The role of CD4^{+}T cells in Host Protective responses against cutaneous Leishmaniasis using Genome-wide Transcriptomics

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Thesis submitted to the University of Cape Town in fulfilment of the degree

Doctor of Philosophy

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April 2013

DECLARATION

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

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Liezel Catharine Smith

DEDICATION

This thesis is dedicated to my parents, Leon and Florence Swarts, and to my beloved husband, Clyde and son, Nicholas.

ACKNOWLEDGMENTS

This dissertation would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the developing and shaping of my scientific career and in the preparation and completion of this study. My sincerest gratitude goes to my supervisor, Professor Frank Brombacher for his guidance and motivation during this work. His knowledge and vision have been a great inspiration.

My co-supervisor, mentor and friend, Dr. Anita Schwegmann, has been an excellent teacher, motivator and councillor at times. Anita, your motivation, enthusiasm, guidance and constant encouragement will always be cherished.

Thank you to Dr. Reto Guler for his help with the critical reading of this dissertation. The University of Cape Town Animal Unit staff, the Genotyping team, CPGR and Omics institute for performing the microarray experiments. A special thank you I would especially like to thank Mr Andrew Einhorn and my dear friend Umeshree Govender for their help with microarray statistics and analysis; Dr Natalie Nieuwenhuizen and Dr Claire Hoving for their lab expertise and advice and the rest of the Division of Immunology.

My deepest gratitude to Wendy Green, Fadwah Booley, Rayaana Fredericks, Nasiema Allie, Jaisubash, Ramona Hurdayal, Suraj Parihar, Sumayah Salie, Toinette Labuscagne, Ronnie Dreyer and countless others in the lab, who always made time to listen me and for their kind words of encouragement and providing me with a vibrant and stimulating environment. Those intriguing, scientific tea time discussions will always be remembered.

I would like to acknowledge the financial support of the University of Cape Town and the Medical Research Council, in particular, Dr Thabi Maitin and Ms Lebogang Montewa who has been

invaluable during this dissertation. I would like to thank the MRC for giving me the opportunity to be part of the internship programme and to attend their training courses and for their excellent advice and support.

Most importantly, I would like to thank the supporting "pillars" in my life for their constant support and care: my lovely husband Clyde and our son, Nicholas ("the greatest bloke alive") who is my unconditional love and support, for all the loving support and sacrifices that they willingly made for me and waited for me. Additional thanks to my parents and family who never questioned my unusual arrival or working hours, their constant support throughout the years; and my friends who tolerated my scarceness. Finally an answer to that one question that was always avoided...."How is the thesis?"

Last but certainly not least, to my Lord and Saviour Jesus Christ, in Whom I find my purpose and reason for being.

Lastly, I would like to acknowledge all the mice that were sacrificed in the name of science.

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List of Symbols and Abbreviations

Abbreviation	Description
-/-	Knockout
<	Less than
>	More than
^{0}C	Degrees Celsius
γc	Common gamma chain
7aad	7-Aminoactinomycin D
Ab	Antibody
AP	Alkaline phosphatase
APCs	Antigen presenting cells
BSA	Bovine serum albumin
CD	cluster of differentiation
CCL5	Chemokine ligand 5
CCR5	Chemokine receptor 5
Cre	Cre recombinase
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme linked immuno-sorbent assay
FACS	Fluorescence-Activated Cell Sorting
Fc	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidise
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IgG2b	Immunoglobulin 2b
IL	Interleukin
IL-4Rα	Interleukin-4 receptor alpha
IL-4Rα$^{-/-}$	IL-4Rα deficient
IL-4Rα$^{-/lox}$	Hemizygous for IL-4Rα (considered wild type controls)
IL-13Rα	Interleukin-13 receptor alpha
JAK	Janus Kinase
mAbs	Monoclonal antibodies
mRNA	Messenger RNA
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

Pen/Strep	Medium containing penicillin and streptomycin
PMA	Phorbol 12-myristate 13-acetate
PNP	P-Nitrophenyl phosphate
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean
SLA	Soluble Leishmania Antigen
STAT	Signal transducer and activator of transcription
TGF-β	Tumour growth factor beta
TH	T-helper ($CD4^+$) cell
TNF	Tumour necrosis factor
UCT	University of Cape Town
WT	Wild type

Abstract

Leishmania major is a protozoan parasite and infection in the human host causes severe cutaneous Leishmaniasis. The study aims to determine how signaling via the IL-4Rα on $CD4^+$Tcells causes susceptibility to *L. major*. We compared gene expression patterns early during infection in $CD4^+$ T cells in the absence or presence of IL-4Rα signaling. Non-healer BALB/c mice with a deletion of the IL-4Rα on all cells (IL-4R$\alpha^{-/-}$) or $CD4^+$ T cells only (iLCK^{cre}IL-4R$\alpha^{lox/-}$) and their controls (wild-type (WT) C57BL/6, WT BALB/c and littermate IL-4R$\alpha^{lox/-}$) were subcutaneously infected with *L. major*. As expected, the C57BL/6 "healer" mice produced a predominant TH1 response, whereas the iLCK^{cre}IL-4R$\alpha^{-/lox}$ mice and susceptible BALB/c mice produced a TH2 response. No significant differences in footpad swelling or parasite burden was observed amongst the groups at this early time point, except for the BALB/c mice which had a higher number of parasites. Activated $CD4^+$ T cells were successfully isolated to greater than 99% purity by FACS sorting, and high quality RNA extracted with no genomic DNA contamination. Activated T cell and regulatory T cell marker expression on linearly amplified RNA from three independent biological experiments was evaluated by RT-PCR to confirm conformity to expected profiles given the genotype of mice and samples were hybridized to Affymetrix Exon arrays at the Centre for Proteomic and Genomic Research (CPGR) and Illumina Bead chip® arrays at the RIKEN Omics Science Centre, Japan. Lists of significantly differentially expressed (DE) genes were generated by comparing expression data from the IL-4R$\alpha^{-/-}$ groups to wild type groups' expression data. The DE gene lists were considered to be biologically relevant as determined by the appearance of many expected genes for this particular study (e.g. CCR5 and H2-T10). Forty novel candidate genes either associated with cell proliferation, differentiation and immune response were identified using various bioinformatics tools such as network analysis. Confirmation of the DE gene expression of candidate genes of interest was done using RT-PCR. This study provides possible targets for manipulation as a foundation for potentially novel therapeutic targets in Leishmaniasis in humans.

Chapter 1

Introduction

1.1 Innate and Adaptive Immunity

The body's defense against infectious organisms and other invaders is called the immune system (Roitt *et al*., 2001). During an immune response the immune system, in a series of steps attacks organisms and foreign substances that invade the body to cause disease. The immune system is divided into two categories, namely innate and adaptive immunity (Figure 1). Innate immunity refers to the body's nonspecific defense mechanisms immediately or within hours of an antigen's appearance in the body (C A Janeway, 1992). These include physical barriers such as skin, immune cells that attack foreign cells or chemicals in the blood. Adaptive (acquired) immunity is antigen-specific and a more complex response than the innate response and it is orchestrated by B and T cells (Roitt *et al*., 2001). When the antigen has been processed and recognized, the immune system creates an army of immune cells specifically designed to attack. An adaptive immune response also results in immunological memory, which confers lifelong protective immunity to reinfection with the same pathogen (Male *et al*., 2006).

Both innate and adaptive immune responses depend upon the activities of leukocytes, which develop and mature in the bone marrow, where they then migrate and guard the peripheral tissues. Lymphocytes are the key players in the immune system, and are found in the lymphoid organs positioned throughout the body. Bone marrow is the ultimate source of all blood cells, including lymphocytes. The thymus is a lymphoid organ where T lymphocytes or T cells mature, and then migrates to other tissues. B lymphocytes or B cells become activated in peripheral lymphoid organs and mature into plasma cells which make and release antigen-specific antibodies such as IgM, IgG, IgE, IgA or IgD antibodies. B-cells can function as antigen presenting cells (APCs) as they can process and present antigens on MHC-II molecules to $CD4^+$ T-cells and possess APC co-stimulatory molecules (Rothenberg *et al*., 2006). The spleen contains specialized compartments and serves as a meeting ground where immune defense confronts antigens. Figure 1 is an illustration depicting the main difference between the innate and adaptive immune system as well as the interface between the two arms.

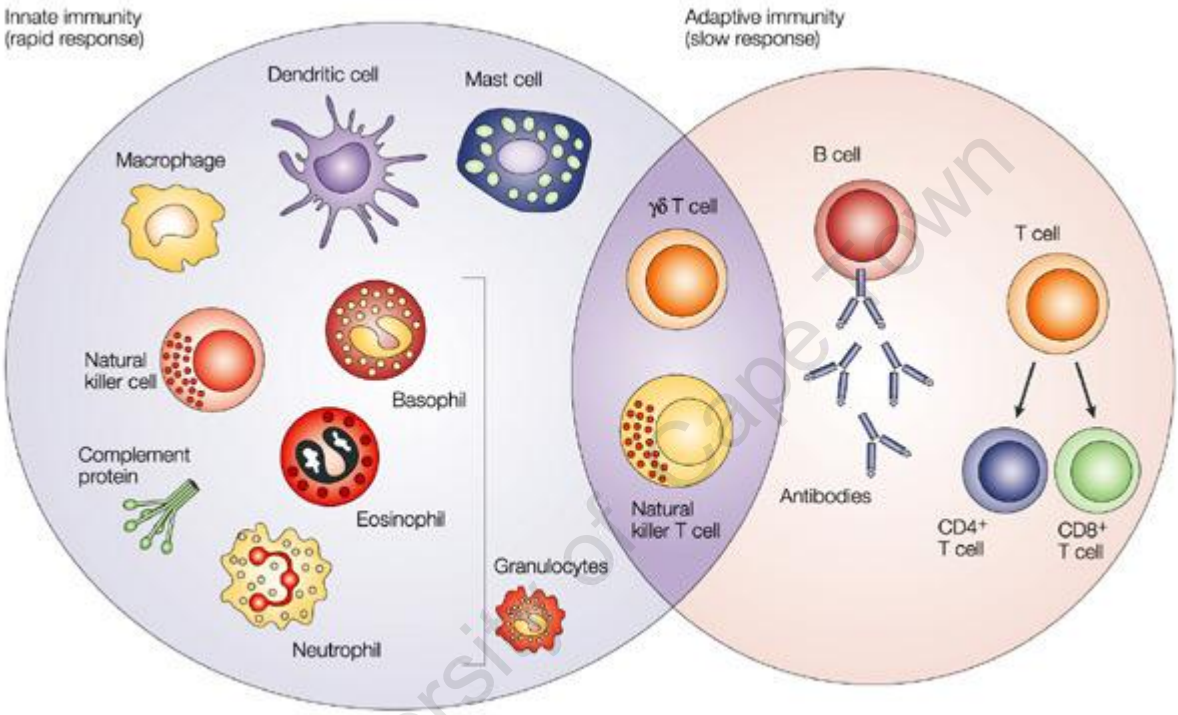


Figure 1 The Innate and adaptive Immune system

The immune system is the body's mechanism of protection against invaders such as viruses, bacteria and parasites and comprises of two parts, namely innate and adaptive. The first line of defense against infection is called the innate response and consists of many cellular components including granulocytes, macrophages, dendritic cells, natural killer cells and mast cells. The second arm is called the adaptive immune response which is orchestrated by lymphocytes (T and B cells) and is defined by specificity and memory. The interface between the two arms of immunity consists of the natural killer T cells and γδT cells. Illustration taken from previous publication, Nature Reviews Cancer 2004.

1.1.1. Cytokines

Cytokines are chemical messengers that play a key role in modulation of immune responses. Cells communicate with one another by releasing and responding to cytokines, and they are essential for the regulation of the cells of both innate and adaptive immune responses (Male D *et al.*, 2006). These proteins are secreted by immune and non-immune cells and act on other cells to coordinate appropriate immune responses such as regulation of lymphocyte turnover, differentiation and activation. Cytokines include a diverse assortment of interleukins, interferons, and growth factors, and can have multiple effects on various different cells.

The production of cytokines is transient and it contributes to many roles in the cell including activation, proliferation, cell differentiation, cell recruitment and release of effector molecules (Brombacher 2012 and Tato, C.M. & O'Shea, J.J, 2006). The host's immune response depends on the cytokines that are produced and are therefore indicators of disease progression. Cytokines can be produced by a variety of cell types and often have overlapping functions on effector cells (Brombacher 2012). A cytokine cascade can also result in an increase or decrease of other cytokines. Some receptor subunits are shared by cytokines, hence their functional redundancy (Durum *et al*., 1998). Therefore, understanding the role of cytokines and the importance of their expression profiles are important. Research has indicated that the use of gene-deficient (knockout) and transgenic mice strains provide an essential tool in our understanding of cytokine function (Durum *et al*., 1998). However, in the absence of a specific gene, compensatory mechanism not normally present may be activated (Louis *et al*., 1998). We now make use of mice that are deficient for cytokines or their receptor subunits and more recently, mice with cell-specific deletions or transgenes to study specific diseases.

1.1.2. T cell differentiation

The immune system's response to any foreign substance, or antigen, involves a cascade of events orchestrated by specialized immune cells. T cells play central roles in cell-mediated and humoral immunity. Several different subsets of T cells have been discovered, each with distinct functions. For a long time it was believed that TH1 / TH2 paradigm dominated.

The major lineages for $CD4^+$ T helper cells are TH1 and TH2; however other subsets have been identified. These subsets include TH17, TfH and $CD4^+CD25^+$ regulatory T cells and they regulate immune responses and play a crucial role in preventing autoimmunity and excessive effector responses to pathogens (Figure 2). Additional effector T cell subsets may include TH5 and TH9, which produce IL-5 and IL-9 respectively (Zhu *et al*., 2010). TH17 cells are IL-17 producing effector $CD4^+$ T cells that require TGF-β, IL-6 and IL-23 for development. The TH17 lineage has a role in autoimmunity and defence against extracellular bacteria (Bettelli *et al*., 2006**;** Mangan *et al*., 2006) and the crucial transcription factor for this lineage is the orphan nuclear receptor (RORγt) (Ivanov *et al.*, 2006). TH17 cells play a crucial role in clearing extracellular microbes (Huang *et al.*, 2004), autoimmunity (Koenders *et al.*, 2005), and certain asthmatic symptoms (Kolls *et al.*, 2003).

TfH cells assists in the formation of germinal centers, affinity maturation and promote immunoglogulin class switch recombination. The primary function of TfH cells is to assist the B cells make antibody responses to T cell-dependent antigens. Bcl6 is a transcriptional repressor that is expressed by TfH cells. These cells may produce TH1 or TH2 cytokines depending on the conditions of their activation (Zhou *et al.*, 2009, Fazileau *et al.*, 2009).

Regulatory T cells are a specialized subpopulation of T cells that are essential for maintaining peripheral tolerance as well as acting to suppress activation of the immune system and thereby maintain homeostasis of the immune system and tolerance to self

antigens (Zhu & Paul, 2008). The critical role that regulatory T cells play within the immune system is evidence by the severe autoimmune syndrome that results from genetic deficiency in regulatory T cells (Sakaguchi *et al.,* 2006; Sakaguchi *et al.*, 2008, Belkaid *et al.*, 2009 and Yamaguchi *et al.*, 2006). Regulatory T cells develop in the thymus and are defined by the expression of the forkhead family transcription factor FoxP3 (forkhead box p3). Expression of this 'master regulator,' FoxP3 is required for regulatory T cells development and appear to control a genetic program specifying this cell fate. Functions of these cells include, maintaining tolerance to self and control deviation, prevention of runaway responses to pathogens or allergens, they help with the maintenance of the balance with microbial flora and facilitate tumors escape from immune monitoring (Feuerer *et al*., 2009). A study has shown that deficiencies in FoxP3 underlie the lymphoproliferation and multiorgan autoimmunity of *scurvy* mutant mice and human patients with immunodysregulation polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome (Ziegler, 2006).

A large majority of FoxP3 expressing regulatory T cells are found within the MHC-II restricted $CD4^+$ T helper cell population and express high levels of the IL-2Rα chain (CD25) (Belkaid *et al*., 2002). To identify and monitor regulatory T cells, a number of different methods have been employed. Initially, high expression of CD4 and CD25 surface markers was used (ie. $CD4^+CD25^+$cells). However, it is known that CD25 is also expressed on non regulatory T cells. The additional expression of FoxP3 allowed for a more specific analysis of regulatory T cells ($CD4^+$CD25 cells) (Vignali *et al.*, 2008). The high level of expression of CD25 (IL-2Rα) on regulatory T cells suggests the importance of IL-2 for these cells. The immunosuppressive cytokines TGF-β and IL-10 have also been implicated in regulatory T cells function. TGF-β plays a major role in regulatory T cell differentiation and development and it induces FoxP3 expression (Chen *et al.*, 2003). IL-2- mediated Stat5 activation is also a requirement for the induction of FoxP3 expression. Therefore, both TGF-β and IL-2 are a prerequisite for the function and survival of regulatory T cells even after they have differentiated.

In addition, Regulatory T cells include three distinct subtypes of $CD4^+$ T cells that are termed inducible regulatory T cells. These subtypes are distinct in the following manner. T regulatory 1 (T_R1) cells secrete high levels of IL-10, no IL-4 and no or low levels of IFN-γ. T helper 3 (T_H3) cells secrete high levels of TGF-β and $CD8^+$ regulatory T cells, although $CD8^+$ T cells are normally associated with cytotoxic T-lymphocyte function and IFN-γ production, a subtype of these cells secrete IL-10 (Mills 2004).

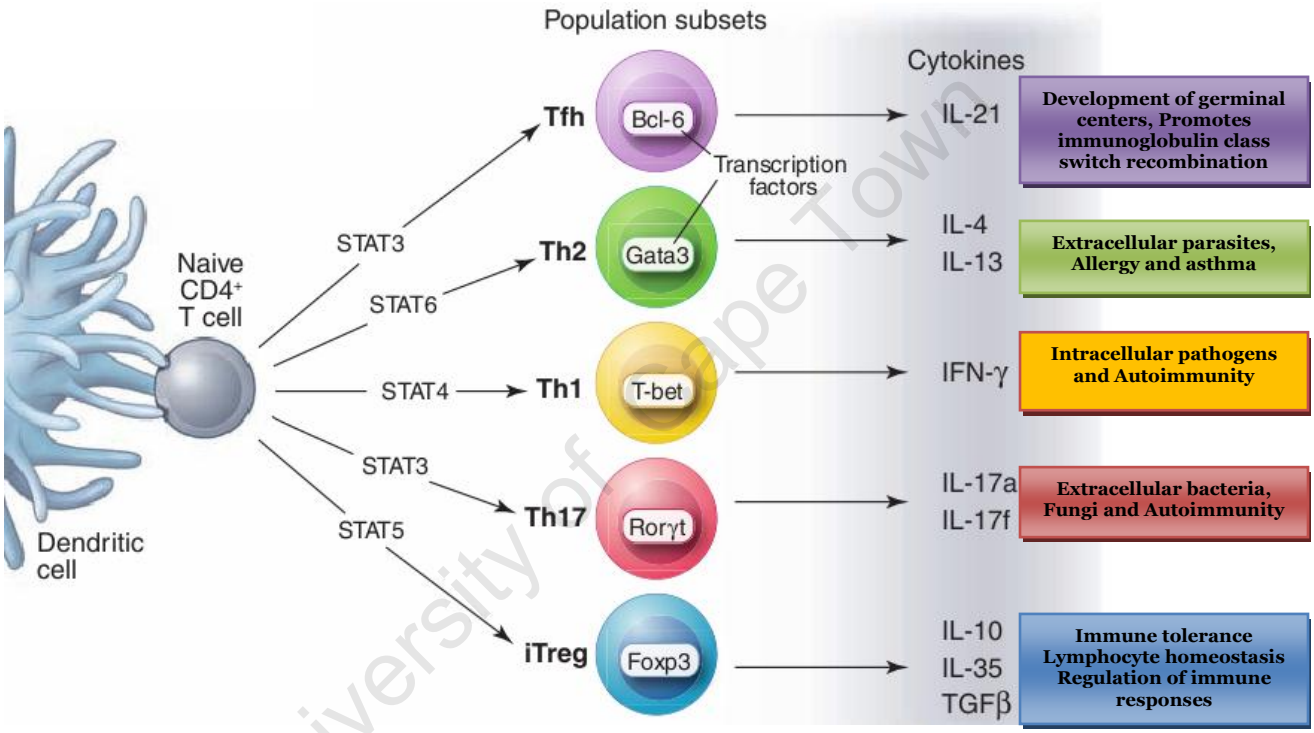


Figure 2 Summary of the different T helper cell differentiation

Differentiation into different effector $CD4^+$ T cell lineages, T helper (TH) 1, TH2, TH17, TfH (T follicular helper cells) and regulatory T cells is initiated through an interaction of dendritic cells with uncommitted (naïve) $CD4^+$ T helper cells. The effector cell types are characterized by their synthesis of specific cytokines and their immune-regulatory functions, as indicated on the right. Illustration adapted from a previous publication (O'Shea and Paul, 2010).

The mechanism of how regulatory T cells function is important and has been grouped into four basic modes of action. Firstly, suppression by inhibitory cytokines, secondly, suppression by cytolysis, thirdly, suppression by metabolic disruption and fourthly, suppression by modulation of dendritic cell (DC) maturation or function (Vignali *et al*., 2008). IL-10, IL-35 and TGF-β form part of the inhibitory cytokines. Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. Metabolic disruption includes high affinity CD25-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition amongst others. Targeting dendritic cells includes mechanisms that modulate DC maturation and/or function such as lymphocyte activation gene-3 (LAG3)–MHC-class-II-mediated suppression of DC maturation, and cytotoxic T lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule, by DCs. Figure 3A illustrates the four mechanisms used by regulatory T cells.

For a number of years it was believed that only a TH1 / TH2 paradigm dominated and that the immune system's response to any foreign substance, or antigen, involves a cascade of events orchestrated by specialized immune cells, leading to either a TH1 or a TH2 response. However, other lineages for $CD4^+$ T helper cells have been identified (Zhu *et al*., 2010). Dendritic cells, a type of immune cell, have two key functions in the initial, innate immune response. First, they produce cytokines that help to kill viruses and bacteria and secondly, they ensure that pathogens and other foreign substances are highly visible to specialized helper T cells which coordinate the longer-term adaptive immune response (Scott & Hunter, 2002). Dendritic cells recognize different types of offending substances and are able to guide the immune system to make the most appropriate response.

TH1 cells mediate immune responses against intracellular pathogens (Mosmann, 1989 and Paul, 1994). TH1 related cytokines production is important for host control of parasite burden in Leishmaniasis. In addition, these cells play an important role in resistance to mycobacterial infections and may induce some autoimmune diseases. Specific cytokines, IFN-γ, lymphotoxin α (LT α) and IL-2 are a few of the cytokines that are produced by the body during a TH1 response (Kelso, 1995). Protective IgG antibodies are also generated to help rid the body of foreign antigens and allergens (Mohammadi *et al*., 2006). The primary function of IFN-γ produced by the TH1 cells is the activation of macrophages to increase their microbiocidal activity (Suzuki Y, 1988). IL-2 production is important for CD4 T-cell memory as well as the stimulation of CD8 cells during the priming phase (Williams MA *et al*, 2006).

