$ *L. major* parasites were found within cells in cryosections of brain tissue from IL81 infected CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice, but not in control mice (Figure 3.13B), indicating that *L. major* parasites traversed the immunological blood-brain barrier to reside in the brains of these mice. Together, these results demonstrate that the absence of IL-4Rα signaling on DCs in *L. major* infected CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice results in a substantial increase in parasite numbers in peripheral organs. Moreover, based on the combined results of these experiments, the presence of *L. major* parasites at peripheral sites seems likely to be a consequence of disease-related parasite dissemination and not a result of late parasite replication subsequent to injection, since parasites were not detected in these sites at earlier time points.

3.8 IL-4Rα-deficient CD11b$^+$ DCs are the major infected cell type in footpad, lymph node and spleen

3.8.1 Viability and localization of GFP$^+$ L. major IL81 parasites

Since CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice showed enhanced dissemination of *L. major* parasites in peripheral organs, we sought to investigate if specific cell populations were important in harboring and disseminating the parasites. CD11c^{cre}IL-4R$\alpha^{-/lox}$ and littermate control mice were infected with GFP-expressing *L. major* IL81 parasites and GFP positivity was used to track infected cell populations in different organs by flow cytometry. To first confirm that GFP$^+$ cell populations were indeed associated with live *L. major* parasites and not harboring fragments of GFP$^+$ amastigotes, a variety of cell populations, namely; dendritic cells, macrophages, neutrophils and B cells, were FACS sorted and plated out for limiting dilution assays. Parasite replication occurred in all GFP$^+$ cell populations indicating that GFP positivity was a good marker for viable parasites associated with cells (Figure 3.14A). In contrast, GFP$^+$ parasites were not detected in sorted CD4$^+$ and CD8$^+$ T cells (data not shown). To further confirm that cells contained intracellular GFP$^+$ parasites, confocal microscopy was performed on sorted cells at 4 week after infection. Confocal imaging revealed that GFP$^+$ parasites associated with phagocytes, such as macrophages, were intracellular, whereas GFP$^+$ parasites associated with lymphocytes, such as B cells, were only attached to the surface of the cells (Figure 3.14B).

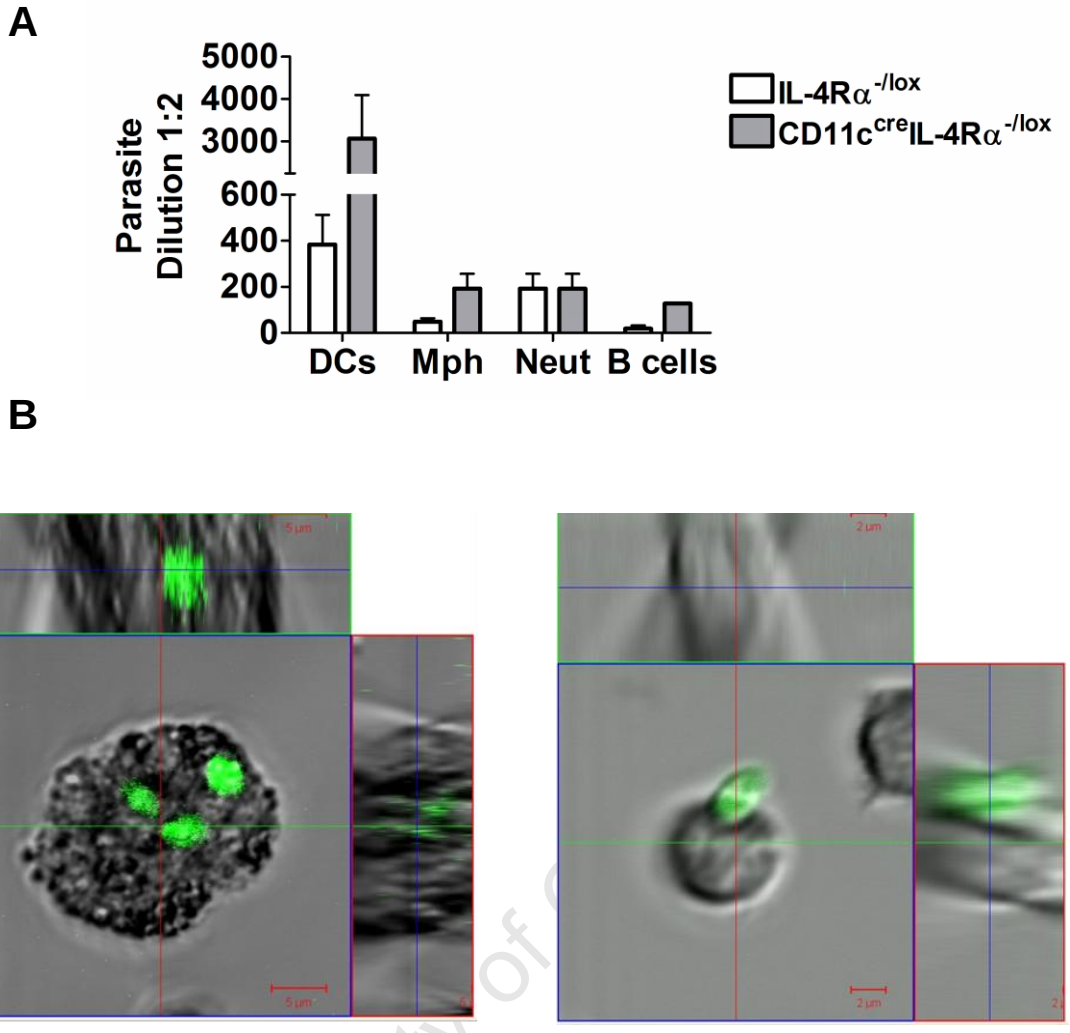

Figure 3.14: GFP$^+$-*L. major* parasites in immune cell populations in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.
Experimental mice were infected subcutaneously with GFP-expressing *L. major* IL81 promastigotes into the hind footpad.
(A) At week 4 after infection, single-cell suspensions of lymph node cells were surface stained for dendritic cells (DCs-CD11c$^+$CD11b$^+$MHCII$^+$), macrophages (Mph-CD11b$^+$MHCII$^+$CD11c$^-$), Neutrophils (Neut-GR1$^+$CD11c$^-$) and B cells (CD19$^+$, CD11c$^-$) and FACS sorted for these immune cell populations. Sorted cells were plated out in two-fold dilutions to determine viable parasite burden by limiting dilution assay.
(B) At week 4 after infection, footpad macrophages (CD11b$^+$MHCII$^+$CD11c$^-$) and lymph node B cells (CD19$^+$CD3$^-$CD11c$^-$) were isolated on a cell sorter, seeded into chamber slides and the localization of GFP$^+$ parasites were viewed and analysed by LSM 510 confocal microscopy. A representative of one of two individual experiments is shown with mean ± SEM.

3.8.2 Cellular infiltration in CD11c^{cre}IL-4Rα$^{-/lox}$ mice during L. major infection

Cutaneous Leishmaniasis is characterized by a localized inflammatory response at the site of infection and the immediate draining lymph node (dos Santos *et al.*, 2008). We therefore evaluated cellular infiltration as an indication of inflammation in the footpad and draining popliteal lymph node at day 3 and week 4 after infection with GFP$^+$ *L. major* IL81 parasites (Figure 3.15). In comparison to littermate mice, CD11c^{cre}IL-4Rα$^{-/lox}$ mice

developed an earlier, stronger inflammatory response in the lymph nodes at the initial stages of infection (day 3), with recruitment of a significantly higher number of total cells into the lymph node (Figure 3.15A). By week 4 the total number of lymph node cells was comparable between the two groups (Figure 3.15B). During early infection (day 3), there was no difference in cell numbers in the footpad between CD11c^{cre}IL-4Rα$^{-/lox}$ and control mice (Figure 3.15A) However by week 4, footpads of CD11c^{cre}IL-4Rα$^{-/lox}$ mice had exacerbated inflammation as shown by the significantly higher cell numbers when compared to control mice (Figure 3.15B). This suggests that the continuous recruitment of immune cells to the site of infection contributes to the uncontrolled inflammatory response developing in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, the effects of which are seen in the increased footpad swelling (Figure 3.5), tissue destruction and footpad necrosis.

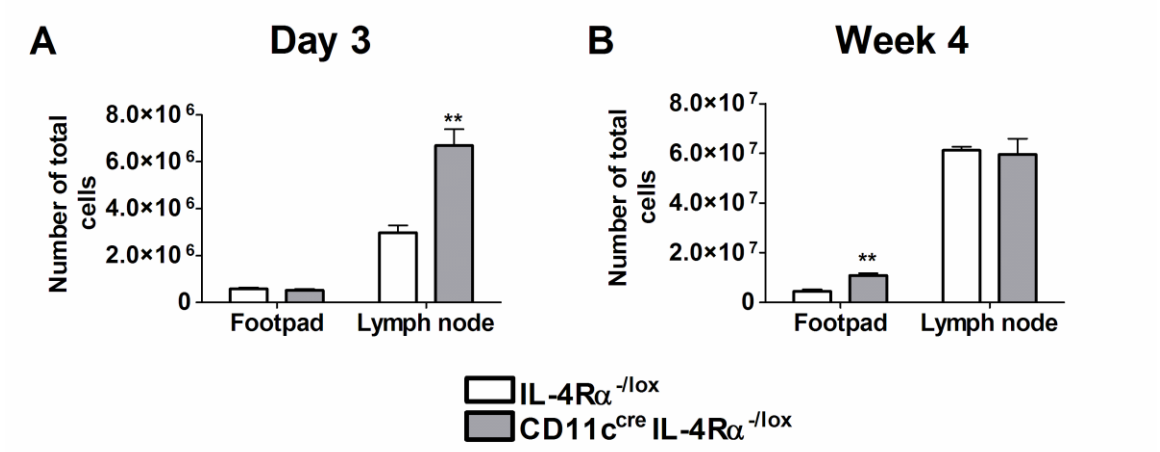

Figure 3.15: Total cell numbers in footpads and lymph nodes of *L. major*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice.
CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 promastigotes into the hind footpad.
(A-B) Footpads and lymph nodes were analysed for cell infiltration at day 3 **(A)** and week 4 **(B)** after infection in CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice. A representative of one of two individual experiments is shown with mean ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (**, $p \leq 0.01$).

