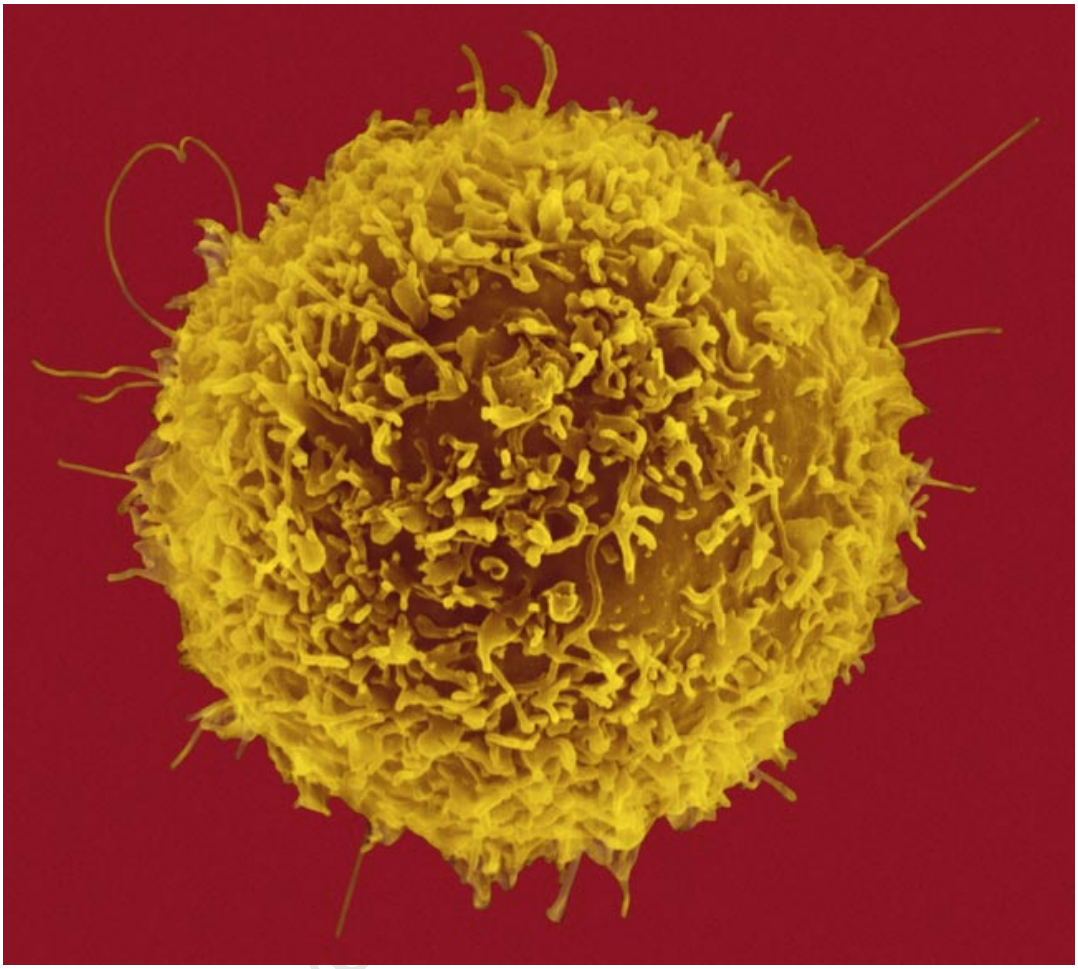
Investigating the role of B and T lymphocytes in the course of murine *Leishmania Major* infection



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Declaration

I, Andrew Einhorn, 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 nor 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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Andrew Einhorn

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Abbreviations

AP	- Alkaline phosphatase
APC	- Antigen Presenting Cell
BCA	- Bicinchonic Acide Protein Estimation
Be1/Be2	- B-effector 1/ B-effector 2
BSA	- Bovine Serum Albumen
Cre	- Cyclization recombinase
DC	- Dendritic Cell
d/e	- Differentially Expressed
ELISA	- Enzyme-linked Immunosorbant Assay
FACS	- Fluorescence-activated cell sorting
FCS	- Fetal Calf Serum
IFN-γ	- Interferon Gamma
iNOs	- Inducible Nitric Oxide synthase
i.p.	- Intraperitoneal
Ig	- Immunoglobulin
KO	- Knockout
IL	- Interleukin
IL-4Rα	- Interluekin 4 Receptor alpha
L. major	- Leishmania Major
LMR	- Leishmania Response locus
MHC	- Major Histocompatibility Complex
MVA	- M (fold change) versus A (average intensity) plot
PMA	- Phorbol 12-myristate 13-acetate
PNP	- 4-Nitrophenylphosphate
R	- Statistical software package used for microarray analysis
SLA	- Soluble Leishmania Antigen
TCR	- T cell Receptor
TLR	- Toll-like Receptor

Abstract

This thesis is composed of two parts. The first is an investigation of the role of B-effector cells in the course of murine *Leishmania Major* (*L. major*). The second is a bioinformatic analysis of microarray slides of activated CD4 T-cells from *L. major* infected BALB/c and C57BL/6 mice in order to investigate the genetic determinants of their divergent disease phenotypes. Overall this thesis is an investigation of the role of B- and T- lymphocytes during the course of *L. major* infection in mice.

B-cells are not traditionally thought to play a role in the pathogenesis of Leishmania major infection. It is well documented that T-helper 1 (Th1) and T-helper 2 (Th2) responses lead to resistance and susceptibility to *L. major* respectively. Recent studies have now shown that B-cells are capable of producing key T-cell differentiating cytokines such as IL-4 and IFN-γ. More specifically, two new B-cell populations have been identified: B effector 1 (Be1) and B effector 2 (Be2) cells, which produce IFN-γ and IL-4 respectively. Using mice lacking the IL-4Rα on B-cells, and thus incapable of producing Be2 cells, we investigate the contribution of Be2 cells to BALB/c susceptibility. We confirm that the deletion of the IL-4Rα on B-cells renders the previously susceptible BALB/c mice resistant to *L. major* strain LV39 and further confirm the phenotype using the more the virulent IL81 strain. We demonstrate that mice lacking Be2 cells exhibit a diminished Th2 response and an enhanced Th1 response, suggesting that B-cells, in addition to their role in antibody production, may also play a role in shaping T-helper responses *in vivo*. In the case of *L. major*, this provides evidence for a novel link between B cells and the cellular immune response.

For many years the divergent phenotypes of BALB/c and C57BL/6 mice during *L. major* infection have provided the paradigm for the Th1/Th2 dichotomy. Whilst this disease model has helped elucidate the role of many key genes including IL-4 and IFN-γ, the genetic determinants of this divergence have not yet been established. By analysing the differential gene expression of activated CD4 T-cells from BALB/c and C57BL/6 mice at three weeks post-infection with *L. major*, we produced a list of 372 differentially expressed genes. By mapping these to known Leishmania Response loci, we narrowed the list to 72 candidate genes. Based on the extent of differential expression we conducted literature searches on 28 of these genes and produced a list of 9 candidate genes most likely to be implicated in genetic determination of BALB/c susceptibility and C57BL/6 resistance.

Part I

1 Introduction

The *Leishmania major* (*L. major*) parasite is transmitted by the bite of a female sand fly. The sand fly bite results in damaging of the microvasculature, which in turn creates a hemorrhagic pool. This initiates a local inflammatory response and recruitment of monocytes and neutrophils to the site of the wound (Belkaid et al., 2000; Kamhawi et al., 2000). Simultaneously, *L. major* parasites in their promastigote phase enter the wound, typically numbering between 1,000 - 10,000 highly enriched metacyclic promastigotes (Rogers et al., 2004). Sand fly saliva has been shown to facilitate the initiation of infection by inducing monocyte chemo-attractants (Teixeira et al., 2005). This is further facilitated by the parasite itself which induces macrophages to secrete MCP1 and CXCL1 – chemo-attractants for monocytes and neutrophils respectively (Racoosin and Beverley, 1997).

At the outset of infection, the wound site is occupied predominantly by neutrophils. By week two post-infection, there is a large influx of monocytes into the infection site and this triggers a more detrimental inflammatory response (Leon et al., 2007). These monocytes differentiate into dendritic cells (DCs) which are thought to be responsible for the uptake and transport of Leishmania parasite to the lymph node (Leon and Ardavin, 2008) for presentation to antigen-specific T cells. While in healer strains such as C57BL/6, DCs initiate a protective response by producing Th1-inducing IL12, in susceptible strains - through mechanisms which are yet to be properly understood - this process leads to the induction of a susceptible Th2 response (Sypek et al., 1993; Vieira et al., 1994).

The Leishmania model has played an important role in furthering our understanding of the Th1/Th2 cellular immune response. The T-helper 1 (Th1) response observed in the healer strain C57BL/6 and the Th2 response observed in the susceptible strain BALB/c gave the first insights into how differing T-helper responses could influence disease outcomes. In the T-helper 1 response, CD4 T-cells are activated under the influence of IL12 and mature to become IFN-γ producing T helper cells. In the T-helper 2 response, CD4 T-cells are activated under the influence of interleukin 4 (IL-4) and go on to produce IL-4, IL-5 and IL-13. These different cytokine panels play different

instructional roles and afford the body at least two polarized methods of handling different types of pathogens.

1.1 The Role of B-cells in T-cell Priming

The extent to which B-cells are involved in T-cell priming is still a matter of debate. Some studies suggest that T-cell priming can occur efficiently in the absence of B-cells (Cunningham et al., 2002). Furthermore, $IgM^{-/-}$ mice – mice lacking membrane-bound IgM and hence mature B-lymphocytes – have been shown to launch fully-fledged Type 1 and Type 2 CD4 T-helper cell responses upon administration of antigen in adjuvant (Epstein et al., 1995; Phillips et al., 1996). Immune competent CD8 T-cell responses have also been observed in B-cell deficient $IgM^{-/-}$ mice (Asano and Ahmed, 1996).

While these studies demonstrate that B-cells are not integral to T-cell priming, they do not rule out the possibility that B-cells can contribute to the shaping of the T-cell response. Using $IgM^{-/-}$ mice, several studies have investigated their impact on T-cell responses. Whilst the above studies demonstrated that B cells are not required for successful T-cell responses, some studies have shown that B-cells impact the measure and extent of the Th1 and Th2 responses (Langhorne et al., 1998; Mastroeni et al., 2000). Other reports suggest that it is less about a Th1 or Th2 bias, and more that B-cell deficient mice show an overall reduction in antigen specific T-cells in circulation (Bergmann et al., 2001; Rivera et al., 2001). One study showed that B-cells are more efficient at presenting protein antigens compared with peptide antigens, (Constant et al., 1995), suggesting the extent to which they participate in T-cell priming might be dependent upon the antigen breakdown of the pathogen involved.

While there are an abundance of studies that implicate B-cells in T-cell priming, it is still not clear to what extent and how they influence the T-helper cell response, whether or not they lead to a Th1 or a Th2 bias, and whether they - together with other antigen presenting cells (APCs) - govern the T-helper cell response, or simply serve to amplify and sustain an existing T-helper cell response. The purpose of this research is to clarify, in the case of one disease model (*L. major*) whether B-effector cells contribute to disease outcome.

1.2 The Role of B-cells in *Leishmania Major*

B-lymphocytes are traditionally thought to play a minimal role in the outcome *L. major* infection. The nature of the T-helper response is thought to be the predominant determinant of phenotype. However, several pieces of evidence suggest the contrary. Firstly, anti-IgM treated mice show enhanced resistance against cutaneous infection (Sacks et al., 1984). Secondly, *in vitro* antigen presentation by B-cells elicited high levels of IL-4 production by T-cells in contrast to macrophages from the same mouse, which elicited no IL-4 production but high levels of IFN-γ (Rossi-Bergmann et al., 1993). Thirdly, B-cell deficient $IgM^{-/-}$ mice showed resistance against infection with the LV39 strain in contrast to their wildtype BALB/c counterparts (Ronet et al., 2008). It was further shown that $IgM^{-/-}$ mice reconstituted with B cells from BALB/c mice reverted to a susceptible phenotype, but that $IgM^{-/-}$ mice reconstituted with $IL10^{-/-}$ B-cells remained resistant (Ronet et al., 2010). IL10 is known to down-regulate IL12 production by dendritic cells and could thus be responsible for diminishing the healer Th1 response (O'Garra and Murphy, 2009). The tentative conclusion from this array of studies is that B cells may play a role in susceptibility to *Leishmania*, possibly through their production of IL10 and subsequent down-regulation of the key Th1 factor IL12, or through their production of other differentiating cytokines such as IL-4 and IFN-γ.

1.3 The Role of Antibody in *Leishmania Major*

For a long time it has been known that antibody isotypes and titer correlate with lesion size in dogs and humans during various types of *Leishmania* infection (Lindsay, 2002). In particular, studies of *Leishmania infantum* in dogs showed that high IgG1 and IgG2 titers correlate with symptomatic and asymptomatic disease outcomes, respectively (Ozbilge et al., 2006). Several studies have been conducted to examine the role of antibodies during murine *L. major* infection, but the evidence is inconclusive. One study showed that sera transfers from infected BALB/c mice to resistant $IgM^{-/-}$ mice had no noticeable effect on disease progression (Ronet et al., 2008). Another showed that sera transfers from infected BALB/c mice to resistant B-cell deficient JhD mice led to exacerbated pathology, ostensibly on account of elevated IL10 secretion by macrophages at the site of infection (Miles et al., 2005).

Several studies on other cutaneous Leishmania strains suggest that antibody may play an important role in disease progression in mice. One study of *L. amazonensis* showed lowered CD4 T-cell numbers and smaller lesion size in B-cell deficient JhD mice

compared with their susceptible wildtype counterparts (Wanasen et al., 2008). Adoptive transfer of immune sera from infected mice restored CD4 T cell activation and reverted the mice to a susceptible phenotype. Two other studies have shown that ligation of immunoglobulins with Fc receptors on inflammatory macrophages led to the production of IL10 (Kane and Mosser, 2001; Yang et al., 2007). IL10 is known to prevent macrophage activation(Lang et al., 2002), and these studies demonstrated that the elevated levels of IL10 led to reduced levels of IL12 and TNFa along with enhanced parasite replication and proliferation *in vitro* (Kane and Mosser, 2001). In spite of the fact that these experiments were done *in vitro*, and used a different strain of Leishmania (*L. amazonensis*), they still provide a theoretical framework whereby antibody interaction with macrophages could alter the course of *L. major* infection in mice.

The literature on the role of B cells in T-cell priming contains many seemingly contradictory results. That being said, two major conclusions can be drawn from existing literature. Firstly, there are enough independent pieces of evidence suggesting that B cells are implicated in T cell priming that the hypothesis (i.e. that B cells play a role in *L. Major* disease outcome) can be taken seriously. Secondly, B cells known as B effector cells are capable of producing T-helper cell differentiating cytokines such as IL-4 and IFN-γ. Analysis of the role of the latter shall be the primary focus of this research.

1.4 Purpose of this Research

It remains to establish whether and to what extent B effector cells can alter the course of T helper cell response *in vivo*. In order to do this, we used a novel BALB/c mouse deficient for IL-4Rα signaling on B-cells (mb1^{cre}IL-4Rα$^{-/lox}$) by gene targeting and site-specific recombination using the cre/loxP system (Hoving et al., 2012). Because IL-4Rα signaling on B-cells is crucial to the development of Be2 cells (Harris et al 2005), these mb1^{cre}IL-4rα$^{-/lox}$ mice are incapable of producing Be2 cells. The experimental design is thus a comparison between the mb1^{cre}IL-4Rα$^{-/lox}$ mouse which is expected to produce Be1 cells only and its heterozygous littermate, the IL-4Rα$^{-/lox}$ mouse which is capable of producing both Be1 and Be2 cells. Upon infection with *L. major*, we observe first if there is a divergent phenotype, and if there is, investigate the shape of the T-helper cells response and the mechanism behind these diverging phenotypes.

2 Materials and Methods

Ethics Statement

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.

Mice Used

Mice with a global (IL-4Rα $^{-/-}$) (Mohrs et al., 1999) or B cell specific (MB1^{Cre}IL-4R α $^{-/lox}$) (Hoving et al., 2012) deletion of IL-4Rα, together with its littermate control (IL-4Rα $^{-/lox}$), IgM$^{-/-}$ mice (Kitamura and Rajewsky, 1992) and experimental controls (BALB/c and C57BL/6) were used for this project. All mice were bred and housed under specific-pathogen-free conditions at the University of Cape Town, South Africa and experiments were approved by the University's Animal Ethics Committee. The mice were matched for age and gender and were maintained under barrier conditions in the biosafety level 2 facilities in individually ventilated cages. Prior to experiment, the mice were genotyped by PCR.

2.1 L. major Passage

L. major parasites (LV39 MRHO/SU/59/P and IL81 MHOM/IL/81/Friedlin) were passaged in susceptible BALB/c mice aged 6-8 weeks. Mice were anaesthetized using Anaket (12 mg/ml) and Rompun (0.16 %) in 1xPBS, and infected in the left-hind footpad with 2 x 10^5 - 2 x 10^6 promastigote parasites suspended in 50 ul PBS using a 26 guage needle. Mice were sacrificed at 6 - 8 weeks post-infection, and infected footpads and popliteal lymph nodes removed for *ex vivo* culture. Footpads and lymph nodes were cultured in 10ml Schneider's medium (20% FCS) in culture flasks (Corning Life Sciences) at room temperature. Parasite cultures were split every 5-7 days: Cultures were checked for contamination before 9ml of supernatant containing parasite was aspirated from flask, washed, and frozen at -80 °C for soluble leishmania antigen (SLA). Corning flasks were replenished with 9ml Schneider's medium (20% FCS). *In vitro*

cultures were split a minimum of two times before use for infection, and a maximum of eight times.

2.2 L. major Infection

L. major parasites were pooled and centrifuged at 500 rpm for 10 mins to pellet debris. Supernatant was then centrifuged at 3000 rpm for 5 minutes to pellet parasites. Parasites were resuspended in 1xPBS and centrifuged at 3000 rpm for 5 minutes. The wash step was repeated 3 times. Parasites were resuspended in 3-5 ml 1xPBS and counted using a *Leishmania* counting chamber. Parasites were then resuspended at 4 x 10^7 parasites/ml and mice infected in the left hind footpad with 50 ul of parasite.

2.3 Disease Progression and Parasite Burden

Disease progression was monitored using footpad swelling. Mouse footpad swelling was measured using calipers (Kroeplin Langenmesstechnik) prior to infection, then once weekly post-infection. Uninfected footpads typically measured 1.8-2.1 mm. Infected mice were sacrificed using halothane asphyxiation followed by cervical dislocation.

Lymph Nodes: Infected lymph nodes were removed using a scalpel and dissecting forceps and placed in 1ml DMEM complete supplemented with 5% FCS. Lymph nodes were mashed through a 40 um cell strainer (Becton Dickinson) into a 50ml Falcon tube in order to create a single cell suspension, and resuspended in 6.4ml DMEM 5% FCS per lymph node in suspension.

Footpads: The skin and toes of infected footpads was removed using scissors and dissecting forceps. Footpads were placed in a 10 ml Greiner Tube containing 2ml Schneiders medium (20% FCS), homogenized using a polytron homogenizer, and topped up to 6.4ml per footpad.

Parasite numbers were measured using limiting diltuion assay (Mohrs et al., 1999). Dilutions were done in flat-bottom 96-well plates (BD Biosciences). Each well except for the first row was filled with 100 ul Schneiders (20% FCS). 200 ul of suspension from either lymph node or footpad homogenates was seeded in the first row in duplicate. 2-fold serial dilutions were then made across 96-well plates. Plates were wrapped in tin foil and stored at RT. After 7 days, wells were examined for the presence of parasites. The number of parasites present for a particular sample is given by the 2^(6+number of wells containing parasite).

2.4 Enzyme-linked Immunosorbant assays (ELISA)

Cytokine Elisa

Anti-cytokine antibodies (IFN-γ, IL-4, IL10, IL12 - see Appendix D for company/cat#) were diluted in 1xPBS or Carbonate Coating Buffer (15mM $NaCO_3$, 35mM $NaHCO_3$, 71mM NaCl in 1xPBS – pH 9.5). Wells were coated with diluted antibody (50 ul per well) in a 96-well plate and incubated overnight at 4°C. Plates were blocked using 2% milk powder in 1xPBS and incubated at 4°C overnight. 50 ul of serum/supernatant was added to each well: standards diluted 1:3 and samples diluted 1:3 (Dilution buffer: PBS 1% BSA). Plates were incubated overnight at 4°C. Biotinylated secondary antibody was diluted in dilution buffer and 50 ul added to each well and incubated at 37°C for 2 hours. 50 ul of streptavidin was added and incubated for 1 hour at 37°C. 50 ul of PNP substrate was added (1mg/ml) and allowed to develop. Absorbance was read at 405nm using (reference wavelength 492 nm) and stopped using 25 ul of 1M NaOH.