Host defense against extracellular pathogens are mediated by TH2 cells (Mosmann *et al*., 1986). These cells produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin and are important in the induction and persistence of asthma and other allergic diseases. The positive feedback cytokine for TH2 is IL-4 (Le Gros *et al.*, 1990) and is also the major mediator of IgE class switching in B cells (Kopf M *et al.*, 1993). IgE binds to FcεRI on basophils and mast cells and leads to the production of several cytokines including IL-4, IL-13 and tumor necrosis factor (TNF). Recruitment of eosinophils is mediated by IL-5 which also has an effect of mast cells and lymphocytes. During an allergic reaction, IL-9 induces the production of mucin on epithelial cells (Longphre M *et al.*, 1999). The IL-10 that is produced by TH2 cells, suppresses TH1 cell proliferation as well as dendritic cell function (Moore KW *et al.*, 2001). The expulsion of helminthes and the induction of airway hypersensitivity are mediated by the cytokine IL-13 (Wynn TA *et al.*, 2003 and Urban JF *et al.*, 1992).

The TH1 / TH2 paradigm is of relevance to this study as the differentiation has been shown to play a role during *L. major* infection (Radwanska *et al.*, 2007) (Figure 3B). Typically, IL-12 causes the activation of naïve $CD4^+$ T cells to differentiate into TH1 via the Stat4 pathway to activate the transcription factor T-bet. As mentioned earlier, these cells produce IFN-γ as the hallmark cytokine. Conversely, IL-4 leads to the activation of naïve $CD4^+$ T cells to activate the transcription factor GATA3 via the STAT6 pathway to drive the TH2 differentiation. This leads to the production of IL-4, IL-13 and IL-5 amongst other TH2 cytokines. IL-4 is needed for the induction of most Th2 responses (Forbes, *et al.*, 2010 and Van Panhuys, *et al.*, 2008). These subsets, TH1 and TH2 can also be negatively regulated by the cytokines IFN-γ and IL-4, which suppress differentiation of TH2 and TH1 respectively. Table 1 lists the major differences between mouse TH1 and TH2 cells and Figure 2 illustrates the different effector $CD4^+$ T cell lineages.

Table 1 TH1 vs TH2 Major differences

	TH1	TH2
Response to	**Microbial and intracellular pathogens**	**Extracellular parasites and allergic reactions**
Initiating cytokines	IL-12, IL-18	IL-4
Antibodies	IgG2a IgG2b	IgE IgG1
Transcription factors	T-bet , STAT-4	GATA3, STAT-6
Cytokines produced	IFN-γ (IL-2)	IL-4 (IL-2), IL-13, IL-5.
Inhibited by cytokines	IL-4, IL-10, TGF-β	IFN-γ, TGF-β

A

B

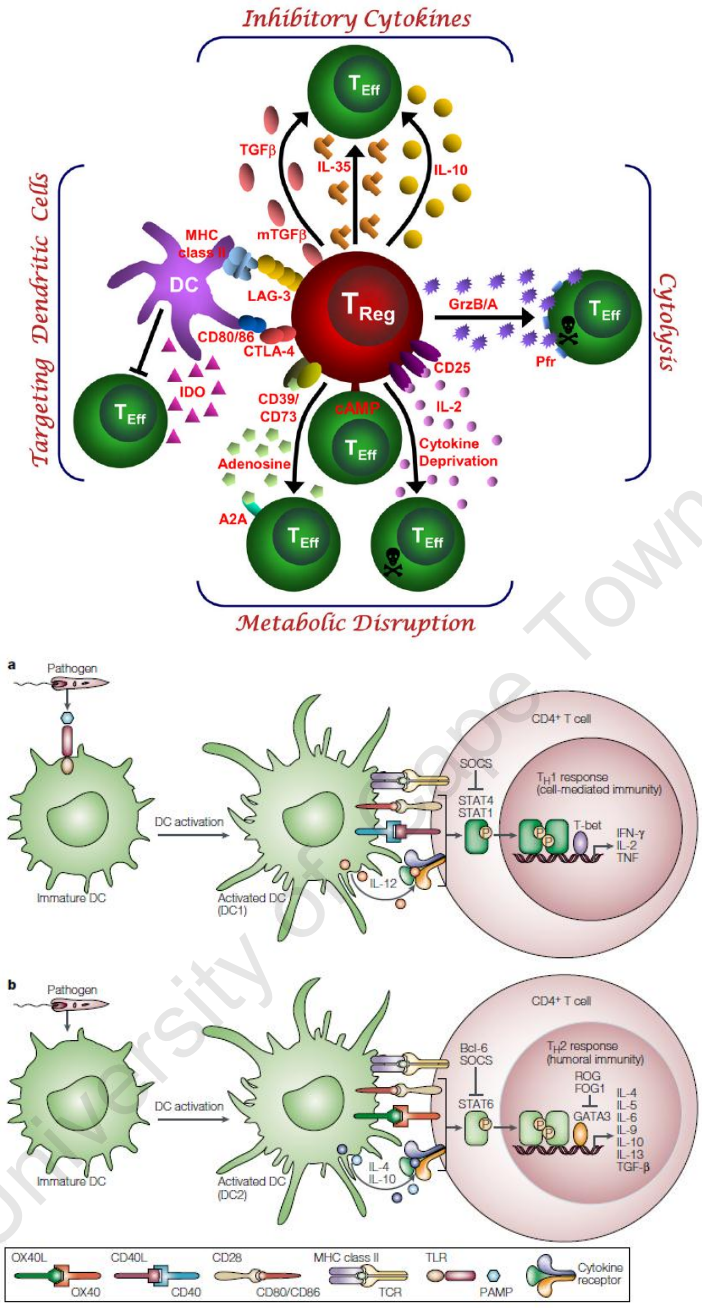


Figure 3 Regulatory T cell mechanism and TH1 / TH2 differentiation

A. Basic mechanism used by regulatory T cells. Illustration of the various regulatory T cell mechanisms centered on the four basic modes of action. These include inhibitory cytokines, cytolysis, metabolic disruption and targeting dendritic cells. **B**. TH1 / TH2 lineages. Illustration of TH1 and TH2 cell differentiation, antigen presentation and its interaction with its co-stimulatory molecules. Illustrations were taken from Vignali *et al*., 2008 and Sacks *et al*., 2002.

1.2. IL-4 Receptor alpha (IL-4Rα)

Interleukin 4 (IL-4) is a cytokine that induces differentiation of naive helper T cells to TH2 cells and was originally identified in 1982. The gene for IL-4 together with the genes for IL-5 and IL-13 are localized of chromosome 11 (Morgan *et al*., 1992). It has been shown that basophils (Min *et al*., 2004), mast cells (Plaut *et al*., 1989), γδT cells (Ferrick *et al,*. 1995), NK1.1+ T cells (Yoshinoto and Paul 1994), eosinophils (Sabin *et al*., 1996) and conventional T cells (Launois *et al*., 1995; Noben-trauth *et al*., 2000) are all sources of IL-4 and these cells have been shown to initiate TH2 differentiation. IL-4-independent TH2 differentiation has also been previously described (Mohrs *et al*., 2000).

IL-13 is a cytokine secreted by many cell types, but especially TH2 cells. This cytokine is produced by many cell types including dendritic cells, NK cells, mast cells, basophils and T cells (McKenzie *et al.,* 1993; de Saint-Vis *et al.,* 1998; Hoshino *et al.,* 1999; Heller *et al.,* 2005). It has been shown in studies by Noben-Trauth *et a.l* in 1999 that IL-13 plays a role in the susceptibility of BALB/c mice during *Leishmania major* infection.

These two cytokines (IL-4 and IL-13) are closely related and has similar functions in that they play a role in the induction of TH2 responses and the inhibition of TH1-associated cytokines such as IFN-γ and TNF (Paul, 2010). Both share a common receptor subunit, the interleukin-4 receptor alpha (IL-4Rα) (Duschl & Sebald, 1996) (Figure 4) through which they signal.

The mouse IL-4Rα gene is located on chromosome 7 (accession number M29854) and is encoded by 12 exons (Mosley *et al*., 1989). The IL-4Rα chain consists of two separate chains, the 140kDa IL-4-binding chain and the common gamma chain, γc. There are two types of IL-4 receptor complexes that function as heterodimers. Firstly, the type I receptor consists of the IL-4Rα and the γc, which is also a component of the receptors for IL-2, IL-7,

IL-9, IL-15 and IL-21 (Leonard and Lin 2000; Hershey, 2003) while the type II receptor consists of the IL-4Rα and the IL-13Rα.

This receptor (IL-4Rα) is very closely linked to the activities of IL-4, in that it plays a major role in the regulation of the differentiation of naïve $CD4^+$ T cells into a TH2 phenotype as well as a role in class switching to IgG1 and IgE (Coffman *et al*., 1986).

The components of the IL-4Rα complexes are constitutively associated with the Janus tyrosine kinases (JAKs). JAK1 associates with IL-4Rα (Yin *et al*. 1994) while JAK3 associates with the γc (Russell *et al*., 1994; Nelms *et al*., 1999). Upon ligand binding the receptor subunits dimerize leading to the activation of JAKs. Activated JAKs phosphorylate tyrosine residues in the cytoplasmic domain of the IL-4Rα, which can in their phosphorylated state act as docking sites for signaling molecules. The IL-4Rα dedicated signal transducer and activator of transcription 6 (STAT6) is recruited to the phosphorylated IL-4Rα where it also becomes phosphorylated by JAKs (Tadeka *et al*., 1996). Phosphorylated STAT6 monomers dimerize and translocate to the nucleus to bind STAT6 binding elements in promoters of IL-4 or IL-13 responsive genes (Tadeka *et al*., 1996). The cytoplasmic domain of IL-13Rα1 also binds to STAT3 and IL-4 or IL-13 binding leads to the phosphorylation of this transcription factor. Recently, it was discovered that IL-4Rα could also signal through Syk kinase to enhance phagocytosis and cell adhesion (Ennaciri and Girard, 2009). The roles of IL-4, IL-13 and their receptors in type II immunity forms a central part of the studies presented here and is therefore discussed further in the following section.

In order to further characterize the role of these cytokines, mice lacking the IL-4Rα globally as well as on specific cell types using the Cre-loxP system were generated in our lab (Dewals *et al*., 2009; Herbert *et al*., 2004; Horsnell *et al*., 2007; Mohrs *et al*., 1999; Radwanska *et al*., 2007).

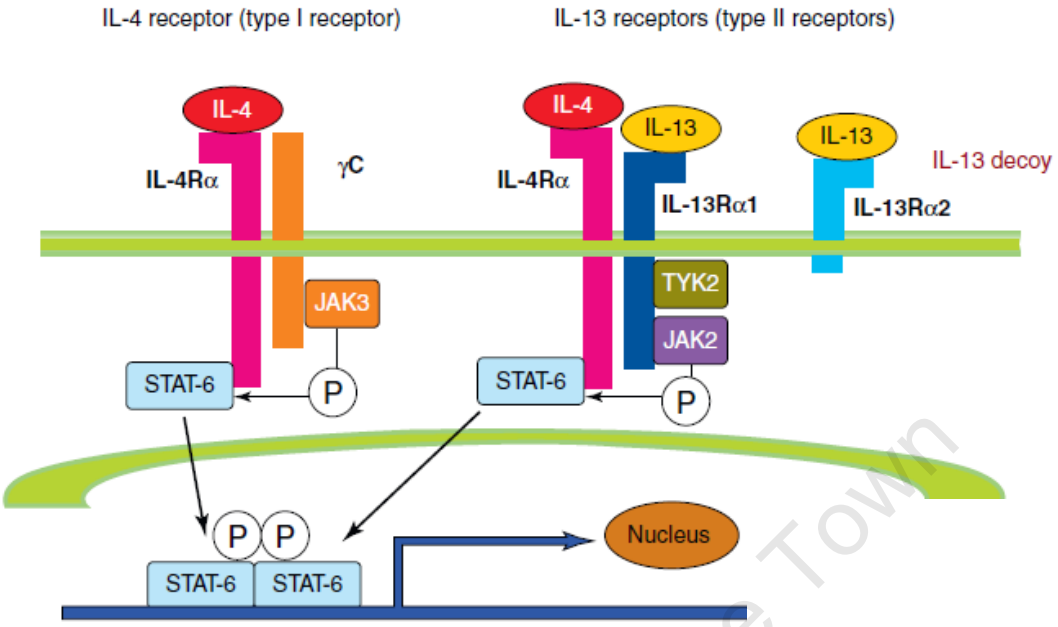


Figure 4 **IL-4 and IL-13 receptor complexes**

IL-4 interacts with the IL-4Rα subunit in combination with either γc (Type I) or IL-13Rα1 (Type II). IL-13 interacts with IL-13Rα1 in combination with IL-4Rα or with IL-13Rα2. Illustration adapted from previous publication, Holgate, 2012.

This strategy employed to generate these cell-specific IL-4Rα knockout mice is illustrated in Figure 5. Using these IL-4Rα knockout mice, a role for IL-4 and IL-13 was established during *Leishmania major* infection (Radwanska *et al*., 2007), *Nippostrongylus brasiliensis* infection (Horsnell *et al*., 2011; Horsnell *et al*., 2007), *Schistosoma mansoni* infection (Dewals *et al*., 2010; Dewals *et al*., 2009; Herbert *et al*., 2004; Leeto *et al*., 2006; Marillier *et al*., 2010) and allergy (Kirstein *et al*., 2010). For the purpose of this study, we investigated the role of $CD4^{+}$T cells host protective responses against cutaneous leishmaniasis using

genome-wide transcriptomics with specific interest in the absence of IL-4Rα on $CD4^+$T cells.

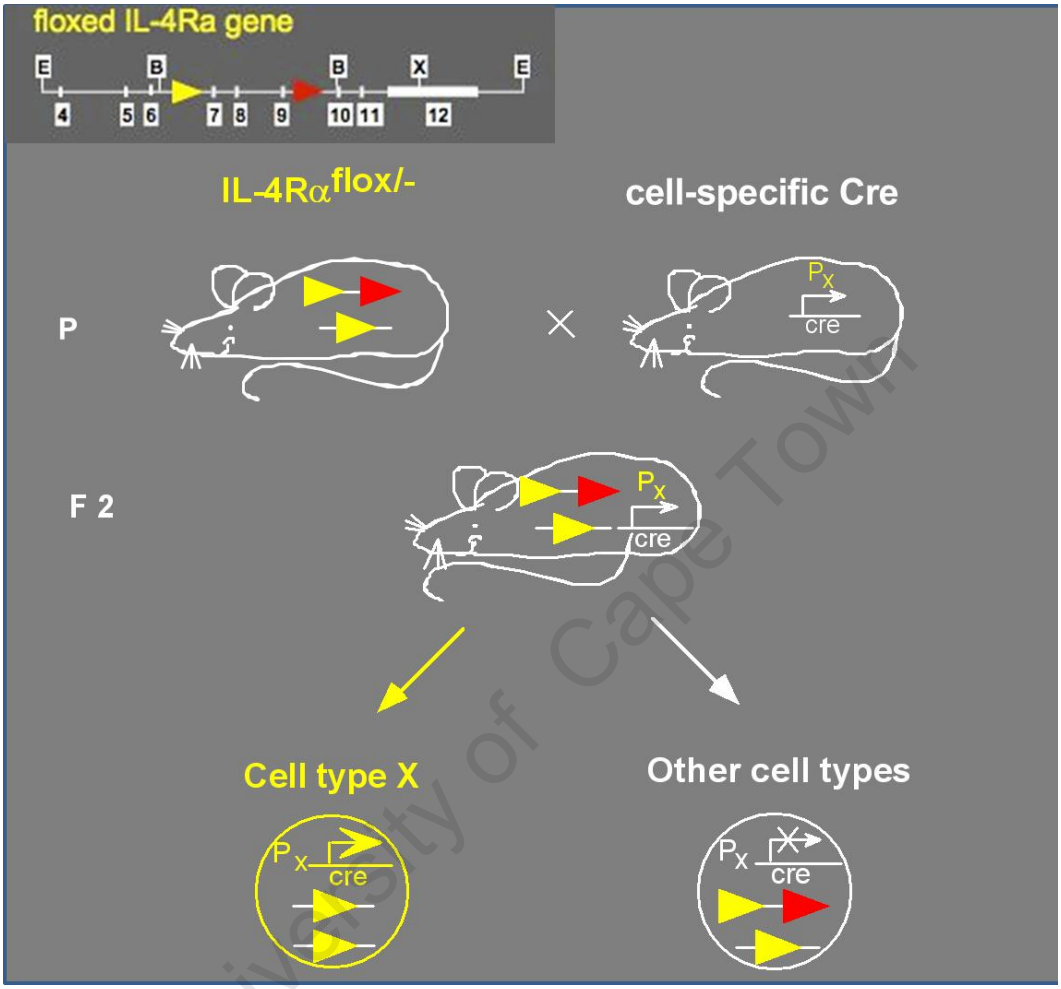


Figure 5 Breeding strategy to generate IL-4Rα knockout mice.

IL-4Rα$^{-/-}$ mice were mated with transgenic mice possessing an LCK promoter driving Cre recombinase, resulting in iLCK^{cre}IL-4Rα$^{-/-}$ offspring. These offspring were mated with mice containing IL-4Rα flanked by loxP sites (IL-4Rα$^{lox/lox}$) to produce mice with iLCK driven cre-mediated deletion of IL-4Rα (iLCK^{cre}IL-4Rα$^{-/lox}$). Illustration adapted from Radwanska *et al.,* 2007.

1.3. *Leishmania major* / Leishmaniasis

1.3.1 Background

Leishmaniasis is a disease caused by a protozoan parasite that is transmitted by the bite of the *Phlebotomus* genus of sand fly. Leishmaniasis is found mainly in the subtropics and tropics. Various forms of Leishmaniasis are determined by which part of the body is affected (Table 2). In visceral leishmaniasis, the parasite affects the organs of the body. The most common form is cutaneous leishmaniasis, where the skin is the predominate site of infection. Cutaneous leishmainiasis can involve the mucocutaneous membranes, a condition called mucocutatneous leishmaniasis.

Table 2 Etiological agents of the various forms of Leishmaniasis

Type	Etiological species
Cutaneous	**Americas – *L. tropica mexicana, L. braziliensis,* and *L. amazonensis*** **Old World (Europe, Asia, and Africa) – *L. tropica, L. major, L. infantum,* and *L. aethiopica***
Mucocutaneous	**Americas – *L. braziliensis*** **Old World (Europe, Asia, and Africa) – *L. aethiopica***
Visceral	**India, Kenya – *L. donovani*** **South Europe and North Africa – *L .infantum*** **Americas – *L. chagasi***

The global impact of leishmaniasis has been severely underestimated, and the scope of endemic areas and number of recorded cases of the disease are on the increase (Dujardin *et al*., 2008). Leishmaniasis is listed by the World Health Organisation as one of the top six diseases, with an estimated 12 million people infected that is endemic in 88 countries (http://www.who.int/leishmaniasis/cutaneous_leishmaniasis/en/).Cutaneous leishmaniasis currently affects approximately 1.5 million people annually (Desjeux, 2001). Despite the disease not being fatal it is important to study diseases of poverty, like leishmaniasis. They often cause high morbidity however low mortality. Therefore, the

burden of disease becomes largely invisible. Those that are affected by the disease are often isolated due to the social stigma associated with the disease.

1.3.2 Leishmania/HIV co-infection

It is increasingly more important to study Leishmania due to the increase in HIV infections. Leishmaniasis is one of the opportunistic infections which affects HIV-infected individuals. The visceral form of the disease appears to be the most common form associated with co-infection with HIV (Ezra *et al*., 2010). Figure 6 illustrates the global distribution of reported cases of *Leishmania* / HIV co-infection and the distribution of leishmaniasis cases. Approximately 31 countries have reported *Leishmania* / HIV co-infection in 1999. Having both pathogens concomitantly in the same system, the macrophage enhances the effects that influence the expression and multiplication of either one or both pathogens (Cruz *et al.,* 2008).

A vicious circle is established whereby the protozoan parasite *Leishmania* induces a more robust HIV-1 production and the virus mediates a greater parasitic replication. In co-infected patients there is a depletion of both the cellular and humoral responses to *Leishmania*, this further increases the risk of disease progression (Moreno *et al.*, 2000). HIV-infected individuals have nonfunctional T lymphocytes and this is problematic for *Leishmania* infections (Cruz *et al.,* 2008).

The overlap in the geographical areas with high risk of both HIV and leishmaniasis is increasing, with the spread of leishmaniasis (typically a rural disease) into urban areas and the increased spread of HIV into rural areas. Leishmaniasis accelerates the onset of AIDS by cumulative immuno-suppression and by stimulation of the replication of the virus. In addition, since visceral leishmaniasis can be spread intravenously, sharing of needles by intravenous drug users is a direct way of spreading leishmaniasis.

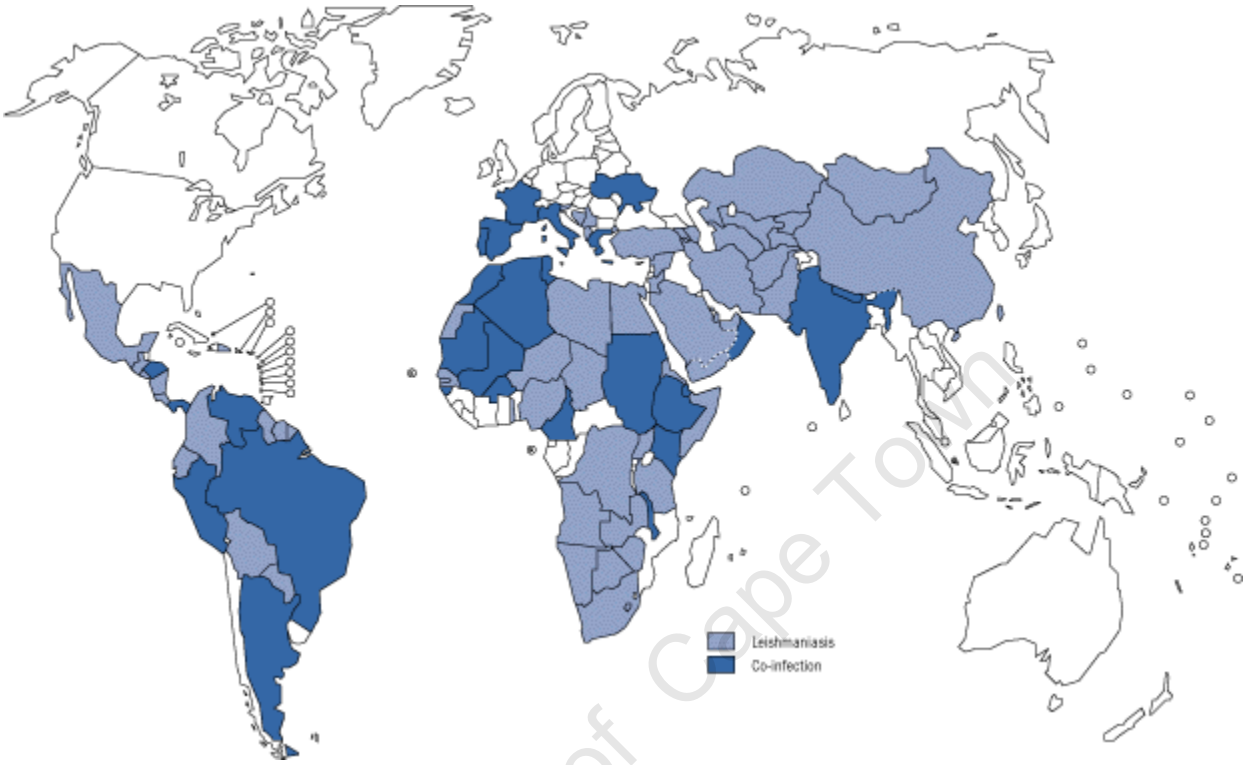


Figure 6 **Global distributions of reported cases of leishmaniasis and *Leishmania*/HIV co-infection.**

WHO-compiled a depiction of endemic regions of Leishmaniasis (light blue) and those areas were HIV co- infection has significantly complicated the clinical outcome. Illustration was taken from http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish/en/index.html.