3.8.3 Cellular populations infected with GFP$^+$-L. major parasites in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice

Total footpad and lymph node cells were analyzed for specific cell populations harboring GFP$^+$ *L. major* parasites at both early (day 3) and late time points (week 4) following infection. At day 3 after infection, a large proportion of phagocytic cells, particularly, plasmacytoid DCs (pDCs), macrophages and neutrophils, had infiltrated the infected footpad (Figure 3.16A). By week 4 after infection, numbers of infiltrating cells had increased substantially, with conventional DCs (cDCs) also now present in high numbers

(Figure 3.16B). The number of infiltrating cells was significantly higher in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice compared to IL-4R$\alpha^{-/lox}$ mice (Figure 3.16B). At the early time point (day 3), macrophages were the dominant cell type infected with GFP$^+$ *Leishmania* in the footpad, with similar numbers in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice and littermate controls (Figure 3.16C). This was in contrast to the draining lymph node, where conventional and plasmacytoid DCs were predominantly infected (Figure 3.16D), suggesting that these cells were more important in delivering the parasites to the lymph node. Significantly increased numbers of infected DCs were already found in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice compared to controls (Figure 3.16D). At week 4 after infection, the footpad harbored particularly high numbers of GFP$^+$ *L. major* infected macrophages, cDCs and neutrophils (Figure 3.16E), while in the draining lymph node, the cDCs were still the dominant infected cell type (Figure 3.16F). Again the number of infected cells was significantly higher in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice (Figure 3.16E and 3.16F) compared to littermate controls.

Conventional DCs form a highly heterogeneous cell population consisting of various subsets (e.g. myeloid, lymphoid, follicular and plasmacytoid) (Scott and Hunter, 2002), therefore we performed further FACS staining at early and late time-points after infection to determine if any particular DC subset was important in harboring and disseminating *L. major* in infected mice. At day 3 (Figure 3.17A), CD11b$^+$ DCs were the main subset infected with *L. major* in the footpads but in the LN there was a large population of CD103$^+$ DCs (tissue DCs) that harbored *L. major* parasites (Figure 3.17B). We also observed similar populations of infected cells at day 7 after infection. Later, at week 4, CD11b$^+$ DCs were the main infected DC population in both the footpad and lymph node (Figure 3.17C and 3.17D). CD11b$^+$ DCs were the main cell type containing *L major* parasites in the spleen at 4 weeks, with significantly increased numbers of infected cells in CD11c^{cre}IL-4R$\alpha^{-/lox}$ mice compared to controls (Figure 3.17E and 3.17F).

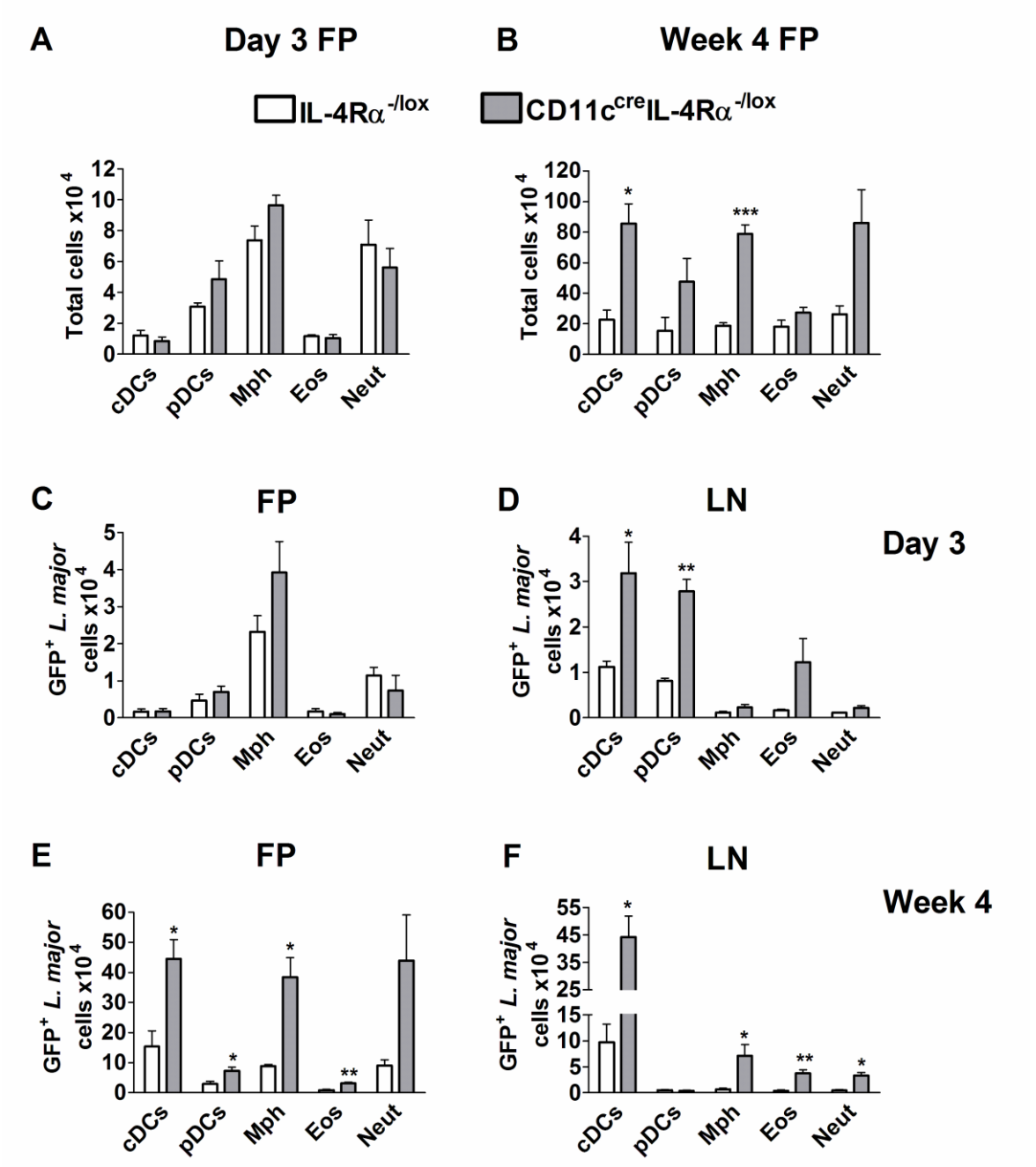

Figure 3.16: Various cell populations infected with *L. major* parasites in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 promastigotes into the hind footpad.

(A-B) Footpad cells were analysed for total immune cell populations at day 3 **(A)** and week 4 **(B)** after infection by FACS staining.

(C-D) At day 3 after infection, the number of cells containing GFP$^+$ *L. major* parasites was identified within the indicated cell populations derived from footpad **(C)** and lymph node **(D)**.

(E-F) At week 4 after infection, the number of cells containing GFP$^+$ *L. major* parasites was identified within the indicated cell populations derived from footpad **(E)** and lymph node **(F)**.

Cell populations were differentiated based on the following markers; conventional dendritic cells (cDCs; CD11c$^+$MHCII$^+$), plasmacytoid DCs (pDCs; CD11c$^+$PDCA$^+$SiglecH$^+$), macrophages (Mph; CD11b$^+$MHCII$^+$CD11c$^-$), Eosinophils (Eos; SiglecF$^+$CD11c$^{-/int}$), Neutrophils (Neut; GR-1^{high}SSC^{high}FSC^{high} CD11c$^-$). A representative of one of two individual experiments is shown with mean ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$). FP = Footpad and LN = Lymph node.