Antibody Elisa

ELISA was carried out to quantify the amount of antigen-specific IgG1, IgG2a, IgG2b and IgG3 present in the serum samples from infected animals. ELISA plates (NUNC) were coated with 50 ul SLA at 25 ug/ml in coating buffer (1xPBS) and incubated overnight at 4°C. Plates were blocked with 200 ul of blocking buffer (PBS 2% milk powder) overnight at 4°C. Samples were diluted 1:3 with a beginning concentration of 1:1 or 1:10 and incubated at 4°C overnight. 50 ul of secondary alkaline phosphatase (AP) conjugate isotype (IgG1, IgG2a, IgG2b, IgG3) – see Appendix D for company/cat# - was added and incubated for 3 hours at 37°C. After three sets of washing using wash buffer, 50 ul of purinenucleosidephosphorylase (PNP) substrate solution (1 mg/ml) was added to each well. Reaction was stopped using 25 ul NaOH and absorbance read using VersaMax ELISA microplate reader at 405 nm (reference wavelength 492 nm). Optical density (OD) was plotted against dilution to ascertain relative quantities of antibody present in the different samples using Softmax Pro.

2.5 SLA Preparation

L. major parasites from culture were stored in PBS in 2 ml eppindorfs at -80°C. For the extraction of the soluble proteins used in cell stimulations and antibody ELISA, the following procedure was followed. Parasites were thawed on ice and centrifuged at 500 rpm for 10 minutes to pellet. Parasites were resuspended in sterile PBS containing

protease inhibitor (Sigma Aldrich), and suspension was transferred to a 12 ml Greiner tub. To lyse, parasites were sonicated 4 times for 20 seconds at 20 second intervals. Lysate was transferred to a sterile eppindorf and centrifuged at 8000 rpm for 10 minutes to pellet the debris. The resulting supernatant was filtered through a 0.22 um filter syringe and the protein concentration was determined using a BCA assay according to manufacturer's instructions. 500 ul aliquots were made and stored at -80°C.

2.6 Cell Restimulation

The cell restimulations were carried out to quantify the amount and type of cytokines produced by knockout mice relative to their wildtype counterparts. Three primary restimulations were conducted: unstimulated (i.e. media only), αCD3, and SLA. For the unstimulated samples, 1 x 10^6 cells were resuspended in a final volume of 500ul DMEM Complete 10% FCS in a 48-well plate. For the αCD3 restimulation, plates were coated with 200 ul of 20 ug/ml αCD3 in PBS and incubated at 37°C for 30 minutes. PBS was aspirated and cells resuspended in 500ul DMEM Complete 10% FCS were plated in the coated wells. For the SLA restimulation, cells were resuspended in DMEM Complete 10% FCS containing SLA at a concentration of 50 ug/ml. Samples were incubated at 37°C for 72 hours. Plates were centrifuged at 1600 rpm for 5 minutes and supernatants transferred to duplicate plates and stored at -20°C for later use in the cytokine ELISA.

2.7 Fluorescence Activated Cell Sorting (FACS)

Staining of Cell Surface Markers

Extra-cellular fluorescence-activated cell-sorting (FACS) was used to quantify cell populations from the lymph node, measure relative abundance of surface markers on cells, or separate cell populations physically by sorting. The staining procedure for all three processes was the same, and the quantities outlined below are sufficient for 2-5 x 10^6 cells. For quantities of cells larger than 5 x 10^6, dilution volumes were scaled up appropriately. Cells were kept at 4°C at all times. Cells were pelleted in FACS tubes by centrifuging at 1600rpm for 5 minutes, and resuspended in 50ul staining antibody diluted in ice-cold FACS buffer containing 1% heat-inactivated rat serum and 10 ug/ml 2.4.G.2 Fc Block (FACS Buffer = PBS + 0.1% BSA). Cells were incubated on ice in dark

for 20 minutes. Cells were subsequently washed in ice-cold FACS buffer. If biotinylated antibodies were used, streptadvidin-antibody conjugates were added in a volume of 50 ul (1% rat serum, 1% Fc Block) and incubated for 15 minutes on ice in dark. Cells were washed three times in FACS buffer and resuspended in a volume of 200ul for acquisition on the flow cytometer (BD Biosciences Facscalibur). Resulting data was analysed using FlowJo (Ver. 9.5.2).

Intracellular FACS

In conjunction with extra-cellular staining, intra-cellular FACS was used to quantify relative amounts of intra-cellular proteins between different samples or cell populations. Cells were restimulated with PMA/Ionomycin to increase intracellular cytokine production. They are then stained with surface markers, after which they are fixed and then stained intracellularly.

Stimulation: 2 x 10^6 cells per sample were pelleted in FACS tubes by centrifuging at 1600 rpm for 5 minutes. Supernatant was flicked off and cells resuspended in 500 ul DMEM Complete 5% FCS containing PMA (50 ng/ml), Ionomycin (250 ng/ml) and Monensin (200 uM). Cells were incubated for 4 hours at 37°C.

Intracellular staining: Cells were stained with the appropriate extracellular antibodies according to the extracellular staining protocol above. Cells were then washed and resuspended in 200 ul ice-cold PBS. An equal volume of 4% paraformaldehyde in PBS was then added to achieve a final concentration of 2%, and incubated at 4°C o/n. Following incubation, cells were centrifuged, resuspended in 25 ul permeabilisation buffer containing 2% rat serum, 1% 2.4.G2 Fc block, and incubated on ice for 10 minutes. Cytokine antibodies were diluted at 2x final concentration in permeabilisation buffer containing 2% rat serum and 1% Fc block, and 25 ul of stain was added to each sample (total volume 50 ul). Cells were left to stain on ice for 45 minutes. Cells were washed in permeabilisation buffer and resuspended in FACS buffer for acquisition on the flow cytometer (BD Biosciences FACScalibur).

2.8 Sera Collection

Sera was collected from $MB1^{Cre}$ and BALB/c mice at 3 - 8 weeks post infection with low dose (2 x 10^5) *L. major* IL81 using the cardiac puncture method for blood collection. In brief, infected mice were anaesthetised using the standard dose of ketamine HCl (Anaket) and xlyazine (Rompun). The cardiac puncture was conducted using a 1 ml

syringe and a 22 gauge needle according to the procedure outline by Hoff et al (Hoff, 2000). 1 - 1.5 ml of antibody containing blood was extracted per mouse and stored in 1.5 ml gel separator tubes (Microtainer, BD, USA). Samples were centrifuged at 8000 rpm for 15 minutes at 4°C to extract the red blood cells and isolate the antibody-containing sera. Sera was either used directly for transfers or otherwise stored at -80°C in the gel separation tubes.

2.9 Sera Transfers

5 groups of 7 mice were infected for the purposes of this experiment:

Table 2. Groups of Mice Used for Sera Transfer Experiments

	Mouse Strain	**Sera Received**	**Type**
Group 1	$MB1^{Cre}$	None	Control
Group 2	BALB/c	None	Control
Group 3	$IgM^{-/-}$	None	Control
Group 4	$IgM^{-/-}$	MB1Cre	Experiment
Group 5	$IgM^{-/-}$	BALB/c	Experiment

Prior to any sera transfers taking place, mice were infected using low dose (2×10^5) IL81 *L. major* parasite according to the infection procedure detailed previously. Sera transfers were conducted on days 3, 10, 17, 24 and 31 post-infection. Sera collected from infected $MB1^{Cre}$ mice was thawed on ice and pooled. Each recipient mouse received 200ul of antibody-containing immune sera intraperitoneally (i.p) on each date of transfer. Similarly, sera collected from infected BALB/c mice was transferred into the $IgM^{-/-}$ mice designated recipients of BALB/c sera. Footpad swelling was monitored throughout the infection using Vernier Callipers and mice were killed at 6 weeks post infection for quantification of parasite burden.

3 Results

3.1 Generation of the B-cell Specific IL-4Rα knockout mice

Mice expressing Cre recombinase under control of the mb1 promoter (Hobeika et al., 2006) were backcrossed to BALB/C for 9 generations, then intercrossed with global IL-4Rα$^{-/-}$ BALB/C mice (Mohrs et al., 1999) to generate mb1^{cre}IL-4Rα$^{-/-}$ mice on a BALB/C genetic background. These mice were intercrossed with floxed IL-4Rα$^{lox/lox}$ BALB/C mice (exon 6 to 8 flanked by loxP) (Herbert et al., 2004) to generate mb1^{cre}IL-4Rα$^{-/lox}$ BALB/C mice. Characterization of the mb1^{cre}IL-4Rα$^{-/lox}$ mice has been described previously (Hoving et al., 2012). These studies demonstrated that IL-4Rα was efficiently depleted on all B-cells in the spleen, bone marrow and peritoneal cells of the mb1^{cre}IL-4Rα$^{-/lox}$ mice. In contrast, CD4, CD8 and NK T-cells as well as macrophages were shown to express the IL-4Rα at levels comparable to the heterozygous littermate IL-4Rα$^{-/lox}$.

3.2 Mb1^{cre}IL-4Rα$^{-/lox}$ mice Do Not Express IL-4Rα on B-cells

The expression of IL-4Rα was tested on major lymphocyte populations (B and T-cells) using FACS to investigate whether the B cell specific depletion of IL-4Rα remained stable after *Leishmania* infection. Single cell suspensions were prepared from the draining popliteal lymph nodes of mice infected with *L. major* LV39 at week 8 post infection. Total cells were stained with anti-CD4, anti-CD19 and anti-IL-4Rα antibodies. As shown in Figure 3.1, IL-4Rα was efficiently depleted on B cells from the LN in mb1^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.1B) whilst other cell types, such as T cells, had comparable IL-4Rα expression in mb1^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate controls (Figure 3.1C). Mb1^{cre}IL-4Rα$^{-/lox}$ mice show a marked reduction in IL-4Rα expression on lymph node cells as a whole in comparison with the wildtype control (Figure 3.1A). Upon further analysis of the lymphocyte subsets, mb1^{cre}IL-4Rα$^{-/lox}$ mice exhibit normal levels of IL-4Rα expression on CD4 T-cells but essentially complete abrogation of expression of IL-4Rα on CD19+ cells compared with the littermate control (Figure 3.1B-C).

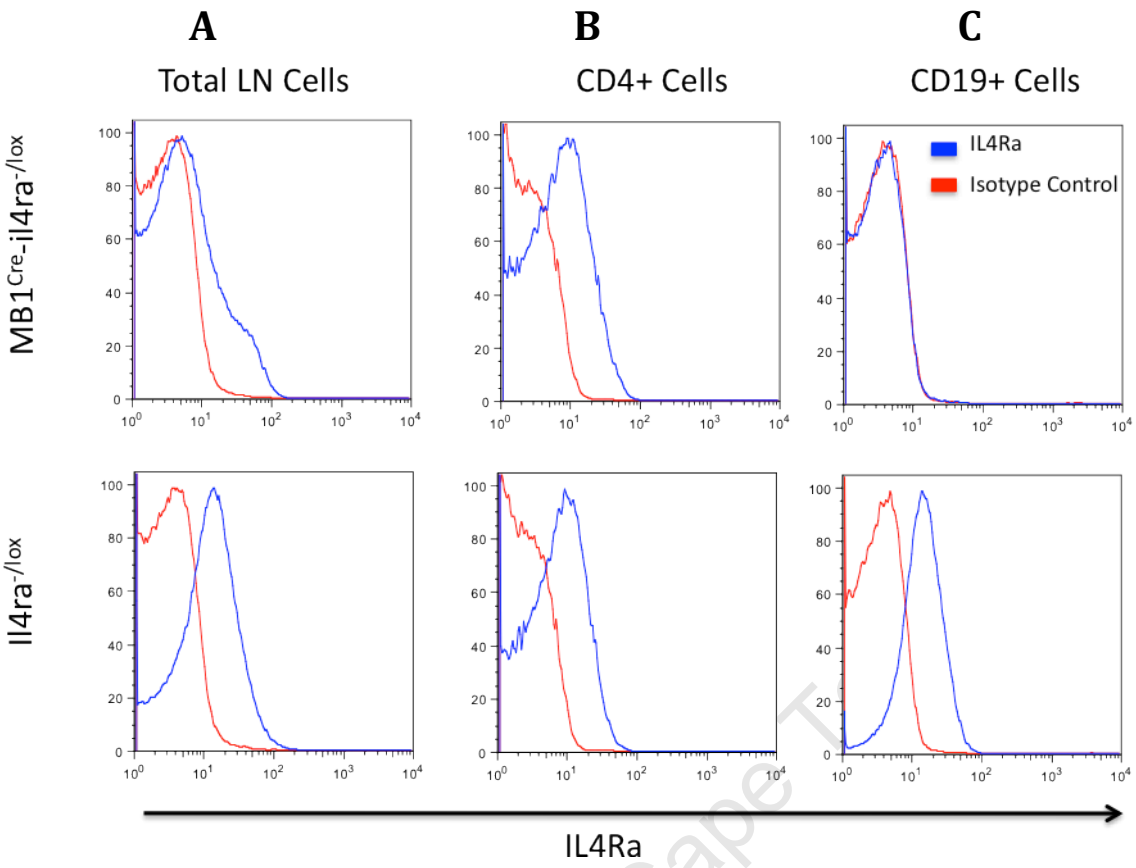


Figure 3.1. B-cells in mb1^{Cre}IL-4Ra$^{-/lox}$ do not express IL-4Ra. Mice were infected with 2x10^6 *L. major* LV39 and expression of IL-4Ra was analysed by FACs on popliteal lymph node cells isolated from mb1^{Cre}IL-4Ra$^{-/lox}$ mice and heterozygous littermate IL-4Ra$^{-/lox}$ at week 8 post infection. Lymphocyte populations were gated as B cells (CD19+CD3-) and CD4 T cells (CD4+CD8-) and histogrammes of IL-4Ra expression on CD4 or CD19 population was analyzed using FlowJo. A representative sample of three separate experiments is shown.

3.3 Mb1^{cre}IL-4Rα$^{-/lox}$ mice are resistant to *L. major* strains LV39 and IL81

In order to investigate the role of IL-4Rα signaling on B cells during cutaneous Leishmania, mb1^{cre}IL-4Rα$^{-/lox}$ mice were infected with *L. major* LV39 (Figure 3.2) and the more virulent IL-81 strain (Figure 3.3) in the left hind footpad (strains MRHO/SU/59/P and MHOM/IL/81 respectively).

3.3.1 Mb1^{cre}IL-4Rα$^{-/lox}$ exhibit reduced footpad swelling

Upon infection with *L. major* LV39, the footpad swelling in the mb1^{cre}IL-4Rα$^{-/lox}$ mice is consistent with earlier findings in our laboratory (Revaz-Breton et al, manuscript in preparation). Whereas the IL-4Rα$^{-/lox}$ mice show susceptibility to infection (increased and sustained footpad swelling, lesion development after 7-8 weeks), the mb1^{cre}IL-4Rα^{-}

$^{/lox}$ mice seemed to control lesion development, and footpad swelling trajectory more closely resembled that of the resistant C57BL/6 mice, with swelling peaking at around 3-4 weeks post infection and thereafter reducing (Figure 3.2A; At 8 weeks: mb1^{cre}IL-4Rα$^{-/lox}$ = 2.46mm; IL-4Rα$^{-/lox}$ = 3.5mm; pval = 0.006)

We followed this experiment with an infection using the more virulent *L. major* IL-81 parasite strain and found that mb1^{cre}IL-4Rα$^{-/lox}$ mice remained resistant to infection (Figure 3.3A; At 6 weeks: mb1^{cre}IL-4Rα$^{-/lox}$ = 2.3mm; IL-4Rα$^{-/lox}$ = 3.2mm; pval = 0.0049). Similar to the *L. major* LV39 infection, footpad swelling peaked at around 4 weeks post-infection, and reduced thereafter, mirroring the trajectory of the resistant C57BL/6 control mice. This result suggests that the mb1^{cre}IL-4Rα$^{-/lox}$ phenotype is consistent across different strains of *L. major*.

3.3.2 Mb1^{cre}IL-4Rα$^{-/lox}$ exhibit reduced parasite burden

In all experiments, parasite burden was quantified using limiting dilution assay (Mohrs et al., 1999). Parasite burden was measured at two time points, once at 3 weeks post infection and once at 8 weeks post infection (end of experiment). In the case of *L. major* strain IL81, parasite burden was measured at 3 weeks and 6 weeks post infection due to the shorter duration of the infection.

Parasite burden was measured in both the footpad and the draining lymph node of infected mice. During infection with *L. major* LV39, parasite burden in the LN of mb1^{cre}IL-4Rα$^{-/lox}$ and littermate control mice was comparable at both 3 weeks and 8 weeks after infection (Figure 3.2B). The resistant C57BL/6 mice show a parasite burden in the lymph node two orders of magnitude lower than the littermate control at both time points after infection.

Parasite burden measured in the footpads of mb1^{cre}IL-4Rα$^{-/lox}$ and littermate control mice was comparable at 3 weeks post-infection. However, at 8 weeks post-infection, the resistant mb1^{cre}IL-4Rα$^{-/lox}$ mice showed significantly lower parasite burdens in the footpad (1/100th) compared to the littermate control (Figure 3.2C).

The same phenomenon was observed during infection with *L. major* IL81 strain. Lymph node parasite burden did not differ significantly between littermate control and mb1^{cre}IL-4Rα$^{-/lox}$ at either 3 or 6 weeks after infection. However, parasite burden in the footpad was markedly reduced in mb1^{cre}IL-4Rα$^{-/lox}$ mice in comparison with littermate control mice at 6 weeks post-infection, consistent with the reduced footpad swelling observed in these mice (Figure 3.3B-C).

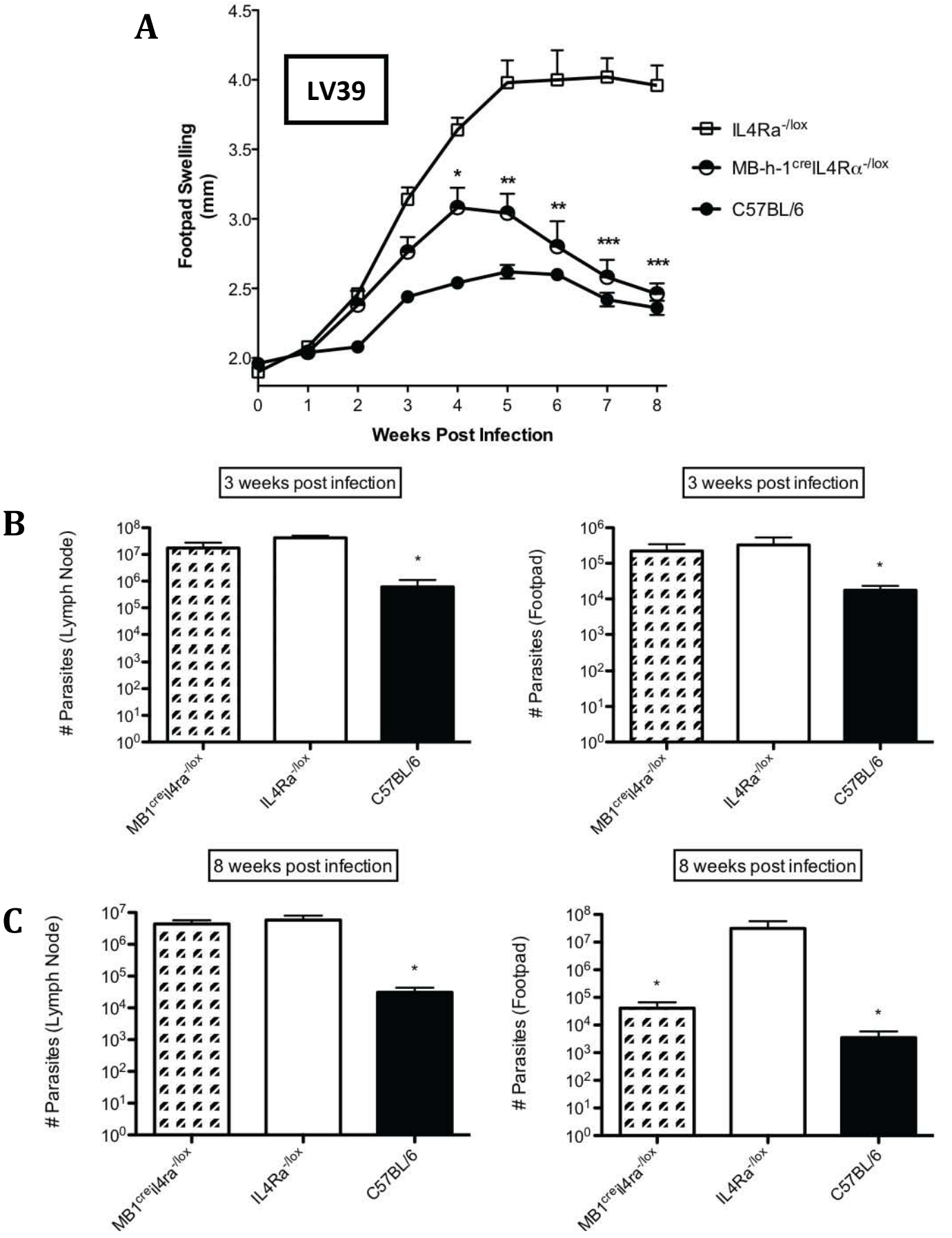


Figure 3.2: Mb1^{cre}IL-4ra$^{-/lox}$ phenotype upon infection with 2x10^6 L Major LV39. Groups of 12 mb1^{cre}IL-4ra$^{-/lox}$, control IL-4Ra$^{-/lox}$ and C57Bl6 were infected with 2x10^6 L Major parasite in their left hind footpads. (A) Footpad swelling was measured over the course of 8 weeks using callipers (Kroeplin Langenmesstechnik). (B) Parasite burden at 3 weeks post infection. (C) Parasite burden at 8 weeks post infection. For all figures: Mean +- SEM; * => p-value of <0.05 relative to the IL-4Ra$^{-/lox}$; ** < 0.01; *** < 0.001. Results are reflective of two or more separate experiments.