1.3.3. Life cycle

Infection begins with metacyclic promastigote parasites being transmitted to the host during a blood meal of the sandfly vector (*Phlebotomus papatasi*) (Figures 7 and 8). The parasites are phagocytosed by phagocytes including neutrophils, macrophages and dendritic cells and survive in phagolysosomes, where they differentiate into amastigotes. These amastigotes grow and divide, infecting other macrophages while waiting to be ingested by a feeding sandfly.

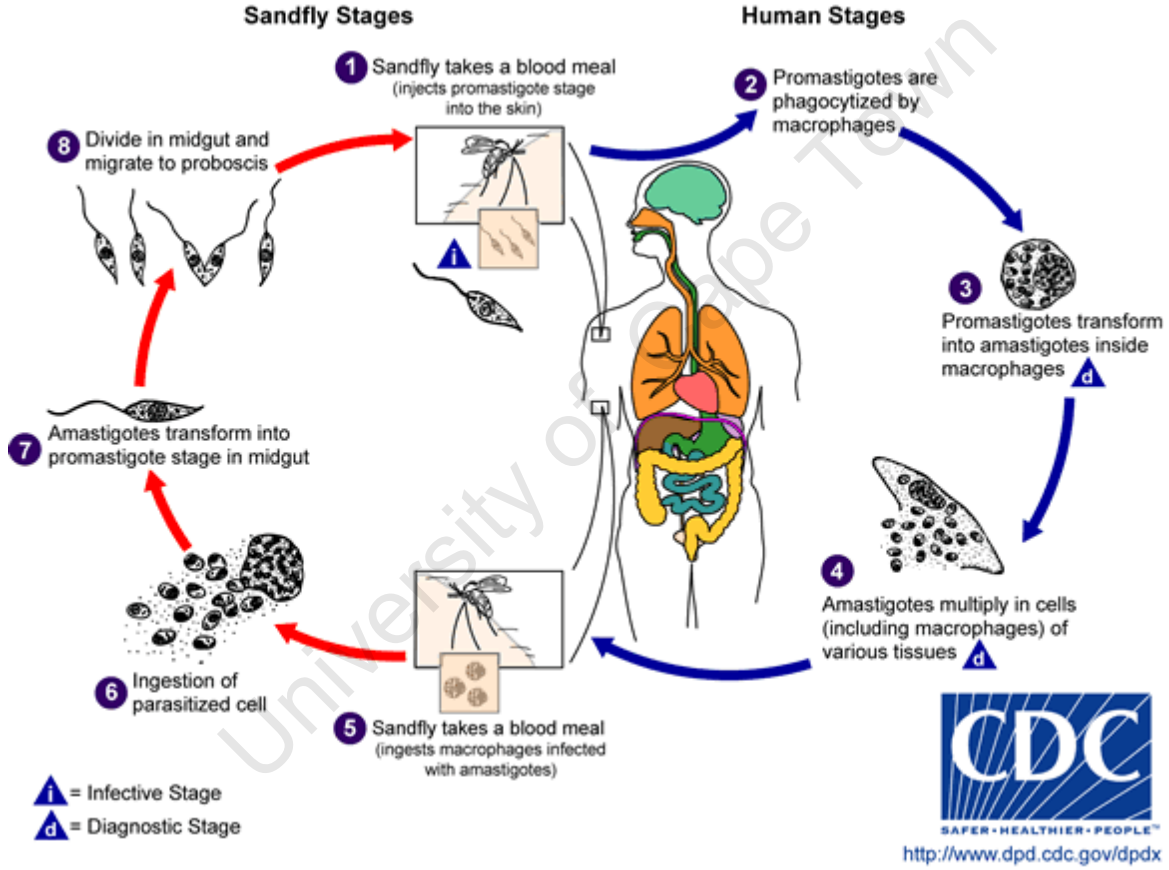


Figure 7 Life cycle of Leishmania parasites.

Parasites are transferred between hosts by a sandfly vector during blood meals (step 1), they then become phagocytosed by macrophages (step 3) where they reside within the phagosome and replicate as amastigotes (step 4) and develop the disease phenotype. Infected macrophages may then be taken up by a naive sandfly (step 5-8) to repeat the cycle. Illustration taken from http://en.wikipedia.org/wiki/File:Leishmania_LifeCycle.gif.

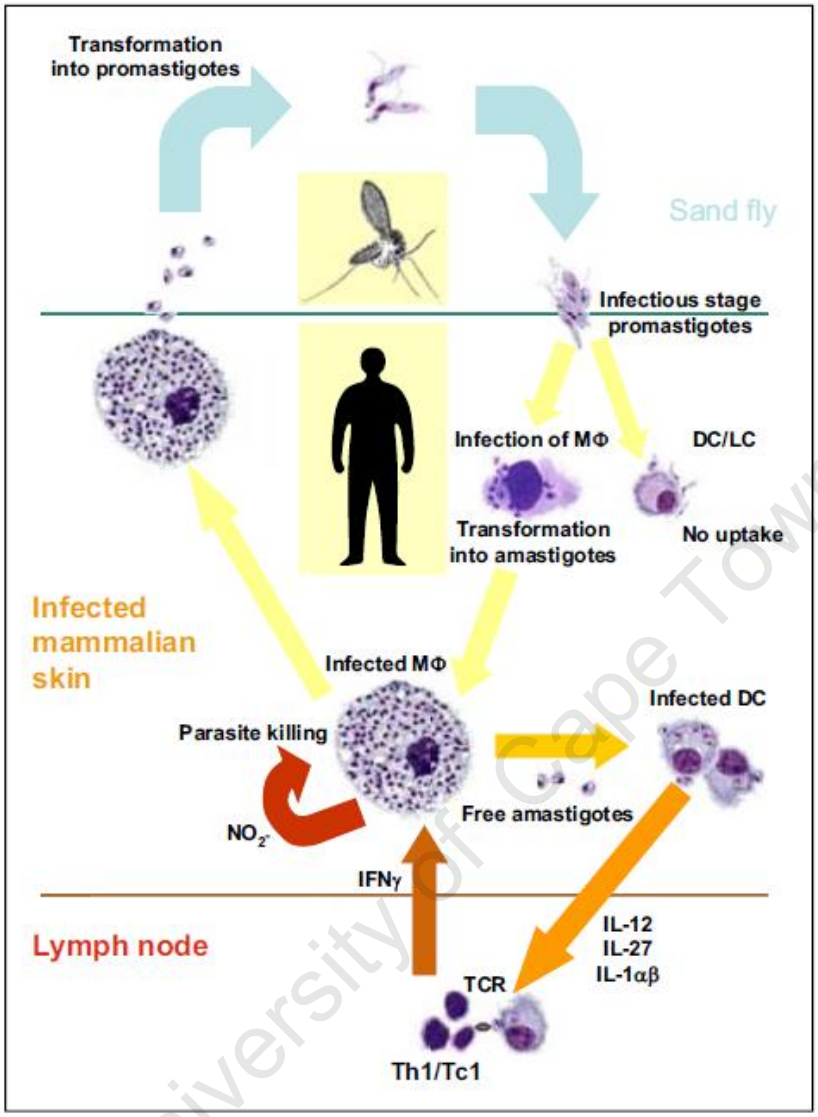


Figure 8 ***Leishmania major*** **infection and resulting immunity**

The immune response in brief after the parasite has been taken up by the resident macrophages in the skin. Once the uptake occurs, the promastigotes transforms into obligate amastigotes where they replicate. These macrophages lyse and release the amastigotes which then infect dendritic cells. This lead to its activation and that of the T cells to differentiate into IFN-γ producing TH1 cells which in turn induce the macrophages to upregulate iNOS to eliminate the intracellular organism. The metacyclic promastigotes can be transmitted when the sand fly takes another blood meal. This illustration was taken from Barral-Netto *et al.*, 2007.

1.3.4. Immune response to *L. major*

It is generally accepted that protective immunity against *L. major* is dependent on an IL-12-driven type-1 response mainly characterized by the production of interferon-gamma (IFN-γ) by $CD4^+$ TH1 cells (Gorak, Engwerda, & Kaye, 1998; Sypek *et al*., 1993; Walker *et al*., 1999). IFN-γ in turn mediates protection by inducing NOS-2 expression and nitric oxide (NO) production in classically activated macrophages (Liew and O'Donnell, 1993). In contrast, conventional opinion and early studies with *L. major* (Heinzel *et al*., 1989; Leal *et al*., 1993.) would suggest than an IL-4 driven TH2 response and associated cytokines like IL-13 counter-regulate TH1 responses and consequently it would be expected that a TH2 response would be detrimental to the outcome of disease. Indeed susceptible BALB/c mice deficient for IL-4 (Kopf *et al*., 1996a), IL-13 (Matthews *et al*., 2000), the IL-4 receptor-alpha (IL-4Rα) (Mohrs *et al*., 1999), or STAT6 (Stamm *et al*., 1998) are able to contain infection with *L. major*.

TH2 cytokines like IL-4, IL-9, IL-10 and IL-13 are susceptible factors as identified by fellow researchers from our group and others during the last few years (Arendse *et al*., 2005; Kopf *et al*., 1996; Mohrs *et al*., 1999; Stamm *et al*., 1998; Alexander *et al*., 2002). Even though, *Leishmania major* is the organism that has been most intensely studied and from which the original TH1 / TH2 paradigm of immunological development during infectious disease was determined, the exact role of IL-4 and IL-13 is still not clear. Firstly, TH2 differentiation can be independent of IL-4 (Mohrs *et al*., 2000). Secondly, both cytokines can have beneficial functions (Mohrs *et al*., 1999) and are needed for effective drug therapy and vaccination (Alexander *et al*,. 2000; Stäger *et al*., 2003). Thirdly, the IL-4/IL-13 dependency for susceptibility is reliant on the Leishmania strain used (Noben-Trauth *et al*., 1996 and Noben-Trauth, 2000) and possibly on the chronicity of the disease (Mohrs *et al*., 1999), as summarized in the literature (Alexander J and Brombacher F, 2012). Recent evidence shows that IL-4Rα responsive non-T cells are critical for transforming from a non-healer to a healer strain in cutaneous leishmaniasis (Radwanska *et al*., 2007).

The murine model of infection for *Leishmania major* (*L. major*) has been used extensively to unravel mechanisms underlying T cell differentiation into TH1 or TH2 cells (Sacks & Noben-Trauth, 2002). Essentially, most laboratory mouse strains (such as C57BL/6 mice) are able to control *L. major* infection, raising a polarised TH1 response following infection this is driven by IL-12 and the production of IFN-γ (Sacks & Anderson, 2004; Sacks & Noben-trauth, 2002). These resistant C57BL/6 mice was also found to produce IL-4 early during infection, however they still developed and unimpaired TH1 biased response (Scott *et al*., 1996). Due to a genetic predisposition, BALB/c mice develop a TH2 response (with the production of IL-4 and IL-13) following infection with *L. major* and are susceptible to infection with the development of progressive lesions and systemic disease. The *L. major* mouse model has been a useful tool, not only in identifying the importance of TH1 polarisation in resistance to the disease, but also in identifying the processes involved in controlling TH1 differentiation *in vivo* (Sacks and Anderson 2004). Studies have shown that global IL-4Rα deficient mice showed resistance to *L. major* infection during the acute phase of infection however, the mice continued to develop necrotic lesions during the chronic phase. In contrast, the T cell-specific IL-4Rα KO (knock out) mice developed resistance to *L. major* infection during the acute as well as chronic phases of infection (Mohrs *et al*., 2000 and Radwanska *et al*., 2007).

Studies using $CD4^+$T cell specific IL-4Rα deficient mice on a susceptible BALB/c background, demonstrated that loss of IL-4Rα responsiveness on $CD4^+$T cells together with the presence of IL-4Rα responsiveness on other non-$CD4^+$ cell types, lead to clinical immunity in "non-healer" BALB/c mice infected with *L. major* (Radwanska *et al*., 2007). This suggests that during *L. major* infection, signaling via the IL-4Rα on $CD4^+$T cells induces a cascade of events that ultimately results in susceptibility and disease progression. These studies have highlighted that IL-4Rα signaling on cells other than T cells may have a protective role during *L. major* infection.

Thus, the aim of this study is to identify the gene expression patterns in activated $CD4^+$T cells and regulatory T cells that are associated with resistance or susceptibility to *L. major*, with the long term goal that these genes (and their encoding protein products) can serve as targets for rational drug design. To do this we will use DNA microarrays to analyze the gene expression profiles of IL-4Rα responsive and non-responsive $CD4^+$ T cells (activated and regulatory) isolated from the draining lymph nodes of susceptible WT BALB/c mice and resistant mice $CD4^+$T cell-specific IL-4Rα KO BALB/c ($iLCK^{cre}$IL-$4R\alpha^{lox/-}$) mice respectively at three weeks after infection with *L. major.* Control mouse groups will include WT C57BL/6, global IL-4Rα KO (IL-$4R\alpha^{-/-}$) and IL-$4R\alpha^{lox/-}$ mice. This differential microarray strategy comparing genetically resistant and susceptible mice was used previously by researchers from our group to identify genes as potential drug targets with a low false positive rate during *listeria monocytogenes* infection (Schwegmann *et al*., 2007).

1.3.5 The role of regulatory T cells in Leishmaniasis

$CD4^+CD25^+$ Regulatory T cells are a major source of IL-10 and play an important role in the regulation of immune responses. Regulatory T cells are essential for the suppression of detrimental pathogenic responses especially to self antigens, however, they may also lead to the suppression of beneficial responses. In C57BL/6 mice infected with *L. major*, the regulatory T cells are recruited to the site of infection. Here the regulatory T cells function in a way to suppress the effector cells to eliminate the parasites (Belkaid *et al.*, 2000). The depletion of $CD25^+$ cells in this mouse strain enhances the production of IFN-γ by $CD4^+$ T cells in the lesions. This resulted in clearance of the parasites. Similarly, in a susceptible BALB/c mouse strain, treatment with anti-CD25 increases resistance to *L. major* infection (Gray *et al.*, 2006). By regulating the biased TH2 response in susceptible BALB/c mice infected with *L. major*, the $CD4^+CD25^+$ regulatory T cells play a significant disease controlling role (Xu *et al.*, 2003). Other studies demonstrated that the transfer of naïve $CD4^+CD25^+$T cells in SCID mice reconstituted with $CD4^+CD25^-$ T cells, suppressed *L. major* disease development. This suggests that $CD4^+CD25^+$ T cells also inhibit TH2 responses and

susceptibility to *L. major* infection (Xu *et al.,* 2003). Therefore, this study is aimed at identifying the role of regulatory T cells in *Leishmania* infection as current evidence suggests that the balance between regulatory T cells and effector cells is critical for immunity to *Leishmania*.

Aims of the project

The key goal of this project is to identify the transcriptional differences in activated $CD4^+$ T cells and regulatory T cells that do or do not express IL-4Rα that results in either a protective or non-protective immune response during leishmaniasis. Comparative gene expression profiling of activated $CD4^+$ T cells and regulatory T cells will identify genes that are important for host protection or disease progression and will help us elucidate the molecular mechanisms of how these activated T cell subsets are primed to guide the immune response to be either protective or non-protective. A deeper understanding of these mechanisms may result in rational approaches for effective drug and vaccine development as a long-term goal. This aim was to be achieved by performing comparative *L.major* infections in mice lacking IL-4Rα on $CD4^+$ T cells (Radwanska *et al*., 2007) and assessing the transcriptional profile of the popliteal lymph node T cells at three weeks post infection.

Specific objectives

- Complete a minimum of three comparative *L. major* infections comprising from $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$, BALB/c and C57BL/6 mice at 3 weeks post infection.
- Isolate activated and regulatory T cells at three weeks post infection.
- Purify total RNA isolated from activated and regulatory T cells and profile the gene expression of these by DNA microarray
- Analyze the microarray using bioinformatics to identify genes and pathways important for host protection or disease progression
- Validate the microarray results by quantitative RT- PCR

Chapter 2

Materials and Methods

2.1 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: 012/033). All efforts were made to minimize suffering of the animals.

2.2 Mice

Mice with a global (IL-4Rα $^{-/-}$) (Mohrs *et al*., 1999) or T cell specific (iLCK^{cre}IL-4R α $^{-/lox}$) (Radwanska *et al*., 2007) deletion of IL-4Rα, together with its littermate control (IL-4Rα $^{-/lox}$) and experimental controls (BALB/c and C57BL/6) were used for this project (Sacks *et al*., 2002). 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.

2.3 Tail vein bleed

After restraining the mouse, the tail was heated under an infrared fluorescent lamp to dilate the veins. The tail was extended and an incision was made across the lateral tail vein with a disposable scalpel blade (Swann-Morton). From the incised site approximately 200µl of blood was collected in microtainer serum separator tube. Gentle pressure was then applied to the wound to ensure hemostasis. The serum from this blood was used for antibody titers.

2.4 CD4^{+} T cell-specific IL-4Rα deletion in iLCK^{cre} IL-4Rα$^{-/lox}$ BALB/c mice

The IL-4Rα is found on haematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte and brain tissue (Nelms *et al.* 1999). In order to study the effects of IL-4 on specific cell types it is necessary to target the IL-4Rα gene specifically, this is accomplished by using Cre/loxP recombination (see Figure 9). Cyclization recombinase (Cre) inserted downstream of the promoter recognises a pair of loxP binding sites flanking the gene of interest (Exon 7 through 9 of IL-4Rα). Cre-recombinase brings the two loxP sites together, removing the intervening DNA which encodes the transmembrane, soluble and extra cellular membrane proximal regions (Nagy 2000). To specifically target IL-4Rα deletion on CD4^{+}T cells (iLCk^{cre}IL-4R$^{\alpha-/lox}$), Cre-recombinase expression was driven using the T cell-specific LCK promoter (Gu *et al*., 1994; Radwanska *et al*., 2007). LCK protein tyrosine is a kinase involved in the T cell signal transduction pathway (Janeway and Travers 1996 and Chiang *et al*., 2001). Introduction of a neo/tk selection cassette flanked by two loxP sites (yellow arrowhead), and one loxP site 5' of exon 10 resulted in the generation of the conditional "floxed" mouse after Cre-mediated recombination. An intercross between the floxed strain and LCK^{cre}, knockin strain facilitated the generation of CD4^{+}T cell-specific IL-4Rα-deficient mice. Numbers indicate exons of the IL-4Rα; B, E and X, restriction sites for BamHI, EcoRI and XhoI (Herbert *et al*., 2004).

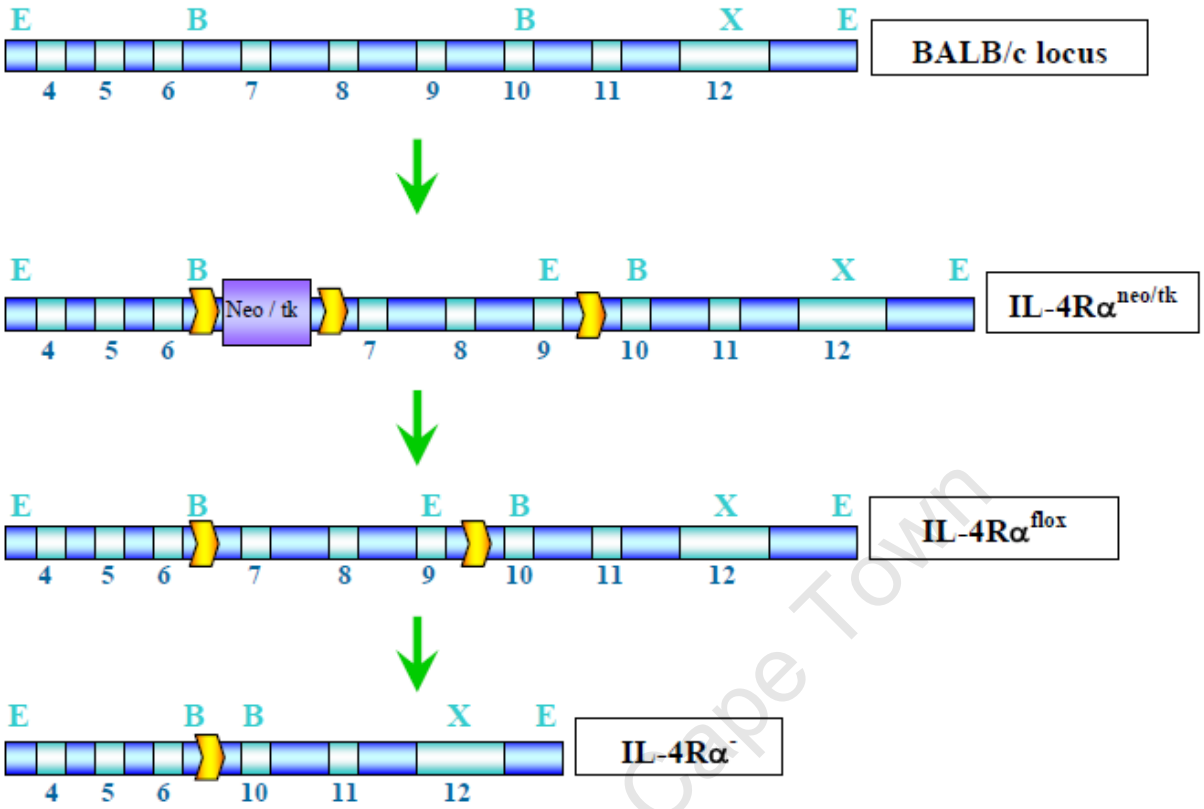


Figure 9 IL-4Rα gene locus and targeted deletion

Introduction of a neo/tk selection cassette flanked by two loxP sites (yellow arrowhead), and one loxP site 5' of exon 10 resulted in the generation of the conditional "floxed" mouse after Cre-mediated recombination. An intercross between the floxed strain and LCK^{cre}, knock-in strain facilitated the generation of T cell-specific IL-4Rα-deficient mice. Numbers indicate exons of the IL-4Rα; B, E and X, restriction sites for BamHI, EcoRI and XhoI (Herbert *et al.*, 2004).

Table 3 Primers for genotyping IL-4Rα knockout mice

Target	Primer	Product size (bp)
WT forward	5'-TGACCTACAAGGAACCCAGGC-3'	600
WT reverse	5'-CTCGGCGCACTGACCCATCT-3'	
KO forward	5'-GCTGCTGACCTGGAATAACC-3'	471
KO reverse	5'-CCTTTGAGAACTGCGGGCT-3'	
loxP forward	5'-CCCTTCCTGGCCCTGAATTT-3'	188
loxP reverse	5'-GTTTCCTCCTACCGCTGATT-3'	
iLCK^{Cre} forward	5'-CAGGTATGCTCAGAAAACGCCTGG-3'	450
iLCK^{cre} reverse	5'-GAGGGTGGAATGAAACTCTCGGT-3'	

2.5 Culturing and preparation of *Leishmania major*

After 5-6 weeks post infection, passage BALB/c mice infected with $2x10^6$ *L. major* MRHO/SU/59/P was sacrificed by cervical dislocation and infected footpads and the draining popliteal lymph nodes were harvested and placed in 5ml Schneider's medium (Sigma) supplemented with 20% FCS in a tissue culture flask. The flask was then incubated at room temperature in a humidified chamber for 7 days. From days 3-4, motile parasites become apparent. Parasites were counted daily to determine the growth curve in 0.1% glutaraldehyde in Neubauer chamber with depth of 0.05mm (conversion factor $2x10^4$). At days 6 -7, parasites should be at ~ 10^8/ml in the stationary phase. To prepare parasites for infection, supernatants were transferred from the culture flask to a 50ml falcon tube and centrifuged at 500 rpm for 5 minutes to pellet the debris. The supernatant was then transferred to a new 50 ml falcon tube and centrifuged at 3000 rpm to pellet the parasites. Parasites were then washed twice in HBSS with protein inhibitor (see appendix) and counted as described above. Finally the parasites were resuspended in the required volume for infection.