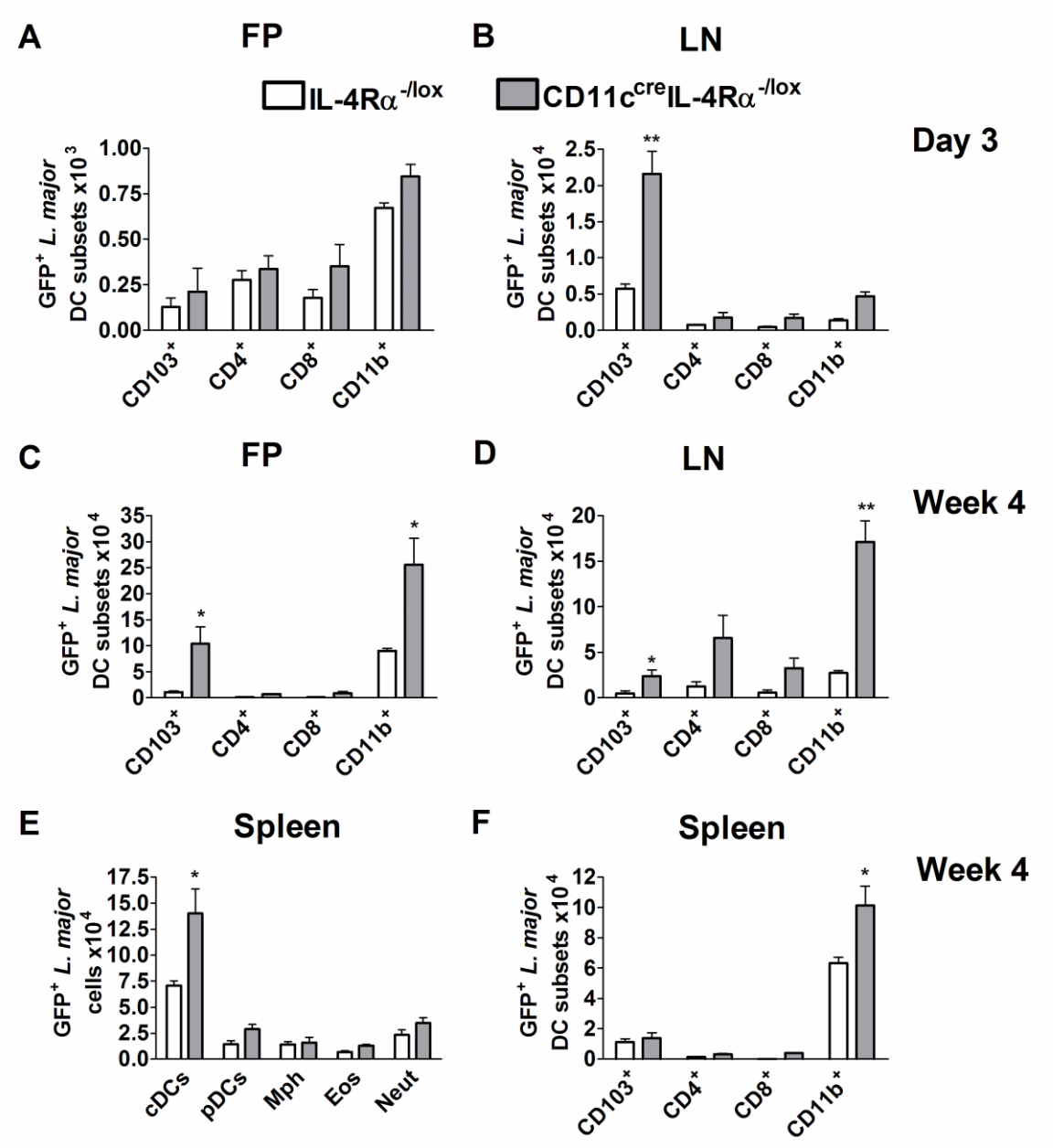

Figure 3.17: CD11b$^+$ inflammatory dendritic cells disseminate *L. major* parasites from the site of infection.

CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 promastigotes into the hind footpad.

(A-B) The number of cells containing GFP$^+$ *L. major* parasites were identified within cDC subsets derived from footpad **(A)** and lymph node **(B)** at day 3 after infection.

(C-D) The number of cells containing GFP$^+$ *L. major* parasites were identified within cDC subsets derived from footpad **(C)** and lymph node **(D)** at week 4 after infection.

(E-F) Spleens were harvested to analyse GFP$^+$ *L. major* parasites in the indicated cell populations **(E)** and DC subsets **(F)** at week 4 after infection.

Cell populations were differentiated based on the following markers; conventional dendritic cells (cDCs; CD11c$^+$MHCII$^+$), plasmacytoid DCs (pDCs; CD11c$^+$PDCA$^+$SiglecH$^+$), CD103$^+$ DCs (CD11c$^+$CD103$^+$), CD4$^+$ DCs (CD11c$^+$CD4$^+$CD3$^-$), CD8$^+$ DCs (CD11c$^+$CD8$^+$CD3$^-$), CD11b$^+$ DCs (CD11c$^+$CD11b$^+$MHCII$^+$), macrophages (Mph; CD11b$^+$MHCII$^+$CD11c$^-$), Eosinophils (Eos; SiglecF$^+$CD11c$^-$), Neutrophils (Neut; GR-1^{high}CD11c$^-$). A representative of one of two individual experiments is shown with mean ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$). FP = Footpad and LN = Lymph node.

3.8.4 Infiltration of CD11b$^+$ dendritic cells in spleen of CD11c^{cre}IL-4Rα$^{-/lox}$ mice during L. major infection

Seeing that CD11b$^+$ DCs were the preferred host cells for *L. major* parasites, we next examined the numbers of CD11b$^+$ DCs infiltrating the spleen during infection with GFP-expressing *L. major* IL81 parasites. We found that overall numbers of CD11b$^+$ DCs were increased to a similar degree in spleens of both CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls at day 1 and day 3 as well as by week 4 after infection (Figure 3.18). This suggested that differences in parasite killing in infected DCs and not migration of infected DCs might be responsible for the increased numbers of GFP$^+$ infected cells in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.16 and Figure 3.17).

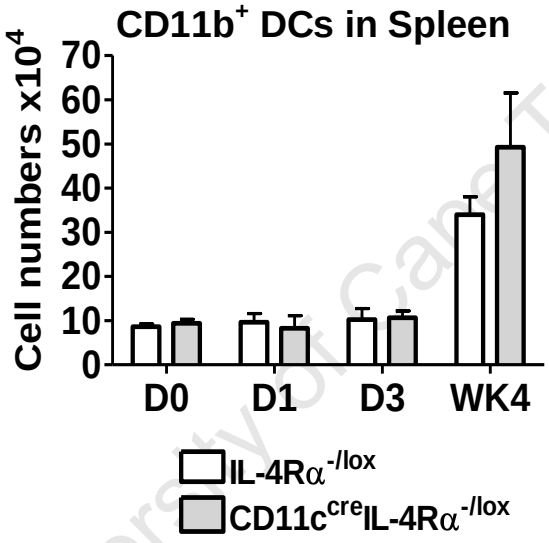

Figure 3.18: Infiltration of CD11b$^+$ dendritic cells in spleen during *L. major* IL81 infection in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.
Mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 (MRHO/SV/59/P) strain into the hind footpad. The number of infiltrating CD11b$^+$ DCs in spleen of CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice was quantified by flow cytometry at Day 0 (D0), Day 1 (D1), Day 3 (D3) and Week 4 (WK4) after infection.
A representative of one of two individual experiments is shown with mean ± SEM.

3.8.5 Immunohistochemistry confirms the presence of GFP$^+$ parasites in dendritic cells from peripheral tissues of CD11c^{cre}IL-4Rα$^{-/lox}$ mice

In order to supplement the flow cytometry data and confirm the presence of higher numbers of intracellular GFP$^+$ *L. major* parasites in DCs from CD11c^{cre}IL-4Rα$^{-/lox}$ mice *in vivo*, we performed confocal microscopy on lymph node and spleen cryosections. Fluorescent staining of dendritic cells and macrophages in spleen tissue confirmed that dendritic cells were a major reservoir of intracellular GFP$^+$ *L. major* parasites in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, with a higher concentration of green fluorescent foci (GFP$^+$) than macrophages (Figure 3.19A).

Quantification of intracellular GFP$^+$ *L. major* amastigote parasites in dendritic cells revealed significantly increased numbers of parasites per field of view in CD11c^{cre}IL-4Rα$^{-/lox}$ mice in both the lymph node (Figure 3.19B) and spleen (Figure 3.19C) compared to littermate control mice. Moreover, quantitative analysis further revealed that dendritic cells in spleen (Figure 3.19C) from CD11c^{cre}IL-4Rα$^{-/lox}$ mice had higher GFP$^+$ *L. major* parasite loads in comparison to the amount of intracellular GFP$^+$ parasites in macrophages. Taken together, the finding that CD11b$^+$ DCs constitute the major source of infected cells in LN and spleen and the well-established migratory properties of DCs suggests that they may play a role in the systemic spread of parasites in both CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate controls. Moreover, these results suggest that differences in the survival and/or growth of parasites within IL-4Rα deficient CD11b$^+$ DCs, as opposed to migration of these DCs, could be responsible for the increased parasite loads in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

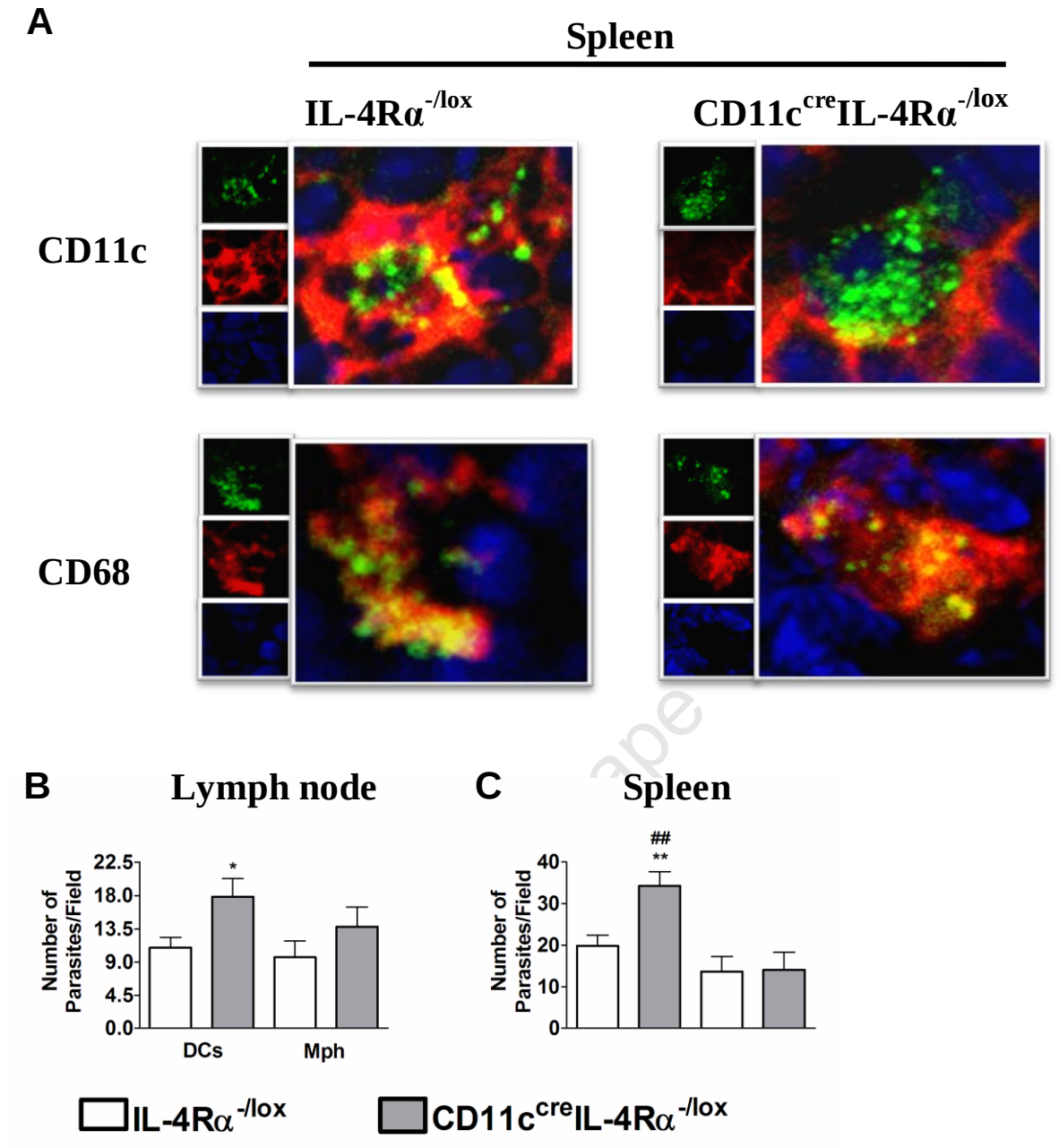

Figure 3.19: Dendritic cells predominantly harbor GFP$^+$ *L. major* parasites in lymph nodes and spleen in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

Mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-expressing *L. major* IL81 promastigotes into the hind footpad.