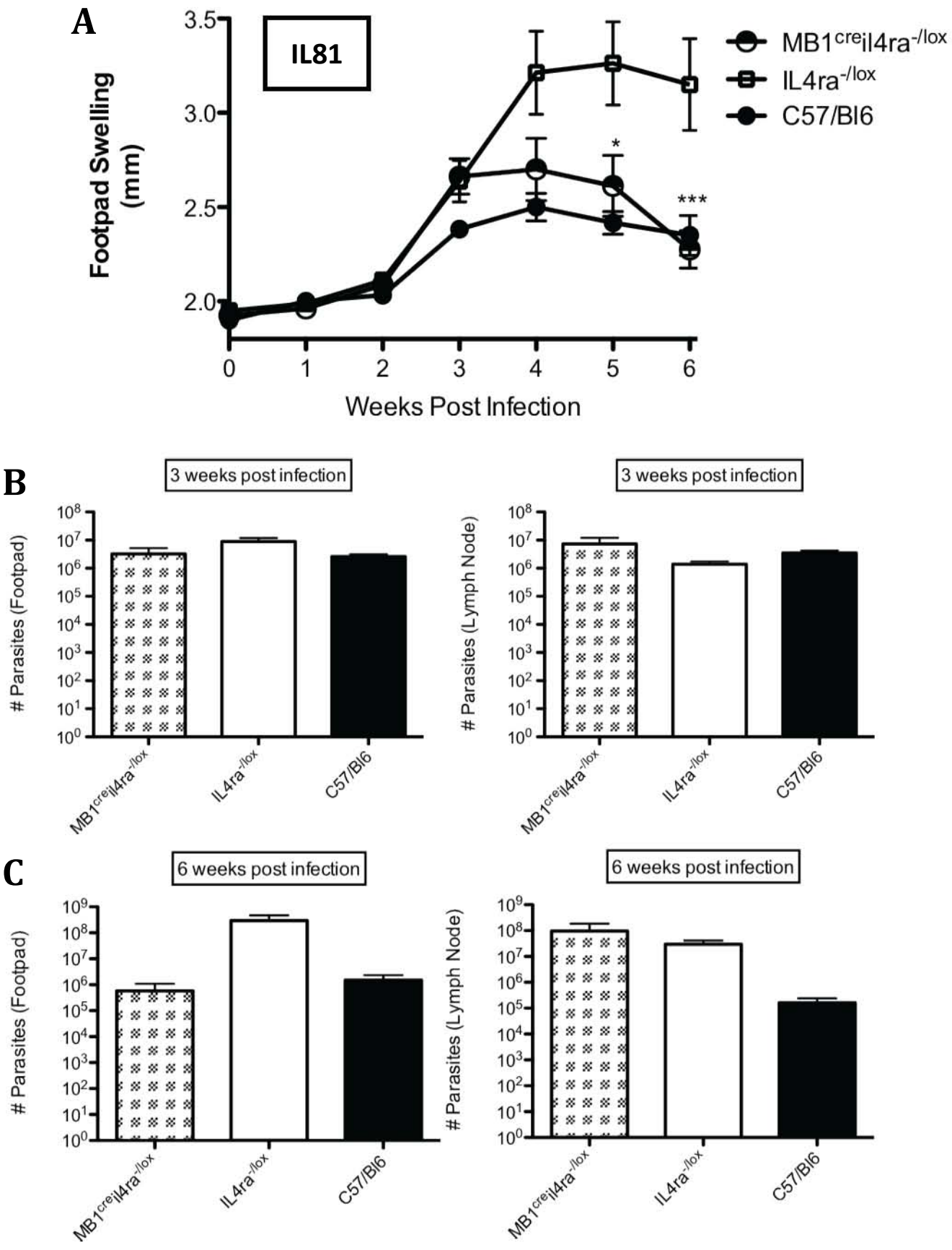


Figure 3.3: Mb1^{cre}IL-4ra$^{-/lox}$ phenotype upon infection with 2x10^5 L Major IL81. Groups of 12 mb1^{cre}IL-4ra$^{-/lox}$, control IL-4Ra$^{-/lox}$ and C57Bl6 were infected with 2x10^5 L Major parasite IL81 in their left hind footpads. (A) Footpad swelling was measured over the course of 6 weeks using callipers (Kroeplin Langenmesstechnik). (B) Parasite burden at 3 weeks post infection. (C) Parasite burden at 6 weeks post infection. For all figures: Mean +- SEM; * => p-value of <0.05 relative to the IL-4Ra$^{-/lox}$; ** < 0.01; *** < 0.001. Results are reflective of two separate experiments.

3.4 Mb1^{cre}IL-4Rα$^{-/lox}$ exhibit a Th1 biased cytokine response

During infection with both the *L. major* LV39 and the *L. major* IL81 strains, the cytokine profile was quantified at 3 weeks post infection. Cells from infected lymph nodes were restimulated at 37°C for 72 hours in the presence of either αCD3 or soluble Leishmania antigen (SLA). Supernatants were then tested for the quantity of IFN-γ, IL-4 and IL10 using ELISA (Figures 3.4 and 3.5).

During *L. major* LV39 infection, mb1^{cre}IL-4Rα$^{-/lox}$ mice displayed significantly higher IFN-γ production upon αCD3 stimulation in comparison to the heterozygous littermate IL-4Rα$^{-/lox}$ mice (Figure 3.4A). Consistent with previous observations (Revas-Breton, unpublished data), control C57BL/6 mice also showed significantly elevated levels of IFN-γ (Figure 3.4A). Both mb1^{cre}IL-4Rα$^{-/lox}$ and C57BL/6 mice showed significantly higher IFN-γ production than the littermate control mice upon SLA stimulation (Figure 3.4A). Conversely, IL-4 production was significantly reduced in the mb1^{cre}IL-4Rα$^{-/lox}$ mice when compared with IL-4Rα$^{-/lox}$ under αCD3 stimulation(Figure 3.4B). As expected, IL-4 production in C57BL/6 was also significantly lower than in the IL-4Rα$^{-/lox}$ under both αCD3 and SLA restimulations.

In the case of the *L. major* IL81 infection, , the trend towards increased Th1 cytokines in mb1^{cre}IL-4Rα$^{-/lox}$ mice was the same. Upon restimulation of LN cells with both αCD3 and SLA, the mb1^{cre}IL-4Rα$^{-/lox}$ mice showed a stronger IFN-γ response and a reduced IL-4 response compared with the heterozygous littermate control mice (Figure 3.5 A-B). Taken together, these results demonstrate that mb1^{cre}IL-4Rα$^{-/lox}$ mice appear to control infection with *L. major* and exhibit a pronounced Th1 cytokine response compared with the littermate control during infection.

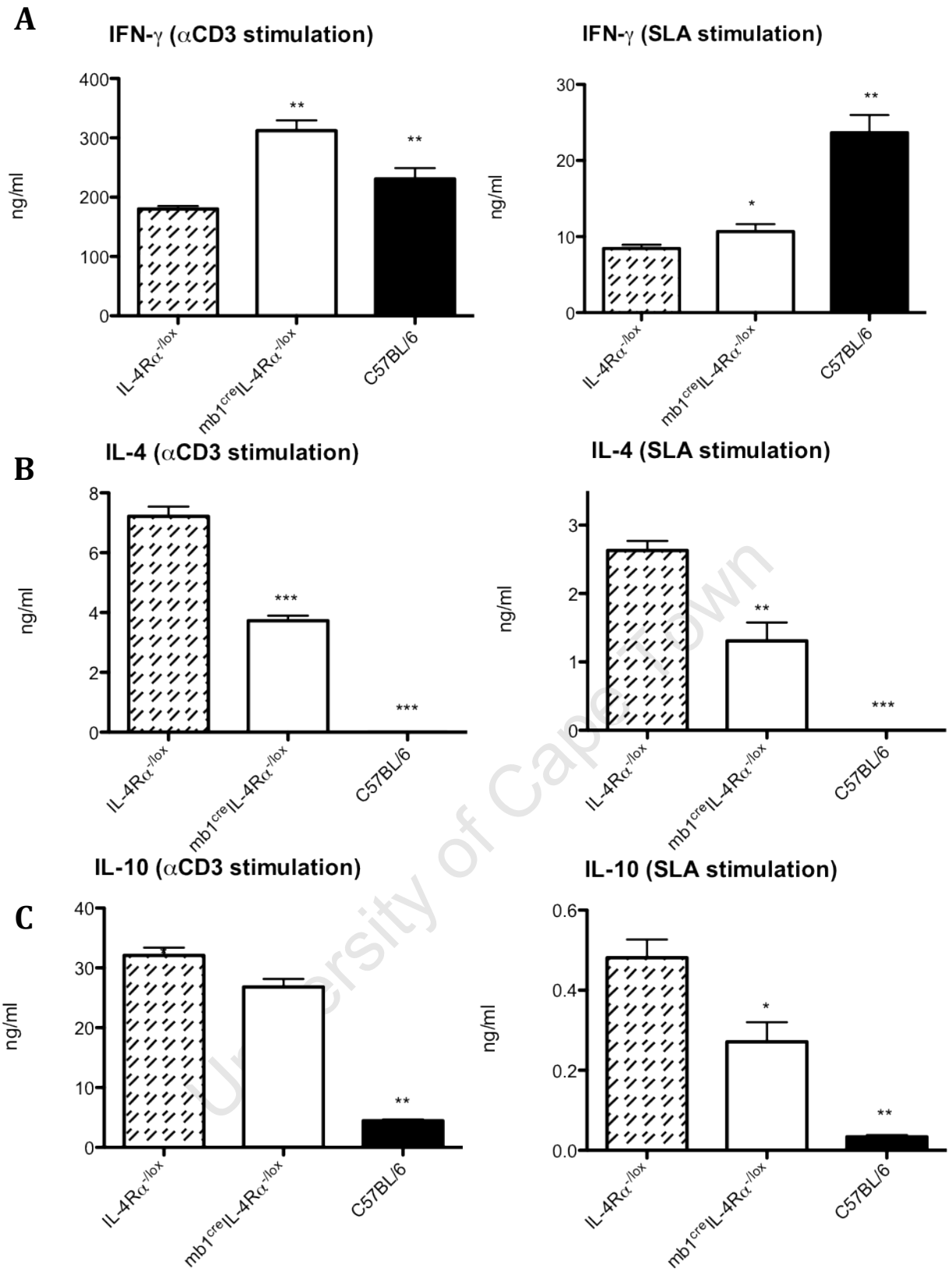


Figure 3.4: Th1 immunity is enhanced in mb1^{cre}IL-4Ra$^{-/lox}$ mice in response to acute *L. major* LV39 infection. Groups of 12 mice were infected subcutaneously with 2×10^6 stationary phase metacyclic *L. major* LV39 promastigotes into the hind footpad. At week 3 post infection, total LN cells were stimulated with 20ug/ml of αCD3 and 100ug/ml soluble Leishmania antigen (SLA) for 72 hrs. The production of IFN-γ (A), IL-4 (B) and IL-10 (C) in cell supernatants was measured by ELISA. A representative of one of two individual experiments are shown. Data is expressed as mean +- SEM; Statistical analysis was performed defining differences to IL-4Rα$^{-/lox}$ mice as significant (*, $p \leq 0.05$, **, $p \leq 0.01$; ***, $p \leq 0.001$.

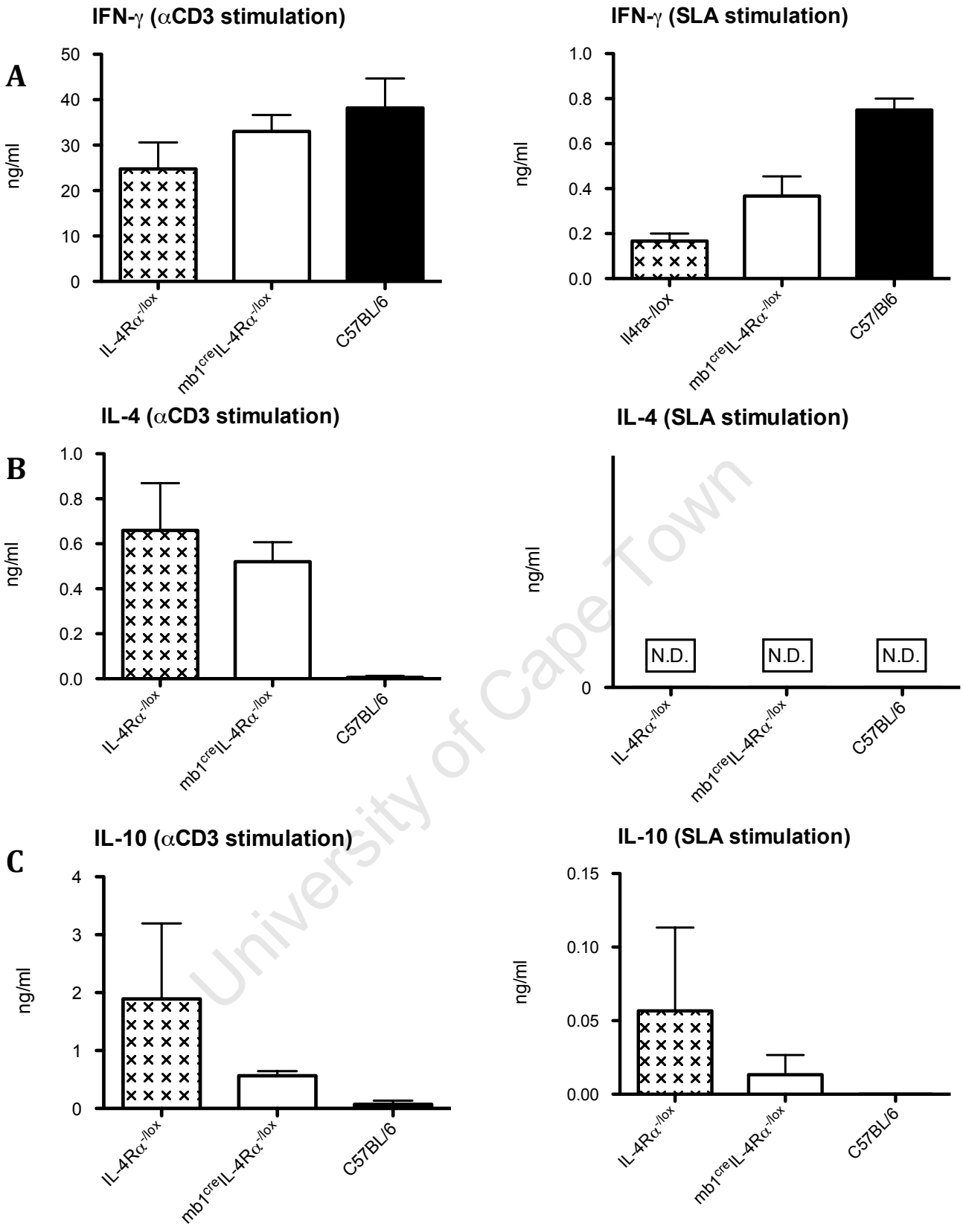


Figure 3.5: Th1 immunity is enhanced in mb1^{cre}IL-4Rα$^{-/lox}$ mice in response to acute *L. major* IL81 infection. Groups of 12 mice were infected subcutaneously with 2×10^5 stationary phase metacyclic *L. major* IL81 promastigotes into the hind footpad. At week 6 post infection, total LN cells were stimulated with 20ug/ml of αCD3 and 100ug/ml soluble Leishmania antigen (SLA) for 72 hrs**.** The production of IFN-γ (A), IL-4 (B) and IL-10 (C) in cell supernatants was measured by ELISA. Data from one experiment. Data is expressed as mean +- SEM. N.D = not detected.

3.5 Mb1^{cre}IL-4Rα$^{-/lox}$ produce lower levels of IL10

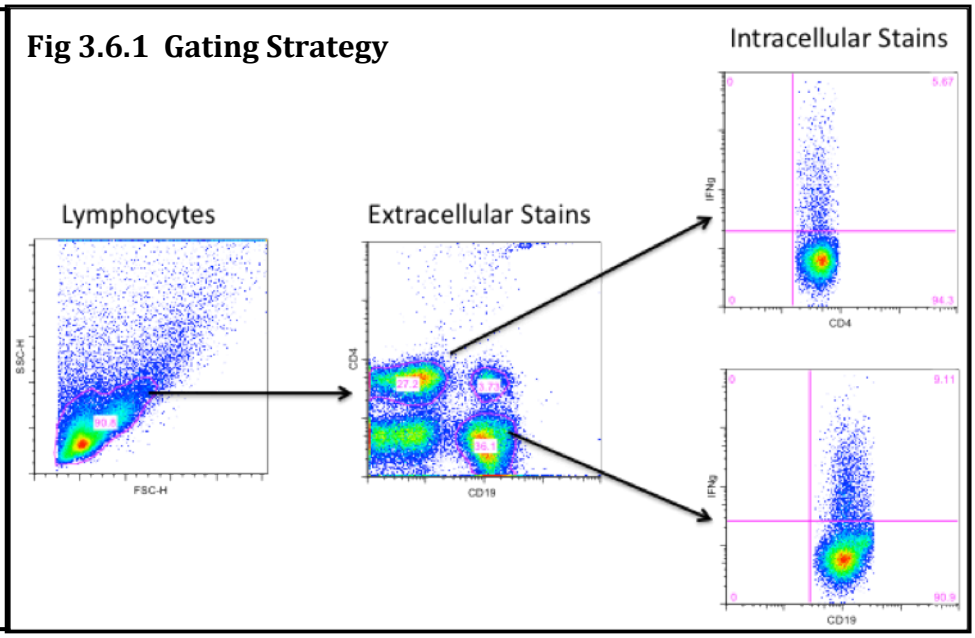
A paper published in the Journal of Immunology in 2010 (Ronet et al., 2010) showed that IL10 production by B cells contributes to Th2 differentiation in BALB/C mice. We therefore investigated IL10 production in the mb1^{cre}IL-4Rα$^{-/lox}$ mice lacking Be2 cells. During both *L. major* LV39 and IL81 infections, levels of IL10 production was decreased in the mb1^{cre}IL-4Rα$^{-/lox}$ mice compared with the littermate control mice, and more closely resembled the almost undetectable levels of IL10 production in the resistant C57BL/6 mice (Figure 3.4C, Figure 3.5C). IL10 is known to enhance Th2 responses by inhibiting macrophages from priming T-cells to differentiate into Th1 cells (O'Garra and Murphy, 2009). Take together, these data indicate that in addition to expressing an enhanced Th1 response, the mb1^{cre}IL-4Rα$^{-/lox}$ mice also appear to produce less IL10, which may be another contributing factor in their resistant phenotype.

3.6 Mb1^{cre}IL-4Rα$^{-/lox}$ exhibit a polarized B-effector 1 response

Next we examined the cytokine production by B-cells using intracellular FACS. Lymph node cells extracted from the popliteal node at 3 weeks post infection with *L. major* LV39 were restimulated for 4 hours with PMA, Ionomycin and Monensin. Cells were then surface stained for B and T cell populations using markers CD19 and CD4, respectively, and then stained intracellularly for IFN-γ, IL-4 and IL12.

The gating strategy for B and T cells is shown Figures 3.6.1. Mb1^{cre}IL-4Rα$^{-/lox}$ mice appear to contain a higher percentage of IFN-γ producing B-cells (9.11% of total B cells) compared with the IL-4Rα$^{-/lox}$ (7.46% of total B cells) (Figure 3.6.2A), whilst levels of IL-4 producing B-cells in the mb1^{cre}IL-4Rα$^{-/lox}$ mice (2.83%) were reduced in comparison to the littermate control (3.75%). Approximately, 1.1% of C57BL/6 B-cells are IL-4 positive (Figure 3.6.2B). The percent of IL-12 producing B cells was similar between mb1^{cre}IL-4Rα$^{-/lox}$ (2.8%) and the littermate control (2.58%) (Figure 3.6.2C). Together, these data suggest that mb1^{cre}IL-4Rα$^{-/lox}$ mice have a larger population of B-effector 1 cells relative to their littermate control.

Figure 3.6.1: Gating of B and T cells for intracellular staining. Groups of six mice were infected with 2x10^6 LV39 parasite. Lymph node cells were extracted at 3 weeks post-infection and surface stained using aCD4 and aCD19 to identify T and B cells respectively. Intracellular stains conducted included IFN-γ, IL-4 and IL12.