2.6 *Leishmania major* infection

Prior to infection, mice were bled to access naïve antibody titers by ELISA. *L. major* LV 39 (MRHO/SU/59/P) strain was maintained by continuous passage in BALB/c mice and cultured *in vitro* as previously described (Radwanska *et al*., 2007). Six mice per group were anesthetized and infected with 50 µl of $2x10^6$ stationary phase metacyclic promastigote stage *L. major*, subcutaneously into the left hind footpad. Infected footpad swelling was measured weekly as an indication of disease progression using a pocket thickness gauge (Mitutoyo, Japan) until mice were to be sacrificed. Necrosis was also noted and recorded. Mice with a footpad swelling measurement of greater than 5mm were euthanized in accordance with the animal ethics regulations.

2.7 Quantification of parasite burden

Parasite burden was determined from infected organs by the limiting dilution assay (LDA) and quantitative real-time PCR (Nicolas, Prina, Lang, & Milon, 2002). At three weeks post infection, the mice were sacrificed by cervical dislocation and sterilized by spraying liberally with 70% ethanol. The infected footpads and draining popliteal lymph nodes were harvested and placed in 2ml Schneider's medium supplemented with 20% FCS and DMEM supplemented with 10% FCS respectively. The footpad was homogenized and a single cell suspension was prepared from the lymph node to a final volume of 6.4 ml. Two-fold serial dilutions were prepared in Schneider's medium (Sigma) with 20% FCS in a flat bottomed tissue culture grade 96 well plate, and the cultures were examined for parasites after 7 days at room temperature. The number of parasites per footpad or draining lymph node was calculated as follows: parasite burden = (geometric mean of titre from quadruplicate cultures) × (reciprocal fraction of the homogenized footpad inoculated into the first well). The titre was the reciprocal of the last dilution in which parasites were observed (Carrion *et al*., 2011).

2.8 Lymphocyte isolation

Post sacrifice the draining popliteal lymph nodes was harvested from the mice and placed in ice cold DMEM supplemented with 5% FCS. Single cell suspensions were made from the pooled lymph nodes per group, by teasing lymph nodes through a 40µM filter (Becton Dickinson) or metal sieve using the plunger of a syringe into a 5.0 ml falcon tube. Single cell suspension was collected by centrifugation at 1600 rpm for 5 minutes at 4°C in a 50ml falcon tube. Cells were washed twice in media. The pelleted cells were resuspended in 10ml media and stained with Trypan blue (1 in 10 dilution) (Sigma-Aldrich, Irwine, UK) to assess the number of cells per group. Cells were counted using a Neubauer counting chamber (Surface: 0.0025 mm^2, Tiefe: 0.1 mm).

2.9 Extracellular Surface staining

Extracelluar surface staining was performed to set up gating strategy for FACS sorting of the activated T cells and regulatory T cells. Lymphocytes were isolated as described above and resuspended in FACS buffer (PBS supplemented with 0.1% BSA and 0.05% Sodium Azide). Activated T cells ($CD4^+CD44^{med\text{-}hi}CD62L^{lo}$) and regulatory T cells ($CD4^+CD25^+$) were quantified in the lymph node using fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC) monoclonal antibodies A list of the specific dilution and catalogue numbers are listed in the appendix. Cells from one of the control groups (BALB/c) were used for this part of the experiment. A volume of 500 µl popliteal lymph node cells ($1x10^7$ cells /ml) from the BALB/c control group was aliquoted into each FACS tube and centrifuged at 1600 rpm at 4°C for 10 minutes to pellet the cells. These cells where then incubated with 2% rat serum diluted in FACS buffer on ice for 5 minutes to prevent non specific binding. Cells were stained for the surface markers for 15 minutes at 4°C in the dark and were resuspended in FACS buffer containing 7AAD (1:1000) (Sigma, St. Louis, USA) and acquired using the FACS Calibur (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo v7.2 (Tree Star, Inc., Ashland, Oregon, USA)

2.10 FACS Sorting of $CD4^+$ T cells and $CD4^+CD25^+$ regulatory T cells from popliteal lymphnodes

2.10.1 Activated T cell Isolation using CD4 Microbeads

Lymph node $CD4^+$ T cells were positively selected using anti-CD4 coupled to microbeads (MACS, Miltenyi Biotec). Single cell suspensions prepared from lymph nodes were incubated with anti-CD4 microbeads and passed through an LS separation column (max 10^8 cells). The magnetically labeled $CD4^+$ cells were retained in the column while unlabeled cells ran through. After removal from the magnetic field, $CD4^+$ cells were eluted. These cells were then stained for surface marker expression for activated T cells ($CD4^+CD44^{med-hi}CD62L^{lo}$) supplemented with 4% rat serum then resuspended in 7AAD and FACS Sorted using the FACS Vantage. A purity of >98% was obtained, which was confirmed by FACS.

2.10.2 Regulatory T cells isolation using $CD4^+CD25^+$ regulatory T cell Isolation Kit (MACS Miltenyi Biotec)

Lymph node $CD4^+CD25^+$ regulatory T cells were isolated in a two-step procedure. First, $CD4^+$ T cells were pre-enriched by depletion of unwanted cells. Then, $CD25^+$ cells were positively selected from the enriched $CD4^+$ T cell fraction. For the isolation of $CD4^+$ T cells, non-CD4 T cells were indirectly magnetically labeled with a cocktail of Biotin-conjugated antibodies and Anti-Biotin microbeads. In parallel, the cells were labeled with $CD25^-$PE. The cell suspension was then loaded onto a MACS column (LD), which was placed in the magnetic field of a MACS Separator. The magnetically labeled non-$CD4^+$ T cells were retained in the column, while the unlabelled, $CD4^+$ T cells ran through. For the isolation of $CD4^+CD25^+$ cells, the $CD25^+$ PE labeled cells in the enriched $CD4^+$ T cell fraction were magnetically labeled with Anti-PE microbeads. The cell suspension was loaded onto a column which was placed in the magnetic field of the MACS Separator. The magnetically labeled $CD4^+CD25^+$ cells were retained in the column, while the unlabelled cells ran through. After removal of the column from the magnetic field, the retained $CD4^+CD25^+$ cells were eluted as the positively selected cell fraction and once again separated over a new

column, to achieve highest purities. The cells were resuspended in 7AAD and FACS Sorted using the FACSVantage. A purity of >98% was obtained, which was confirmed by FACS.

2.11 Flow cytometry

The following antibodies were used for flow cytometry: CD3-FITC, CD4-PerCP, CD8-PE, CD19-biotin, CD86-FITC, CD11c-PE, MHCII-APC, CD44-FITC, CD62L-APC, CD4-FITC and CD25-PE (all BD Bioscience Erembodegem, Belgium). For intracellular cytokine staining, popliteal lymph node cells from *L.major* infected mice were seeded at $2x10^6$ cells/well and stimulated at 37° C for 4 hours with phorbal myristate acetate (Sigma-Aldrich) (50ng/ml), ionomycin (Sigma-Aldrich) (250ng/ml) and monensin (Sigma-Aldrich) (200μM) in DMEM/10% FCS. Cells were stained, fixed and permeabilized, and intracellular cytokines were stained with anti-IL-10, anti-IL-12, anti-IFNγ, and anti-IL-13 and isotype controls (BD Bioscience) (all PE-labelled). Cells were acquired on a FACS Calibur machine (BD Immunocytometry systems, San Jose, CA, USA) and data were analyzed using Flowjo software (Treestar, Ashland, OR, USA).

2.12 Antibody detection by Enzyme-linked Immunosorbent Assays (ELISA)

Blood samples were taken by tail vein bleeding at day zero and three weeks post infection in gel separation tubes (Microtainer, BD, USA). Samples were centrifuged at 8000 rpm for 15 minutes at 4°C. Leishmania-specific IgG1, IgG2a and IgG2b were measured by indirect ELISA using 5μg/ml Soluble Leishmania Antigen (SLA) for coating (overnight at 4°C) on nunc maxisorb ELISA plates (Nunc, Reskilde Denmark). The plates were blocked for 3 hours at 37°C, washed 3 x, serum added in serial dilutions and incubated overnight at 4°C. Alkaline Phosphatase (AP)-labelled goat-anti-mouse isotype-specific Abs (Southern Biotechnology, Birmingham, USA) were added for 1 hour at 37°C. Total IgE was measured by sandwich ELISA using clone 84.1C as a coat and anti-mouse IgE (Southern Biotechnology, USA) for detection (Nieuwenhuizen *et al*., 2007). Total IgE, IgG1, IgG2a and IgG2b levels were measured for naïve and *L. major* treated mice (days 0 and 21) by sandwich ELISA. Anti-mouse IgE (84.1C), IgG1, IgG2a and IgG2b mAbs were used for coating, purified recombinant mouse IgE, IgG1, IgG2a and IgG2b (BD Pharmingen) were

used as standards and anti-mouse isotype-specific Abs (Southern Biotechnology, USA) for detection. 4-Nitrophenylphosphate (PNP, Fluka, Switzerland) dissolved at 1mg/ml in substrate buffer was added to all samples and the absorbance measured at 405nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany).

2.13 RNA Preparation from FACS sorted Cells

Sorted activated and regulatory T cells were lysed in 500 µl TriReagent® (Molecular Research, USA) and stored at -80°C. To extract the RNA, thawed samples were incubated at room temperature for 5 min to ensure complete dissociation of nucleoproteins. To each sample, 0.1 ml of chloroform was added and samples were vortexed for 15 seconds and incubated at room temperature for a further 15 min prior to centrifugation at 12000 rpm for 15 min at 4°C. The RNA contained in the upper aqueous layer was purified using the miRNeasy 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) on an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The Agilent assays were performed by the Centre for Proteomic and Genomic Research, Cape Town.

2.14 cDNA Synthesis

For each sample, 20 ng of extracted RNA (>200 nt fraction) was converted to cDNA via a poly-A and random priming method and linearly amplified and purified using the Ovation Pico WTA System (Cat. No. 3300, Nugen, USA) in conjunction with the DNA Clean & Concentrator Kit (Cat. No. D4005, Zymo Research, USA). The purity, concentration and yields were assessed using the Nanodrop.

2.15 Quantitative Real-time Polymerase Chain Reaction

The transcription level of selected genes (Type 1 and Type 2) was confirmed by quantitative real-time RT-PCR. This was performed on cDNA samples to determine gene expression levels of marker genes. Each reaction contained SYBR® Green Master Mix (Cat. No. 04913850001, Roche, Germany), primers and cDNA template. Reactions were prepared in 96- or 384-well plates (Roche, Germany) using a QIAgility robot (Qiagen, USA) and PCRs were performed in a LightCycler® 480 (Roche, Germany). To generate PCR standards, PCR products from each amplified gene were purified using the QIAquick PCR Purification Kit (Cat. No. 28104, Qiagen, USA) and subsequently quantified on a Nanodrop ND1000 (Thermo Scientific, USA). Using the formula: pmoles of ds DNA = (ug of ds DNA) x 1515 ÷ (product size in bp), the concentration to which the purified sample had to be diluted to obtain 1 x 10^6 molecules/µl was determined. Standard curves were prepared using serial ten-fold dilutions of the known amounts of starting material for each gene. Gene expression levels were normalized to either the rs12 or HPRT house-keeping gene. PCR specificity was confirmed via melt-curve analysis (a single sharp peak indicated a single homogenous product). 2-ΔΔCt method was used as the relative quantification algorithm in real-time RT-PCR of a target transcript in comparison to a reference gene. The threshold cycle (CT), defined as the cycle at which PCR amplification reached a significant value, was given as the mean value. The relative expression of each mRNA was calculated by ΔCT method (where ΔCT was the value obtained by subtracting the CT value of the endogenous control gene from the CT value of the target gene). All PCR reactions were done in triplicate. The fold change in the transcription level of target genes was calculated using the $2^{-\Delta\Delta}$Ct method. Primer sequences and PCR conditions are indicated in Table 4.

Table 4 Primers used for the quantitative real-time PCR Pre-microarray validation

No	Genes	Sequences	Primer length	Product length	Annealing Temp	Ext Time (sec)	Acquisition Temp
1	IL-4-Forward	5'-TCG GCA TTT TGA ACG AGG TC-3'	20	216	60	9	80
1	IL-4-Reverse	5'-GAA AAG CCC GAA AGA GTC TC-3'	20				
2	IFN-γ-Forward	5'-GCT CTG AGA CAA TGA ACG CT-3'	20	227	60	10	82
2	IFN-γ-Reverse	5'-AAA GAG ATA ATC TGG CTC TGC-3'	21				
3	GAPDH-Forward	5'-TTC ACC ACC ATG GAG AAG GC-3'	20	236	60	9	80
3	GAPDH-Reverse	5'-GGC ATG GAC TGT GGT CAT GA-3'	20				
4	Rs12-Forward	5'-GGA AGG CAT AGC TGC TGG AGG T-3'	22	365	60	10	80
4	Rs12-Reverse	5'-CGA TGA CAT CCT TGG CCT GA-3'	20				

2.16 Microarray design and overview

Amplification of RNA was performed using the WT-Ovation Pico assay. The purified cDNA was fragmented and subsequently labeled using the FL-Ovation™ cDNA Biotin Module. The fragmentation step is an enzymatic process producing single stranded cDNA fragments ranging from 50 – 100 bases. To ensure that the fragmentation step was successful, the samples were analysed using the Agilent Bioanalyser. The samples were labeled using an enzymatic process where a biotin-labeled nucleotide is attached to the 3-hydroxyl end of the fragmented cDNA.

2.16.1 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. After 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, Cape Town.

2.16.2 For Regulatory T cell microarray

2 µg of amplified cDNA was biotin-labeled using the Encore™ BiotinIL Module (Cat No. 4210, Nugen, USA) and purified using the Qiagen Mini Elute Spin Column (Cat. No. 28004, Qiagen, USA). Samples were then hybridized to Illumina® MouseWG-6 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA) as per the manufacturer's indications. BeadChips were scanned with the Illumina® BeadArray Reader and signal intensities were recorded as iDAT (image data) files for each chip. The above steps were performed by Dr. Roy Sugata at the RIKEN Omics Science Centre, Japan.

2.17 Microarray data analysis

For both the Affymetrix exon array (activated T cells) and Illumina bead array (regulatory T cells), the iDAT files were converted to data tables using Affymetrix Expression Console™ Software (goo.gl/QnfMw) and Illumina® Genome Studio Version 3 software (www.illumina.com). Quality control of the data was also done using this software. Samples were normalized by the quantile normalization method using the Bioconductor package, Limma (Smyth & Speed, 2003) and clustered via the hierarchical clustering method using Spotfire® software (spotfire.tibco.com). The above steps were also performed by the Centre for Proteomic and Genomic Research, Cape Town (activated T cells) and Dr. Roy Sugata at the RIKEN Omics Science Centre, Japan (regulatory T cells). A python program was written by a fellow lab member, Mr Andrew Einhorn, to generate lists of differentially expressed genes from the normalized data using user-defined criteria for fold-change and p-value cut-offs. Significance of differential expression was determined using a two-tailed Student's t-test. The python program required an input of a minimum expression cut off-value which was calculated by determining the expression level at which genes were classified as 90 % 'present' by the Genome Studio software (www.affymetrix.com and www.illumina.com). Including a minimum cut-off reduced the amount of noise in the list of differentially expressed genes. Genes were only excluded if expression values in both datasets being compared fell below the minimum. The python program also appended annotations to each gene using the Affymetrix and Illumina annotation files (www.affymetrix.com and www.illumina.com). Annotated gene lists were loaded onto Ingenuity Pathway Anaysis (IPA) software (Ingenuity® Systems, www.ingenuity.com) and Babelomics (http://babelomics.bioinfo.cipf.es) for network, pathway and functional analyses. Automated literature searches using user-defined search terms were performed using MILANO online software (Rubinstein & Simon, 2005). Figure 10 depicts the experimental approach to obtain the lists of differentially expressed genes.

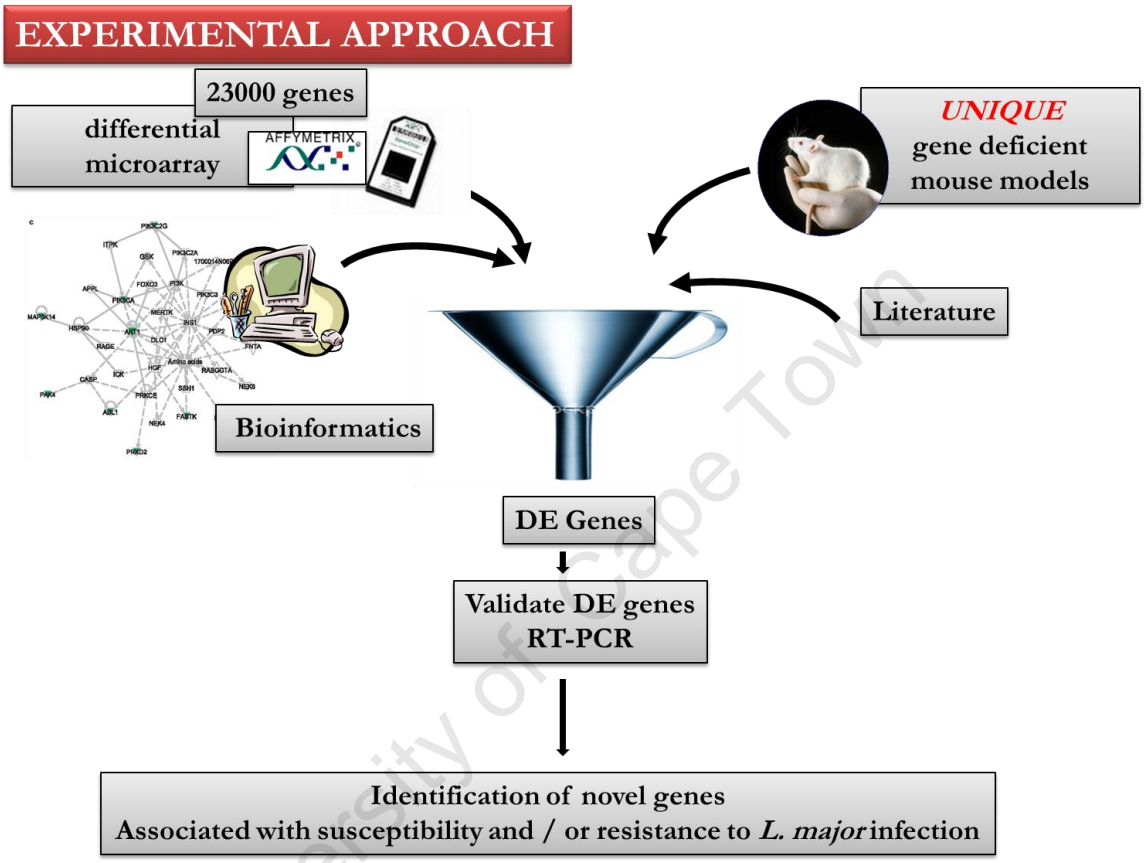


Figure 10 **Experimental approach to obtain list of differentially expressed genes**

2.18 Statistical analysis

Values are shown as mean ± SD or SEM and significant differences were determined using the unpaired one or two-tailed Student's t-test or one-way ANOVA using a Dunnett's Multiple Comparison post test. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively (Graphpad Prism™).

2.19 Calculating the fold change from the microarrays

Activated T cell Microarray was performed using affymetrix platform. The expression values received were log transformed values. Firstly, data was converted to linear, then triplicate values were averaged, data was then normalized to a control, BALB/c. The normalized individual values were then plotted using Graphpad prism to perform statistical analysis.

For the regulatory T cell microarray was performed using Illumina platform. The expression values received were linear values. This data was already linear. Triplicate values were averaged; data was then normalized to a control, BALB/c. The normalized individual values were then plotted using Graphpad prism to perform statistical analysis.

2.20 Validation of candidate genes using quantitative RT-PCR

To confirm the differential expression of the candidate genes, quantitative RT-PCR using the same RNA samples used in the microarray, was performed. The method used was as described above. All PCR reactions were done in triplicate. The fold change in the transcription level of target genes was calculated using the $2^{-\Delta\Delta}$Ct method. Primer sequences and PCR conditions are indicated in Table 5.

Table 5 Primers used for the quantitative real-time PCR- Validation Post-microarray validation

No	Genes	Sequences	Primer length	Product length	Annealing Temp	Ext Time (sec)	Acquisition Temp
1	IL-18r1-Forward	5'-GGCCAGGTGCAGTCTGTAGCAA-3'	22	275	60	11	78
1	IL-18r1-Reverse	5'-TGAGATGCTTGGTGGGAAACCTGAT-3'	25				
2	STAT4-Forward	5'-GCTGCAGCCAACATGCCTATCCAG-3'	24	322	60	13	77
2	STAT4-Reverse	5'-GGTGGGAGGTTCTCGTAAGCAAGA-3'	24				
3	Rnf130-Forward	5'-GGACAGGAACCAGCGTCGTC-3'	20	444	60	18	82
3	Rnf130-Reverse	5'-GTGCGAGGAGTGAGCTCCCCA-3'	21				
4	Rapgef4-Forward	5'-ACATCTGAAGTGCCCAGAGTGATGT-3'	25	257	60	11	84
4	Rapgef4-Reverse	5'-GGTGCTGGCGTGCCAACAATG-3'	21				
5	CCR5-Forward	5'-GCAGTTTCGGAGCAGTGTTGCTT-3'	23	355	60	14	79
5	CCR5-Reverse	5'-AAGCTGGGGCTGTAGCAGACTGA-3'	23				
6	CCL5-Forward	5'-CCTCACCATATGGCTCGGACACCA-3'	24	79	60	3	80
6	CCL5-Reverse	5'-TCCTTGACGTGGGCACGAGG-3'	20				
7	B2M-Forward	5'-TCACGCCACCCACCGGAGAA-3'	20	265	60	11	80
7	B2M-Reverse	5'-TGTCTCGATCCCAGTAGACGGTC-3'	23				

Chapter 3

Results

Results

3.1.1 Comparative *L. major* infection

In order to investigate the role of IL-4Rα-responsive T cells during cutaneous Leishmaniasis, comparative *L. major* infection experiments were done in non-healer BALB/c mice with a deletion of the IL-4Rα on all cells (IL-4Rα$^{-/-}$) or T cells only (iLCK^{cre}IL-4Rα$^{lox/-}$), their littermate controls (wild-type (WT) BALB/c and IL-4Rα$^{lox/-}$) and healer C57BL/6 mice. Six mice per group were infected subcutaneously in the hind footpad with 2×10^6 stationary phase metacyclic promastigotes of *L. major* LV39 (MRHO/SV/59/P). Disease progression was monitored by measuring the footpad swelling, parasite burden and systemic antibody responses. Three independent biological experiments were carried out.

3.1.2 Footpad swelling and Parasite burden

Footpad swelling was monitored weekly using a pocket thickness gauge (Mitutoyo, Japan) until the end point of the experiment. At three weeks post-infection parasite burden in footpads was determined by the limiting dilution method. Significant differences in footpad swelling were observed for the iLCK^{cre}IL-4Rα$^{lox/-}$ and IL-4Rα$^{-/-}$ groups when compared to the BALB/c mice at the last time point (Figure 11A). Parasite burden in footpads was similar among all the mouse groups, with the susceptible BALB/c mice displaying a small but significant higher parasite burden as compared to the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ and C57BL/6 mice (Figure 11B).