(A) After 4 weeks of infection, crysections of lymph node and spleen were stained with Hoechst nuclear stain (Blue) and mAb against CD11c$^+$ dendritic cells or CD68$^+$ macrophages (red). The figure shows representative micrographs of spleen cryosections showing distribution and localization of GFP$^+$ *L. major*-infected (green) CD11c$^+$ dendritic cells and CD68$^+$ macrophages (red) from CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate mice (original magnification ×400). Insets show individual channels.

(B-C) The number of GFP$^+$ amastigote parasites in dendritic cells and macrophages from CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate control mice stained with mAb against CD11c$^+$ DCs and CD68$^+$ macrophages, were quantified from multiple lymph node **(B)** and spleen **(C)** cryosections of individual mice by confocal microscopy. Data is expressed as mean ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$) or to infected dendritic cells (##, $p \leq 0.01$). DCs = Dendritic cells and Mph = Macrophages.

3.9 IL-4Rα-deficient dendritic cells have impaired IL-4-mediated instruction and reduced iNOS expression in CD11c^{cre}IL-4Rα$^{-/lox}$ mice

3.9.1 Impaired dendritic cell instruction in L. major-infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice

To test the relevance of IL-4-mediated DC instruction *in vivo* in our model, CD11c^{cre}IL-4Rα$^{-/lox}$ mice and controls were infected with *L. major* IL81 and at 4 weeks post infection total LN cells were restimulated with SLA to measure production of IL-12p40 and IL-10 cytokines in the supernatant. Lymph node cells from infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice produced significantly reduced IL-12p40 but increased IL-10 compared to littermate IL-4Rα$^{-/lox}$ mice (Figure 3.20A).

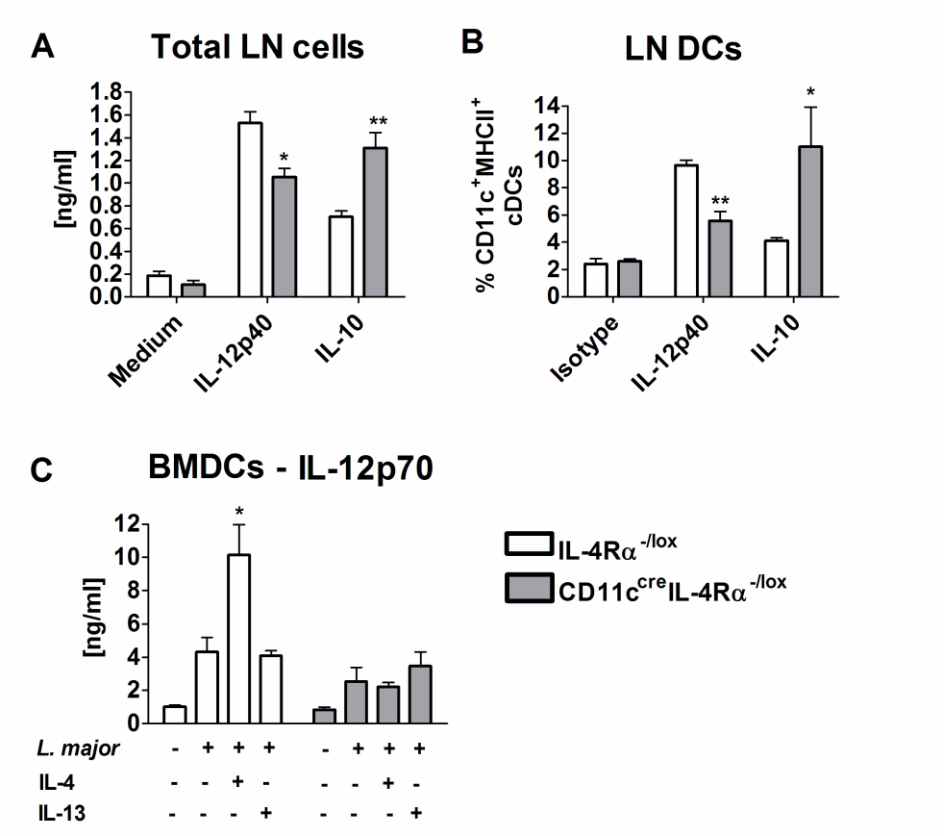

Figure 3.20: Abrogation of IL-4Rα expression on dendritic cells impairs IL-4-mediated dendritic cell instruction during *L. major* infection.

Mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-*L. major* IL81 promastigotes into the hind footpad for 4 weeks.

(A) Total lymph node cells were restimulated with SLA and production of IL-12p40 and IL-10 was determined by ELISA at week 4 after infection.

(**B**) Total lymph node cells were restimulated with PMA/Ionomycin/Monensin and intracellular levels of IL-12p40 and IL-10 in dendritic cells was determined by flow cytometry at week 4 after infection.

(C) BMDCs were infected with *L. major* in the presence or absence of recombinant IL-4 or IL-13. Culture supernatants were collected after 48 hours to determine IL-12p70 levels by ELISA.

A representative of one of two individual experiments is shown with mean values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$).

Moreover, intracellular cytokine staining revealed that DCs from CD11c^{cre}IL-4Rα$^{-/lox}$ mice produced less IL-12p40 and more IL-10 than those from littermate IL-4Rα$^{-/lox}$ controls (Figure 3.20A and Figure 3.20B). In addition, differences in IL-12p70 production were detected *in vitro* (Figure 3.20C). *L. major*/IL-4 stimulated BMDCs derived from IL-4Rα$^{-/lox}$ mice showed increased IL-12p70 production in agreement with previous reports (Lutz *et al.*, 2002), whereas IL-4 had no additive effect on IL-12p70 production in BMDCs from CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.20C). IL-13 did not increase IL-12p70 production, as previously shown (Lutz *et al.*, 2002) in CD11c^{cre}IL-4Rα$^{-/lox}$ mice or control animals.

3.9.2 mRNA expression of specific cytokines on dendritic cells isolated from L. major-infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice

CD11c^{+}MHCII^{+} DCs from the lymph nodes of *L. major*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ and littermate control mice were positively selected using MACs beads then gated as shown (Figure 3.21A) and FACS sorted to a purity of 99%. We assessed characteristic dendritic cell morphology by Diff-Quick staining (Figure 3.21B). mRNA was isolated from sorted DCs and converted to cDNA after which RT-PCR was performed. Quantification of mRNA demonstrated decreased expression of the genes for IL-12p40 and the Th1-promoting cytokine IL-18 in sorted LN DCs from CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to controls (Figure 3.21A and 3.21B). In contrast, there was a trend towards increased mRNA expression of IL-10 (Figure 3.21C) as well as significantly increased mRNA expression of IL-23 (Figure 3.21D) and activin A (Figure 3.21E), cytokines which are involved in promoting Th2 responses by inducing Th17 cells and alternative activation of macrophages, respectively (Ogawa *et al.*, 2006; Lopez Kostka *et al.*, 2009). Together, these results indicate that IL-4Rα-mediated DC instruction occurs *in vivo*, with IL-4Rα deficient DCs producing more IL-10, which could subsequently lead to reduced IL-12 and IL-18 responses after exposure to *L. major* parasites, resulting in an impairment of protective Th1 responses.