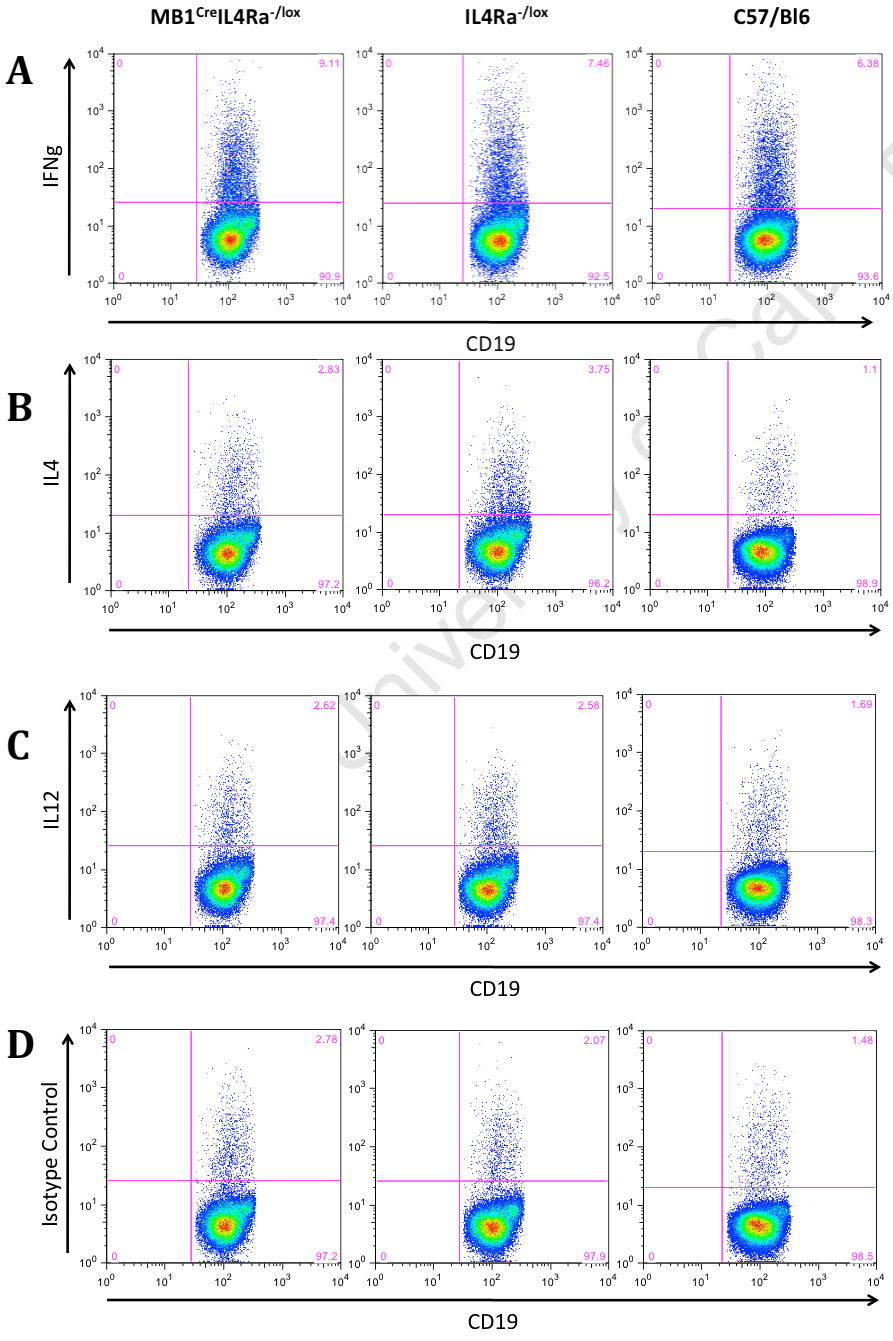


Figure 3.6.2: Intracellular cytokine expression of IFN-γ, IL-4 and IL-12 of B-cells. Cells extracted from lymph nodes of mb1^{Cre}IL-4Rα$^{-/lox}$, IL-4Ra$^{-/lox}$ and C57/Bl6 mice at 3 weeks post infection with 2x10^6 LV39 parasite were surface stained for CD4 and CD19, followed by intracellular staining for IFN-γ (A), IL-4 (B) and IL12 (C) and istoype control (D). Cells were gated for CD19+CD4- B cells. Data shown is from a single experiment. Six mice pooled per group.

3.7 B-Cell Surface Markers

In addition to understanding the composition of B-cell cytokine production, we were interested in analysing B cell function in association with T cell priming since B cells themselves are antigen-presenting cells (Kurt-Jones et al., 1988). In particular, MHCII, CD80/86, and CD21 were analysed. CD80 and CD86 form a complex on the B-cell surface and are known to interact with CTLA-4 and CD28 on T-cells (Freeman et al., 1991; Chen et al., 1994). CD21 forms part of the B-cell receptor complex together with CD19 and CD81. Together, these proteins are known to enhance the signaling cascade within B-cells when confronted with antigen (Aubry et al., 1992; Fearon and Carroll, 2000). It is thus of interest to understand whether B-effector 1 and B-effector 2 cells exhibit differential expression of any of these proteins.

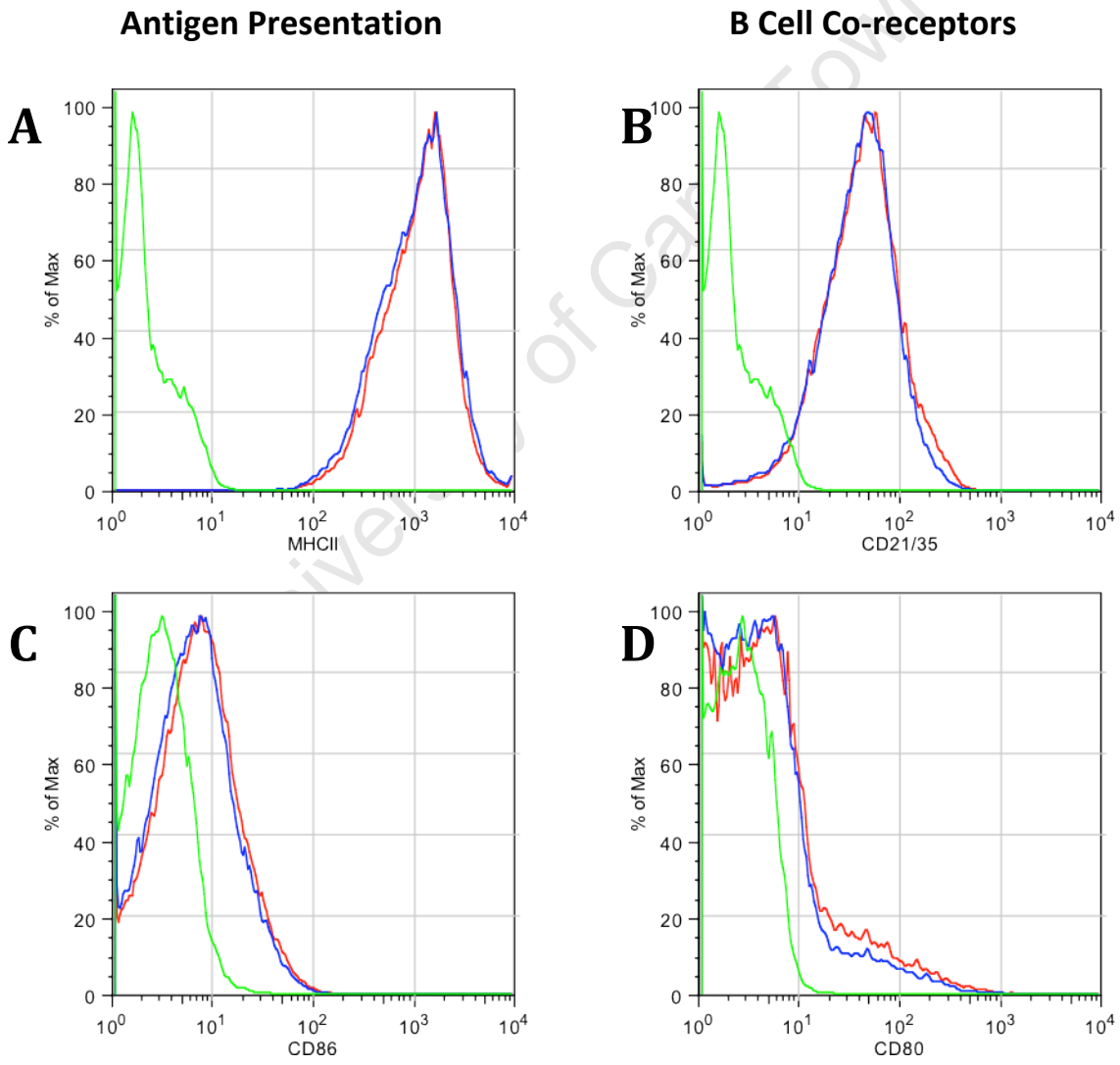


Figure 3.7: B-cell MHCII and Co-receptor expression. Mb1^{CRE}IL-4rα$^{-/lox}$ (red), IL-4Ra$^{-/lox}$ (blue) and isotype controls (green) were stained for B-cell markers: (A) MHCII (B) CD21/35 (C) CD86 and (D) CD80. Cells shown constitute CD19+ live cells extracted from the popliteal lymph nod at 3 weeks post-infection with 2x10^6 LV39 and all graphs represent populations from the same biological experiment. 6 mice per group.

LN cells were extracted at 3 weeks post infection with LV39 and stained for B-cells in addition to CD21, CD80 and CD86 (Figure 3.7). MHCII is expressed in abundance on B-cells, which is expected during infection (Figure 3.7A). T-cell co-stimulatory molecules CD21, CD86 and CD80 are also expressed on both sets of B-cells (Figure 3.7B-D).

There are no apparent differences between mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ suggesting that Be1 and Be2 cells do not differentially express these molecules. However, it is significant that these molecules are in fact present. They confirm that the Be2 cells are activated in a state where they can present antigen to T-cells (MHCII) and activate T-cells (CD80/86/21). In such a state, and in conjunction with our prior data about differing levels of IL-4 and IFN-γ production between mb1^{cre}IL-4Rα$^{-/lox}$ and BALB/C mice, the evidence suggests that these B-cells are both able to activate T-cells and secrete the polarizing cytokines that are required to effect a polarized T-helper cell response.

3.8 Mb1^{cre}IL-4Rα$^{-/lox}$ exhibit a polarized immunoglobulin response

Given that the IL-4Rα is known to play a role in antibody class-switching (Geha et al., 2003), we sought to examine and compare Ig responses between the mb1^{cre}IL-4Rα$^{-/lox}$ mice and its heterozygous littermate. Antibody titers of IgG1, IgG2a, IgG2b, IgG3 and *Leishmania* specific IgE were measured at week 8 after infection by ELISA (Figure 3.8A-E).

Titers of IgG1 and IgG3 were greatly reduced in the mb1^{cre}IL-4Rα$^{-/lox}$ mice compared with the littermate control (Figure 3.8A and 3.8C). Indeed, the levels of these two antibody isotypes were even lower in mb1^{cre}IL-4Rα$^{-/lox}$ than in resistant C57BL/6 mice. IgG2a and IgG2b, on the other hand, were expressed at higher levels in mb1^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3.8B and 3.8D). Leishmania specific IgE levels in the mb1^{cre}IL-4Rα$^{-/lox}$ mice were also greatly diminished compared with littermate controls (Figure 3.8E).

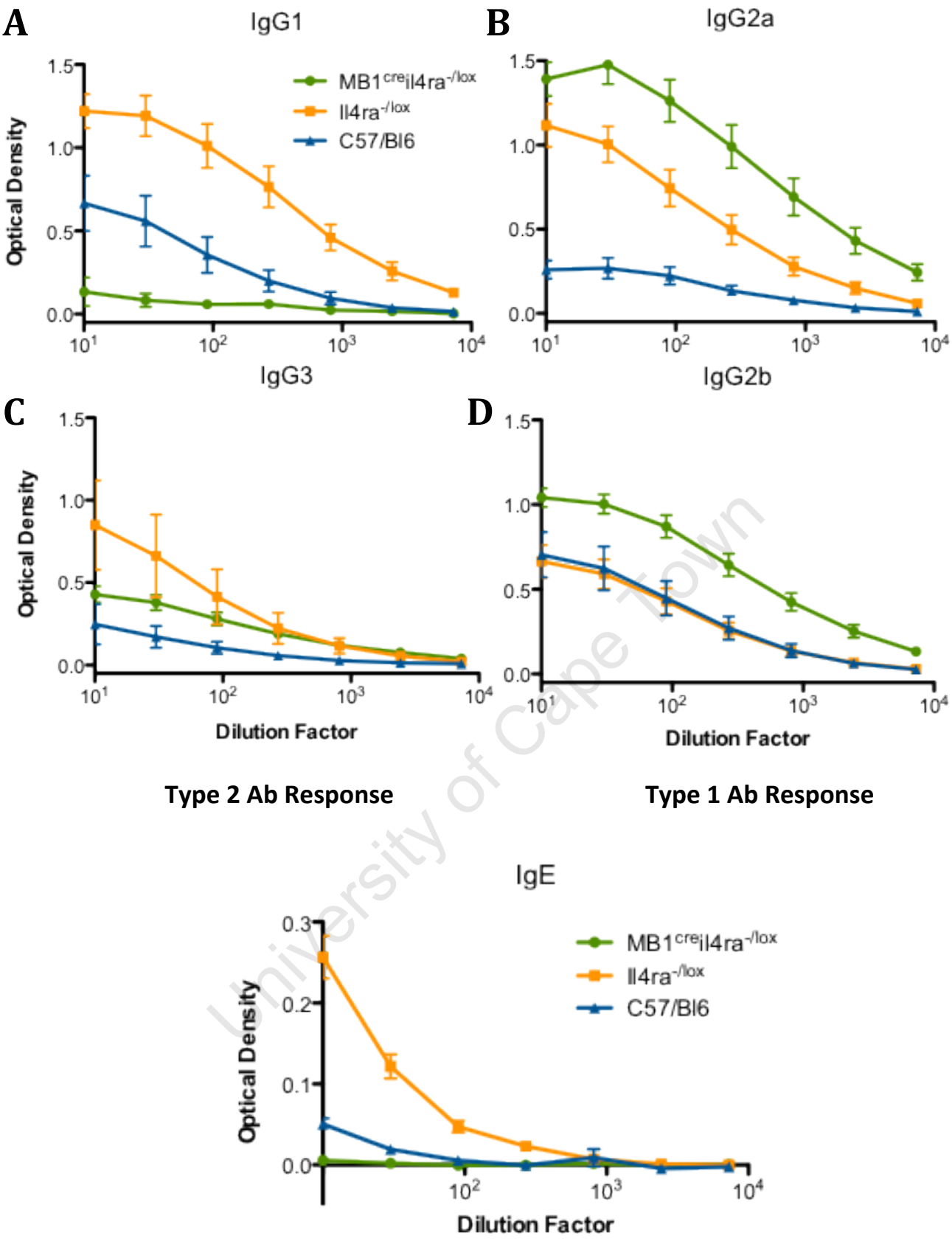


Figure 3.8. MB1^{CRE}IL-4ra$^{-/lox}$ antibody response. MB1^{CRE}IL-4ra$^{-/lox}$, IL-4Ra$^{-/lox}$, and C57Bl6 were bled at 4 weeks and 8 weeks post infection with 2x10^{6} LV39 and sera extracted for immunoglobulin isotype analysis. 96-well plates were coated with SLA (25ug/ml) and IgG/IgE ELISAs conducted in the usual way. Type 2 antibodies IgG1 and IgG3 (A and C) are shown on left and Type 1 antibodies IgG2a and IgG2b (B and D) are shown on the right hand side. (E) Relative IgE titers for three mouse strains. Plots are representative of three or more experiments at both 4 and 8 week time points. 6 individual mice per group.

3.9 Results of Pilot Sera Transfer Experiments

We next sought to investigate if serum transfers from infected mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice into IgM$^{-/-}$ mice could affect immune responses to *L. major* infection in the recipient IgM$^{-/-}$. IgM$^{-/-}$ are BALB/c mice lacking mature B cells due to a disruption in the IgM transmembrane domain and exhibit a partially susceptible response to *L. major* infection (Ronet et al., 2008).

In these experiments, we were principally interested in comparing IgM$^{-/-}$ mice that had been reconstituted with either mb1^{cre}IL-4Rα$^{-/lox}$ sera or IL-4Rα$^{-/lox}$ sera. For the purposes of this experiment, mice were infected with 2x10^5 *L. major* IL81 and footpad swelling was measured over the course of 6 weeks as an indication of disease progression. IgM$^{-/-}$ recipient mice were reconstituted with mb1^{cre}IL-4Rα$^{-/lox}$ sera or IL-4Rα$^{-/lox}$ sera on days 3, 10, 17, 24 and 31 post-infection.

As shown in Figure 3.9.1A, it appears that the IgM$^{-/-}$ mice reconstituted with IL-4Rα$^{-/lox}$ sera developed increased footpad swelling compared with IgM$^{-/-}$ mice reconstituted with mb1^{cre}IL-4Rα$^{-/lox}$ sera. While the absolute swelling was higher, the error margins were sizeable enough that the result could not be rendered significant at the 5% level. Additionally, while footpad swelling in the mb1^{cre}IL-4Rα$^{-/lox}$ reconstituted IgM$^{-/-}$ mice appeared to be less, no differences in parasite burden in the footpad was observed (Figure 3.9.1B).

Figure 3.9.2 shows the quantity of IgG1, IgG2a, IgG2b, and IgG3 contained in sera both from donors mb1^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ and from recipient IgM-/- mice 3 days post sera transfer. This is an important control as we want to establish that recipient mice do contain titers of the antibody we are interested in. As Figure 3.9.2A shows, IL-4Rα$^{-/lox}$ shows the highest level of IgG1, with the IgG$^{-/-}$ recipient show reasonably high quantities too. Mb1^{cre}IL-4Rα$^{-/lox}$ and IgG$^{-/-}$ recipient, on the other hand, contain little to none of this antibody. A similar contrast can be seen with IgG2B (Figure 3.9.2D). Whilst titers are not as high in recipient mice, the anitobodies are nonetheless present.

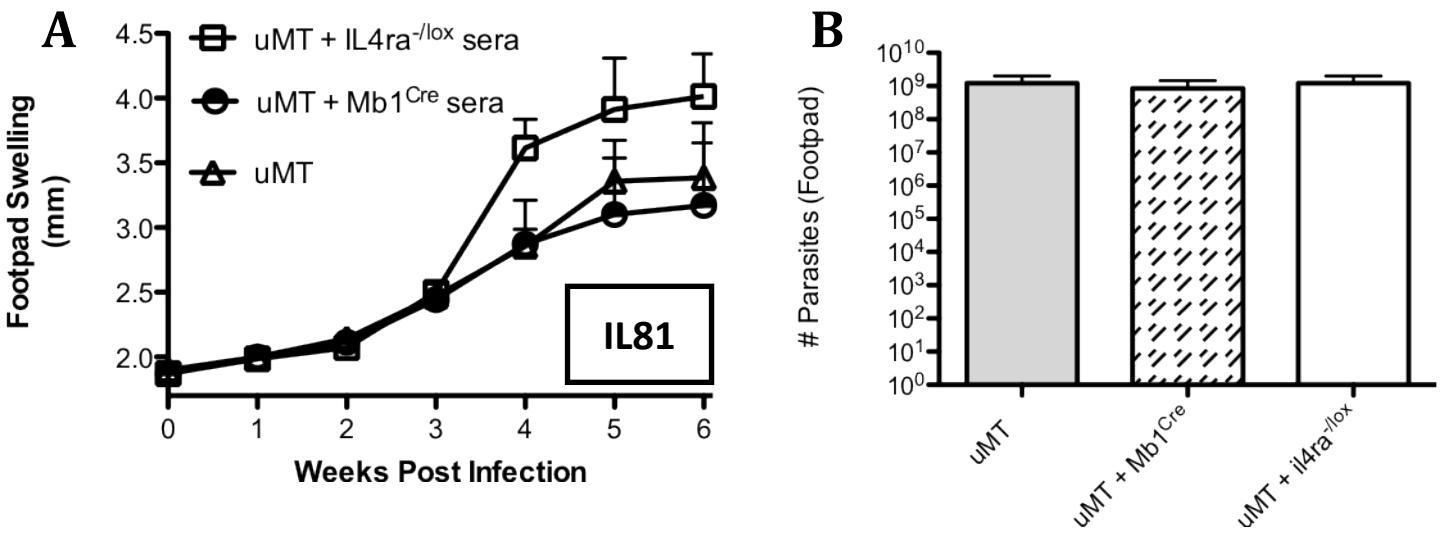


Figure 3.9.1: Disease progression in IgM$^{-/-}$ mice following sera transfer from mb1^{Cre}IL-4Ra$^{-/lox}$ mice and littermate mice. Three groups of 7 IgM$^{-/-}$ mice were infected with 2x10^5 L major IL81 parasites in the left hind footpad. Group 1 was reconstituted weekly with sera from infected mb1^{Cre}IL-4Ra$^{-/lox}$ mice (IgM$^{-/-}$ +Mb1^{Cre}), Group 2 weekly with sera from infected IL-4Ra$^{-/lox}$ mice (IgM$^{-/-}$ + IL-4Ra$^{-/lox}$), Group 3 provided the IgM$^{-/-}$ control. (A) Footpad swelling was measured weekly over the course of 6 weeks. (B) Parasite burden was measured in the footpads at 6 weeks post infection using limiting dilution assay. For all figures: Mean +- SEM; * => p-value of <0.05 relative to the IL-4Ra$^{-/lox}$; ** < 0.01; *** < 0.001. Results are reflective of one experiment.