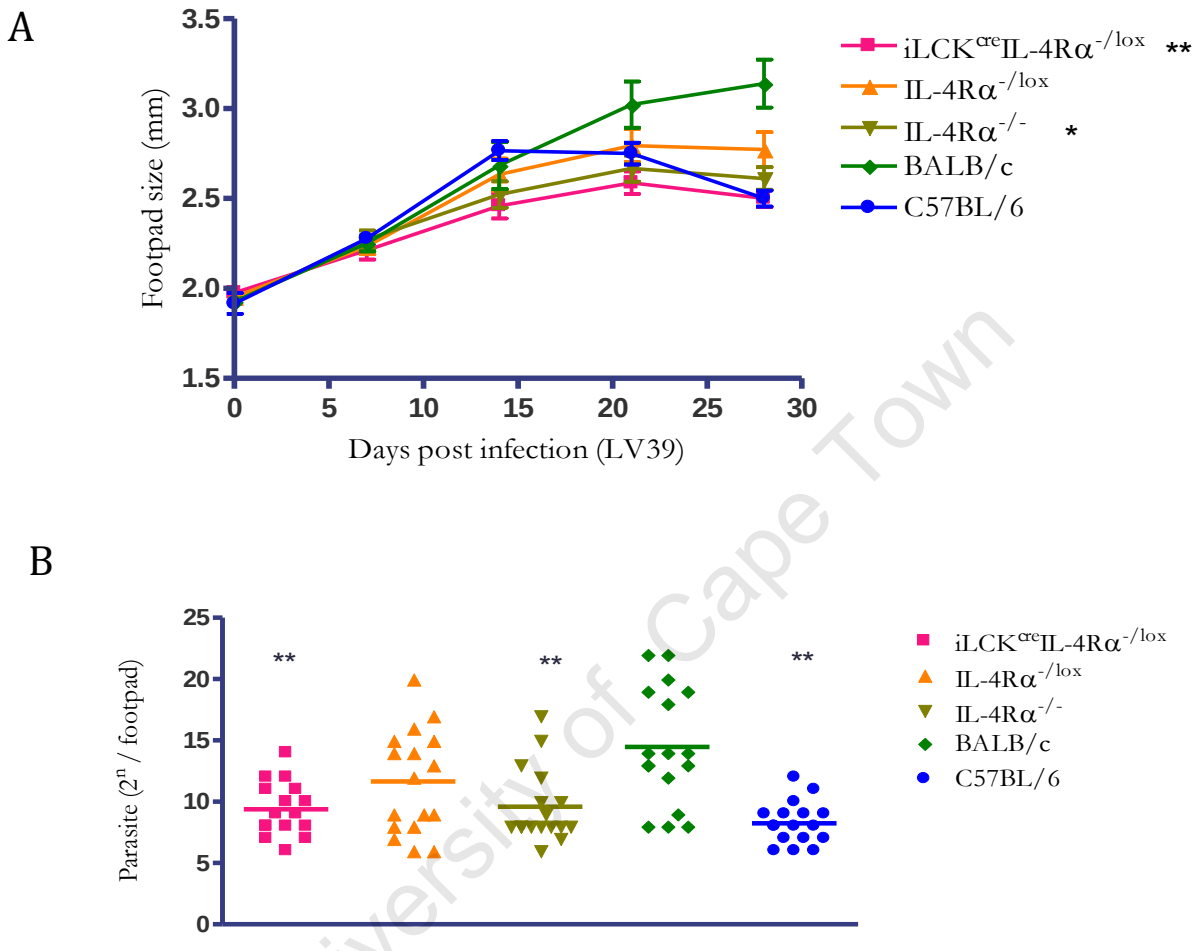


Figure 11 Footpad swelling and parasite burden during acute *L. major* infection

Six mice per strain were infected subcutaneously in the hind footpad with 2 × 10^6 stationary phase metacyclic promastigotes of *L. major* LV39 (MRHO/SV/59/P). A. Footpad swelling was monitored weekly using a pocket thickness gauge (Mitutoyo, Japan). Pooled data of three experiments. Statistics: BALB/c vs. iLCK^{cre}IL-4Rα$^{-/lox}$ p< 0.01 **, BALB/c vs. IL-4Rα$^{-/-}$ p<0.05 *. B. At three weeks post-infection parasite burden in footpads was determined by the limiting dilution method. A representative of one of three experiments is shown. Statistical significance was determined by comparing all groups to the BALB/c control group ** P<0.01 (Anova)

3.1.3 Antibody responses to *L. major* infection

In order to assess TH1 or TH2 differentiation and the development of the type 1 or type 2 immune responses during the acute phase of leishmaniasis, the levels of antigen–specific IgG2b and total IgE was measured in the sera of each mouse group by sandwich ELISA and using SLA. Total IgE was detected at significantly higher levels in both the susceptible BALB/c mice and the IL-4Rα$^{lox/-}$ littermate controls as compared to the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ and C57BL/6 mouse groups. The levels of total IgE were equivalent between the iLCK^{cre}IL-4Rα$^{lox/-}$ and C57BL/6 mice, whereas in the IL-4Rα$^{-/-}$ mice the total IgE level was significantly impaired and was at the detection level of the ELISA (Figure 12). This is as previously reported, as the presence of IL-4Rα on B cells is needed to produce IgE (Radwanska *et al.*, 2007). Since IgE production is strictly dependent on IL-4 signaling (Shimoda *et al*., 1996) this data indicates very strong signaling via the IL-4Rα in the BALB/c and the IL-4Rα$^{lox/-}$ mice during the acute phase of *L. major* infection, which is associated with TH2 associated type 2 immune responses. Similarly, since C57BL/6 mice are known to mount a TH1 associated immune response to *L. major* infection and that the SLA-specific IgE levels were equivalent between the iLCK^{cre}IL-4Rα$^{lox/-}$ and C57BL/6 mice, this data suggested that the iLCK^{cre}IL-4Rα$^{lox/-}$ may also have developed TH1 associated immune response despite its susceptible BALB/c genetic background. It was therefore expected that the iLCK^{cre}IL-4Rα$^{lox/-}$ and C57BL/6 mice have high SLA-specific IgG2b levels, which is indicative of a type 1 response (Mohrs *et al*., 1999). However, no clear type 1 immune response was observed between the various mouse groups (Figure 13).

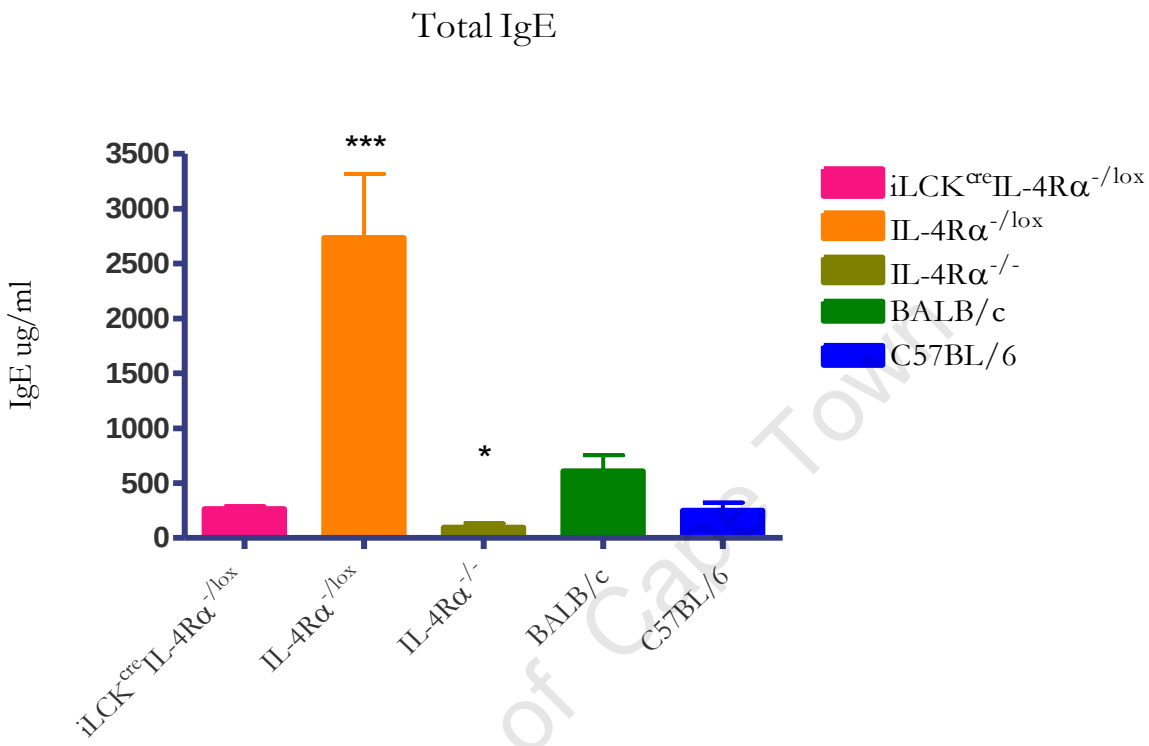


Figure 12 Total IgE production.

Six mice per strain were infected with 2x10^6 *L. major* LV39 (MRHO/SV/59/P) metacyclic promastigotes parasites and the serum levels of total IgE from individual mice were analysed at three weeks post infection by ELISA. Pooled data of three experiments is shown. All strains were compared to the BALB/c strain to determine statistical significance. *P<0.05 and *** P<0.001

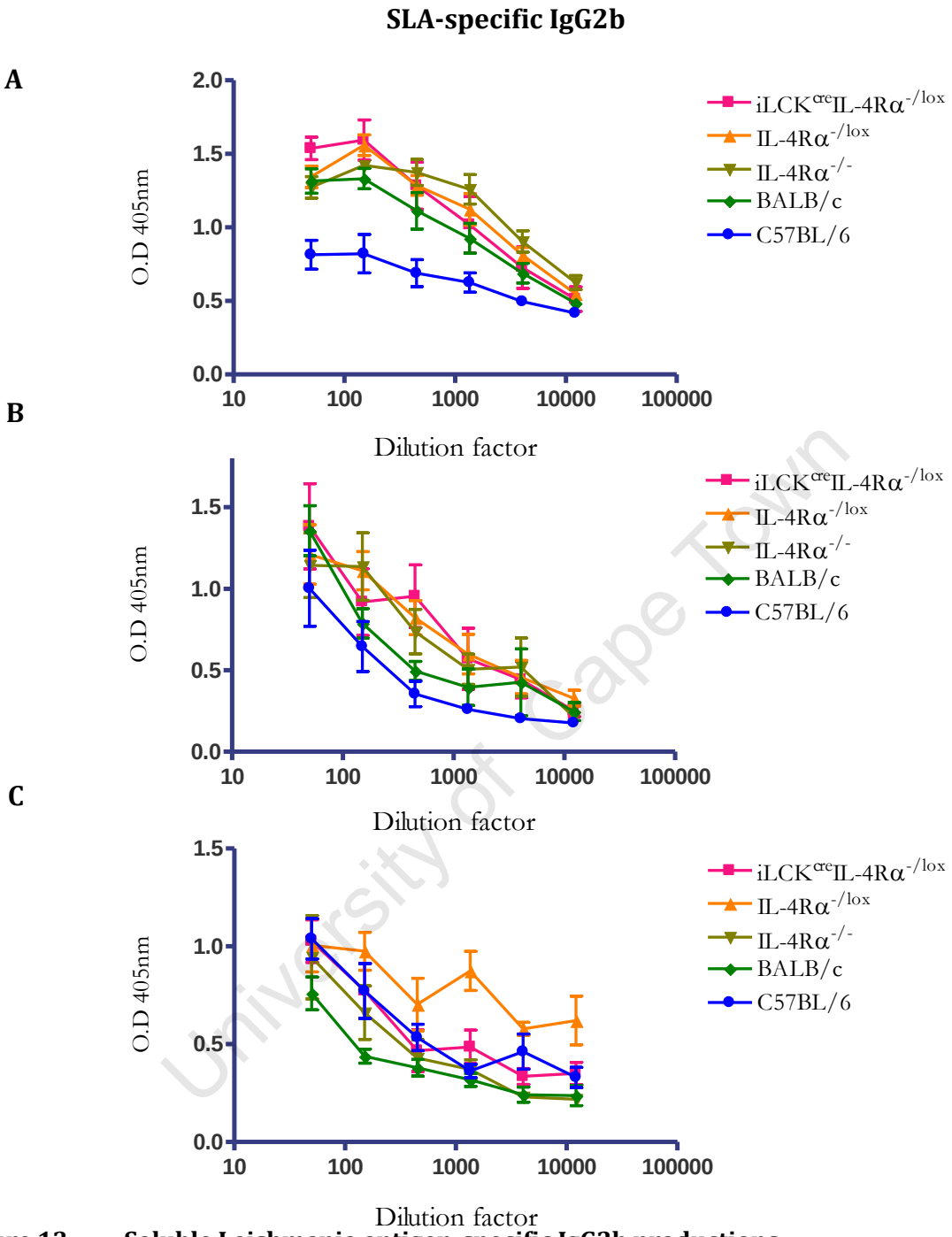


Figure 13 Soluble Leishmania antigen-specific IgG2b productions.

Six mice per strain were infected with 2x10^6 *L. major* LV39 (MRHO/SV/59/P) metacyclic promastigotes parasites and the serum levels of SLA-specific IgG2b from individual mice were analysed at three weeks post infection by ELISA. Each graph (A-C) represents an independent biological experiment.

3.1.4 Popliteal Lymph node T cell isolation

At three weeks post-infection, $CD4^+$ $CD44^{med-hi}CD62L^{lo}$ activated T cells and $CD4^+$ $CD25^+$ regulatory T cells were isolated from the draining lymph nodes of $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$, BALB/c and C57BL/6 mice that were infected *L. major* LV39 (MRHO/SV/59/P).

A single round of FACs sorting using the strategy depicted in Figures 14 yielded a cell purity of >99% for the $CD4^+CD44^{med-hi}CD62L^{lo}$ activated T cells (Table 6). Two rounds of FACs sorting was needed to isolate the $CD4^+CD25^+$ regulatory T cells to a cell purity of >97%. It is important to note that one limitation of this study was that it was not possible to isolate a 100% pure regulatory T cell population. This was because it would require that the isolated $CD4^+$ T cells be stained for both FoxP3 in addition to CD25. FoxP3 is a transcription factor that act as the master regulator for natural regulatory T cells and its detection by FACs analysis would require intracellular staining, which would result in the loss of RNA from the cells. In order to obtain RNA from the FACs sorted cells for microarray and quantitative RT-PCR, the cells needed to be viable. To determine the percentage of true $CD4^+CD25^+FoxP3^+$ T cells within the FACS sorted $CD4^+CD25^+$ regulatory T cells, an aliquot of the $CD4^+CD25^+$ regulatory T cells were intracellularly stained for FoxP3.

On average 75% - 85% of the the $CD4^+CD25^+$ regulatory T cells were $FoxP3^+$ (Figure 15). For the $CD4^+CD25^+$ regulatory T cells isolated from $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$ and BALB/c mice, greater than 80% were $FoxP3^+$. However for the $CD4^+CD25^+$ regulatory T cells isolated from C57BL/6 mice, only 74% were $FoxP3^+$ cells.

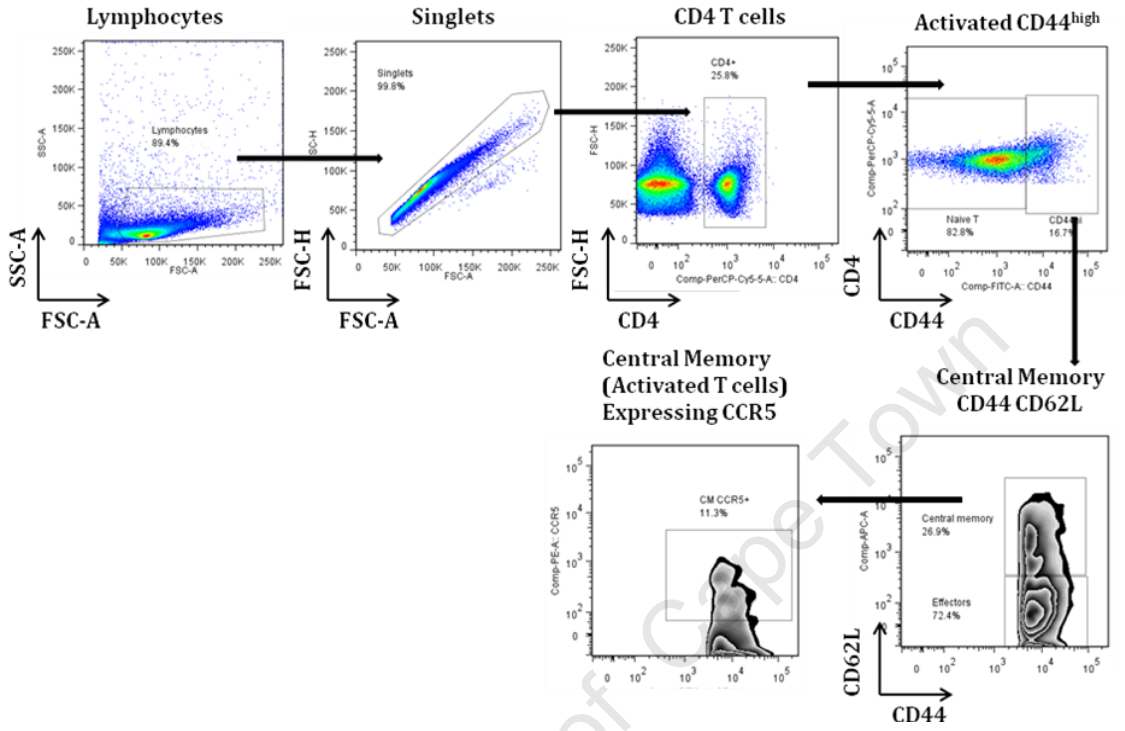


Figure 14 Flow cytometry gating strategy to isolate CD4+ CD44$^{med\text{-}hi}$ CD62L^{lo} activated T cells as well as expression of CCR5 (see p152) .

Mice were infected with 2x10^{6} *L. major* LV39 (MRHO/SV/59/P) metacyclic promastigotes parasites. At three weeks post-infection, mice were killed and CD4^{+}CD44^{med_hi}CD62L^{lo} activated T cells were isolated.

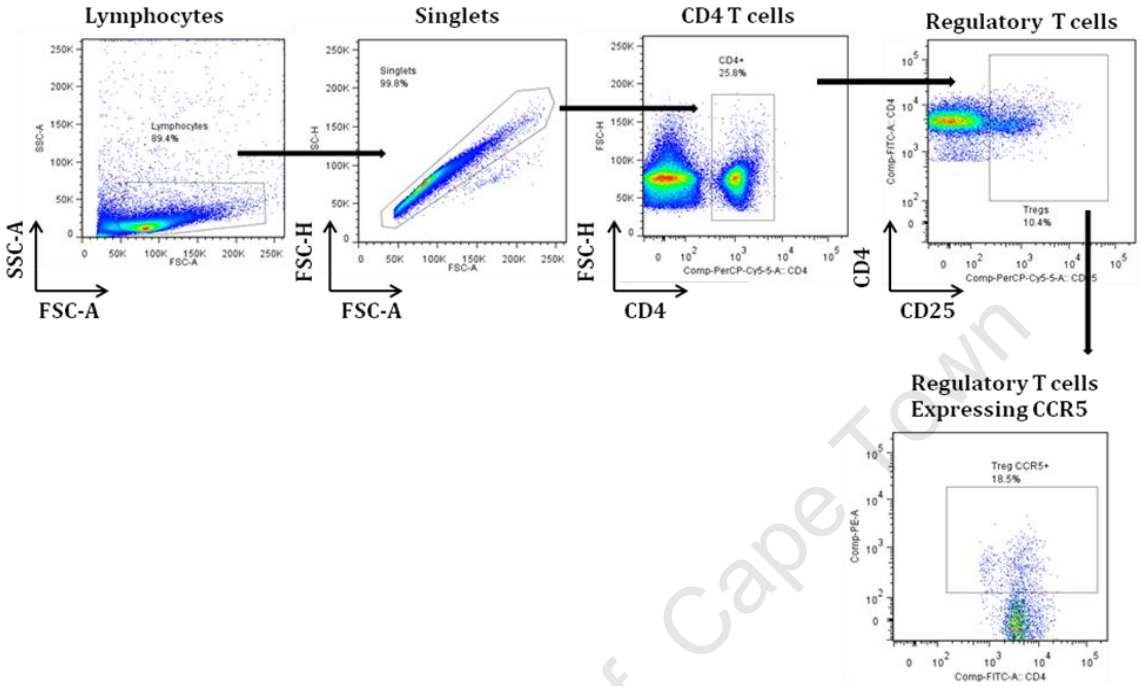
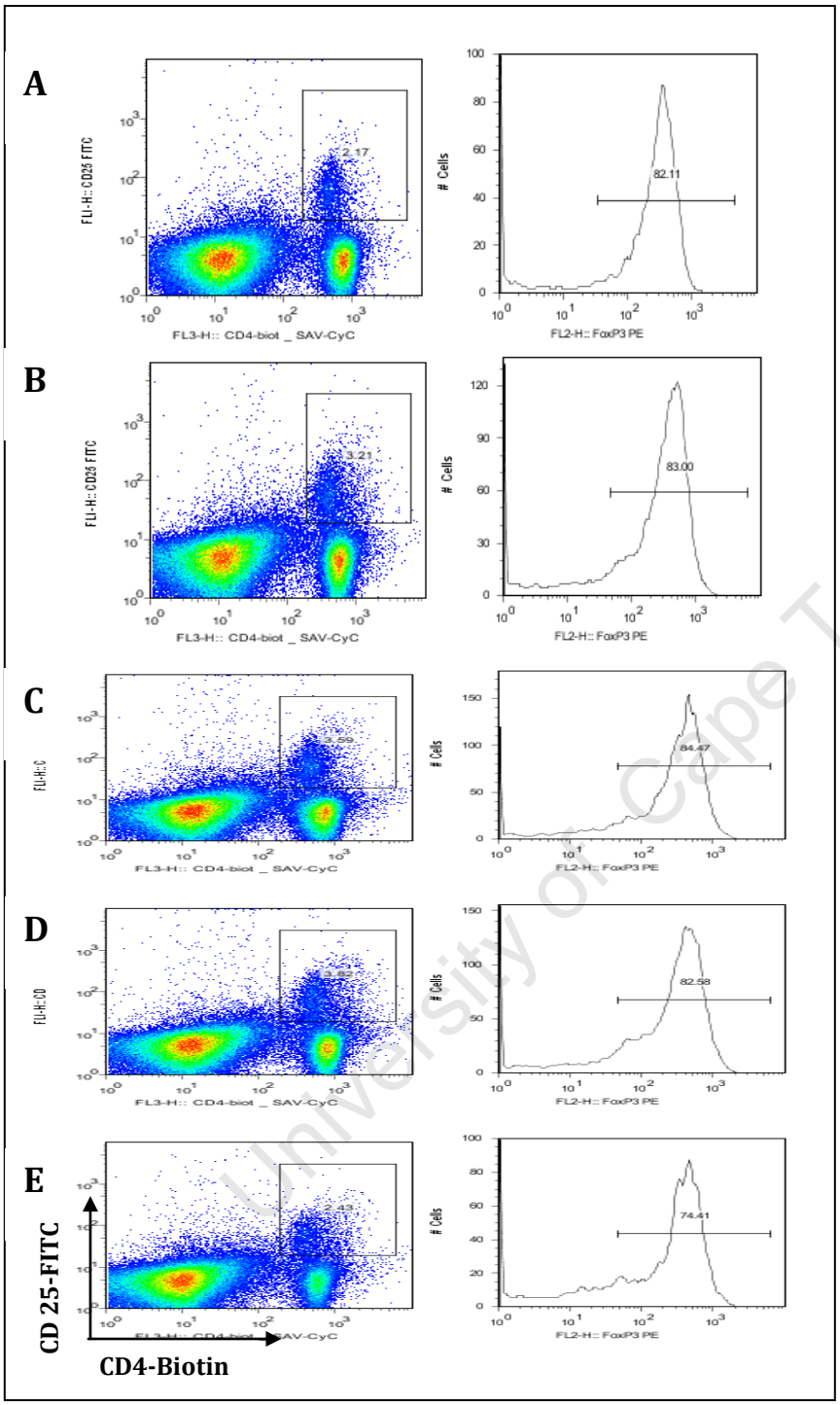


Figure 15 Flow cytometry strategy to obtain regulatory T cells as well as expression of CCR5 (see p152).