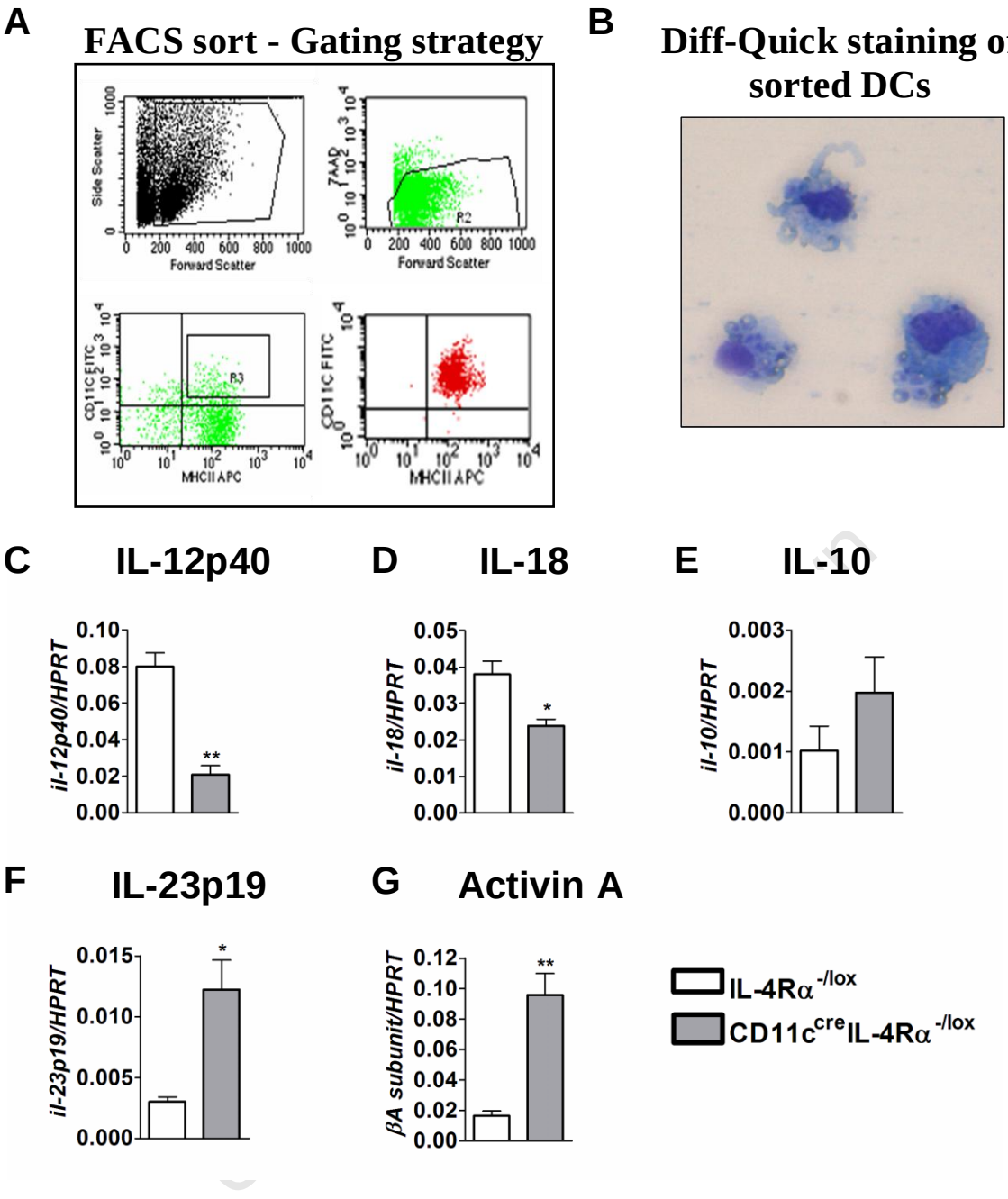

Figure 3.21: mRNA expression of specific genes in dendritic cells isolated from *L. major*-infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice at week 4 after infection.

Mice were infected subcutaneously with 2×10^6 stationary phase metacyclic GFP-*L. major* IL81 promastigotes into the hind footpad.

(A-B) CD11c$^+$MHCII$^+$ DCs from lymph nodes of *L. major* IL81 infected mice were gated as shown and FACS sorted to 99% purity. Cell morphology was assessed by Diff-Quick staining **(B)**.

(C-G) mRNA was isolated from sorted DCs and converted to cDNA. cDNA was used in SYBR-Green RT-PCR to measure mRNA expression of IL-12p40 **(C)** and IL-18 **(D)**, IL-10 **(E)**, IL-23p19 **(F)** and Activin A **(G).** Expression was normalized against the housekeeping gene *HPRT*.

A representative of one of two individual experiments is shown with mean ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$, **, $p \leq 0.01$).

3.9.3 Reduced expression of iNOS in IL-4Rα-deficient dendritic cells from CD11c^{cre}IL-4Rα$^{-/lox}$ mice

It is well known that macrophage killing by iNOS-catalyzed nitric oxide production is a key feature of an effective anti-leishmanial immune response. However, a recent publication has implicated a population of iNOS^{+}-producing CD11b^{+} inflammatory DCs in controlling *Leishmania* infection (De Trez *et al.*, 2009). We therefore examined iNOS production by DCs in CD11c^{cre}IL-4Rα$^{-/lox}$ mice. In hypersusceptible CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, a significantly reduced percentage of CD11b^{+} DCs produced iNOS compared to IL-4Rα$^{-/lox}$ littermate controls (Figure 3.22A). In addition, intracellular levels of iNOS expression were also lower (Figure 3.22B). Together, these data demonstrate that IL-4Rα-deficient CD11b^{+} DCs have impaired killing effector functions, which most likely contributes to the increased parasite burdens observed in these DCs in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice. Moreover, iNOS-producing DCs appear to influence control over *L. major* infection in BALB/c mice.

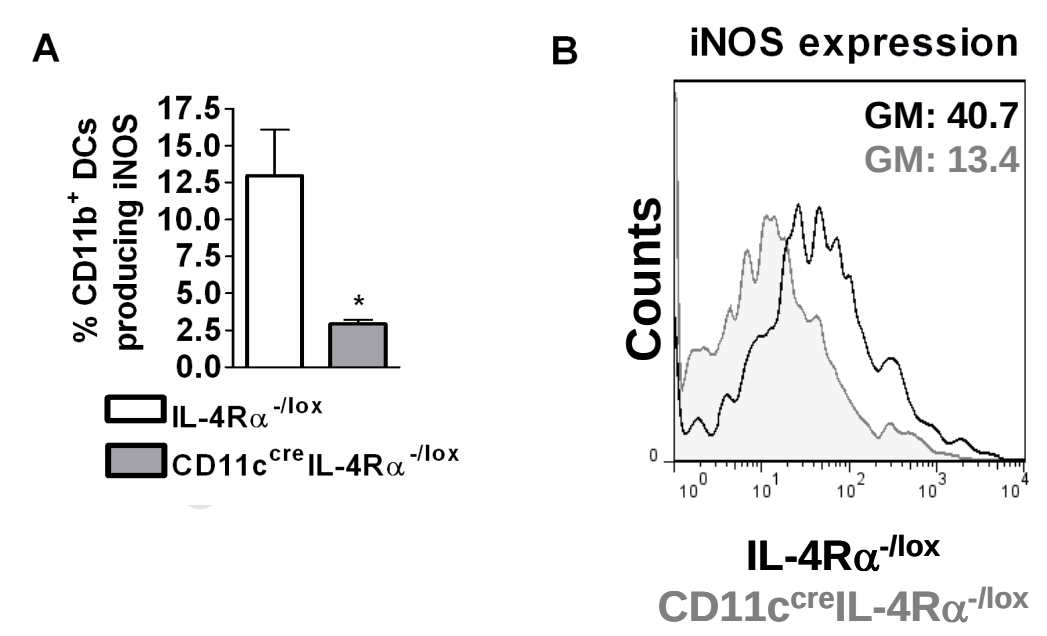

Figure 3.22: Abrogation of IL-4Rα expression in dendritic cells impairs iNOS expression.
CD11c^{cre}IL-4Rα$^{-/lox}$ and control mice were infected subcutaneously with 2×10^{6} stationary phase metacyclic GFP-*L. major* IL81 promastigotes into the hind footpad. Total lymph node cells were surface-stained and gated for CD11b^{+}CD11c^{+}MHCII^{+} DCs followed by intracellular staining for iNOS.
(A) Percentage of CD11b^{+} inflammatory DCs producing iNOS.
(B) Intracellular iNOS expression shown as population shifts in CD11b^{+}CD11c^{+}MHCII^{+} inflammatory DCs from CD11c^{cre}IL-4Rα$^{-/lox}$ and control mice.
A representative of one of two individual experiments is shown with mean values ± SEM. Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice (*, $p \leq 0.05$).

CHAPTER 4:

DISCUSSION

CHAPTER 4

DISCUSSION

Understanding the underlying mechanisms of pathogenesis and immune control in murine models of cutaneous Leishmaniasis will aid in the design of effective therapeutics and vaccines. During acute *L. major* infection in mice, Th1 cells provide protection through production of IL-12, IFN-γ and TNF, which promotes classical activation of macrophages and killing of intracellular parasites by effector nitric oxide production (Sypek *et al.*, 1993; Wei *et al.*, 1995; Guler *et al.*, 1996). In contrast, Th2 cells promote infection by secretion of IL-4, IL-13, IL-5 and IL-9 cytokines, which counter-regulate Th1 responses, impair classical activation of macrophages and induce arginase I production by alternatively activated macrophages that favour parasite growth and survival (Kopf *et al.*, 1996; Matthews *et al.*, 2000; Arendse *et al.*, 2005; Holscher *et al.*, 2006).

Although several studies have clearly established that IL-4 is a key cytokine in the development of non-healing disease in BALB/c mice (Sadick *et al.*, 1990; Launois *et al.*, 1995; Kopf *et al.*, 1996; Himmelrich *et al.*, 2000), apparently contradictory evidence also suggests that IL-4 has the ability to instruct protective Th1 responses (Noben-Trauth *et al.*, 1996; Noben-Trauth *et al.*, 1999; Biedermann *et al.*, 2001). Usually, *L. major* parasites down-modulate protective immunity and establish non-healing disease in susceptible strains by suppressing the induction of IL-12 production in macrophages and dendritic cells (Carrera *et al.*, 1996; Belkaid *et al.*, 1998; Moll *et al.*, 2002; Liu *et al.*, 2009). The term "instruction theory" was coined when exogenously administered IL-4, but not IL-13, was found to increase DC-derived IL-12 production induced by pathogen products, via signaling through the type I IL-4 receptor complex (Hochrein *et al.*, 2000; Biedermann *et al.*, 2001; Lutz *et al.*, 2002). Several studies also indicate that both IL-4 and IL-13 play a role in promoting DC maturation via signaling through the type II IL-4 receptor complex (Lutz *et al.*, 2002; Padilla *et al.*, 2005). As it was unclear how IL-4 induces IL-12 production in DCs, studies investigated IL-10 as a possible mediator of this process since this immunosuppressive cytokine was shown to inhibit IL-12 production by DCs (D'Andrea *et al.*, 1993). Indeed, Yao and colleagues clearly demonstrated that the mechanism behind DC instruction was inhibition of IL-10 by IL-4, leading to higher levels of IL-12 and increased protective Th1 responses (Yao *et al.*, 2005).

Most *in vitro* and *in vivo* studies on the effects of IL-4 have been conducted with exogenously administered IL-4, but the consequence of IL-4-mediated DC instruction

during disease *in vivo* had not been demonstrated. To address these issues, dendritic cell-specific IL-4Rα-deficient ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$) BALB/c mice were generated by gene-targeting using the cre/*lox*P recombinase system under control of the CD11c locus. These mice were found to have abrogated IL-4Rα expression on DCs and alveolar macrophages, with other cell types maintaining normal IL-4Rα expression and function (Hurdayal and Nieuwenhuizen *et al.* Submitted manuscript).