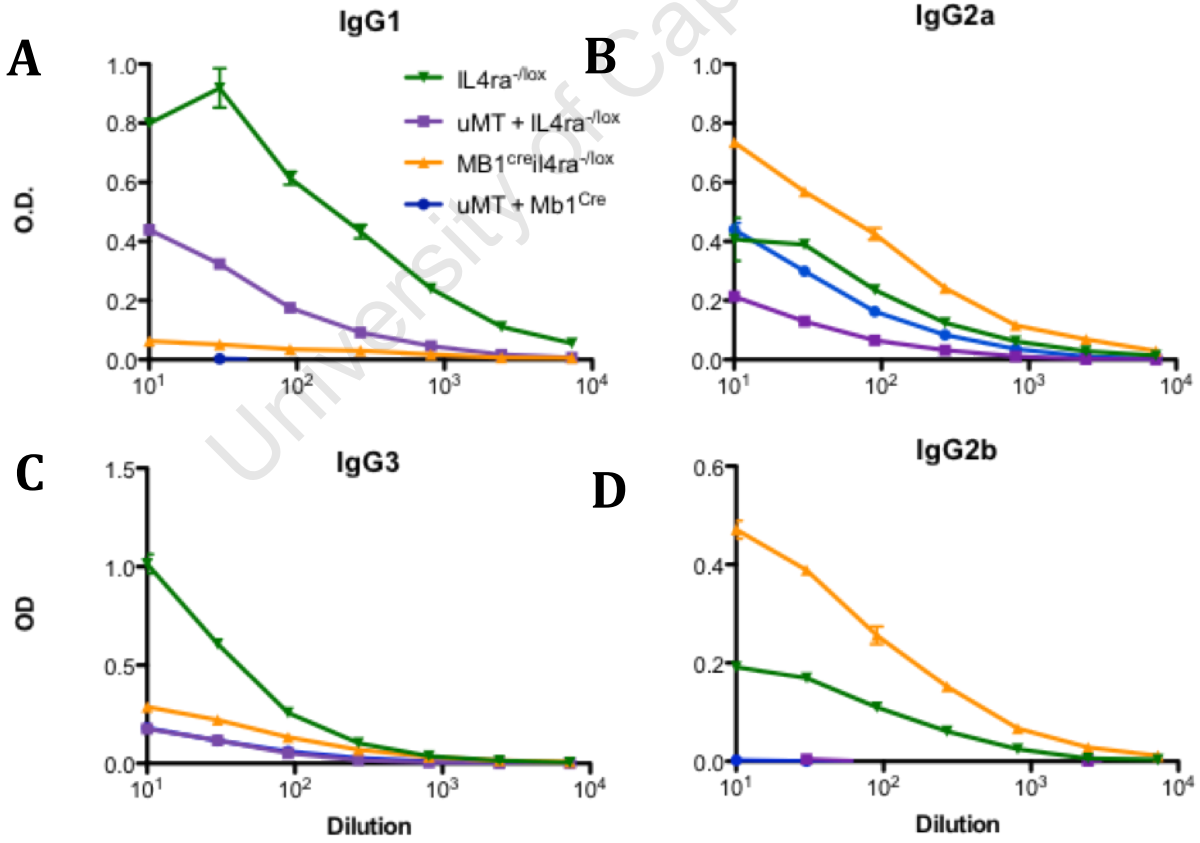


Figure 3.9.2: IgG antibody titers in sera transfer mice. Three groups of 7 IgM$^{-/-}$ mice were infected with 2x10^5 L major IL81 parasites in the left hind footpad. Group 1 and 2 were reconstituted with sera from 6 week infected mb1^{Cre}IL-4Ra$^{-/lox}$ and IL-4Ra$^{-/lox}$ mice. **(A-D)** Green and orange lines show IgG titres from infected IL-4Ra$^{-/lox}$ and mb1^{Cre}IL-4Ra$^{-/lox}$ mice respectively at 6 weeks post-infection. Blue and purple lines show the IgG titers in the IgM$^{-/-}$ mice 5 days post transfer from infected mb1^{Cre}IL-4Ra$^{-/lox}$ and 4Ra$^{-/lox}$ mice respectively.

4 Discussion

4.1 Cellular Immune Response

We investigated the role of B-effector cells in the course of *L. major* infection (Harris et al., 2000). In particular, we used the mb1^{cre}IL-4Rα$^{-/lox}$ mouse to examine how a mouse which could not produce B-effector 2 cells differed phenotypically from its littermate control (IL-4Rα$^{-/lox}$ BALB/c mouse able to produce both B-effector 1 and 2 cells) upon infection with *L. Major*. We confirmed earlier results observed in our lab that (, in contrast to wildtype IL-4Rα$^{-/lox}$, mb1^{cre}IL-4Rα$^{-/lox}$ mice appear to control infection with LV39 (Revaz-Breton et al, manuscript in preparation). Furthermore we showed that this phenotype extends to the more virulent IL81 strain of *L. major*. In both the case of LV39 and IL81 infections, footpad swelling at week 3-4 began to level off and subside in mb1^{cre}IL-4Rα$^{-/lox}$ mice whereas the wildtype control continued to experience increasing swelling and eventually necrosis. This result was further confirmed by quantifying parasite burden in the footpad; mb1^{cre}IL-4Rα$^{-/lox}$ mice showed significantly reduced parasite burden in the footpad at 6 and 8 weeks post infection in the case of IL81 and LV39 strains respectively.

In addition to establishing this phenotype we examined the cytokine milieu in the infected lymph nodes. We found that at the point at which the mb1^{cre}IL-4Rα$^{-/lox}$ phenotype diverges from the IL-4Rα$^{-/lox}$ control (typically around 3 weeks post infection), the cytokine milieu in the mb1^{cre}IL-4Rα$^{-/lox}$ mouse showed a Th1 bias in contrast to the IL-4Rα$^{-/lox}$ which showed a Th2 bias, akin to results observed *in vitro* ((Harris et al., 2005a; Harris et al., 2005b). In particular, the mb1^{cre}IL-4Rα$^{-/lox}$ mouse showed elevated levels of IFN-γ production and reduced levels of *IL-4* production relative to littermate control. We showed that both an αCD3 restimulation and an SLA restimulation produce the same result. These observations provide preliminary support to Harris' hypothesis that B effector cells can influence the nature of the T-helper response, and hence determine the outcome of infections with diseases such as *L. major* which require a polarized Th1 response in order to control and clear infection.

Having observed divergence not only in phenotype but in cytokine composition in the lymph node of the mb1^{cre}IL-4Rα$^{-/lox}$ mouse, we then attempted to ascertain whether or

not B-cell cytokine production was the major cause of these differences. We used extra cellular FACS to label cells with T and B cell markers (CD4, CD19) and stained intra-cellularly for cytokines *IL-4*, IFN-γ and IL12. We discovered that a greater portion of B-cells from the mb1^{cre}IL-4Rα$^{-/lox}$ mouse produce IFN-γ compared with the IL-4Rα$^{-/lox}$ counterparts, and significantly less IL-4. This corroborates Harris' model (Harris et al., 2005b) which suggests that B cells require IL-4Rα signaling on their cell surface before differentiating into IL-4-producing Be2 cells. Without Be2 cells, the mb1^{cre}IL-4Rα$^{-/lox}$ seems to compensate by producing a larger number of Be1 cells, the cumulative result being more IFN-γ and less *IL-4* produced by the mb1^{cre}IL-4Rα$^{-/lox}$ mouse. While this does not prove that the B effector cells are the sole cause of the healer phenotype observed, it implicates them as contributors to shape of the T helper response *in vivo*.

4.2 Parasite Burden in Lymph Node

It is perhaps worth noting in these concluding remarks that while the mb1^{cre}IL-4Rα$^{-/lox}$ exhibit significantly lower parasite burden in the footpad compared with IL-4Rα$^{-/lox}$ at the end point of the experiment, the parasite burden in the lymph nodes of the two mice remains comparable throughout. While it is beyond the scope of this thesis to explore this phenomenon, what we can observe is that the battleground in the footpad appears to be different from that in the lymph node, and that although the same set of B- and T-lymphocytes are governing the body's response to the infection, they do so with differing levels of success in these two sets of tissue. It is possible that the *L. major* parasite, having co-evolved with the mammalian immune system, prefers – even depends – on the immune system to proliferate. As such the lymph node may act as a reservoir for the parasite while skin and muscle tissue are contested in a different way.

4.3 Humoral Immune Response

In order to create the mb1^{cre}IL-4Rα$^{-/lox}$ mouse, our lab selectively deleted the *IL-4*Rα on B-cells using the Cre-LoxP system. This system put the Cre gene on an mb1 promoter, a promoter that is expressed exclusively and pervasively in early B-cell development (Hoving et al., 2012). The desired, and observed result, is that B-cells do not produce functional surface *IL-4*Rα. While according to the mechanism established by Harris (Harris et al., 2005b) this deletion renders the mb1^{cre}IL-4Rα$^{-/lox}$ mouse unable to

produce B-effector 2 cells, it also has another unintended effect, namely the altering of the antibody composition of this mouse.

The *IL-4*Rα is a key signalling protein in the class-switching process (Ezernieks et al., 1996; Geha et al., 2003). *IL-4*Rα is required in order for antibody producing B-cells to differentiate into IgG1 and IgE producers. While the humoral immune response has not previously been considered a defining factor in *L. major* pathogenesis (Ronet et al., 2008), the stark contrast between mb1^{cre}IL-4Rα$^{-/lox}$ antibody response and control IL-4Rα$^{-/lox}$ antibody response merited some further investigation. In particular, in order to conclude that B effector cells are indeed responsible for altering the T helper response, we need to demonstrate that the divergent antibody repertoires between these two mice are not behind the observed phenotype.

While fully investigating the antibody response was outside the scope of this thesis, we were able to conduct a pilot experiment which produced some interesting results. To summarize the nature of the antibody response during *L. major* infection, IL-4Rα$^{-/lox}$ mice tend to produce a polarized type 2 response - namely high levels of IgG1, IgG3 and IgE. After knocking out the *IL-4*Rα on B-cells, we observe greatly diminished levels of these three antibody isotypes and instead see elevated levels of IgG2a and IgG2b, indicative of a type 1 antibody response.

In order to assess whether these differences affect the pathogenesis in *L. major* infection, we undertook a series of antibody transfer experiments. In particular, we used IgM$^{-/-}$ recipient mice (devoid of B-cells and therefore antibodies) and treated them with immune sera either from infected mb1^{cre}IL-4Rα$^{-/lox}$ mice or from infected IL-4Rα$^{-/lox}$ mice. Recipient IgM$^{-/-}$ mice were infected with a low dose of IL81 and subsequently subjected to regular intraperitoneal doses of antibody-containing immune sera. The IgM$^{-/-}$ receiving IL-4Rα$^{-/lox}$ sera provided the control (i.e. no B-cells but a normal Type 2 biased antibody response) while the IgM$^{-/-}$ receiving mb1^{cre}IL-4Rα$^{-/lox}$ sera provided the experiment (i.e. no B-cells but a type 1 biased antibody response). We should reiterate that due to time constraints this experiment was conducted only once. Nonetheless, the results are of interest. The IL-4Rα$^{-/lox}$ sera recipients appeared to experience a more exacerbated trajectory of disease with increased footpad swelling relative to the IgM$^{-/-}$ control. The mb1^{cre}IL-4Rα$^{-/lox}$ sera

recipients, in contrast, appeared to showed no difference in comparison with the $IgM^{-/-}$ control.

The implication here is that while we have established reasonable evidence that B effector cells participate in shaping and influencing the T-helper cell response during the course of *L. major* infection (corroborating (Harris et al., 2000), they may not be the sole contributor to the observed $mb1^{cre}$IL-4R$\alpha^{-/lox}$ phenotype. These preliminary sera-transfer results corroborate earlier evidence (Miles 2005, Wanasen 2008) that antibody – IgG1 and IgE in particular – may contribute to the susceptible IL-4R$\alpha^{-/lox}$ phenotype. By knocking out the *IL-4*Rα on B-cells in the $mb1^{cre}$IL-4R$\alpha^{-/lox}$ mouse, we may inadvertently have reduced the potency with which *L. major* manifests by removing the susceptibility factors IgG1 and IgE.

4.4 Future Research: Improved Sera Transfer Experiments

As discussed in the literature review of this thesis, the role of antibody in Leishmania infection is a matter of some dispute (Miles et al., 2005; Ronet et al., 2008; Wanasen et al., 2008). This pilot experiment, if confirmed at a later date, would provide evidence that the humoral immune response does indeed have a role to play in the course of *L. major* infection.

While we observed differences between $mb1^{cre}$IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$-sera recipients, over the course of the experiment we established that the protocol for these sera transfers could be improved. We observed that, in spite of conducting sera transfers almost weekly, recipient mice never achieved antibody titers comparable to those of the donor mice (data not shown). This is not surprising. In a mouse with a total volume of 2.5ml of blood, a transfusion of 0.4-0.7ml of antibody containing sera would lead to antibody concentrations one third that of the donor mice. Additionally, in $mb1^{cre}$IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice, antibodies are created on a continual basis whereas in the $IgM^{-/-}$ recipient mice, antibodies are reabsorbed and degraded in the body without being replaced. The antibody titer in these $IgM^{-/-}$ recipients thus resembles a pulse-rate, with titer rising sharply upon sera transfer and then decaying over time until the next transfer. This contrasts with titers in a normal antibody producing mouse which are typically elevated and sustained.

Future transfer experiments could make use of the GradiFrac System (Amersham Biosciences) whereby immune sera is passed through the GradiFrac G column in order to concentrate antibodies before administering them to recipient mice. With lower volumes required, antibody titers could be increased and sera transfers could be done more regularly, helping to more accurately resemble the situation in a normal mouse. Protocol aside, the sera transfer experiment opens up a new possibility, namely that the phenotype observed does not result exclusively from the activity of B effector cells, but is either entirely a result of a divergent antibody response, or otherwise some combination of the activity of B effector cells and of the changed humoral immune response.

4.5 Future Research: Bone Marrow Chimera Experiments

While we have demonstrated that mb1^{cre}IL-4R$\alpha^{-/lox}$ mice exhibit a changed cytokine milieu as a result of the *IL-4*Rα deletion on B cells, we have not elucidated the mechanism by which the influence is exerted. It is, for example, possible that the polarized Type 1 antibody response in the mb1^{cre}IL-4R$\alpha^{-/lox}$ mouse indirectly feeds back to dendritic cells and macrophages (Kane and Mosser, 2001; Yang et al., 2007) thus changing the nature of the T-helper response via these antigen presenting cells. It is also possible that our original hypothesis stands, namely that B effector cells directly prime T helper cells through the production of IFN-γ and *IL-4*. In order to definitively test the latter hypothesis, we have developed the bone marrow chimera experimental setup outlined below.

We are asking the question: Is *IL-4* produced by B cells responsible for shaping the susceptible T-helper 2 response observed in BALB/c mice? Because no transgenic mice currently exist that have this property (i.e. B-cells that do not produce *IL-4*), one way to test this is to create a mixed bone marrow chimera. This is a standard procedure whereby mice lacking B cells (IgM$^{-/-}$) are irradiated and reconstituted with *IL-4* deficient B cells from *IL-4*-/- mice. The result is a proxy for a wildtype BALB/c mouse whose B-cells cannot produce *IL-4*. If these bone marrow chimeras control *L. major* infection in the same way the mb1^{cre}IL-4R$\alpha^{-/lox}$ mice do, we will have definitively shown that the *IL-4* produced by B effector 2 cells is indeed responsible for the Th2 biased cellular immune response and therefore the susceptibility of the BALB/c mouse to *L. major*.

The experimental setup is as follows:

Table 4. Bone Marrow Chimera Experimental Setup

Group	No. Mice	Description	Reconstitution
Group 1	8	Mice w/ normal B cells	80% IgM$^{-/-}$ 20% BALB/c
Group 2	8	Mice w/ IL-4 knock-out B cells	80% IgM$^{-/-}$ 20% *IL-4*-/-
Group 3	8	Mice w/ IL-4Rα knock-out B cells	80% IgM$^{-/-}$ 20% B *IL-4*Rα-/-

If the B effector cell hypothesis is correct, then we would expect Group 1 to be susceptible to *L. major* infection, Group 2 to control infection in a similar way to mb1^{cre}IL-4Rα$^{-/lox}$ mice, and Group 3 to control infection in a similar way to Group 2. If, however, Group 1 and 2 are both susceptible but Group 3 is not, this would lend further evidence to the counter-hypothesis that it is not the B-effector 2 cells behind the susceptibility but some alternative B cell mechanism such as the antibody response.

4.6 Conclusion

In this piece of research we confirmed the resistant phenotype of the mb1^{cre}IL-4Rα$^{-/lox}$ mouse upon infection with *L. major* LV39 and extended this phenotype to the more virulent strain of *L. major* IL81. This phenotype raises interesting questions about the underlying mechanism. We have shown that the cytokine milieu in the mb1^{cre}IL-4Rα$^{-/lox}$ shows a Th1 bias (more IFN-γ, less *IL-4*) in comparison with the control IL-4Rα$^{-/lox}$ mouse. We showed that the cytokine producing B-cells in the mb1^{cre}IL-4Rα$^{-/lox}$ mouse tend to contain more IFN-γ and less *IL-4*, underlining the changed composition of B-effector cells in the mb1^{cre}IL-4Rα$^{-/lox}$ mouse. While this lends evidence toward a B effector cell mechanism belying the resistant phenotype of the mb1^{cre}IL-4Rα$^{-/lox}$ mouse, we also showed that mb1^{cre}IL-4Rα$^{-/lox}$ and BALB/c mice show contrasting antibody repertoires. Mb1^{cre}IL-4Rα$^{-/lox}$ produce relatively high levels of IgG2a and IgG2b but dramatically reduced levels of IgG1 and IgE. We conducted a pilot sera-transfer study which suggests that the divergent antibody repertoire of the mb1^{cre}IL-4Rα$^{-/lox}$ mouse may indeed contribute to its resistant phenotype. This lends evidence to work first done by (Miles et al., 2005) on the role of the humoral immune response in *L. major*, as well as clinical observations made by other researchers (Ozbilge et al., 2006).

While this series of experiments has not fully clarified the mechanism by which the mb1^{cre}IL-4Rα$^{-/lox}$ phenotype is generated, it nonetheless provides the groundwork for

potentially interesting further experimentation. In particular, the combination of sera transfer experiments and mixed bone marrow chimera experiments outlined above should at the very least elucidate whether it is the changed antibody repertoire or the changed B effector cell population that is driving the phenotype. In the event it is the former, we will have shown - contrary to what was previously understood (Ronet et al., 2008) - that the humoral immune response is a key agent in *L. major* pathogenesis. In the event it is the latter, we will have shown in a live disease model how B effector cells can influence the shape of the T helper response and hence change disease outcome.

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Part II

5 Introduction

It had been a long-observed phenomenon that different strains of lab mice were either susceptible or resistant to *Leishmania Major* (*L. major*). The paragon of the resistant response was the black C57BL/6 mouse, which, upon infection, experienced initial swelling, and parasite replication followed by reduced swelling and dramatic reduction in parasite numbers. The susceptible counterpart was the white BALB/c mouse, which, upon infection, experienced ever-increasing footpad swelling, parasite replication and eventual necrosis at the site of infection. For a long time this dichotomy remained a mystery. Not until the advent of cytokine detection assays was it discovered that C57BL/6 mice produced an immune response overwhelmingly characterized by the macrophage activating factor IFN-γ, while the susceptible BALB/c mouse instead exhibited low levels of IFN-γ and high levels of what was then known as the B-cell stimulant factor (BSF), or what is now known as interleukin 4 (Mosmann and Coffman, 1989; Morris et al., 1993). It was subsequently shown that the primary producers of these cytokines were activated CD4+ T-cells. Because CD4 T cells exacted their influence through the activation of other cells such as B-cells and macrophages rather than through direct interaction with the pathogen, they came to be known as T-helper cells. The stark contrast between the T-helper cell population in C57BL/6 mice and BALB/c mice helped to establish the T-helper1/T-helper2 (Th1/Th2) paradigm whereby the former was characterized by the activation of macrophages and the latter characterized by the suppression of macrophage activation and the stimulation of IgG production by B-cells (Locksley et al., 1987; Scott et al., 1988).