Mice were infected with $2x10^6$ *L. major* LV39 (MRHO/SV/59/P) metacyclic promastigotes parasites. At three weeks pot-infection, mice were killed and $CD4^+CD25^+$ regulatory T cells were isolated



Sample	% FoxP3^{+} Regulatory
iLCK^{cre}IL-4Rα$^{lox/-}$	82.11
IL-4Rα$^{lox/-}$	83.00
IL-4Rα$^{-/-}$	84.47
BALB/c	82.58
C57BL/6	74.41

Figure 16 Percentage of CD4⁺ CD25⁺ regulatory T cells that are FoxP3⁺.

CD4⁺CD25⁺ Regulatory T cells from **A**. iLCK^{cre}IL-4Rα$^{lox/-}$, **B**. IL-4Rα$^{lox/-}$, **C.** IL-4Rα$^{-/-}$, **D**. BALB/c and **E**. C57BL/6 mice were stained intracelluarly for the transcription factor, FoxP3. The percentages obtained are illustrated in the box on the right of this figure.

Table 6 Summary of yield and purity of sorted $CD4^{+}CD44^{med\text{-}hi}CD62L^{lo}$ activated T cells

Experiment	Sample	Yield (no of cells)	Purity (%)
A	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	89225	99.68
	$IL\text{-}4R\alpha^{lox/-}$	110887	99.81
	$IL\text{-}4R\alpha^{-/-}$	210596	99.61
	BALB/c	125430	99.86
	C57BL/6	70002	99.70
B	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	132161	99.83
	$IL\text{-}4R\alpha^{lox/-}$	113565	99.49
	$IL\text{-}4R\alpha^{-/-}$	147745	99.36
	BALB/c	266488	99.93
	C57BL/6	169255	99.81
C	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	205116	99.83
	$IL\text{-}4R\alpha^{lox/-}$	427970	99.91
	$IL\text{-}4R\alpha^{-/-}$	254514	99.87
	BALB/c	216640	99.88
	C57BL/6	142106	99.87

Table 7 Summary of yield and purity of sorted $CD4^{+}CD25^{+}$ regulatory T cells

Experiment	Sample	Yield (no of cells)	Purity (%)
A	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	131378	99.66
	$IL\text{-}4R\alpha^{lox/-}$	203879	99.73
	$IL\text{-}4R\alpha^{-/-}$	489951	99.83
	BALB/c	360343	99.77
	C57BL/6	143770	99.64
B	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	302681	99.01
	$IL\text{-}4R\alpha^{lox/-}$	284165	99.31
	$IL\text{-}4R\alpha^{-/-}$	403907	99.50
	BALB/c	349880	99.19
	C57BL/6	249199	99.22
C	$iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$	78754	98.33
	$IL\text{-}4R\alpha^{lox/-}$	132363	98.86
	$IL\text{-}4R\alpha^{-/-}$	173470	98.6
	BALB/c	188130	98.95
	C57BL/6	58755	97.23

3.1.5 Purification of RNA from activated and regulatory T cells

Six mice per group were infected with $2x10^6$ stationery phase *L major* LV39 (MRHO/SV/59/P) metacyclic promastigotes into the hind footpad. At 3 weeks post-infection, $CD4^+CD44^{med-hi}CD62L^{lo}$ activated T cells and $CD4^+CD25^+$ regulatory T cells were isolated from the draining lymph nodes and FACS sorted to >98% purity. Total RNA was extracted using the Qiagen RNeasy Plus Micro Kit which contains proprietary "gDNA Eliminator" spin columns that efficiently removes any contaminating genomic DNA.

The quality and quantity of the RNA was checked by BioAnalyzer analysis and no contaminating genomic DNA was detected (Figure 17). The RNA Integrity Number (RIN) for the RNA samples values ranged from 7.5 - 9.6 for the activated T cells (Table 8) and 6.6 - 9.3 for the regulatory T cells (Table 9). The RIN values for two samples, $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$ (experiment B) and BALB/c (experiment C), was not calculated due to an error in the BioAnalyzer software. Good quality RNA is indicated by 2 distinct peaks corresponding to the 28S and 18S rRNA whereas degradation is indicated by decreased signal intensities for both rRNA peaks. The electropherograms for both these samples displayed prominent peaks that were comparable to the other samples. Hence, it was assumed that the quality was equivalent to the other samples. The BioAnalyzer analysis for these two samples was not repeated, in order to conserve as much RNA for the downstream microarray and PCR analyses. The electropherograms for these two samples displayed prominent peaks that were comparable to the other samples. Therefore it was assumed that the quality was equivalent to the other samples. The RIN is an indication of the level of RNA degradation (Schroeder *et al*., 2006) and a RIN of ≥ 7 is considered acceptable for microarray. This meant that the RNA isolated from $IL\text{-}4R\alpha^{lox/-}$ regulatory T cells (experiment A) and potentially $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$ activated T cells (experiment B) and BALB/c activated T cells (experiment C) may not be of high enough quality for efficient cDNA conversion and microarray analysis. However, since the method of cDNA synthesis and linear amplification used a whole transcriptome approach, transcripts with a compromised poly-A tail can be successfully amplified (Tariq *et al*., 2011). This allows for RNA samples that are partially degraded, to be successfully converted to cDNA, linearly amplified and

then successfully used in downstream gene expression analyses including microarrays (www.nugeninc.com).

The yield of RNA for each sample isolated from activated T cells or regulatory T cells ranged from 6.7 to 13µg and 7.1 to 12.6µg respectively. The RNA was successfully converted to cDNA and linearly amplified using the Ovation® Whole Transcriptome Amplification System (Nugen, USA), and yields exceeding 3ug were obtained for all of the samples (Tables 8 and 9). Since minimum cDNA yield of approximately 3ug was required for each microarray, and electrophoerograms indicated pure cDNA with no genomic DNA contamination (data not shown), the cDNA was considered to be of sufficient high quality and purity for use in the microarray as well as the validation quantitative RT-PCR experiments.

Table 8 Summary of the RNA yields from activated T cells

Experiment	Sample	Yield (no of cells)	RNA yield (pg/ul)	RIN*	cDNA yield (ug)
A	iLCK^{cre}IL-4Rα$^{lox/-}$	89225	4802	8.1	10.401
	IL-4Rα $^{lox/-}$	110887	4682	8.8	9.036
	IL-4Rα $^{-/-}$	210596	8346	9.6	7.242
	BALB/c	125430	7078	7.7	6.705
	C57BL/6	70002	3660	7.6	8.964
B	iLCK^{cre}IL-4Rα$^{lox/-}$	132161	3702	N/A	13.011
	IL-4Rα $^{lox/-}$	113565	4799	8.5	12.159
	IL-4Rα $^{-/-}$	147745	6800	8.9	11.145
	BALB/c	266488	7853	8.5	11.949
	C57BL/6	169255	6527	9.0	11.625
C	iLCK^{cre}IL-4Rα$^{lox/-}$	205116	9630	8.4	11.721
	IL-4Rα $^{lox/-}$	427970	14418	8.4	12.228
	IL-4Rα $^{-/-}$	254514	9456	8.5	10.479
	BALB/c	216640	2737	N/A	12.927
	C57BL/6	142106	6107	8.7	11.652

* RNA Integrity Number

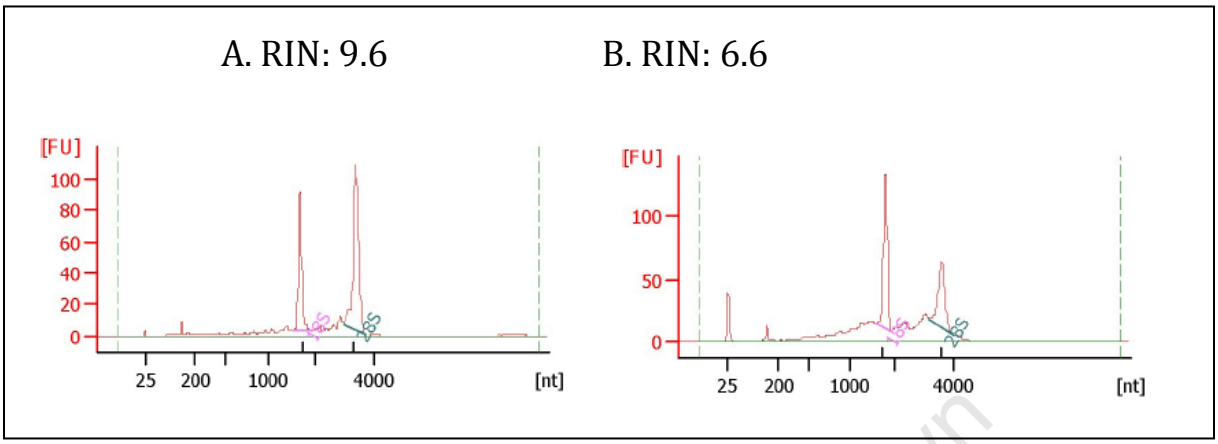


Figure 17 Representative Bioanalyzer electropherograms of RNA Samples. The integrity of RNA extracted from sorted T cells was assessed using a capillary electrophoretic approach. The samples displaying the highest (A) and lowest (B) RNA Integrity Numbers (RIN) are shown. Good quality RNA is indicated by 2 distinct peaks corresponding to the 28S and 18S rRNA whereas degradation is indicated by decreased signal intensities for both rRNA peaks as well as an increase in shorter fragments. The RIN scale ranges from 1 to 10.

Table 9 Summary of the RNA yields from regulatory T cells

Experiment	Sample	Yield (no of cells)	RNA yield (pg/ul)	RIN	cDNA yield (ug)
A	iLCK^{cre}IL-4Rα$^{lox/-}$	131378	4998	7.7	7.098
	IL-4Rα $^{lox/-}$	203879	9777	6.6	7.257
	IL-4Rα $^{-/-}$	489951	15245	6.9	12.576
	BALB/c	360343	11625	7.9	9.72
	C57BL/6	143770	4914	7.1	2.916
B	iLCK^{cre}IL-4Rα$^{lox/-}$	302681	8896	8.8	12.384
	IL-4Rα $^{lox/-}$	284165	7892	8.9	12.33
	IL-4Rα $^{-/-}$	403907	9001	8.7	9.972
	BALB/c	349880	10899	8.8	10.932
	C57BL/6	249199	7595	8.7	12.597
C	iLCK^{cre}IL-4Rα$^{lox/-}$	78754	2754	9.1	12.027
	IL-4Rα $^{lox/-}$	132363	4756	9.1	11.412
	IL-4Rα $^{-/-}$	173470	5248	8.9	12.033
	BALB/c	188130	6876	9.3	11.655
	C57BL/6	58755	1925	8.9	9.75

In order to determine if the RNA isolated from each mouse group was "biologically representative" of the observed phenotypic data (Figures 11-13) and reported *in vivo* data (Radwanska *et al*., 2007), the induction of IFN-γ and IL-4 was measured by quantitative real-time RT-PCR. C57BL/6 mice are reported to be genetically resistant to *L. major*, characterized by elevated IFN-γ levels and the induction of a strong TH1 response during infection. By contrast, susceptible BALB/c mice develop a progressive disease accompanied by high levels of IL-4 and TH2-associated cytokines (Radwanska *et al*., 2007). IFN-γ expression is associated with a typical TH1 response, whereas, IL-4 expression is associated with a TH2 response (Alexander, Satoskar, & Russell, 1999). An aliquot of the RNA that was to be used for microarray analysis was used to make cDNA for the RT-PCR analysis. The expression profiles of the IFN-γ and IL-4 for all three independent biological experiments are shown in Figures 18 – 19.

As expected, the level of IFN-γ was the highest in the activated and regulatory T cells isolated from C57BL/6 mice, and was on average 5-fold higher as compared to the BALB/c, iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, and IL-4Rα$^{-/-}$ mouse groups. The expression of IFN-γ was at similar reduced levels in both the activated and regulatory T cells for the WT BALB/c, iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, and IL-4Rα$^{-/-}$ mouse groups.

In contrast, the level of IL-4 was 2-fold higher in the BALB/c activated T cells as compared to the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$ and IL-4Rα$^{-/-}$ mouse groups, and 4-fold higher than the activated T cells from C57BL/6 mice. The expression of IL-4 was equivalent in the activated T cells for the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$ and IL-4Rα$^{-/-}$ mouse groups.

The level of IL-4 was almost 5-fold higher in the BALB/c regulatory T cells as compared to the iLCK^{cre}IL-4Rα$^{lox/-}$ and IL-4Rα$^{-/-}$ mouse groups, and 2-fold higher than the IL-4Rα$^{lox/-}$ regulatory T cells. The expression of IL-4 was 4-fold higher in the BALB/c regulatory T cells as compared to the from C57BL/6 regulatory T cells.

Furthermore, the amplification of the RT-PCR products was very efficient and robust, indicating that the quality of the RNA was high and can be confidently used for microarray analysis.

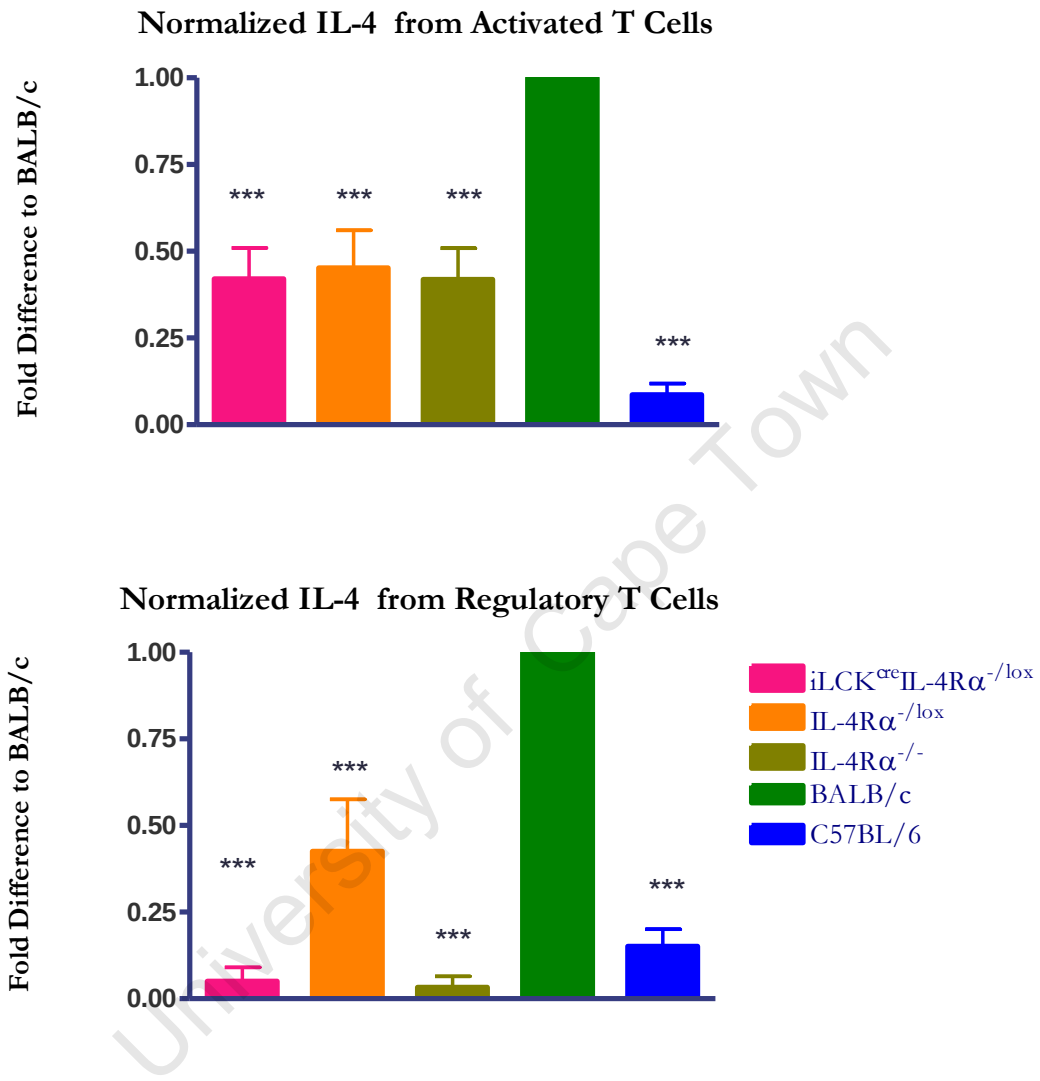


Figure 18 Expression levels of IL-4 expression in activated ($CD4^+CD44^{med\text{-}hi}CD62L^{lo}$) and regulatory ($CD4^+CD25^+$) T cells isolated from *L. major*-infected mice at three weeks post infection. For each mouse group, T cells were isolated from pooled popliteal lymph nodes of 6 mice and sorted to > 98% purity. Total RNA was extracted, converted to cDNA and then linearly amplified and used for quantitative RT-PCR. Gene expression data was normalized to the rs12 housekeeping gene, and then compared to the BALB/c group. Each graph represents the average and standard deviation for three independent biological experiments *** P<0.001

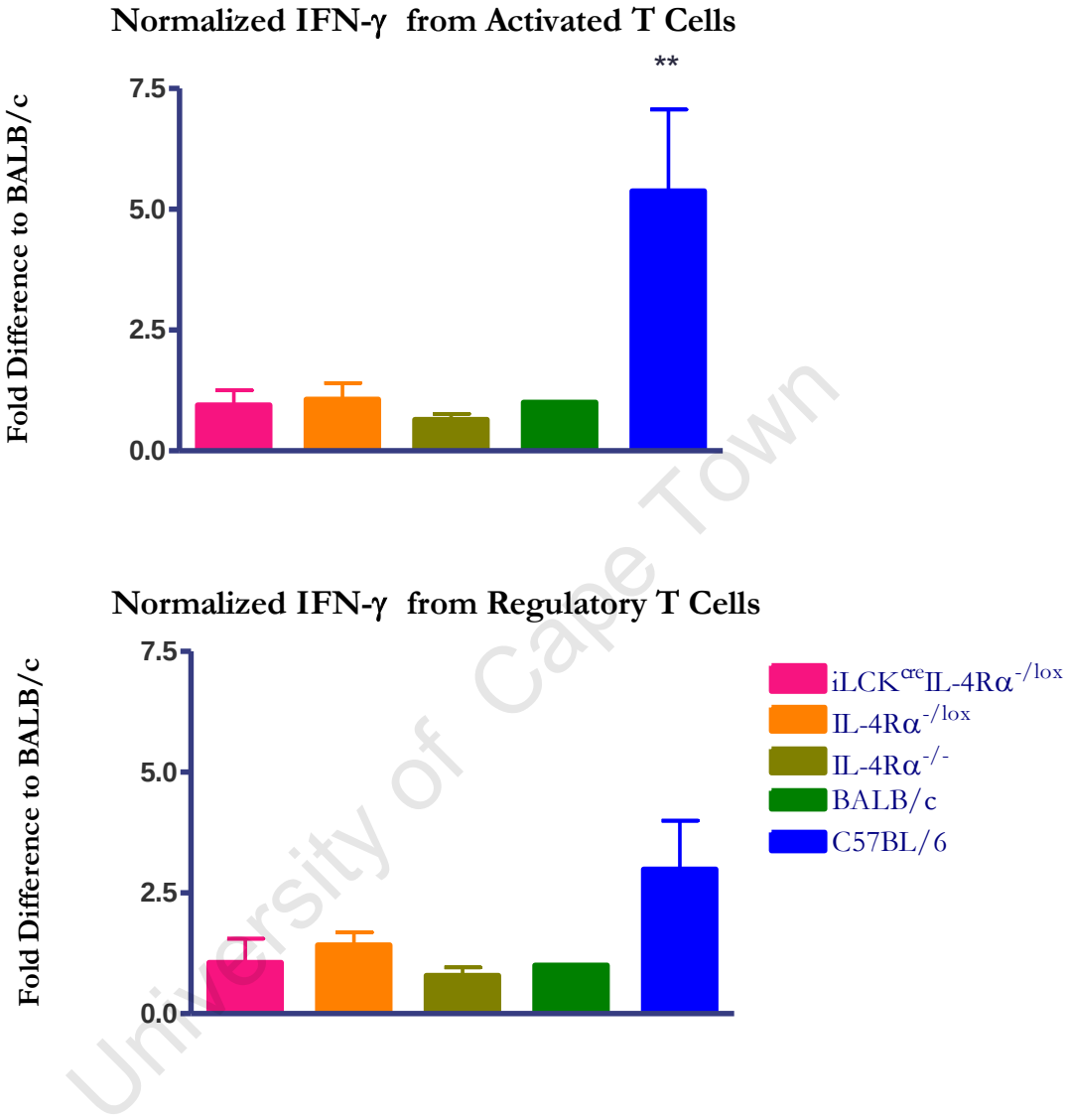


Figure 19 IFN-γ expression in activated (CD4^{+}CD44^{med-hi}CD62L^{lo}) and regulatory (CD4^{+}CD25^{+}) T cells isolated from *L. major*-infected mice three weeks post infection. For each mouse group, T cells were isolated from pooled popliteal lymph nodes of 6 mice and sorted to > 98% purity. Total RNA was extracted, converted to cDNA and then linearly amplified and used for quantitative RT-PCR. Gene expression data was normalized to the rs12 housekeeping gene, and then compared to the BALB/c group. Each graph represents the average and standard deviation for three independent biological experiments *** P<0.001

3.2 Gene expression profiling of popliteal lymph node Activated T cells

Transcriptional profiling by microarray was used to assess the differences in activated T cells isolated from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 mice at three weeks post infection with *L. major*.

Transcriptional profiling was performed to identify genes and pathways by which signaling via the IL-4Rα on CD4^{+}Tcells confers host protection or susceptibility to *L. major*. Gene expression patterns were compared early during infection in T cells in the absence or presence of IL-4Rα signaling.

3.2.1 Quality Control of microarray data

The activated T cell microarray was performed using a GeneChip Mouse Exon 1.0 ST Array on an Affymetrix platform at the Centre for Proteomics and Genomic Research (CPGR) in Cape Town, South Africa. Before extracting biologically meaningful results from the microarray data, it was necessary to assess the quality of the data using various quality control metrics. Before extracting biologically meaningful results from the microarray data, it was necessary to assess the quality of the data using various quality control metrics. The quality scores for all microarrays were within the required specifications outlined in Table 10, indicating no technical problems with respect to hybridization, washing, staining and specificity. For all microarrays, the individual background was consistently low and homogenous within each array, and the signal to noise ratio consistently high.

Table 10 Control metrics used to assess quality of microarray data

Quality Control metric	Description
Sample Quality	A sample / hybridization should be flagged and possibly removed when several metrics are outliers within the distribution of samples. If none of these metrics consistently denotes an outlier sample, the researcher should confidently proceed with downstream analysis.
Pos_vs_neg_auc	This is the "area under the curve" or AUC value for a ROC curve which plots the detection of positive controls against the false detection of negative controls.
All Probe set Mean	The mean of the signal of all probe sets included in the analysis.
All Probe Set RLE Mean	Mean absolute relative log expression (RLE). The signal of each probe set is compared to the median signal value of this probe set of the study. The metric is the mean of these differences from all the probe sets.