Infection of $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice with *L. major* LV39 and IL81 parasites revealed IL-4Rα signaling on DCs to be highly important in protection against cutaneous Leishmaniasis. Compared to IL-$4R\alpha^{-/lox}$ littermate controls and WT BALB/c mice, $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice showed dramatically worsened disease progression, with greater footpad swelling and necrosis, and significantly higher parasite burdens both locally and in visceral organs such as the spleen and liver. As expected, genetically resistant C57BL/6 mice effectively controlled infection, as did global IL-$4R\alpha^{-/-}$ mice, which have been shown to be resistant during the acute phase of *L. major* infection, with disease progression in the chronic phase only (Mohrs *et al.*, 1999; Radwanska *et al.*, 2007).

Progressive disease during *L. major* infection in BALB/c mice has been attributed to the predominance of $CD4^+$ Th2 cells, Th2 cytokines and type 2 antibody responses (Himmelrich *et al.*, 2000; Matthews *et al.*, 2000; Arendse *et al.*, 2005; Holscher *et al.*, 2006; Radwanska *et al.*, 2007). Analysis of $CD4^+$ T cell cytokine responses in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice revealed a decrease in IFN-γ accompanied by a marked increase in IL-4, IL-13 and IL-10, while augmented secretion of IgG1 and IgE antibodies by B cells confirmed a shift towards a susceptible Th2-type immune phenotype. In agreement with the "instruction theory", inhibition of IL-4 signaling on DCs reduced the protective Th1 immune phenotype while enhancing a disease promoting Th2 phenotype. The reduced Th1 and increased Th2 responses in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice demonstrate that IL-4-mediated DC instruction theory is relevant *in vivo*, with biological quantities of IL-4 acting through DCs to promote Th1 immunity. Total draining lymph node cells from $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice restimulated with *Leishmania* antigen secreted more IL-10 and less IL-12 than those from IL-$4R\alpha^{-/lox}$ mice. DCs from the lymph nodes of $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice also produced more IL-10 and less IL-12, thereby confirming impaired IL-4-mediated DC instruction *in vivo* in $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice and a role for IL-4Rα signaling in mediating IL-10 production by DCs.

Aside from its role in instruction, IL-10 is known to be a susceptibility factor during *L. major* infection, being produced at higher levels in susceptible BALB/c mice, and is capable of suppressing both Th1-mediated effector functions (Heinzel *et al.*, 1991; Kane and Mosser, 2001; Noben-Trauth *et al.*, 2003) and development of delayed type hypersensitivity (DTH) responses (Radwanska *et al.*, 2007). In humans, IL-10 is strongly associated with persistent *L. major* infection (Anderson *et al.*, 2005). Quantification of mRNA expression also revealed decreased expression of the genes for Th1-promoting cytokines, IL-12p40 and IL-18 in DCs from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to littermate control DCs, while expression of the genes for Th2-promoting cytokines, IL-23p19 and Activin A, were significantly increased. IL-23 production by DCs has been shown to induce the expansion of Th17 cells which in turn increases the recruitment of neutrophils to the site of infection that promote susceptibility to *L. major* (Lopez Kostka *et al.*, 2009).

Neutrophils are typically recruited within hours to the site of parasite inoculation with qualitative and quantitative differences being observed between susceptible and resistant mouse strains (Tacchini-Cottier *et al.*, 2000). In BALB/c mice, a sustained recruitment of elevated numbers of neutrophils persists, which does not occur in resistant C57BL/6 mice. Indeed, these professional phagocytes have been shown to enhance susceptibility to *L. major* through production of inhibitory cytokines, such as IL-10 and TGF-β, and by acting as "Trojan horses" for *Leishmania* entry into their final host cells, the macrophages (Tacchini-Cottier *et al.*, 2000; Laskay *et al.*, 2003; van Zandbergen *et al.*, 2004; Charmoy *et al.*, 2007). Along with other studies (Ritter *et al.*, 2009), we also found neutrophils at the site of infection harboring *L. major* parasites and infected cell numbers increased substantially during disease progression. These data illustrate a paradox of neutrophil function in cutaneous Leishmaniasis. Although these cells are actively involved in host innate defense to the parasite, they also contribute to pathology and enhance susceptible Th2 responses during infection (Tacchini-Cottier *et al.*, 2000; Sacks and Noben-Trauth, 2002). Activin A is a pleiotropic cytokine belonging to the TGF-β superfamily, and has previously been found to promote alternative activation of macrophages by inducing Arginase I and decreasing IFN-γ induced expression of iNOS (Ogawa *et al.*, 2006). Given that $CD11b^{+}$ DCs in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had increased Activin A and IL-10, it is possible that Activin A and/or IL-10 could have promoted impaired classical activation of $CD11b^{+}$ DCs as shown by the reduced expression of iNOS in these cells.

IFN-γ plays an important role in mediating protective immunity during *L. major* infection by classically activating macrophages to induce iNOS-mediated NO production for intracellular killing of parasites (Liew *et al.*, 1990; Stenger *et al.*, 1994; Diefenbach *et al.*, 1999; Holscher *et al.*, 2006). iNOS-deficient C57BL/6 mice are highly susceptible to *L. major* infection (Stenger *et al.*, 1994; Wei *et al.*, 1995) and latent Leishmaniasis is reactivated in chronically infected healthy C57BL/6 mice by inhibition of endogenous NOS-2 (Stenger *et al.*, 1996; Holscher *et al.*, 2006; De Trez *et al.*, 2009). Together, these studies indicate that iNOS expression is crucial for the sustained control of *L. major* infection. Induction of iNOS-mediated NO production is counter-regulated by IL-4 and IL-13, which promote the development of alternatively activated macrophages and arginase I production depleting L-arginine as a substrate for iNOS. IL-10 has also been shown to suppress intracellular killing of pathogens in macrophages by suppressing IFN-γ responses (Bogdan *et al.*, 1991; Gazzinelli *et al.*, 1992; Kane and Mosser, 2001) and inducing an alternatively activated macrophage phenotype in the absence of IL-4 and IL-13 (Dewals *et al.*, 2010). Parasites such as *Leishmania* can utilize polyamines generated by arginase I activity for their own growth thus making alternatively activated macrophages a favorable environment for their survival (Brombacher, 2000; Roberts *et al.*, 2004; Kropf *et al.*, 2005; Colotti and Ilari, 2011). Accumulating reports have demonstrated a role for alternative macrophage activation and arginase I expression in influencing susceptibility to *L. major* infection (Kropf *et al.*, 2004; Arendse *et al.*, 2005; Kropf *et al.*, 2005; Holscher *et al.*, 2006). $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice which lack IL-4/IL-13 induced alternative activation of macrophages were found to have increased resistance to infection (Holscher *et al.*, 2006), while neutralization of endogenous arginase I with N-hydroxy-nor-L-arginine completely ameliorated disease progression in BALB/c mice (Kropf *et al.*, 2005). Macrophages from the footpads of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were found to have reduced iNOS expression and increased arginase I expression compared to those from littermate control $IL\text{-}4R\alpha^{-/lox}$ mice, demonstrating a shift towards alternative activation of macrophages most likely as a consequence of increased IL-4, IL-13 or IL-10.

iNOS expression was also decreased in $CD11b^{+}$ DCs from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, possibly as a consequence of reduced IFN-γ and/or increased IL-10 and activin A. Moreover, $CD11b^{+}$ DCs from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had higher *L. major* parasite loads than those from littermate controls correlating with the reduced iNOS expression. Previous studies have revealed that iNOS-producing DCs constitute a major Th1-regulated effector

cell population and contribute to resistance during infection by *L. major* (De Trez *et al.*, 2009), *L. monocytogenes* (Serbina *et al.*, 2003) and *Brucella sp.* (Copin *et al.*, 2007). De Trez *et al.* (2009) demonstrated that inflammatory $CD11b^{+}$ DCs were the main iNOS-producing cells in both the footpad lesion and the draining lymph node of *L. major* infected C57BL/6 mice and that the induction of iNOS expression in these cells required a local Th1 microenvironment. On the other hand, a Th2 microenvironment inhibited the differentiation and activation of $iNOS^{+}$ inflammatory DCs which highlighted an important role for IL-4 and IL-13 as negative regulators in the induction of these DCs. The reduced iNOS expression in both macrophages and DCs in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice is therefore likely to play a role in the uncontrolled survival and replication of *L. major* parasites observed both in the footpad and at peripheral sites.

In susceptible BALB/c mice, *L. major* parasites can disseminate within 24 hours from the site of infection in the footpad to the popliteal lymph nodes as well as to peripheral sites such as the spleen, liver, lungs and bone marrow (Laskay *et al.*, 1995; Schilling and Glaichenhaus, 2001). Resistant mouse strains restrict the spread of the parasites and dissemination is inhibited by the administration of recombinant IL-12 (Laskay *et al.*, 1995). In $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, *L. major* parasites were not detected in spleens at early time points during IL81 infection (day 1 and day 3) but by week 4, parasites were detected in the LN, spleen and liver. During LV39 infection, parasites were also detected at week 8 in the LN, spleen and liver but at week 3, parasites were only detected in LN and not in peripheral sites (spleen and liver). This suggests that parasite dissemination in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice may have occurred at a later stage of infection. While several susceptible mouse strains have been reported to show some increase in dissemination (Guy and Belosevic, 1995; Murray *et al.*, 2002; Kautz-Neu *et al.*, 2011a), disseminated parasite loads in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were dramatic, with relatively higher parasite burdens in the spleens and footpads compared to other strains. Unexpectedly, parasites were even identified within the brain of some of the $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice which has only rarely been reported for this cutaneous strain and with very low levels of parasites detected (Amini *et al.*, 2008). However, dissemination of parasites to the central nervous system (CNS) has been frequently observed in visceral Leishmaniasis in both humans and canines (Nieto *et al.*, 1996; Vinuelas *et al.*, 2001; Abreu-Silva *et al.*, 2003; Petersen and Greenlee, 2011).