To date research into this dichotomy has focused in two primary areas: 1) What are primary determinants of the differing T-helper responses, and 2) How do the different T-helper responses activate the immune system differently.

5.1 Determinants of T-helper 1 and 2 Responses

In the case of *L. Major*, it has been shown that a key determinant of the Th1 response was production of IL12 by dendritic cells and macrophages (Macatonia et al., 1993;

Seder et al., 1993). IL12R signaling on T-cells, together with activation of its TCR and other T-cell co-receptors such as CD28 result in differentiation of that T-cell into an IFN-γ producing Th1 cell through the induction of putative transcription factors Stat 1, Stat 3 and Stat 4 (Jacobson et al., 1995; Magram et al., 1996). There are many factors which cause activated macrophages and dendritic cells to produce IL12, amongst which are microbial components such as endotoxin, unidentified components of viruses, intracellular bacteria such as Listeria, protozoa such as Toxoplasma (Hsieh et al., 1993; Szabo et al., 1995; Trinchieri, 1995).

Similarly it was found that activation of CD T-cells in the presence of IL-4 resulted in IL-4 producing Th2 cells. What the exact source of the initial IL-4 is is still a matter of some dispute. Some experiments suggest that IL-4 might not be required for initial Th2 differentiation but it is the quantity or rather concentration of antigen that determines the T-helper response (Bretscher et al., 1992; Hosken et al., 1995). Other experiments suggest that it may be memory effector T-cells or natural killer cells that produce the early IL-4 (Bradley et al., 1993; Yoshimoto and Paul, 1994).

5.2 *L. major* Susceptibility in BALB/c mice

The apparent resolution of infection in BALB/c mice upon treatment with anti-IL-4 suggests that early IL-4 production by these mice drives the susceptible Th2 response (Sadick et al., 1990; Chatelain et al., 1992). This view is supported by the fact that IL-4 deficient BALB/c mice are rendered resistant to *L. major* infection (Kopf et al., 1996; Mohrs et al., 1999). Additionally, there is evidence that the IL-4 production can be confined to a small set of CD4+ T cells expressing the VB4Ba8 T-cell receptor (TCR) that recognise the Leishmania antigen LACK (Launois et al., 1997). VB4 deficient mice exhibit stronger Th1 responses and appear to control their lesions (Himmelrich et al., 2000). That this VB4Ba8 IL-4 production is responsible for susceptibility in BALB/c is not, however, clear. Resistant C57BL/6 mice appear to show equal expression of this TCR and experiments using IL-4 linked green fluorescent protein suggest that the C57BL/6 also produces a burst of IL-4 through these cells (Reiner et al., 1993; Julia and Glaichenhaus, 1999). It has thus been suggested that it is not so much the over-production of IL-4 by BALB/c mice that belies their susceptibility but their inability to switch to the Th1 phenotype.

The ability of exogenous IL12 to redirect the Th2 response in BALB/c mice has been widely documented (Heinzel et al., 1993; Sypek et al., 1993). Additionally, the disruption of IL12 production in resistant strains has been shown to render these mice susceptible (Heinzel et al., 1993; Heinzel et al., 1995). It thus seemed plausible that ineffective IL12 signaling on BALB/c T-cells may be responsible for their inability to switch to a Th1 response. However reconstitution of the BALB/c with an Il12R transgene did not render it resistant suggesting another mechanism is at play (Nishikomori et al., 2001).

Other explanations for the sustained Th2 response in BALB/c mice have also been proposed. Early and sustained infiltration at the point of infection by neutrophils in BALB/c but not C57BL/6 mice could promote the Th2 response by some indirect mechanism (Beil et al., 1992). The location of infection may also be another factor with intravenous and intranasal infection of normally resistant strains of mice leading to Th2 responses and susceptibility (Nabors et al., 1995; Constant et al., 2000). The latter hypothesis suggests that different populations of dendritic cell at different points of infection might trigger divergent responses.

While the exact mechanism behind BALB/c susceptibility is not know, and it is further not known whether the responsibility lies with T-cells or some other cell type such as macrophage or dendritic cell, what is known for certain is that it is some form of endogenous genetic difference between BALB/c and C57BL/6 behind the divergent phenotypes. In an attempt to clarify the genetic loci of these differences, studies were conducted on infection of recombinant inbred strains and serial backcross mapping (Noben-Trauth et al., 1999; Lipoldova et al., 2000). In these experiments, susceptible and resistant lab strains were crossed and susceptibility measured in terms of lesion size. Several genetic loci called *Leishmania Major* Response (LMRs) were identified, but these loci collectively contained hundreds of genes and no clear candidates were identified.

5.3 T-helper Cell Effector Mechanisms

The primary genes currently known to be involved in the Th1 response include the cytokines IL12 and IFN-γ, transcription factors Tbet and Stat 4, and the costimulatory molecules CD40 and CD40L. For mice that are deficient in IL12, IFN-γ, Tbet and CD40,

the immune response reverts to susceptible Th2 (Wang et al., 1994; Campbell et al., 1996; Mattner et al., 1996; Szabo et al., 2002). IL12R signaling during T-cell activation, in conjunction with CD40-CD40L interaction between APCs and T-cells, leads to a signaling cascade which results in the transcription factors Tbet and Stat4 within the T-cell. These in turn lead to the transcription of IFN-γ, IL2 and TNF.

IFN-γ has a wide-range of effects, including class switching of naive B-cells to IgG2a producing B-cells, the inhibition of IL-4 production by CD 4 T cells, and - perhaps most importantly in the case of *L. major* - the activation of macrophages to produce inducible nitrogen oxide synthase (iNOS) which result in the production of reactive nitrogen intermediates (RNIs) and killing of intracellular bacteria and parasites. TNF is believed to be an important cofactor in macrophage activation and RNI production (Bogdan et al., 1990).

The key genes involved in the Th2 response are cytokines IL-4, IL5, IL6, IL13 and TGFbeta, transcription factors GATA3 and STAT6, and costimulatory molecules OX40-OX40L. The presence of OX40 signaling and IL-4 during T-cell activation result in a signal cascade that sends STAT6 into the nucleus and production of transcription factor GATA3. GATA3 promotes the production of IL-4 and other Th2 cytokines. BALB/c mice deficient in IL-4 show partial control of *L. major* while BALB/c mice deficient in IL-4Rα are rendered entirely resistant (Noben-Trauth et al., 1999). As the IL-4 and IL3 receptors share the IL-4Rα chain in common, it is believed that IL13 also contributes to Th2 susceptibility. The role of TGFbeta is believed to be ancillary to that of IL-4, but in some circumstances has been shown to suppress the activation of macrophages. In *L. major*, C57BL/6 x BALB/c F1 mice treated with anti-TGFbeta in the chronic stage of infection showed enhanced resistance compared with the control, supporting this hypothesis (Li et al., 1999).

5.4 Summary

For nearly 25 years, *L. major* has provided the core evidence for the T-helper cell paradigm. Endogenous genetic factors result in some strains of lab mice exhibiting resistance to infection (C57BL/6) while others exhibit a susceptible phenotype (BALB/c). This disease model has helped us to elucidate the cells and cytokines that drive different T-helper responses, the pathways via which naive T-cells mature into

active Th1/Th2 cells, and several effector mechanisms by which these T-helper cells exert their influence.

Two questions, however, still remain. Firstly, what gene (genes) is (are) the root cause of the divergent phenotype between BALB/c and C57BL/6? Secondly, while several Th1/Th2 mechanisms have been elucidated and many genes implicated and characterized in the process of establishing these mechanisms, are there any other marker Th1/Th2 marker genes of which we are currently unaware?

The purpose of this microarray analysis is to produce a full genetic read-out of the differences between Th1 and Th2 cells in the hopes a) of understanding what the endogenous genetic differences between BALB/c and C57BL/6 are that lead to their divergent phenotypes, and b) of clarifying whether there are any further genes or effector functions that might be involved in these polarized responses.

5.5 Experimental Design

The preparation and wet lab work for this microarray was conducted by a PhD student in our lab (Liezel Smith, unpublished data). The BALB/c and C57BL/6 microarray chips were controls for a larger experiment comparing IL-4Rα$^{-/-}$ mice with T-cell specific IL-4Rα$^{-/-}$ mice and with the heterozygous littermate IL-4R$^{-/lox}$. The latter three mice form the substance of Liezel's Phd thesis while I conducted the analysis of the control chips. While I conducted the microarray quality control, normalization and gene filtering for all mouse strains, I have restricted the analysis here to BALB/c and C57BL/6 microarray chips only.

BALB/c and C57BL/6 mice were infected with standard dose (2x10^6) *L. major* strain LV39 (MRHO/SU/59/P). At three weeks post infection, mice were sacrificed and activated T-cells extracted from popliteal lymph nodes. Three separate infections were conducted providing three biological replicates and a total of six microarray chips – 3 BALB/c and 3 C57BL/6. The materials, methods, results and discussion that follow concern themselves with these six microarray chips.

6 Methods

Summary of sample preparation (L. Smith, 2010, unpublished data)

6.1 Infection and Extraction of Cells

Groups of six C57BL/6 and six BALB/c mice were infected with a standard dose (2x106) L. major LV39 (MRHO/SU/59/P) metacyclic promastigotes in the left hind footpad. At three weeks post-infection, mice were sacrificed and popliteal lymph nodes extracted for isolation of activated CD4 T cells. Lymph nodes were pooled (BALB/c and C57BL/6 respectively) and a single cell suspension achieved by teasing lymph nodes through a 40 uM filter (Becton Dickinson) and stored in DMEM supplemented with 5% FCS.

6.2 Isolation of Activated CD4 T Cells

Single cell suspensions were incubated with anti-CD4 Microbeads (MACS, Miltenyi Biotec) and then passed through an LS separation column. CD4+ cells remained in the column while CD4- cells were washed through. After removal of the magnetic field, CD4+ cells were eluted and stained for surface markers of activated T cells (CD4+CD44+CD62Lmed-high+), resuspended in 7AAD and sorted by FACS using FACS Vantage. A purity of >98% was obtained in each of the three biological replicates as confirmed by FACS.

6.3 RNA Extraction

RNA was extracted from purified activated T cells using RNeasy Mini Kit (Cat. No. 217004, Qiagen, USA), RNeasy MinElute Cleanup Kit (Cat. No. 74204, Qiagen, USA) and the RNase-Free DNase Set (Cat. No. 79254, Qiagen, USA). RNA quantity was determined using a Nanodrop ND1000 (Thermo Scientific, USA) and the integrity was determined using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513, Agilent Technologies, USA). The Agilent assays were performed by the Centre for Proteomic and Genomic Research, Cape Town.

6.4 Affymetrix Microarray

For Activated T cell microarray 2 µg of amplified cDNA was labeled and hybridized to GeneChip Mouse Exon 1.0 ST Arrays for 18 hours in accordance with the Affymetrix protocol. At the end of hybridization, the arrays were washed and stained using the GeneChip® Fluidics Station 450 and scanned using the GeneChip® Scanner 3000 7G.

The above steps were performed by the Centre for Proteomic and Genomic Research (CPGR), Cape Town.

6.5 Quality Control and Data Analysis

The results of the Affymetrix Array were delivered in the form of .CEL files from CPGR. Quality control and normalization was conducted using the Bioconductor package Oligo (Version 1.22.0, maintainer Benilton Carvalho). The R script used to conduct normalization is contained in Appendix X. In brief, .CEL files were loaded into Oligo using the Affymetrix library package pd.moex.1.0.st.v1 (prepared by Benilton Carvalho). The chips were normalized using the robust multi-array average (RMA) method described by Irizarray (Irizarry et al., 2003).

The following plots were generated as part of the quality control procedures: Box and whisker plots pre and post normalization; Gene expression histograms pre and post normalization; MVA plots with Loess curve fits (Irizarry et al., 2003); and interchip correlation plots. Normalized gene expression data was exported to .CSV files for further analysis.

6.6 Filtering for Differentially Expressed Genes

Differential expression depended on three separate metrics. First, the gene had to exhibit levels of expression above the background noise as measured by the average value of the Affymetrix negative controls (Gene Expression Console, Affymetrix). Second, the gene had to show greater than 1.4x fold change between samples. Finally, a p-value of <0.05 was required in order for the differential expression to be considered significant. Genes not meeting these criteria were filtered from the dataset leaving the differentially expressed genes used in further qualitative and literature-based analysis.

7 Results and Discussion

7.1 Quality Control

All six microarray slides used in this experiment were found to be viable and to exhibit normal gene expression profiles. Boxplots of the data before normalisation showed relative consistency across samples with mean expression values between 7 and 8 with standard deviations of +/- 1 (Figure 7.1A). Note that intensities as measured by Affymetrix Gene Console range from 1 - 32 768. In log transformed space this equates to 0 - 15. Post-normalisation, samples share a mean of ~7 and share a similar standard deviation allowing proper comparison of gene expression between slides (Figure 7.1B).

The distribution of gene expression across the six samples showed small variation (Figure 7.2A). Only the fourth sample, "Bl6 1" differed slightly from the other slides, but with a mean value of 8 and a reasonable distribution of gene expression about the mean, nothing about this slide suggests RNA degradation might have taken place. Post-normalization all samples share a similar distribution of intensities (Figure 7.2B).

In an effort to graphically represent the levels of differential expression between slides, MVA plots were produced. A representative sample of MVA plots is shown in Figure 7.3. The y-axis measures differential expression while the x-axis measures overall abundance of transcript in the samples. As expected, there is a lower level of differential expression between mice of the same strain (Figure 7.3A-B) with the majority of genes falling under a fold change value of 1.5x. Between mice of different strains, a higher level of differential expression is observed (Figure 7.3C-D) and a larger number of genes are differentially expressed at the 1.5x fold change level or higher.

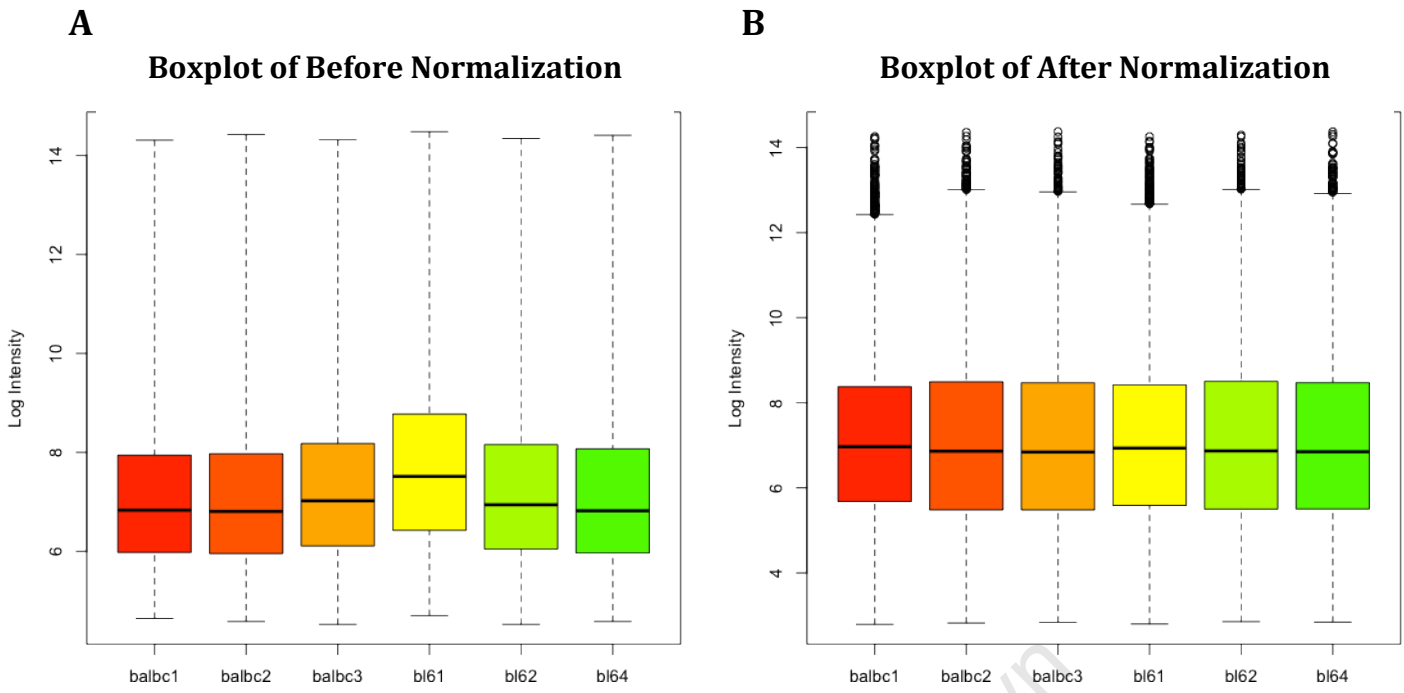

Figure 7.1. Box and Whisker Plots. Expression data for each of six samples is log transformed and then plotted pre and post normalization (A and B respectively). Data was normalized using the robust multi-array average method (Irizarry, 2003) and plotted using the Bioconductor Oligo package boxplot function (Oligo version 1.22.0, Benilton Carvalho)

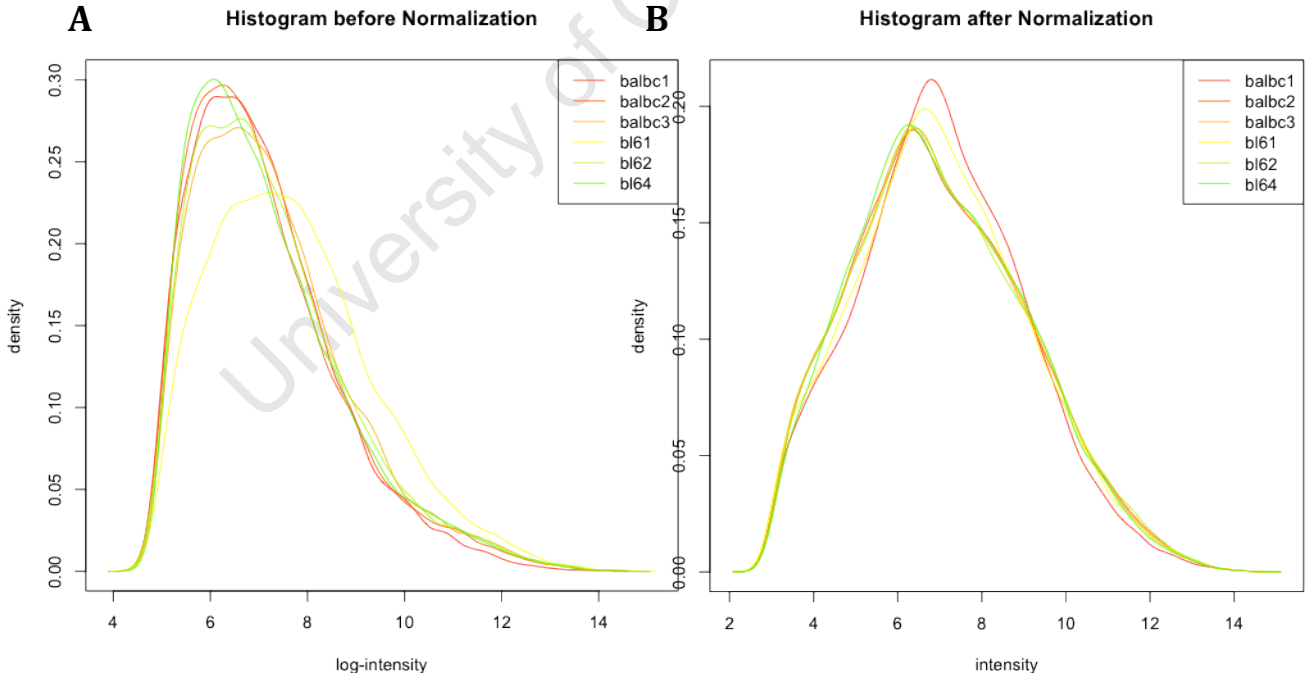

Figure 7.2. Histograms. Histograms for each of the six biological replicates are plotted above, pre and post normalization (A and B respectively). On the x-axis are the log-transformed expression values. On the y-axis the frequency/density of genes at that level of gene expression. Histograms were generated using the Bioconductor package Oligo's histogram function (Oligo version 1.22.0, Benilton Carvalho).