It has been suggested that intracellular parasites can gain access to the CNS via infected leukocytes (Abreu-Silva *et al.*, 2003) and/or disruption to the blood brain barrier caused by inflammation (Petersen and Greenlee, 2011). Studying the mechanisms by which other pathogens such as bacteria invade the CNS may lend insights into *Leishmania major* dissemination. Many intracellular organisms such as *Mycobacterium tuberculosis, Listeria monocytogenes, Brucella* spp. and *Salmonella* spp. appear to make use of the "Trojan-horse" mechanism, using phagocyte-facilitated invasion for entry into the CNS (Drevets and Leenen, 2000; Drevets *et al.*, 2004). In order to investigate which cells might act as a "Trojan-horse" and facilitate dissemination of *L. major* parasites to peripheral sites, including the CNS, in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, animals were infected with GFP-expressing *L. major* IL81 parasites and cell populations containing parasites were identified by flow cytometry.

At day 3 and 7 after infection, macrophages were the predominant cell type harboring *L. major* in the footpad, while plasmacytoid DCs, or pDCs, and cDCs, specifically $CD103^+$ tissue DCs, were the main infected cell type in the lymph nodes, indicating that these DCs could be initially responsible for transporting parasites to the lymph node. At week 4, *L. major* parasites were still detected in macrophages in the footpads as well as $CD11b^+$ DCs and neutrophils, but primarily in $CD11b^+$ inflammatory DCs in the lymph node. The number of infected DCs in both footpad and LN was significantly higher in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to littermate controls.

A previous study also reported that DCs were the primary infected cell population in the draining LN of *L. major* infected mice (Muraille *et al.*, 2003). Our study expands upon these findings by identifying that at the onset of infection it is $CD103^+$ tissue DCs and pDCs that possibly transport parasites to the draining LN and later the inflammatory $CD11b^+$ DCs that become parasitized. pDCs are in fact efficient at cell migration through expression of the chemokine receptor CXCR3, which enables their migration into inflamed lymph nodes in response to chemokines CXCL9 and CXCL10 (Yoneyama *et al.*, 2004). Given the migratory capacity of pDCs and the fact that these cells are inefficient at antigen-presentation due to ineffective capturing, processing and MHC-loading of antigens, it seems likely that pDCs may instead serve as vectors that transport *Leishmania* antigen to the lymph nodes for final presentation by other DC subsets. Interestingly, eosinophils also appeared to phagocytose parasites and carry them to the lymph nodes. Not much is known about the role of eosinophils in *Leishmania* infection, although they are

known to infiltrate the site of infection in the early phase and appear to be able to kill the parasites (Grimaldi *et al.*, 1984; Belkaid *et al.*, 2000; Watanabe *et al.*, 2004).

$CD11b^+$ DCs were also one of the main cell types containing *L. major* parasites in the spleen, with $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice again showing a greater number of infected DCs, and higher parasite loads in the DCs. Numbers of $CD11b^+$ DCs were equivalent in spleens of $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice and littermate controls, indicating that the increased survival and/or growth of parasites in these DCs due to inhibition of iNOS was responsible for the increase in infected cell numbers. Overall, the data suggest that $CD11b^+$ dendritic cells may play a role in disseminating *L. major* parasites to peripheral organs such as the spleen, liver and brain and that their ability to kill parasites is impaired in the absence of IL-4Rα signaling.

Interestingly, a recent study found that infected inflammatory DCs, which are monocyte-derived $CD11b^+$ DCs expressing Ly6C, F480, Ly6G and iNOS, showed a unique ability to disseminate to peripheral sites during *M. tuberculosis* infection (Schreiber *et al.*, 2011). Additionally, $CD11b^+Ly6C^+$ cells were found to be the principal phagocytic cells harboring *L. monocytogenes* in circulation (Drevets *et al.*, 2004; Drevets and Bronze, 2008). A study using DsRed-expressing *L. major* parasites to trace infected cells reported that $CD11b^+$ iNOS-producing inflammatory DCs co-expressing $CD11c^+MHCII^+Ly\text{-}6C^+$ were also the major infected cell population in footpads and draining lymph nodes during infection in C57BL/6 mice (De Trez *et al.*, 2009). This is in agreement with our data and together, these data suggest that inflammatory DCs may disseminate *L. major* and that their effector responses, such as iNOS expression, are important in controlling disease.

Our results demonstrate that DC instruction by IL-4 during *L. major* infection *in vivo* promotes IL-12 production and has other effects on DC phenotype that collectively influence protective Th1 responses and disease outcome. Our findings on the role of IL-4-mediated DC instruction in resistance to *L. major* can be summarized in the hypothetical model shown in Figure 4.1. In the absence of IL-4Rα-signalling on dendritic cells in *L. major*-infected $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice, IL-4 secreted into the microenvironment by Vβ4Vα8 $CD4^+$ T cells, basophils eosinophils and/or mast cells directly polarizes naïve T cells towards Th2 cell differentiation.

However, the Th2 response is exacerbated because DCs are no longer responsive to IL-4 and therefore IL-12 production by DCs is decreased and IL-10 increased. This leads to poor parasite control and increased parasite persistence since IL-12 is the key cytokine that drives a protective Th1 response (Figure 4.1). CD11c^{cre}IL-4Rα$^{-/lox}$ mice are therefore hypersusceptible to *L. major*. Hypersusceptibility is associated with a shift to Th2-type immune responses, with Th2 cytokines (IL-4, IL-13 and IL-10) providing a positive feedback loop for further activation and differentiation of CD4^{+} Th2 cells and induction of alternatively activated macrophages, which express increased arginase but reduced iNOS. Inhibition of iNOS favors *Leishmania* survival and growth leading to strikingly increased parasite loads in peripheral organs of CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

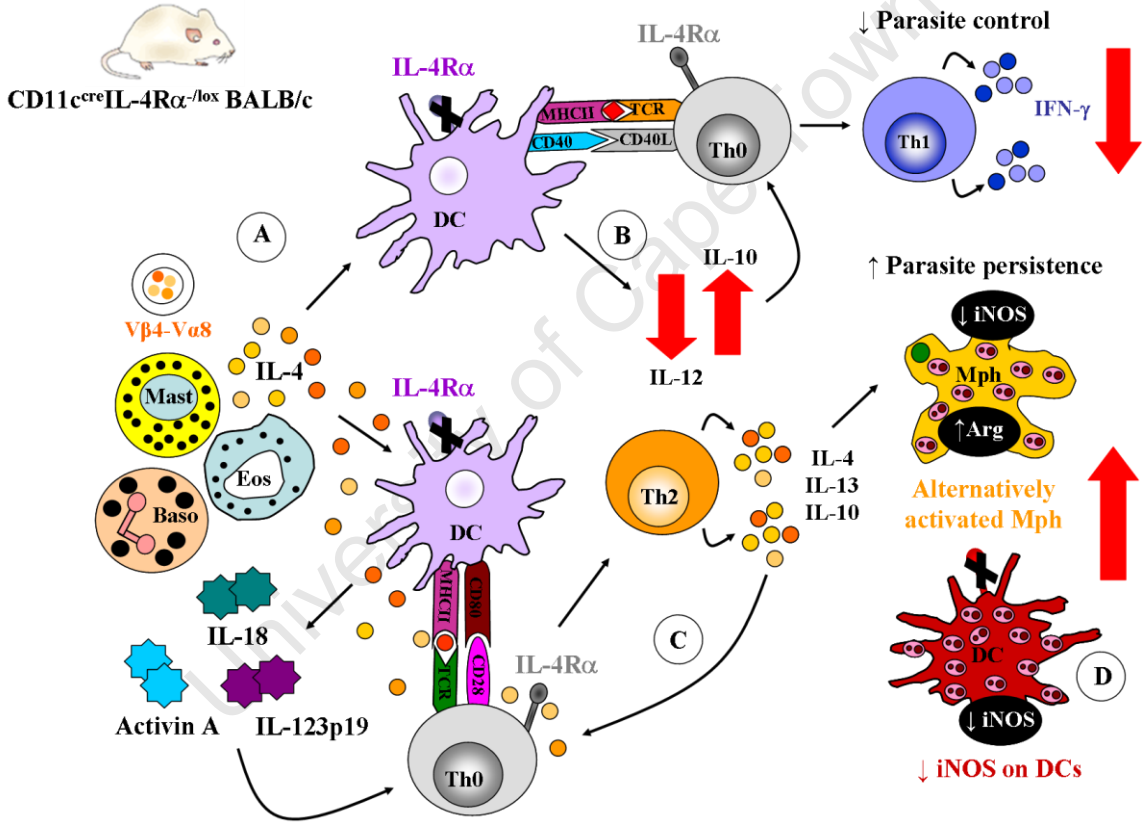

Figure 4.1: Immune responses to *L. major* infection in CD11c^{cre}IL-4Rα$^{-/lox}$ mice
(A) In the absence of IL-4Rα on dendritic cells (DCs) in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, IL-4 secreted by Vβ4Vα8 CD4^{+} T cells, mast cells, eosinophils and/or basophils in the microenvironment prime naïve T cells towards Th2 cell differentiation. **(B)** DCs are no longer responsive to IL-4 (and IL-13) and IL-12 production by DCs is reduced with increased IL-10. Since IL-12 drives protective Th1 responses, CD11c^{cre}IL-4Rα$^{-/lox}$ mice are hypersusceptible to *L. major* infection and protective Th1 responses are reduced. **(C)** Increased Th2 cytokines (IL-4, IL-13 and IL-10) continue to activate naïve cells towards Th2 differentiation and drive induction of alternatively activated macrophages (Mph) which secrete more arginase (Arg) and less iNOS, which favors *Leishmania* growth and survival, leading to higher parasite burdens in CD11c^{cre}IL-4Rα$^{-/lox}$ mice. **(D)** IL-4Rα-deficient DCs also show reduced iNOS expression, which contributes to disease progression due to increased survival of *Leishmania* parasites in infected DCs.