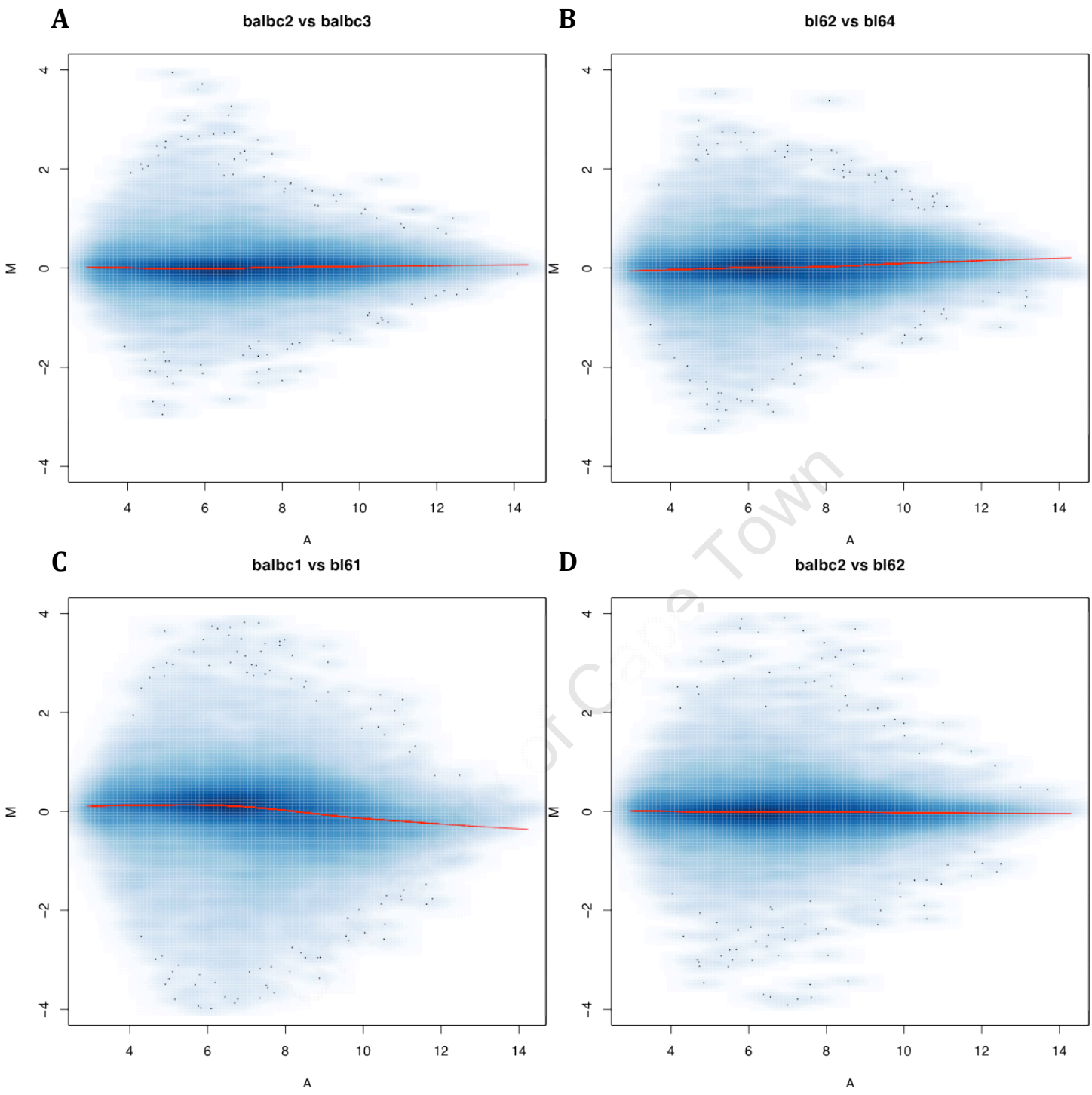

Figure 7.3. **MVA Plots.** Figures 3 A – D show a representative subset of the 15 MVA plots from post-normalization. The y-axis is a measure of the differential expression (great than zero is over expression, less than zero is under expression in the first sample relative to the second sample. The x-axis is a measure of the absolute gene expression in log-transformed space. Figures A and B represent MVA plots between samples from the same strain. Figures C and D represent MVA plots between samples from different strains.

7.2 Differentially Expressed Genes

19,857 mouse genes were arrayed on the Affymetrix GeneChip Mouse Exon ST Array. Table 7.1 below outlines the levels of differential expression between BALB/c and C57BL/6 activated T-lymphocytes given different assumptions about fold change and p-value. A total of 376 genes exhibited both a fold change greater than 1.4x and a p-value less than 0.05, representing 1.9% of total genes arrayed.

Table 7.1 Differentially Expressed Genes by Fold Change and P-value

P - Value	Fold Change										
	1.0	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**1.7**	**1.8**	**1.9**	**2.0**
1.00	9758	6042	3457	2076	1363	936	689	525	415	323	265
0.75	7633	5940	3445	2074	1362	936	689	525	415	323	265
0.50	5150	4690	3130	1976	1327	924	685	522	412	321	264
0.25	2392	2324	1885	1331	957	712	545	430	354	279	234
0.10	987	979	868	681	544	431	359	299	263	218	186
0.08	790	783	705	567	470	374	320	267	235	201	171
0.05	573	569	523	435	376	300	262	228	203	176	152
0.03	341	341	316	270	238	198	177	159	141	125	108
0.01	169	169	158	143	126	113	100	90	84	74	68

7.3 Key Th1/Th2 genes are differentially expressed between BALB/c and C57/BL6

Within the list of differentially expressed genes generated by the microarray, a variety of known Th1/Th2 markers were found (Table 7.2). Canonical Th1 marker genes included IFN-γ, TNF and Stat 4, all up-regulated in C57BL/6 relative to BALB/c. Conversely, canonical Th2 marker genes IL-4 and IL-1r1 were up regulated in BALB/c.

Table 7.2 Key Th1/Th2 Marker Genes Appearing in D/E Gene List

KEY TH1/TH2 MARKER GENES					
	Gene	**Log Intensity**		**Significance**	
	Symbol	**BALB/c**	**C57/BL6**	**Fold Change**	**P Val**
Th1 Markers	Stat4	10.3	10.8	-1.4	0.005
	Ifng	12.1	12.8	-1.7	0.047
	Tnf	9.0	9.9	-1.9	0.005
	Il18r1	10.4	11.5	-2.1	0.002
	Il18rap	10.3	11.3	-2.0	0.009
	Ccr5	8.5	10.7	-4.7	0.001
	Ccl5	8.9	10.2	-2.4	0.025
	Il12rb2	9.9	11.2	-2.4	0.003
	Il2ra	10.2	10.8	-1.5	0.037
Th2 Markers	Il1r1	9.6	7.1	5.6	0.031
	Il4	10.0	7.2	7.1	0.000
CD4 Markers	Cd86	7.3	8.3	-2.0	0.020
	Tlr4	7.3	6.2	2.1	0.008
	Tnfsf14	6.1	6.7	-1.5	0.027
	Ccnd3	7.7	8.2	-1.5	0.011

7.4 Differentially expressed genes map to known Leishmania Response regions

Differentially expressed (d/e) genes were mapped against known Leishmania Response regions (LMRs) (Lipoldova et al., 2000; Havelkova et al., 2006). A total of 72 d/e genes mapped to 12 LMRs across 11 chromosomes (Table 7.3). These LMRs collectively constitute 13% of the mouse genome (Figure 8). A full list of these genes is contained in Appendix B. Based on their level of differential expression and their appearance within known LMRs, 28 of these candidate genes were analysed further through literature searches and the 9 top candidates are discussed in the next chapter.

Table 7.3. 72 differentially expressed genes map to known LMRs

LMR	Chr	Len (cM)	D/E Genes	Paper
3	5	14	4	Lipoldova et al 2000
4	6	24	13	
5	10	20	1	
6	11	8	2	
7	17	10	19	
8	1	27	5	Havelkova et al 2006
9	4	5	4	
10	8	16	3	
11	3	20	9	
13	18	6	2	
14	2	24	6	
15	11	20	4	
Totals		**194**	**72**	

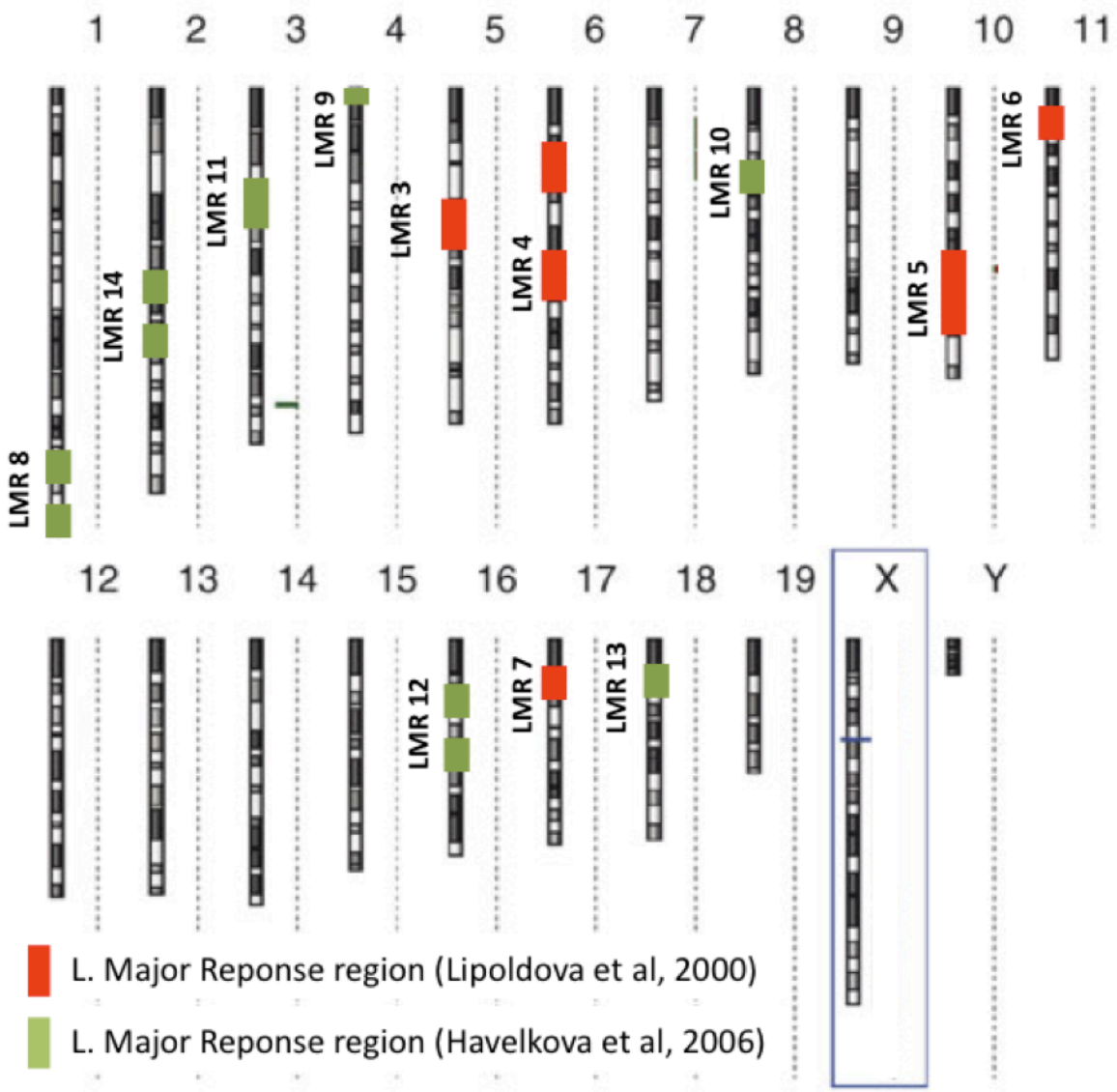

Figure 8: Leishmania Response (LMR) loci on mouse genome. Two sets of LMR discovered by the same research group and published in two separate papers are mapped out above. Diagram of mouse genome from Chong et al (Chong et al., 2007)

8 Discussion

8.1 Differentially Expressed Gene List Contains Key Th1/Th2 marker genes

Several key Th1/Th2 marker genes are differentially expressed in this microarray. Each is discussed in turn in an effort a) to validate the microarray itself and b) to contextualize the discussion of novel marker genes mapping to the LMRs.

STAT4 [1.42x up-regulated in C57BL/6, Chromosome 1]

Stat4 is a transcription factor belonging to the Signal Transducer and Activator of Transcription protein family. It is a well-known marker of the Th1 response due to its role in the up-regulation of IFN-γ in T-cells in response to stimulation with IL12. Its up-regulation in C57BL/6 is thus an expected feature of this microarray. Notably, STAT4 is known to bind to the promoters of cytokines IFN-γ and TNF, and receptors IL18R1, IL18RAP, IL12rB2, all of which were found to be differentially expressed in this microarray (up-regulated between 1.7-2.4x in C57/BL6).

Whilst expected, this result helps to confirm the dichotomy we expect to see, which in turn allows us to more closely examine unexpected or unknown genes and link them to the Th1 or Th2 response with a greater level of confidence.

IFN-γ [1.72x up-regulated in C57BL/6, Chromosome 10]

Interferon Gamma (IFN-γ) is the primary T-helper 1 marker gene. Its differential expression in this microarray is confirmation of the IFN-γ cytokine ELISAs conducted as part of this experiment (Smith, 2011, unpublished data) and suggests that the microarray hybridization and processing was successful.

TNF [1.89x up-regulated in C57BL/6, Chromosome 17]

Tumor necrosis factor (TNF) is a key cytokine involved in systemic inflammation and is known to induce fever, apoptotic cell death, sepsis and inhibit tumorogenesis. TNF is known to synergize with IFN-γ in mediating killing of *Leishmania Major* through the

induction of nitric oxide (Liew et al., 1990). It is a key Th1 marker gene and we thus expect its up-regulation in this microarray.

IL18r1/IL18RAP [2.03-2.37x up-regulated in C57BL/6, Chromosome 1]

IL18r1 and IL18RAP collectively form the IL18 receptor (IL81r). IL18 is known to synergize with IL12 to up-regulate IFN-γ production in T-cells (Tsuji et al., 1999). Both the IL18 and IL12 receptors are up-regulated in C57BL/6 T-cells in this microarray demonstrating a clear Th1 phenotype. Interestingly, IL18r shares homology with IL1r and forms a gene cluster with several other members of the IL1r family on chromosome 1. It is therefore surprising that IL1r is down-regulated 5.63x in this microarray, perhaps suggesting complementary roles for the IL18r and IL1r in the Th1 and Th2 responses respectively.

CCR5 [4.73x up-regulated in C57BL/6, Chromosome 9]

CCR5 is a chemokine receptor protein used by T cells to migrate to the sites of infection. CCR5 has been shown to be characteristic of Th1 lymphocyte (Loetscher et al., 1998). Interestingly, the ligand for CCR5, namely CCL5, is also up-regulated in C57BL/6 and been previously shown to be involved in resistance to infection with *L. major* (Santiago et al., 2004). Evidently the expression and interaction of these two proteins plays a role in Th1 cells locating the site of infection and exerting their influence there.

CCL5 [2.43x up-regulated in C57BL/6, Chromosome 11]

CCL5 is a chemotactic factor secreted by several cells in the immune system, including T cells. The gradient produced by CCL5 at the site of inflammation is used by various cells to migrate into or out of the site of infection. Interestingly, CCL5 has been shown to mediate the immigration of Langerhans cells into the epidermis of skin (Ouwehand et al., 2012). CCL5 is strongly up-regulated in the C57BL/6 T cells, suggesting that Th1 cells may facilitate dendritic cell entry into the site of infection.

CCL5 is interesting for another reason. It has been shown that, with the help of IFN-γ and IL2 (also produced by Th1 cells and observed to be up-regulated in this microarray), CCL5 induces the proliferation of natural killer cells (Maghazachi et al., 1996).With the strong up-regulation of CCL5 in C57BL/6, it is possible that CCL5 facilitates the induction of a more potent NK cell response than that observed in BALB/c. Recent research suggests that NK cells strongly facilitate the killing of intracellular parasite by macrophages (Prajeeth et al., 2011).

IL1R [5.63x up-regulated in BALB/c, Chromosome 1]

The interleukin 1 receptor (IL1R) is the cytokine receptor for various members of the interleukin 1 family. Perhaps most interesting of these members is IL33 which is known to induce T helper cells to produce Type 2 cytokines including IL-5 and IL13 (Kurowska-Stolarska et al., 2008; Saenz et al., 2010).

IL1 signaling is not something that has been studied much in the *L. major* model. It is alluded to in one paper in 1989 where production of IL1 by macrophages was shown to be enhanced by *L. major* infection in BALB/c mice but inhibited by the production of IFN-γ (Cillari et al., 1989).

The dramatic up-regulation of IL1R in BALB/c mice, in conjunction with the studies mentioned above, suggests it may play an important role in BALB/c susceptibility. The discovery that members of the IL1 family might play a role in initiating Th2 responses is relatively recent. Nonetheless, data from this microarray suggest that it may well be one of the primary contributors to BALB/c susceptibility.

IL-4 [7.15x up-regulated in BALB/c, Chromosome 11]

Interleukin 4 (IL-4) is the key T-helper 2 marker gene and is responsible from driving the Th2 response. It is interesting to note the extent of the up-regulation in BALB/c. Whereas IFN-γ is only 1.7x up-regulated in C57BL/6, the contrast is IL-4 levels is more extreme. This corroborates evidence that BALB/c mice do generate relatively high levels IFN-γ during *L. major* infection (Smith, 2011, unpublished data), but commensurately more IL-4, which ultimately drives the Th2 response.

CD86 [2.0x up-regulated in C57BL/6, Chromosome 16]

The discovery that CD86 is up-regulated in C57BL/6 is surprising. In fact, that CD86 is expressed at all on these T-cells is somewhat surprising. CD86, also known as B7-2, is typically expressed by antigen presenting cells and plays an important role in providing costimulatory signals necessary for T cell activation and survival. However, there is some evidence that subsets of T cells do express CD86. In particular, it has been shown that effector memory T cells express CD86 and that in response to stimulation most often produce IFN-γ (Jeannin et al., 1999). Effector memory T cells, however, typically arise due to a previous infection and given that the T cells examined in this microarray have experienced only one infection, it is not clear why memory T cells would be present.

TLR4 [2.07x up-regulated in BALB/c, Chromosome 4]

Toll-like Receptor 4 (TLR4) is one of the cell surface receptors responsible for detecting lipopolysacharide from Gram-negative bacteria and is associated with the triggering of inflammatory and type I interferon responses (Bell, 2008). While expression of TLR-4 on macrophages has been associated with increased parasite clearance in vivo (Kropf et al., 2004), not much is known about the activity of TLR-4 on T lymphocytes.

8.2 Differentially Expressed Genes Map to Known LMRs

The mouse genome is composed of 20 chromosomes which are collectively estimated to be 1355 centimorgans (cM) or 2.7 billion base pairs in length (Mouse Genome Sequencing et al., 2002; Rowe et al., 2003). In an effort to narrow down the chromosomal locations responsible for BALB/c susceptibility to L. major, a series of back crossing experiments were conducted whereby resistant strain STS/A was back-crossed against the susceptible BALB/c and at each step in the back-crossing, resistant progeny were selected(Lipoldova et al., 2000; Havelkova et al., 2006). In this way, the researchers succeeded in narrowing down the location of the resistance genes to

several stretches of the genome composing just under 13% of the total genome (Figure 8).

Our microarray produced a total of 376 differentially expressed genes with fold change greater than 1.4x and p-value of less than 0.05. This constitutes approximately 1.6% of the estimated total number of mouse genes (~23,000 on Affymetrix Exon Array). In an effort to narrow down the number of candidate genes which may be involved in BALB/c susceptibility, we mapped our differentially expressed genes to the LMRs already identified by Lipoldova et al. A total of 72 differentially expressed genes mapped to the LMRs. A full list of these genes is contained in Appendix B.

Of the 72 genes that map to these LMR regions, 28 were analysed further using literature searches. Criteria for selection included extent of differential expression (typically greater than 2x fold change) and, in some cases, known linkages with the immune system. A summary of the most important findings, by gene, are detailed below. Of particular interest are the homeobox only protein (Hopx) located on LMR 4 and the receptor activity modifying protein (RAMP1) located on LMR 8. Hopx is strongly up-regulated in resistant C57BL/6 mice. In studies unrelated to Leishmania, it has been shown to play a key role in the longevity and persistence T-helper 1 cells (Albrecht et al., 2010). RAMP1 is strongly up-regulated in BALB/c mice. It forms part of the receptor for calcitonin gene-related peptide (CGRP). In studies unrelated to Leishmania, CGRP has been shown to suppress Th1 and promote Th2 responses, alter Langerin dermal dendritic cell migration to the lymph node, and upregulate IL-4 production by T-cells (Gonzalez-Rey et al., 2007; Mikami et al., 2011).

IL23R [2.9x up-regulated in C57BL/6, Chromosome 6, LMR4]

IL-23 is a pro-inflammatory cytokine belonging to the IL-12 cytokine family. IL-23 is required for the differentiation of T lymphocytes into a subset of T helper cells known as Th17 (Zhou et al., 2007). While this suggests that there may be a larger contingent of Th17 cell present in the C57BL/6, we do not observe any differential expression of IL17 in this microarray. It is thus not clear, given what is currently known about the IL23R, what (if any) its role may be in protection against L. major.