Moreover, $CD11b^{+}$ DCs in $CD11c^{cre}$IL-4R$\alpha^{-/lox}$ mice also show reduced iNOS expression which also enhances disease progression due to increased survival of *Leishmania* parasites in infected DCs (Figure 4.1). The Th1-inducing effect of IL-4 may not be unique to cutaneous Leishmaniasis since early reports on visceral *Leishmaniasis* caused by *L. donovani* clearly demonstrated that expansion of Th2-type cells and their cytokines did not necessarily contribute to susceptibility in mice (Kaye *et al.*, 1991). Moreover, IL-4-deficient mice were unresponsive to sodium stibogluconate treatment and developed progressive disease indicating that effective treatment of visceral Leishmaniasis was dependent on endogenous levels of IL-4 (Alexander *et al.*, 2000). High levels of IL-4 elicited after vaccination with a liposomal formulation of *Leishmania* have also been shown to instruct a Th1 response and promote resistance to *L. donovani* infection in susceptible BALB/c mice (Mazumdar *et al.*, 2004).

Instruction theory may not be restricted to Leishmaniasis, since other disease models have also demonstrated a protective role for IL-4 in promoting Th1 immune responses. Experimental infections with *Candida albicans* in IL-4 deficient mice led to impaired development of Th1 responses (Mencacci *et al.*, 1998) and a Th1 promoting effect of IL-4 has also been observed in autoimmunity (Erb *et al.*, 1997; Bagley *et al.*, 2000; Radu *et al.*, 2000), tumor immunity (Tepper *et al.*, 1989; Golumbek *et al.*, 1991; Schuler *et al.*, 1999) and contact sensitivity reactions (Salerno *et al.*, 1995; Traidl *et al.*, 1999). There is also evidence to suggest that IL-4 may promote Th1 development in humans, since both human and mouse DCs produce increased levels of bioactive IL-12 after stimulation with IL-4 (Hochrein *et al.*, 2000). A similar effect was observed in human peripheral blood mononuclear cells treated with IL-4 plus lipopolysaccharide or *Staphylococcus aureus* (D'Andrea *et al.*, 1995). Furthermore, stimulation of human monocytes with IL-4 during interaction with T cells resulted in upregulated IL-12 production (Bullens *et al.*, 2001).

Incorporating exogenous IL-4 as an adjuvant for enhancing strong Th1 responses could therefore be utilised to boost vaccine efficiency against cutaneous Leishmaniasis. Accordingly, in parallel studies, we have examined the efficacy of IL-4 as an adjuvant during BMDC-mediated vaccination against *L. major*. In this study, vaccination of BALB/c mice with IL-4Rα-deficient BMDCs failed to immunize animals against acute *L. major* infection, possibly due to reduced IL-12 and increased IL-10 secretion by the vaccinating DCs. Conversely, IL-4-responsive BMDCs (DCs with a functional IL-4Rα) that produced higher amounts of IL-12 along with lower levels of IL-10 successfully

immunized BALB/c mice against infection (Masic *et al.*, 2012). These data demonstrate that IL-4-mediated instruction of DCs is critical in eliciting protective Th1 responses during vaccination of BALB/c mice against cutaneous Leishmaniasis. The role of IL-4Rα signaling on DCs in eliciting immunity to other intracellular pathogens is therefore of interest to vaccination strategies, and an exciting avenue to be explored.

In conclusion, several studies had demonstrated a Th1-promoting effect of IL-4 *in vitro* or *in vivo* with exogenously administered IL-4, but the significance of IL-4-mediated DC instruction during disease *in vivo* had not been demonstrated. A dendritic cell-specific IL-4Rα deficient mouse model ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$ BALB/c mice) allowed us to investigate the *in vivo* effects of IL-4 and IL-13 through IL-4Rα signaling on DCs during cutaneous Leishmaniasis. The results presented in this thesis clearly demonstrate that IL-4Rα mediated instruction of DCs occurs *in vivo* and impairment of IL-4Rα signaling on DCs is severely detrimental to the host. This leads to rapid progression of disease and increased dissemination of parasites to peripheral sites. It is also clear that IL-4Rα signaling has additional, important effects on DC phenotype, which extends far beyond simply promoting IL-12 production during *L. major* infection. This study therefore expands our knowledge both on the role of dendritic cells and on the protective effects of IL-4Rα signaling on dendritic cells during cutaneous Leishmaniasis.

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PICTURE REFERENCES – FIGURE 1.6

Cutaneous: http://www.who.int/leishmaniasis/cutaneous_leishmaniasis/en/index.html,

Mucocutaneous: http://tmcr.usuhs.mil/tmcr/chapter46/leishman3.htm,

Visceral: http://drugline.org/medic/term/visceral-leishmaniasis/,

PKDL: http://www.who.int/leishmaniasis/surveillance/slides_manual/en/index1.html

APPENDIX A

General Reagents:

1. **Anaesthetic**

   1.2 ml Anaket-V (100 mg/ml) (Centaur labs, Isando)

   0.8 ml Rompun (2 %) (Bayer, Germany)

   Dissolve in 8.0 ml PBS (1×) and filter-sterilize

2. **Phosphate Buffered Saline (PBS 10×)**

   80g NaCl (1.37 M)

   2g KCl (0.03 M)

   14.4g H_2PO_4 (0.01 M)

   2.4g KH_2PO_4

   Dissolve in 1 L ddH_2O

***In vitro* Reagents:**

1. **Roswell Park Memorial Institute (RPMI)**

   1 vial RPMI (Sigma-Aldrich)

   2 g $NaHCO_3$ (37 g / L)

   800 ml ddH_2O

   100 units/ml penicillin plus 100 μg/ml streptomycin

   Adjust the pH to 7.2 – 7.4

   Make up to 1 L with ddH_2O and filter sterilize

2. **Complete RPMI**

   10% heat-inactivated fetal calf serum (FCS)

   500 μl 2-β-mercaptoethanol (0.1 mM)

   5 ml L-Glutamine (200 mM)

ELISA Reagents:

1. **ELISA Blocking Buffer**

   20g Milk powder (spar instant) (2 %)

   Make up to 1 L with 1× PBS

2. **ELISA Carbonate Coating Buffer (Cytokine ELISA)**

   100 ml 10× PBS

   1.6g Na_2CO_3

   2.9g $NaHCO_3$

   4.2g NaCl

Make to 1 L in ddH_2O and pH 9.5

3. **ELISA Coating Buffer (Antibody ELISA)**

   100 ml 10× PBS

   8g BSA (Merck)

   Make to 1 L in ddH_2O and pH 9.5

4. **ELISA Dilution Buffer**

   10g BSA (1 %) (Roche)

   0.2g NaN_3 (0.02 %) (Merck)

   Make up to 1 L with 1× PBS

5. **ELISA Substrate Buffer (for Alkaline Phosphatase conjugates)**

   0.2g NaN_3 (0.02 %)

   97 ml di-ethanolamine

   0.8g $MgCl_2.6H_2O$

   700 ml ddH2O

   Adjust the pH to 9.8 and make up to 1 L with ddH_2O

6. **ELISA Substrate Buffer (for Horseradish Peroxidase conjugates)**

   Mix an equal volume of TMB Peroxidase Substrate Solution A and Peroxidase Substrate Solution B (Roche Diagnostics, Mannheim, Germany). Add 50 µl per well.

   50 µl 1 M H_3PO_4 to stop the reaction

7. **ELISA Washing Buffer**

   20g KCL 20g KH_2PO_4

   144g $NA_2HPO_4.H_2O$

   800g NaCl (Merck-BDH)

   50 ml Tween 20 (Sigma)

   100 ml 10 % NaN_3 (Merck)

   Make up to 5 L with ddH_2O

***In vivo* Reagents:**

1. **Schneider's Drosphilia Medium (Sigma-Aldrich)**

   1 bottle Schneider's Drosphilia Medium

   0.4 g sodium bicarbonate

   800 ml ddH_2O

   Mix until dissolved.

   Adjust the pH to at least 9.2

Stir for 10 minutes. Adjust the pH to 6.7

Dissolve 0.6 g anhydrous calcium chloride in 50 ml water. Add to medium slowly.

Equilibrated to a pH of 7.0

Add 100U/ml penicillin G plus 100µg /ml streptomycin

Make up to 1 L with ddH_2O and filter-sterilize.

Add 20 % heat-inactivated FCS before use.

2. **Hank's Balanced Salt solution (HBSS)**

   1 vial HBSS (Invitrogen)

   Dissolved in PBS (1×)

3. **Red Cell Lysis Buffer**

   0.037g EDTA

   8.34g NH_4CL

   1.0g $NaHCO_3$

   Make to 1 L with ddH_2O

4. **Complete Dulbecco's Modified Eagle's Medium (DMEM)**

   1 bottle DMEM (Gibco)

   10 % FCS

   1 % HEPES

   500 µl 2-β-mercaptoethanol (0.1 mM)

   100U/ml penicillin G plus 100µg /ml streptomycin

5. **FACS Buffer**

   1 g BSA (0.1%)

   100 ml 1× PBS

   Make up in 1 L in ddH_2O

6. **MACS Buffer**

   50 ml 1× PBS

   5 g BSA (0.5 %)

   0.372 g EDTA (2 mM)

   Make up to 500 ml in ddH_2O

7. **Permebilization Buffer**

   0.5 g Saponin

   0.055 g $CaCl_2$

   0.0625 g $MgS0_4$

0.25 g NaN3 (0.5g/L)

0.5 g BSA (1g/L)

10 mM Hepes (5 ml of 1 M Hepes)

50 ml 1× PBS

Adjust to pH 7.4.

Make up to 500 ml in ddH_2O. Filter-sterilize.

NO and Arginase assays:

1. **Griess Reagent Standard (1mM $NaNO_2$)**

   6.899 mg $NaNO_2$

   Dissolve in 100 ml of ddH_2O and store at 4^oC.

2. **Griess Reagent 1**

   1 g sulfanilamide

   Dissolve in 100 ml 2.5% phosphoric acid. Cover bottle in foil and store at 4^oC.

3. **Griess Reagent 2**

   0.1 g napthyl-ethylene-diamine

   Dissolve 100 ml 2.5 %phosphoric acid. Cover bottle in foil and store at 4^oC.

4. **2.5 % phosphoric acid**

   3 ml 85 % phosphoric acid

   Dissolve in 90 ml of ddH_2O. Bring up volume to 100 ml.