Klrg1 [2.1x up-regulated in C57BL/6, Chromosome 6, LMR 4]

The killer cell lectin-like receptor G1 (Klrg1) is a known marker for natural killer cells (Lanier, 2006). While natural killer (NK) cells are best known for their recognition of non-self and of tumour cells through their screening of MHC1 expression, they have been shown to play a role in L. major infection. In particular, it has been shown that while NK cells to not directly lyse or induce apoptosis of infected macrophages, they do stimulate macrophages to kill intracellular Leishmania in a IFN-γ, TNF, and iNOs dependent manner (Prajeeth et al., 2011). NK cell activation is driven by dendritic cells in an TLR9, IL12, and IL18 dependent manner. This is corroborated by the up-regulation of IL18r and IL12r upregulation in C57BL/6 in this microarray.

Hpgds [2.2x up-regulated in BALB/c, Chromosome 6, LMR 4]

Human prostaglandin D2 (Hpgds) has been shown to associate with the Th2 response. In particular, in response to anti-CD3 and anti-CD28 stimulation, Th2 lines were shown to upregulate Hpgds (Tanaka et al., 2000), but it has not yet been established what the role of this protein is. We can thus confirm that it appears to be a Th2 marker, but what its role in differentiation or effector functions are is still unclear.

Hopx [3.3x up-regulated in C57BL/6, Chromosome 5, LMR 3]

The homeobox only protein (Hopx) is induced by the expression of the Th1 transcription factor Tbet. Interestingly, it has been shown to be key to the persistence of Th1 cells during infection (Albrecht et al., 2010) and has been shown to render Th1 cells resistant to Fas induced apoptosis. As such, Hopx is certainly a marker for Th1 and thus further confirms the validity of our microarray. However, the fact that it appears on LMR3 makes it a particularly interesting candidate for resistance in C57BL/6. For example, if there are differences in the way Hopx is expressed, or if there exist SNPs on this gene between BALB/c and C57BL/6, these could potentially explain the difference in phenotype between these two mice.

In vitro, Th1 cells are much more sensitive to Fas-mediated apoptosis than Th2 or Th17 cells (Varadhachary et al., 1997; Zhang et al., 1997). If there is some defect in

Hopx expression in BALB/c, or in the promoter region to which Tbet binds, this would result in Th1 cell transience instead of persistence. This is the observed phenotype in BALB/c. They appear able to produce IL12 and IFN-γ but the T-helper response nonetheless seems to default to Th2.

STAP 1 [1.7x up-regulated in C57BL/6, Chromosome 5, LMR 3]

Signal transducing adaptor family member 1 (STAP1) is involved in the signal transduction pathway initiated by cytokines on the surface of lymphocytes (Takahashi-Tezuka et al., 1997). Its function is not well-defined, but given that it is up-regulated in C57BL/6 may suggest that it is more prominently expressed in Th1 cells and is involved in either the Th1 differentiation or effector pathways.

OCIAD 2 [4.6x up-regulated in BALB/c, Chromosome 5, LMR 3]

Ovarian carcinoma immunoreactive antigen-like protein (OCIAD2) is strongly up-regulated in BALB/c versus C57BL/6. It also falls within one of the *Leishmania Major* Response regions on chromosome 5. OCIAD2 is primarily associated with studies of cancer and its expression is associated with smaller tumor size and better prognosis in adenocarcinoma patients (Ishiyama et al., 2006).

The expression of OCIAD 2 has been shown to correlate with the inhibition of TGF-beta signalling in lymphocytes (Classen et al., 2010). Down-regulated or up-regulated with anti TGF-beta? This paper

MHC Class Ib Molecules

	Up-regulated in	Fold Change	Chromosome	LMR
H2-Eb1	BALB/C	2.5x	17	7
H2-DMb1	BALB/C	2.1x	17	7
H2-T22	C57BL/6	1.9x	17	7
H2-K1	C57BL/6	2.5x	17	7
H2-T10	C57BL/6	16.6x	17	7

Five MHC Class Ib molecules were found to be differentially expressed in this microarray, three of them up-regulated in BALB/c and two up-regulated in C57BL/6. The MHC Class Ib molecules are a non-polymorphic family of MHC molecules and their role in immunity is little understood. Some have been implicated in anti-viral immunity (Swanson et al., 2008; Chen et al., 2011), others in the bridging of innate and acquired immunity (Rodgers and Cook, 2005). Very little is known about the specific roles of these proteins so it is difficult to read meaning into their differential expression. That being said, it would be interesting to establish whether in other Th1/Th2 disease models/microarrays these same MHC Class Ib genes are differentially expressed.

RAMP 1 (4.4x up-regulated in BALB/c, Chromosome 1)

Receptor activity modifying protein 1 (RAMP1) is a single-transmembrane-domain protein that is required to transport calcitonin-receptor-like receptor (CALCRL) to the plasma membrane and which then collectively function as a receptor for calcitonin gene-related peptide (CGRP) (Heroux et al., 2007). CGRP is a potent peptide vasodilator produced by peripheral and central neurons (Rosenfeld et al., 1983; Brain et al., 1985). Interestingly, CGRP has been shown to be an important regulator of cutaneous immunity through its effects on dendritic and T cell functions (Mikami et al., 2011). In particular, CGRP has been shown to exert different effects in contact hypersensitivity (CHS) models: It inhibits Th1-type CHS, but promotes Th2-type CHS. It has been shown to inhibit the migration of Langerin dermal dendritic cells to the lymph nodes and upregulates IL-4 production of T-cells in draining lymph nodes.

RAMP 1 is strongly up-regulated in the BALB/c mouse compared with C57BL/6. Taken together with the findings of Mikami et al, this suggests that RAMP1 could be one of the key proteins responsible for BALB/c susceptibility.

GON4l (5.4x up-regulated in C57BL/6, Chromosome 11)

Little is known about the Gon4-like (GON4l) gene. However we highlight its presence in this microarray partly because of its strong up regulation in C57BL6 and partly because it is one of the few transcription factors differentially expressed on this microarray (Lu et al., 2011).

8.3 Conclusion

Microarrays, particularly those comparing different strains of mice, naturally produce a vast number of differentially expressed genes. Using previous work conducted by Lipoldova and Havelkova, we were able to narrow down the regions on the murine genome where BALB/c susceptibility to *L. major* is likely to be determined. From a list of 76 such genes, 28 candidates were further investigated. The two most prominent candidates are Hopx and RAMP1. Further studies will be required to confirm these findings at the protein level and conduct experiments to determine the exact nature of their involvement. Such studies might include the use of conditional knockout mice. For example, if RAMP1 is indeed a BALB/c susceptibility factor, then we would expect the phenotypes of BALB/c T-cell-specific RAMP1-knockout mice and wildtype BALB/c to diverge. Whilst RAMP1$^{-/-}$ mice do exist (Sabharwal et al., 2010), the T cell specific mice do not yet exist and would have to be created using a cre-loxP or similar system.

Confirmation that Hopx or RAMP1 are susceptibility factors in BALB/c mice would not only augment our understanding of T-helper cell differentiation and T-helper effector functions, but would also provide possible targets for treating inflammatory disease in humans.

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Appendix A: Solutions

Anaesthetic
1.2ml Anaket-V (100mg/ml) (Centaur labs, Isando)
0.9ml Rompun (2%) (Bayer, Germany)
8.0ml PBS (1X)

Blocking Buffer
20g Milk powder (spar instant) (2%)
Make up to 1L with 1x PBS

Dilution Buffer
10g BSA (Roche)
0.2g NaN_3 (0.02%) (Merck)
Make up to 1L with 1x PBS

FACS Buffer
0.1% BSA (Roche)
0.05% NaN_3 (Merck)
Made up in 1X PBS

PBS (10x)
80g NaCl (1.37M)
2g KCL (0.03M)
14.4g H_2PO_4 (0.01M)
2.4g KH_2PO_4
Dissolve in 1L ddH_20

Schneiders medium
1x Schneider's media (Sigma)
0.4g Sodium Bicarbonate
0.795g $CaCL_2.2H_20$
Make up to 1L with ddH_20

Substrate Buffer
0.2g NaN_3 (0.02%)
97ml di-ethanolamine
0.8g $MgCl_2.6H_20$
700ml ddH_20
Adjust the pH to 9.8 and make up to 1L with ddH_20

Washing Buffer
20g KCL
20g KH_2PO_4
144g $NA_2HP0_4.H_20$
800g NaCl (Merck-BDH)
50ml Tween 20 (Sigma)
100ml 10% NaN_3 (Merck)
Make up to 5L with ddH_20

Appendix B: Differentially Expressed Genes Mapping to LMRs

Differentially Expressed Genes mapping to LMR Regions						
LMR	**Gene Symbol**	**BALB/C**	**C57BL/6**	**P-val**	**Fold Ch**	**Name**
LMR 3	Rell1	8.2	7.6	0.04	1.5	RELT-like 1
LMR 3	Ociad2	9.3	7.1	0.01	4.6	OCIA domain containing 2
LMR 3	Hopx	7.9	9.7	0.01	3.3	HOP homeobox
LMR 3	Stap1	9.3	10.1	0.02	1.7	signal transducing adaptor family member 1
LMR 4	Igf2bp3	6.0	6.7	0.01	1.7	insulin-like growth factor 2 mRNA binding protein 3
LMR 4	Nod1	7.6	8.1	0.02	1.4	nucleotide-binding oligomerization domain containing 1
LMR 4	Vopp1	8.3	8.8	0.01	1.5	vesicular, overexpressed in cancer, prosurvival protein 1
LMR 4	Hpgds	7.9	6.8	0.01	2.2	hematopoietic prostaglandin D synthase
LMR 4	Gng12	9.5	8.5	0.04	2.0	guanine nucleotide binding protein (G protein), gamma 12
LMR 4	Gadd45a	9.2	8.4	0.00	1.7	growth arrest and DNA-damage-inducible 45 alpha
LMR 4	Il12rb2	9.9	11.2	0.00	2.4	interleukin 12 receptor, beta 2
LMR 4	Il23r	6.3	7.8	0.01	2.9	interleukin 23 receptor
LMR 4	Rassf4	7.0	6.2	0.02	1.8	Ras association (RalGDS/AF-6) domain family member 4
LMR 4	Erc1	8.4	7.8	0.02	1.6	ELKS/RAB6-interacting/CAST family member 1
LMR 4	Cecr5	7.2	7.8	0.00	1.5	cat eye syndrome chromosome region, candidate 5
LMR 4	Klrg1	8.7	9.7	0.02	2.1	killer cell lectin-like receptor subfamily G, member 1
LMR 4	Clec4a3	6.9	6.2	0.05	1.7	C-type lectin domain family 4, member a3
LMR 5	Ifng	12.1	12.8	0.05	1.7	interferon gamma
LMR 6	Nudcd3	8.8	8.2	0.01	1.6	NudC domain containing 3
LMR 6	Bcl11a	7.9	7.2	0.03	1.6	B cell CLL/lymphoma 11A (zinc finger protein)
LMR 7	Tagap	9.2	8.5	0.04	1.7	T cell activation Rho GTPase activating protein
LMR 7	Fgfr1op	9.5	8.7	0.00	1.8	Fgfr1 oncogene partner
LMR 7	Zfp52	8.5	9.7	0.02	2.2	zinc finger protein 52
LMR 7	Dcpp1	6.5	7.1	0.01	1.4	demilune cell and parotid protein 1
LMR 7	Dnase1l2	6.1	6.9	0.00	1.8	deoxyribonuclease 1-like 2
LMR 7	Gfer	8.4	9.0	0.02	1.5	growth factor, erv1 (S. cerevisiae)-like
LMR 7	Fahd1	7.5	6.2	0.01	2.5	fumarylacetoacetate hydrolase domain containing 1
LMR 7	Nme3	9.2	8.6	0.00	1.6	mitogen-activated protein kinase 8 interacting protein 3
LMR 7	Dusp1	11.3	10.2	0.03	2.1	dual specificity phosphatase 1
LMR 7	Pacsin1	8.3	7.7	0.01	1.5	protein kinase C and casein kinase substrate in neurons 1
LMR 7	Dnahc8	9.6	7.7	0.00	3.8	dynein, axonemal, heavy chain 8
LMR 7	H2-Eb1	11.0	9.6	0.04	2.5	histocompatibility 2, class II antigen E beta
LMR 7	H2-K1	8.8	10.1	0.04	2.5	histocompatibility 2, K1, K region
LMR 7	H2-DMb1	9.2	8.1	0.00	2.1	histocompatibility 2, class II, locus Mb1
LMR 7	Msh5	7.4	6.8	0.05	1.5	mutS homolog 5 (E. coli)
LMR 7	Tnf	9.0	9.9	0.01	1.9	tumor necrosis factor
LMR 7	H2-T10	6.8	10.9	0.00	16.6	histocompatibility 2, T region locus 10
LMR 7	H2-T22	11.2	10.3	0.04	1.8	histocompatibility 2, T region locus 22
LMR 7	Ppp1r11	11.8	10.9	0.02	1.9	protein phosphatase 1, regulatory (inhibitor) subunit 11
LMR 8	Fam124b	6.2	6.9	0.03	1.6	family with sequence similarity 124, member B
LMR 8	Zfp617	6.1	7.3	0.00	2.3	zinc finger protein 617
LMR 8	Col6a3	7.9	6.4	0.01	2.9	collagen, type VI, alpha 3
LMR 8	Ramp1	10.3	8.1	0.00	4.4	receptor (calcitonin) activity modifying protein 1
LMR 8	St8sia4	10.5	10.0	0.04	1.4	ST8 alpha-N-acetyl-neuraminide
LMR 9	9330169L03Rik	7.4	8.6	0.02	2.2	RIKEN cDNA 9330169L03 gene
LMR 9	Lyn	8.2	6.6	0.02	3.2	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog
LMR 9	Penk	11.2	10.5	0.01	1.6	preproenkephalin
LMR 9	Tox	10.8	10.0	0.01	1.7	thymocyte selection-associated high mobility group box

LMR	Gene Symbol	BALB/C	C57BL/6	P-val	Fold Ch	Name
LMR 10	Wrn	8.7	9.2	0.02	1.4	Werner syndrome homolog (human)
LMR 10	Tmem66	9.7	10.3	0.00	1.6	transmembrane protein 66
LMR 10	Frg1	11.2	10.7	0.04	1.5	FSHD region gene 1
LMR 11	P2ry14	8.6	6.2	0.01	5.1	purinergic receptor P2Y, G-protein coupled, 14
LMR 11	F630111L10Rik	8.8	6.4	0.04	5.3	RIKEN cDNA F630111L10 gene
LMR 11	P2ry13	7.4	6.2	0.02	2.2	purinergic receptor P2Y, G-protein coupled 13
LMR 11	Ube2v1	7.7	8.3	0.02	1.4	ubiquitin-conjugating enzyme E2 variant 1
LMR 11	Lxn	6.3	8.2	0.00	3.7	latexin
LMR 11	Gon4l	7.3	9.7	0.00	5.4	gon-4-like (C.elegans)
LMR 11	Tnfaip8l2	9.7	10.4	0.02	1.7	tumor necrosis factor, alpha-induced protein 8-like 2
LMR 11	Sv2a	7.5	7.0	0.01	1.5	synaptic vesicle glycoprotein 2 a
LMR 11	Slc22a15	8.3	7.1	0.00	2.4	solute carrier family 22 (organic anion/cation transporter)
LMR 13	Tnfaip8	10.3	9.7	0.02	1.5	tumor necrosis factor, alpha-induced protein 8
LMR 13	Ppic	9.5	8.3	0.01	2.3	peptidylprolyl isomerase C
LMR 14	Kynu	8.6	6.4	0.02	4.6	kynureninase (L-kynurenine hydrolase)
LMR 14	Rnd3	9.0	6.2	0.02	7.0	Rho family GTPase 3
LMR 14	Fmnl2	8.9	7.1	0.02	3.4	formin-like 2
LMR 14	Rapgef4	6.2	7.0	0.01	1.7	Rap guanine nucleotide exchange factor (GEF) 4
LMR 14	Rapgef4	6.1	8.6	0.00	5.4	Rap guanine nucleotide exchange factor (GEF) 4
LMR 14	Chn1	8.4	6.9	0.00	2.8	chimerin (chimaerin) 1
LMR 15	Havcr2	7.1	8.3	0.00	2.3	hepatitis A virus cellular receptor 2
LMR 15	Themis	10.3	11.1	0.04	1.7	T cell immunoglobulin and mucin domain containing 2
LMR 15	Havcr1	7.9	5.9	0.01	3.9	hepatitis A virus cellular receptor 1
LMR 15	Rnf130	9.2	7.3	0.02	3.7	ring finger protein 130

Appendix C: Rscript Used to Normalize Microarray Data

Change working directory to T-cell Microarray

```
library(oligo)                                          # Load oligo library
install.packages("pd.moex.1.0.st.v1",dep=T)             # Get the oligo exon array map
celFiles <- list.celfiles("cel_files", full.names = TRUE)  # Get cel files names
aefs <- read.celfiles(celFiles)                         # Read in the cel files
names <- scan(file="names.txt", what = 'character')     # Read in names of slides
sampleNames(aefs) <- names                              # Change names in  raw data
results <- rma(aefs)                                    # Normalize data using RMA

# Create a histogram of raw data
hist(aefs, lty=1, col=rainbow(18), main = "Histogram before Normalization")
legend("topright", col=rainbow(18), lty=1, legend=names)

# Create histogram of normalized data
hist(results, lty=1, col=rainbow(18), main = "Histogram after Normalization")
legend("topright", col=rainbow(18), lty=1, legend=names)

# Create boxplot of data before normalization
boxplot(aefs, col=rainbow(18), names=names, ylab="Log Intensity")

# Create boxplot of data after normalization
boxplot(exprs(results), ylab="Log Intensity", col = rainbow(18), names = names, main
= "Boxplot of Normalized Results")

# Create correlation plot before normalization
correlationPlot(aefs)

# Create correlation plot after normalization
correlationPlot(results)

# Write results to results file
write.table(exprs(results), file="results/normalised_data.txt")
```

Appendix D:

FACS Antibodies

Antibody	Format	Dilution	Type	Company	Cat #
IL-4Ra	Biotin	1:400	Rat IgG2a	BD-pharmingen	552508
IL-4Ra	PE	1:400	Rat IgG2a	BD-pharmingen	552509
CD4	Percp	1:100	Rat IgG2a	BD-pharmingen	553052
CD4	FITC	1:250	Rat IgG2a	BioLegend	100509
CD19	Biotin	1:250	Rat IgG2a	BD-pharmingen	553784
CD19	PerCP-Cy	1:100	Rat IgG2a	BD-pharmingen	551001
MHC II	Biotin	1:200	Mouse IgG3	BD-pharmingen	553609
CD86	APC	1:100	Rat IgG2a	BD-pharmingen	558703
CD80	PE	1:100	Hamster IgG2	BD-pharmingen	553769
CD28	FITC	1:250	Hamster IgG2	BD-pharmingen	553295
Isotype	PE	1:200	Hamster IgG	BD-pharmingen	554711
Isotype	PE	1:80	Rat IgG2b	BD-pharmingen	556925
Isotype	FITC	1:80	Rat IgG2b	BD-pharmingen	553988
IFNg	PE	1:100	Rat IgG1	BD-pharmingen	554412
IL4	PE	1:100	Rat IgG2b	BD-pharmingen	554389
IL12	PE	1:100	Rat IgG1	BD-pharmingen	554479

Cytokine Elisa Antibodies

	Capture	Detection	Standard	Sensitivity
IL4	1:500	1:1000	250ng/ml	10pg/ml
Type	Rat anti Mouse	Biotin Rat anti Mouse	Recombinant	
Company & Clone	BD Pharmingen 550067	BD Pharmingen 554390	BD Pharmingen 550067	
IFNg	1:500	1:1000	250ng/ml	46pg/ml
Type	Rat anti Mouse	Biotin Rat anti Mouse	Recombinant	
Company & Clone	BD Pharmingen 554640	BD Pharmingen 554415	BD Pharmingen 554618	
IL10	1:500	1:1000	250ng/ml	823pg/ml
Type	Rat anti Mouse	Biotin Rat anti Mouse	Recombinant	
Company & Clone	BD Pharmingen 555057	555084	BD Pharmingen 550070	

Appendix D continued

Cytokine Elisa Antibodies

Immunoglobulin	Capture	Detection	Standard
IgE	1:1000	1:1000	Recombinant
Type	Home Brew Purified (84.1C)	Rat anti-mouse IgE-AP	
Company & Clone	Brombacher Lab	Southern Biotec 1130-04	
IgG1	1:1000	1:1000	Recombinant
Type	Goat anti Mouse	Goat anti Mouse AP	Southern Biotec
Company & Clone	Southern Biotech 1070-01	Southern Biotec 1070-04	
IgG2b	1:1000	1:1000	Recombinant
Type	Goat anti Mouse	Goat anti Mouse AP	Southern Biotec
Company & Clone	Southern Biotec 1090-01	Southern Biotec 1090-04	
IgG2a	1:1000	1:1000	Recombinant
Type	Goat anti Mouse	Goat anti Mouse AP	Southern Biotec
Company & Clone	Southern Biotec 1080-1	Southern Biotec 0103-01	