3.2.2 Identification of differentially expressed genes

Differentially expressed genes in activated T cells were identified and by performing comparative analyses of the microarray data between C57BL/6 versus (vs.) BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c, amongst others. From a previous publication (Radwanska *et al*., 2007), it is known that C57BL/6 mice are resistant and are able to heal during the acute and chronic stages of the disease, whereas BALB/c mice are susceptible and succumb to infection. Even though iLCK^{cre}IL-4Rα$^{lox/-}$ is on a susceptible BALB/c genetic background, it is still able to heal. We sought to investigate how similar the transcriptional response of the iLCK^{cre}IL-4Rα$^{lox/-}$ is to the C57BL/6. By comparing the mRNA expression profiles of

C57BL/6 to BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ to BALB/c, these comparisons will identify genes important for mediating protection during *L. major* infection.

Historically, a fold change cut-off of 2-fold was considered standard for identifying differentially expressed genes in microarray data (DeRisi *et al*., 1996). However, it has emerged that arbitrarily assigning a fold change and p-value cut-off may result in a loss of valuable biological information (McCarthy & Smyth, 2009). When using a p-value cut-off of <0.05 and a differential gene expression cut-off of >2-fold, marker genes such as IFN-γ, Tbx21 and Stat4 were excluded as being differentially expressed, even though they have been published to be differentially regulated in CD4^{+}T cells during TH1/TH2 differentiation and *Leishmania* infection (Zhu & Paul, 2008). Therefore in order to determine the best fold cut-off to use when defining differentially expressed genes, different gene lists were generated using a p<0.05 and different differential expression fold cut-offs, and the lists analyzed. A differential gene expression fold change cut-off of 1.2 combined with a p-value < 0.05 was found to be optimal, and was subsequently used for both the C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparative data analyses to identify differentially expressed genes (Table 11, and Tables A1 and A2 in appendix). A total of 456 genes were differentially expressed in activated T cells from C57BL/6 mice as compared to BALB/c mice, with 46% being up-regulated and 54% being down-regulated in the C57BL/6 activated T cells. In contrast, only 17 genes were differentially expressed in activated T cells from iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to BALB/c mice, with 70% being up-regulated and 30% being down-regulated in the iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells. Interestingly, four genes (Gdpd3, Ccr5, Otud6b and H2-T10) were differentially expressed with the same up- or down-regulatory patterns in the C57BL/6 and the iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells as compared to BALB/c activated T cells (Figure 20).

Table 11 Summary of differentially expressed genes from activated T cell microarray.

		iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c	C57BL/6 vs. BALB/c
DE genes p > 0.05	**Total DE genes**	236	487
DE genes p > 0.05 fold change > ± 1.2	**Total DE genes**	17	456
	# DE genes up-regulated	12	209
	# DE genes down-regulated	5	247
	# DE genes with p<0.05	9	349
	# DE genes with *p* <0.01	4	98
	# DE genes with *p* <0.05	4	9
	# DE genes located in *Lmr* loci	7	201

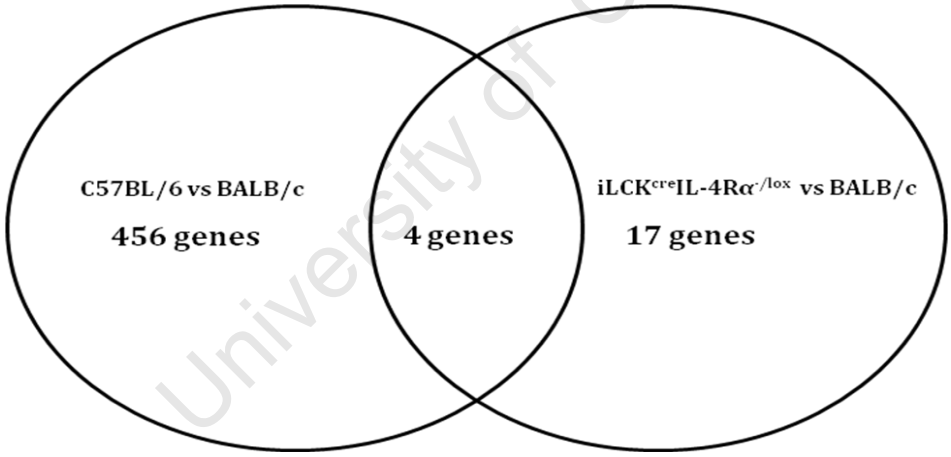


Figure 20 Activated T cells with a p value <0.05 Fc >1.2

Overlapping differentially expressed genes in dataset comparisons comprising the BALB/c control group. Differentially expressed genes in common in both C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Ra$^{-/lox}$ vs. BALB/c dataset comparisons from activated T cell microarray (p-value ≤ 0.05 and Fc > 1.2). Expression data from 3 independent biological replicates were used to generate the gene lists.

3.2.3 Biological relevance and interpretation of microarray data

After having generated the lists of differentially expressed genes, the next step was to determine if the microarray data was biologically relevant, and to interpret the results biologically within the context of the experiment.

3.2.3.1 Differential expression of IL-4Rα

Given that activated T cells from *L. major*-infected from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 were used in this microarray study, the expression of the IL-4Rα gene in each microarray dataset was analyzed for conformity to published data (Figure 21). In the activated T cells of the littermate control IL-4Rα$^{lox/-}$, global knockout IL-4Rα$^{-/-}$ and iLCK^{cre}IL-4Rα$^{lox/-}$, the expression of IL-4Rα was significantly reduced as compared to the susceptible BALB/c mice. These results are in line with the published gene dosage effect of the IL-4Rα alleles, where BALB/c have 2 functioning alleles of the receptor as compared to the knockouts that have only one (IL-4Rα$^{lox/-}$). Surprisingly, in the global knockout IL-4Rα$^{-/-}$ mRNA expression of the IL-4Rα receptor was still observed, even though the gene is not making a functional IL-4Rα protein. A possible hypothesis for this observation is the detection algorithm used for Affymetrix exon array image analysis, which calculated overall gene expression by taking an average across all 12 exons of the IL-4Rα, even though exons 7, 8 and 9 were deleted.

3.2.3.2 Mapping of Differentially expressed genes to *Leishmania major* response (*Lmr*) Loci

Furthermore, 41% of the differentially expressed genes in both C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells in mapped to *Leishmania major* response (*Lmr*) loci (Tables A1 and A2 in appendix). This enrichment was found to highly statistically significant in C57BL/6 vs. BALB/c comparative analysis (Table 12), but not for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c analysis (Table 13).

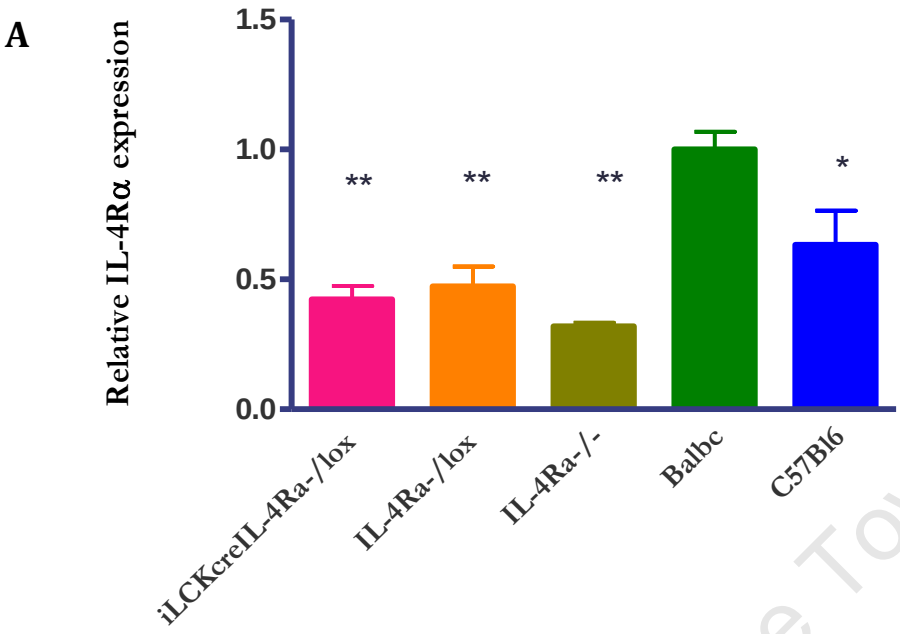


Figure 21 Analysis of IL-4Rα expression levels by microarray

Activated T cells from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative IL-4Rα expression levels post-normalisation of microarray data. All groups were compared to the BALB/c control group which was set to 1. Data are representative of three independent biological experiments. *P<0.05, ** P<0.01

Table 12 Activated T cell microarray of C57BL/6 vs. BALB/c comparison showing linkage to *Lmr* loci. A two-tailed Chi-square test with Yates correction was used to analyze the 2x2 contingency table below.

	Rest of genome	DE genes	Total
# genes in *Lmr*	7091	201	**7292**
# gene not in *Lmr*	16077	286	**16363**
Total	**23168**	**487**	**23655**
Result:	χ^2= 24.950 with 1 degrees of freedom $p < 0.0001$		

Table 13 Activated T cell microarray of iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparison showing linkage to *Lmr* loci. A two-tailed Chi-square test with Yates correction was used to analyze the 2x2 contingency table below.

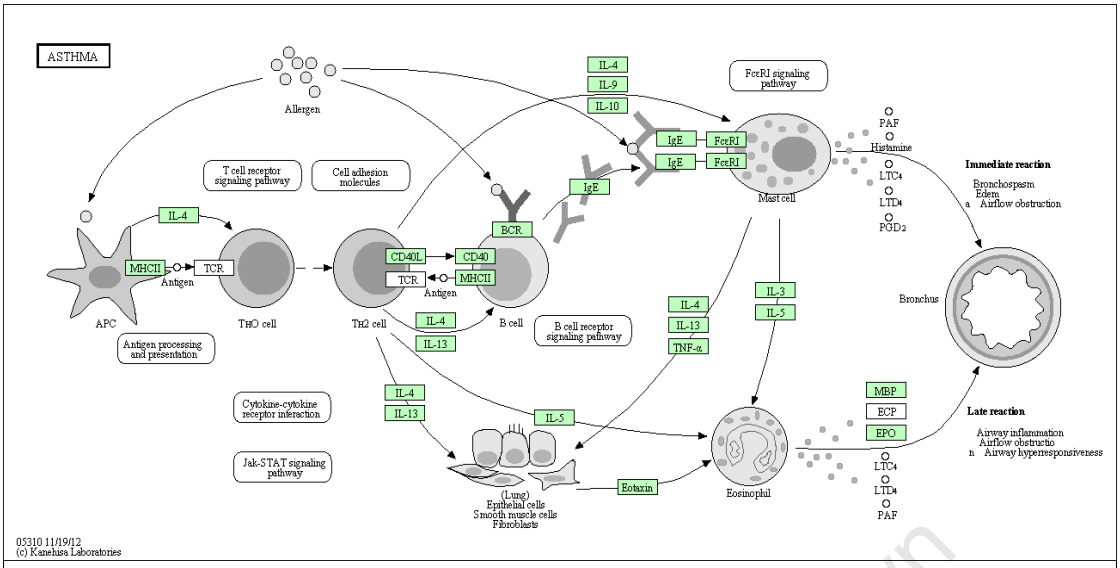
	Rest of genome	DE genes	Total
# genes in *Lmr*	7091	7	**7098**
# genes not in *Lmr*	16077	10	**16087**
Total	**23168**	**17**	**23185**
Result:	χ^2 = 0.893 with 1 degrees of freedom. $p < 0.3446$		

3.2.3.3 Functional clustering of the differentially expressed genes

Functional clustering of the differentially expressed genes into common signaling pathways, functional networks and molecular functions was done using Babelomics (http://babelomics.bioinfo.cipf.es) and Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com).

The top IPA functional networks that were most significant in both the C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparative analysis were cellular growth and proliferation, cell-mediated immune response, cell signaling, inflammatory response, cell-to-cell signaling and interaction, and cell death (Table 14 and Figures 23 and 24). Each network is assigned a score which is derived from the p-value. This score is assigned by IPA according to the fit of that network to the user-defined focus genes. The higher the score, the more statistically significant the clustering of genes in a network is, and the less likelihood that these genes are clustering due to random chance. Similarly, functional clustering using Babelomics revealed that the differentially expressed genes were significantly enriched for cytokine-cytokine receptor interaction, lysosome, transport of synthesized lysosomal enzymes, cell adhesion molecules with interest in the T cell receptor signaling pathway, antigen processing and presentation, Hemapoetic cell lineage, Type 1 diabetes mellitus, Asthma (Jak-STAT signaling), Autoimmune thyroid disease, allograft rejection and graft versus host disease (Figure 22). Babelomics uses a simple enrichment analysis. Once the genes of interest have been selected, the enrichment any type of biologically relevant annotation in these genes is compared to the corresponding distribution of the annotation in the background. FatiGo (Al-Shahrour *et al.*, 2004) is one such tool that is available that use different functionally relevant annotations, such as GO terms and KEGG pathways.

A



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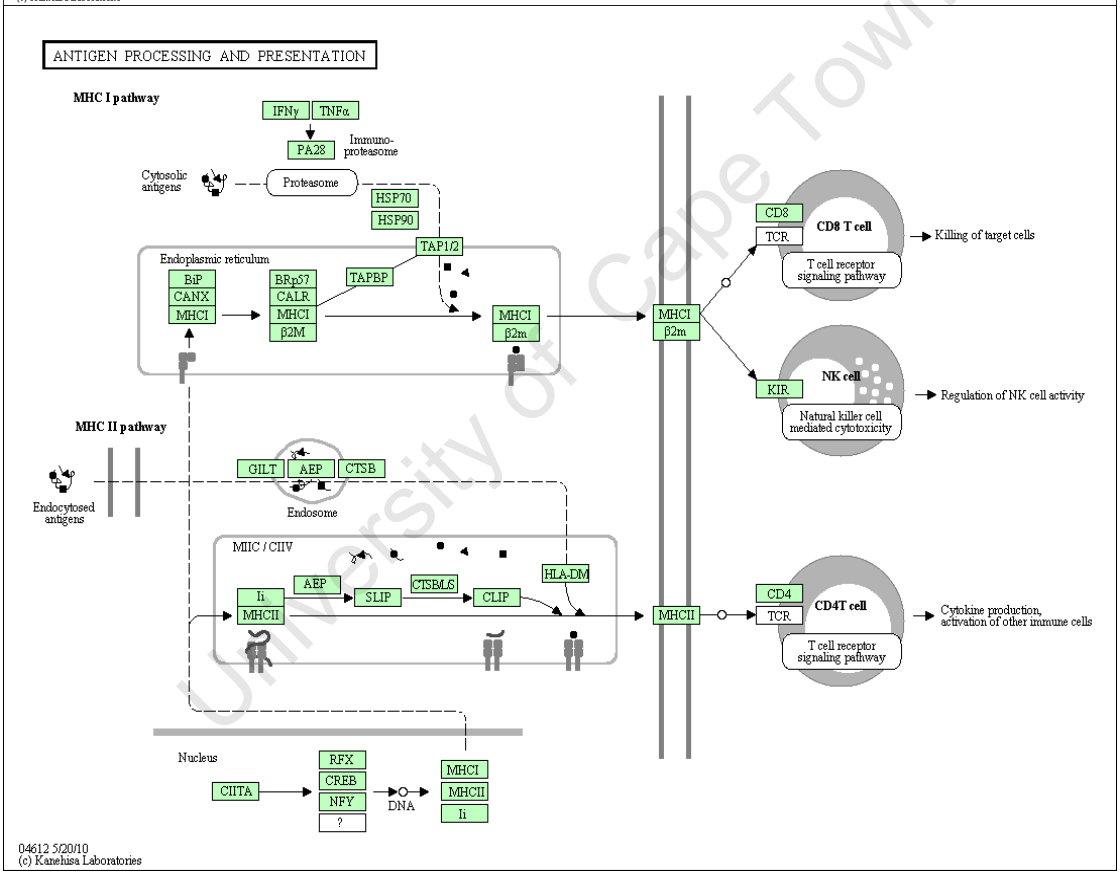

Figure 22 Pathways associated with differentially expressed genes from activated T cell microarray. Babelomics was used to identify known biological relationships among the differentially expressed genes. A) The Jak-STAT signaling and B) Antigen processing and presentation pathways were amongst the pathways that were significantly enriched. (Illustrations were taken from http://babelomics.bioinfo.cipf.es)

Table 14 Summary of the top IPA networks from the activated T cell microarray

C57BL/6 vs. BALB/c		iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c	
Associated network	**Score**	**Associated network**	**Score**
Cellular Growth and Proliferation, Hematological System Development and Function, Cell Death	34	Cellular Function and Maintenance, Cellular Development, Hematological System Development and Function	27
Cellular Movement, Cell Death, Cellular Growth and Proliferation	27	Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism	9
Inflammatory Response, Cell Morphology, Hematological System Development and Function	26	Embryonic Development, Organ Development, Organismal Development	3
Cellular Development, Hematopoiesis, Cell-To-Cell Signaling and Interaction	24		
Cell Cycle, Cellular Development, Cell Death	24		

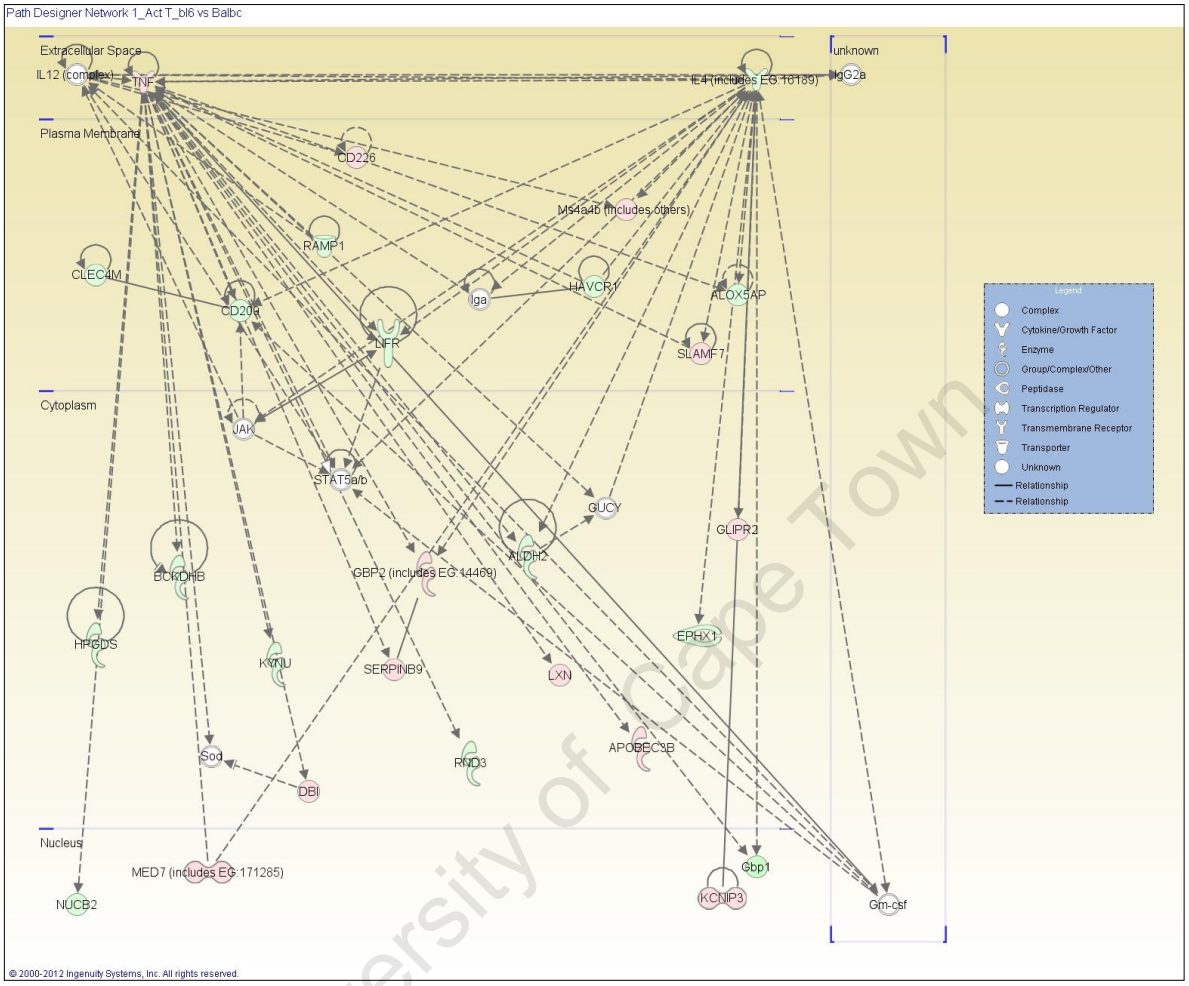

Figure 23 Network analysis of differentially expressed genes in C57BL/6 vs. BALB/c dataset comparisons (FC > 1.2, p < 0.05) from activated T cell microarray.

Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to identify known biological relationships among the differentially expressed genes. Relationships represented may be activation, expression, inhibition, localization, phosphorylation, protein-DNA interaction, protein-protein interaction, proteolysis, regulation of binding, RNA-RNA interactions, transcription or translocation.

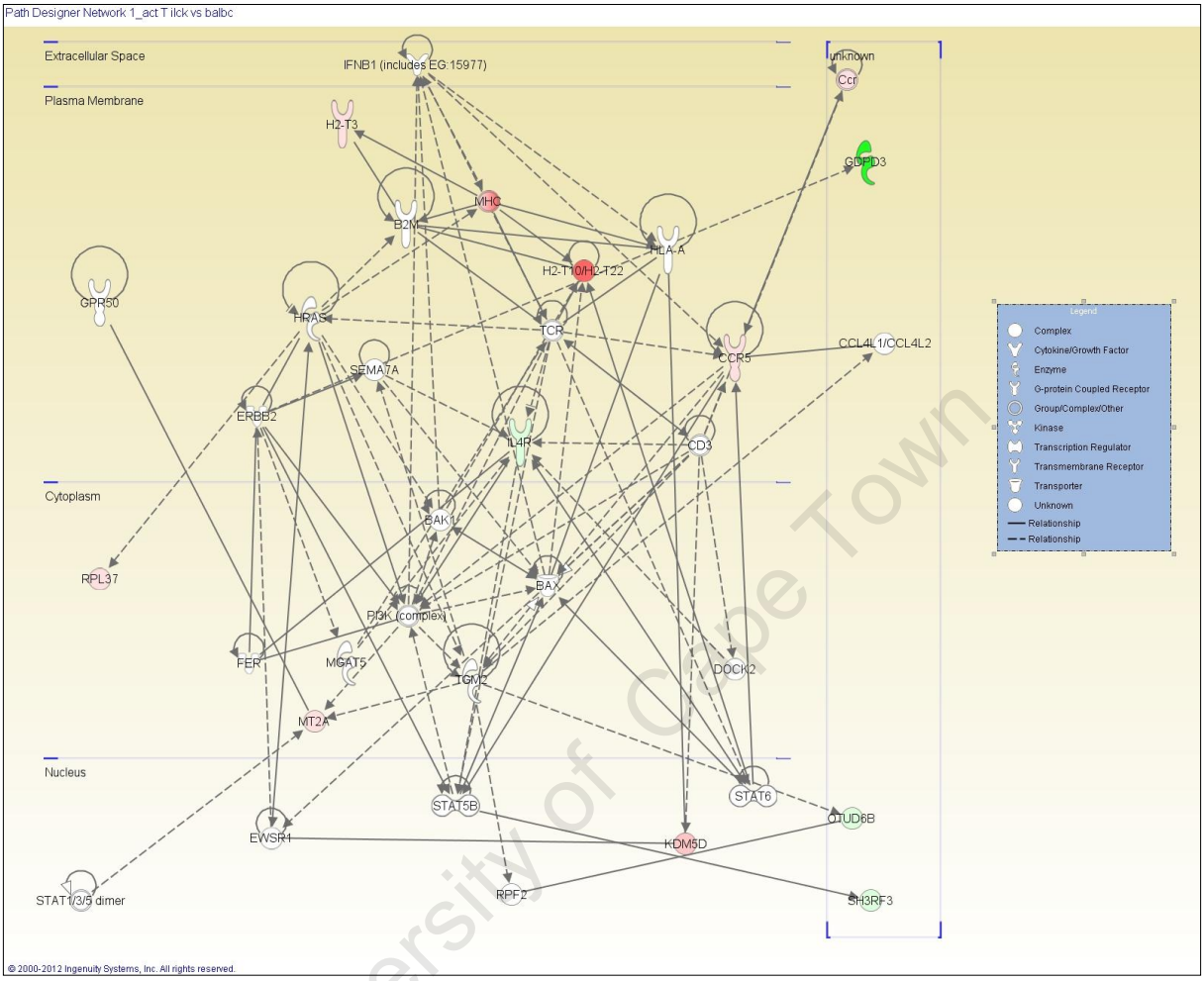

Figure 24 Network analysis of differentially expressed genes in iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c dataset comparisons (FC > 1.8, p < 0.05) from activated T cell microarray.

Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to identify known biological relationships among the differentially expressed genes. Relationships represented may be activation, expression, inhibition, localization, phosphorylation, protein-DNA interaction, protein-protein interaction, proteolysis, regulation of binding, RNA-RNA interactions, transcription or translocation.

In order to identify which DE genes were associated with either TH1 or TH2 differentiation during Leishmaniasis, intensive literature mining for each DE gene was done using the IPA knowledgebase (Ingenuity® Systems, www.ingenuity.com) and the automated literature search tool, MILANO (Rubinstein & Simon, 2005). The molecular interaction networks of the differentially expressed genes with TH1- or TH2-associated molecules were assessed using IPA (Ingenuity ® Systems, www.ingenuity.com). The TH2 molecules assessed were IL-4, IL-13 and the TH2-associated transcription factors, STAT6 and GATA3 (Paul, 2010), and the DE genes having molecular interactions with these TH2 genes are summarized in Table 15. Similarly DE genes having molecular interactions with TH1-assosiated molecules such as IFN-γ, TNF, T-bet and STAT4 were identified and are summarized in Table 15 where, these interactions are depicted by grey shadowing.

Functional annotations such as 'differentiation', 'proliferation', 'activation', 'presentation' and 'recruitment' are regularly used to describe the genes that are important in host defense and immunity against *L.major*. Differentially expressed genes which shared the same or similar combination of functional annotations were identified and are shown in Table 16, along with their molecular interaction with other molecules already known to be associated with TH1/TH2 response.

The above literature mining strategies present only a limited analysis of the gene lists, but were sufficient to identify genes in activated T cells that may be contributing to disease protection or progression during *L. major* infection and / or TH1 / TH2 differentiation.

Table 15 Genes associated with known interactions (grey) with TH2, TH1 and regulatory molecules in activated T cells (Ingenuity ® Systems).

Gene	Gene ID	TH2 molecules						TH1 molecules					Regulatory Molecules	
		IL-4	IL-13	IL-6	IL-2	STAT6	GATA3	IFN-γ	TNF	IL-12	IL-18	STAT4	IL-10	TGF-β
GDPD3	6964245							■						
CTSE	6753067										■		■	
Gbp1	6901952							■	■					
SCG5	6889893											■		
IL4	6788290	■			■	■		■	■					
Ifi202b	6764289			■										
ALOX5AP	6935701								■					
IL1R1	6748884			■									■	
SELL	6754681			■				■						
CCR9	6993138				■									
KYNU	6876735							■						
RAMP1	6751535								■					
CTSH	6991264							■						
HAVCR1	6780570	■						■					■	
RNF130	6780777											■		
GRIA3	7010835								■					
Trim30a/Trim30d	6970066			■	■				■	■				
CCR5	6993154								■					
DNAJC15	6826179							■	■	■				
IL23R	6954418			■	■			■		■		■		■

CYSLTR2	6825872													
GBP2	6901957													
HLA-C	6854990													
Ly6a	6836728													
CCL5	6790288													
IL18R1	6748889													
IL18RAP	6748893													
Ly6a	6836728													
HLA-DRA	6855022													
CTSL2	6813887													
RAMP1	6751535													
CYBB	7015521													
TLR7	7020802													
IL4R	6964160													
A130004G07Rik	6889440													
H2-T3	6855166													
CCR5	6993154													

Table 16 Candidate genes from activated T cell Microarray with relevant biological function (Ingenuity ® Systems).

Gene	Gene ID	Relationship with relevant molecules			Role in cell
		Regulates	Regulated by	Binds	
T cell differentiation					
IL4	6788290	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression
HOPX	6939338	DNA endogenous promoter, DNA promoter, RNA polymerase II	NKX2-5, HNF1A	SRF, Hdac, Sur1	differentiation
IL23R	6954418	STAT3, IFNG, STAT4	IL6, IL21, TGFB1	IL23A, IL23, IL12RB1	growth, expression, function
CCL5	6790288	CCR5, Ca2+, TNF	TNF, lipopolysaccharide, IFNG	CCR5, CCR1, CCR3	chemotaxis, migration, activation
CCR5	6993154	CCR5, Ca2+, CCL4	lipopolysaccharide, CCL5, TNF	CCL4, CCL5, CCL3	chemotaxis, migration, trafficking
CTSE	6753067	HTT, IL18, IL1B	IL10, EBI3, Bleomycin	BAG6	number, degradation and differentiation
CTSL2	6813887	PENK, HTT, CD74	IFNG , lipopolysaccharide, IL13	CST7, UBC	function, number and degradation
TCF4	6861850	CCND1, reporter gene, MYC	CTNNB1, WNT3A, APC	CTNNB1, ID1, ID2	transcription, expression and apoptosis
IL4R	6964160	STAT6, JAK1, JAK3, Cd23, IgE, IL4, IL5, STAT5A, NOS2, IL10, IL13	IL4, STAT6, Interferon Alpha, TNF, TGFB1, Tcr, IL2, TP73, CD40, IFNG	IL4, STAT6, JAK1, IL13, IL2RG, IL13RA1, IRS1, PTPN6, JAK2, SHC1, DOK2, SIRPA, INPP5D, IL13RA2, KAT5	proliferation, differentiation, development, apoptosis, activation, activation in, quantity, growth, S phase, cell cycle progression
A130004G07Rik	6889440	STAT6, JAK1, JAK3	IL4, STAT6, Interferon Alpha	IL4, STAT6, JAK1	proliferation, activation and development
Proliferation					
HLA-DRA	6855022	IFNG, MHC II, LYN	IFNG ,lipopolysaccharide, IL10	HLA-DRB1, CD74	activation, proliferation
IL4	6788290	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression
IL1R1	6748884	NFkB, IL6, IL1B	IL1B, lipopolysaccharide, IL1	IL1B, IL1RAP, IL1A	recruitment, proliferation and infiltration
CTSH	6991264	SFTPB, Surfactant, GS-7119	PRL, IFNG, CTS3	UBC, CSTA	activation and degradation

HAVCR1	6780570	IL4, IFNG, IL10	cisplatin, cyclosporin A, Ren2	TIMD4, UBQLN4, HAVCR1	activation, proliferation and function
Rpl29	6992174	PAPPA	ARNT2, SIM1, IL3	UBC, NEDD8, ANXA2	proliferation, cell cycle progression
MT2A	6978290	Cu2+	heavy metal, triamcinolone acetonide, CLDN7	TFAP2A, MTF1, JUN	homeostasis, apoptosis, proliferation
CCR5	6993154	CCR5, Ca2+, CCL4	lipopolysaccharide, CCL5, TNF	CCL4, CCL5, CCL3	chemotaxis, migration, trafficking
HDGFRP3	6968871	Tubulin	fenofibrate, cisplatin	UBC, Tubulin, Alpha tubulin	proliferation, stabilization, bundling
IL23R	6954418	STAT3, IFNG, STAT4	IL6, IL21, TGFB1	IL23A, IL23, IL12RB1	growth, expression, function
Eef1a1	6857435	phosphatidylinositol 4,5-diphosphate, GTP , GDP	NRG1, cycloheximide, EGF	UBC, SUMO2, SNCA	proliferation, anoikis and transformation
Ly6a	6836728	mineral, Immunoglobulin, Igg	Ifn alpha/beta, IFNG (includes EG:15978), dexamethasone	FGR	number, proliferation, abnormal morphology
CCL5	6790288	CCR5, Ca2+, TNF	TNF, lipopolysaccharide, IFNG	CCR5, CCR1, CCR3	chemotaxis, migration, activation
SLAMF7	6764093	FLT3LG, TXLNA, LTA	Bcr, tretinoin, TGM2	SLAMF7, TRIB2, MAK	cytolysis, proliferation, killing
Rpl29	6992174	PAPPA	ARNT2, SIM1, IL3	UBC, NEDD8, ANXA2	proliferation, cell cycle progression
SLAMF7	6764093	FLT3LG, TXLNA, LTA	Bcr, tretinoin, TGM2	SLAMF7, TRIB2, MAK	cytolysis, proliferation, killing
DBI	6761691	triacylglycerol cholesterol lipid	fenofibrate APP Insulin	UBC TSPO UBXN2A	proliferation
Eef1a1	6857435	phosphatidylinositol 4,5-diphosphate, GTP , GDP	NRG1, cycloheximide, EGF	UBC, SUMO2, SNCA	proliferation, anoikis and transformation
HAVCR1	6780570	IL4, IFNG, IL10	cisplatin, cyclosporin A, Ren2	TIMD4, UBQLN4, HAVCR1	activation, proliferation and function
IL4R	6964160	STAT6, JAK1, JAK3, IRS2, IRS1, Cd23, IgE, IL4 , IL5, STAT5A, NOS2, IL10, FES, IL13	IL4, STAT6, Interferon Alpha, TNF, TGFB1, Tcr, IL2, TP73, CD40, IFNG	IL4, STAT6, JAK1, IL13, IL2RG, IL13RA1,DOK2, IL13RA2	proliferation, differentiation, development, apoptosis, activation, activation in, quantity, growth, S phase, cell cycle progression
A130004G07Rik	6889440	STAT6, JAK1, JAK3	IL4, STAT6, Interferon Alpha	IL4, STAT6, JAK1	proliferation, activation and development
Activation					
HLA-DRA	6855022	IFNG, MHC II, LYN	IFNG ,lipopolysaccharide, IL10	HLA-DRB1, CD74	activation, proliferation

IL4	6788290	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression
P2RY14	6905408	Erk1/2, EGFR, ITGB2	UDP-D-glucose	HNF4A	activation, tyrosine phosphorylation, release
RAMP1	6751535	cyclic AMP, CALCRL, TNF	CALCA, CALC, CALCRL	CALCRL, IAPP, CALCA	binding, production and activation
CTSH	6991264	SFTPB, Surfactant, GS-7119	PRL, IFNG, CTS3	UBC, CSTA	activation and degradation
HAVCR1	6780570	IL4, IFNG, IL10	cisplatin, cyclosporin A, Ren2	TIMD4, UBQLN4, HAVCR1	activation, proliferation and function
IL23R	6954418	STAT3, IFNG, STAT4	IL6, IL21, TGFB1	IL23A, IL23, IL12RB1	growth, expression, function
CYSLTR2	6825872	Ca2+, EGFR, ITGAM	leukotriene D4, E4, C4	eukotriene D4, cysteinyl-leukotriene, montelukast	activation, cell death, release
GBP2	6901957	MMP9, Rac, IRF1	IFNG, IFNB1, lipopolysaccharide	RNA polymerase II, IRF1, STAT1	activation, response, cell spreading
HLA-C	6854990	HLA-B, IFNG, STAT3	IFNG, poly rI:rC-RNA, NEF	B2M, KIR2DL3, Klra4	cytotoxicity, activation, number
CCL5	6790288	CCR5, Ca2+, TNF	TNF, lipopolysaccharide, IFNG	CCR5, CCR1, CCR3	chemotaxis, migration, activation
SLAMF7	6764093	FLT3LG, TXLNA, LTA	Bcr, tretinoin, TGM2	SLAMF7, TRIB2, MAK	cytolysis, proliferation, killing
IL18RAP	6748893	NFkB, IL18, IL6	Il12, Interferon Alpha, STAT4	IL18, IL18R1, MYD88	function, sequestration, activation
F2RL2	6809148	GNA12 F2RL3 EGFR	F2 PROCR PROTEASE	F2R F2 Jam	function activation in formation in
HLA-DRA	6855022	IFNG, MHC II, LYN	IFNG ,lipopolysaccharide, IL10	HLA-DRB1, CD74	activation, proliferation
TLR7	7020802	TNF NFkB Cytokine	lipopolysaccharide resiquimod ssRNA	MYD88 resiquimod imidazoquinolines	production in activation maturation
IL4R	6964160	STAT6, JAK1, JAK3, IRS2, IRS1, Cd23, IgE, IL4 , IL5, STAT5A, NOS2, IL10, FES, IL13,	IL4 , STAT6, Interferon Alpha, TNF, TGFB, Tcr, IL2, TP73, CD40, IFNG	IL4 , STAT6, JAK1, IL13, IL2RG, IL13RA1, IRS1, PTPN6, JAK2, DOK2, IL13RA2	proliferation, differentiation, development, apoptosis, activation, activation in, quantity, growth, S phase, cell cycle progression
A130004G07Rik	6889440	STAT6, JAK1, JAK3	IL4, STAT6, Interferon Alpha	IL4, STAT6, JAK1	proliferation, activation and development
CHRM2	6945419		methoctramine, hyoscyamine		migration, activation in, transformation, inhibition in, apoptosis, survival, cell rounding, mitogenesis in, cell movement, growth

H2-T3	6855166	LCK, Plc gamma, VAV1, CD3E, Ca2+, 1,4,5-IP3, Nfat,	Ifn type 1, dendritic cells, lipopolysaccharide, IL10, IFNG, CD40, RELB, IFNB1, Interferon Alpha, TLR4, Tcr	B2M	priming, activation in
Presentation					
HLA-DRA	6855022	IFNG, MHC II, LYN	IFNG ,lipopolysaccharide, IL10	HLA-DRB1, CD74	activation, proliferation
CTSE	6753067	HTT, IL18, IL1B	IL10, EBI3, Bleomycin	BAG6	number, degradation and differentiation
CLEC4M	6980101	CLEC4M, dextran, E2	mannan,CLEC4M, GP	GP, CLEC4M,ENV	interaction, recognition, binding
CTSH	6991264	SFTPB, Surfactant, GS-7119	PRL, IFNG, CTS3	UBC, CSTA	activation and degradation
H2-T10/H2-T22	6855155	FYN, LCK, VAV1	lipopolysaccharide, dendritic cells, ifn type 1	TCR gamma delta, B2M	priming
HLA-C	6854990	HLA-B, IFNG, STAT3	IFNG, poly rI:rC-RNA, NEF	B2M, KIR2DL3, Klra4	cytotoxicity, activation, number
CYBB	7015521	reactive oxygen species superoxide TNF	NCF1 phorbol myristate acetate NCF2	CYBA NCF2 SPI1	production in generation in apoptosis
H2-T10/H2-T22	6855155	FYN, LCK, VAV1	lipopolysaccharide, dendritic cells, ifn type 1	TCR gamma delta, B2M	priming
Recruitment					
ALOX5AP	6935701	leukotriene, prostaglandin, ALOX5	CSF2, TNF, CEBPB	CEBPE, CEBPA, CEBPD	recruitment
IL1R1	6748884	NFkB, IL6, IL1B	IL1B, lipopolysaccharide, IL1	IL1B, IL1RAP, IL1A	recruitment, proliferation and infiltration

3.2.4 Identification of candidate genes in activated T cells that may confer host protection or susceptibility to *L. major*

Since the aim of the microarray was to identify differentially expressed (DE) genes promoting resistance and or susceptibility during Leishmaniasis, it was hypothesized that these genes may (i) be located on known Leishmania resistance or susceptible loci, (ii) be involved in TH1 / TH2 differentiation, (iii) be involved in known pathways associated with T cell receptor (TCR) signaling, (iv) be involved in the immunological synapse (IS) formation, which aids in the differentiation of T helper cells into a protective TH1 response and or be involved in cell-to-cell signaling and interaction and cellular growth and proliferation etc.

A mining strategy was therefore developed (Figure 25), in which DE genes was identified based on a fold change cut-off of 1.2 and a p value of < 0.05. These lists of DE genes was then used for data mining of the gene ontology database to extract functional data for each differentially expressed gene, describing its cellular location, biological processes and molecular functions. In addition, the strategy included literature mining of the PubMed database to uncover the roles of the DE genes in resistance and susceptibility in Leishmania. After extraction and assignment of functional data, the DE genes were clustered into the "focus" groups based on their gene ontology associations, Babelomics / IPA analysis and previously published functional roles. Candidate genes belonging to two or more of the focus groups were then selected for further analysis. The rationale for selecting genes which intersected with two or more (ie >50%) of the focus functional groups, was that this strategy may increase the probability of selecting candidate genes which have a significant biological impact on the resistance of *L. major* infections. Indeed, the list of differentially expressed genes that intersected with two or more functional groups were found to be enriched with genes previously described in the literature to play a significant role in resistance to *L. major* (Table A5).

Moreover, the significant biological impact for a number of the DE genes during *L. major* infection has been demonstrated in gene deficient mouse models or cell lines. For example, mice deficient for CCR5 displayed skewing towards TH2 differentiation (Wong *et al*., 2003) Therefore, co-clustering of DE genes with these "high biological impact" genes may therefore highlight which of the DE genes should be selected for further analysis. Of the intersection of DE genes, 9 genes were selected for further analysis (Table A5 in appendix).

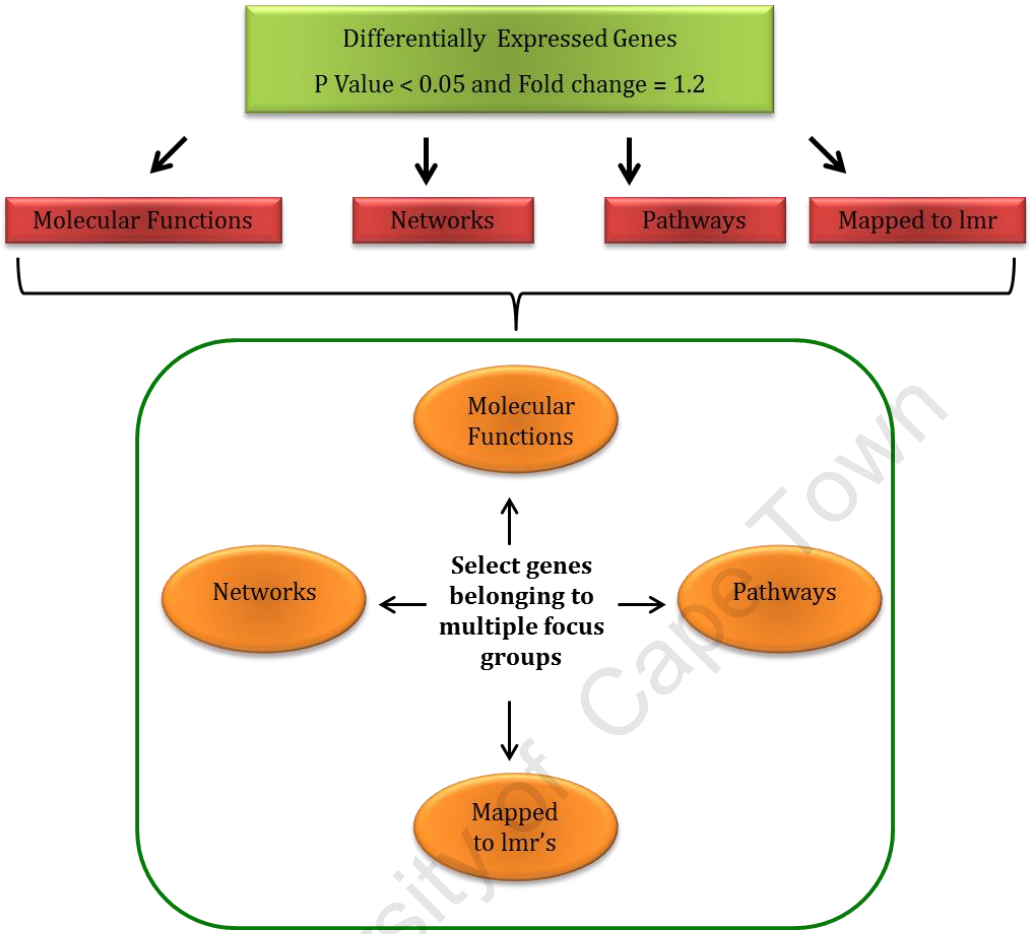

Figure 25 **Differentially expressed genes mining strategy.**

A fold change and p value cut-off of 1.2 and 0.05 was chosen to select the differentially expressed genes. Data mining of the Gene Ontology database, Babelomics and Ingenuity Pathway analysis was employed to extract data for each DE gene describing its cellular localization, biological processes, location on lmr's and molecular functions. In addition, literature mining of the PubMed database is used to uncover the roles of the DE genes in host defense and immunity to intracellular pathogens, TH1 / TH2 differentiation, T cell receptor mechanisms and proliferation. After extraction and assignment of functional data, the DE genes are clustered into the above "focus" groups based on their functional annotations. Genes belonging to more than one focus group were selected for further analysis.

3.3 Gene expression profiling of popliteal lymph node Regulatory T cells

Transcriptional profiling by microarray was used to assess the differences in regulatory T cells isolated from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 mice at three weeks post infection with *L. major*. This was done in order to elucidate the mechanisms by which signaling via the IL-4Rα on CD4^{+}Tcells causes susceptibility to *L. major*. Gene expression patterns were compared early during infection in regulatory T cells in the absence or presence of IL-4Rα signaling.

3.3.1 Quality Control of microarray data

The Regulatory T cell microarray was performed using Illumina® MouseWG-6 v2.0 Expression BeadChips on an Illumina Bead array at the RIKEN Omics Science Centre in Japan. Before extracting biologically meaningful results from the microarray data, the quality of the data was assessed using various quality control metrics.

Quality control of all the regulatory T cell RNA samples was performed by Dr Roy Sugata at the RIKEN Omics Science Centre in Japan and all samples performed well in terms of the QC metrics listed in Table 17.

Table 17 Control metrics used to assess quality of microarray data

Quality Control metric	Description
Hybridization controls	Sample-independent metric. Control oligonucleotides were spiked into the sample and their hybridization efficiency assessed so as to analyse problems with hybridization, washing and staining (Oeser *et al*., 2009).
Perfect match (PM) versus Mismatch (MM)	Sample-independent metric. Signal intensities of PM probes should be higher than MM probes. Deviations point to problems with specificity (Oeser *et al*., 2009).
Housekeeping vs. Background	Sample-dependent metric. Signal intensities of housekeeping genes should be consistent across the arrays and above the background signal(Oeser *et al*., 2009).
Negative controls (Background and Noise)	Sample-dependent metric. The signal intensities for the negative controls should be consistently low (Oeser *et al*., 2009).

Prior to the identification of outlier experiments by clustering (data not shown), interarray normalization was done to adjust for variation in the microarray experiment itself as opposed to biological differences in the samples (Smyth & Speed, 2003). Figure 26 as an example indicating how, post normalization, the intensity distributions were adjusted to be equivalent (and thus comparable) for all samples.

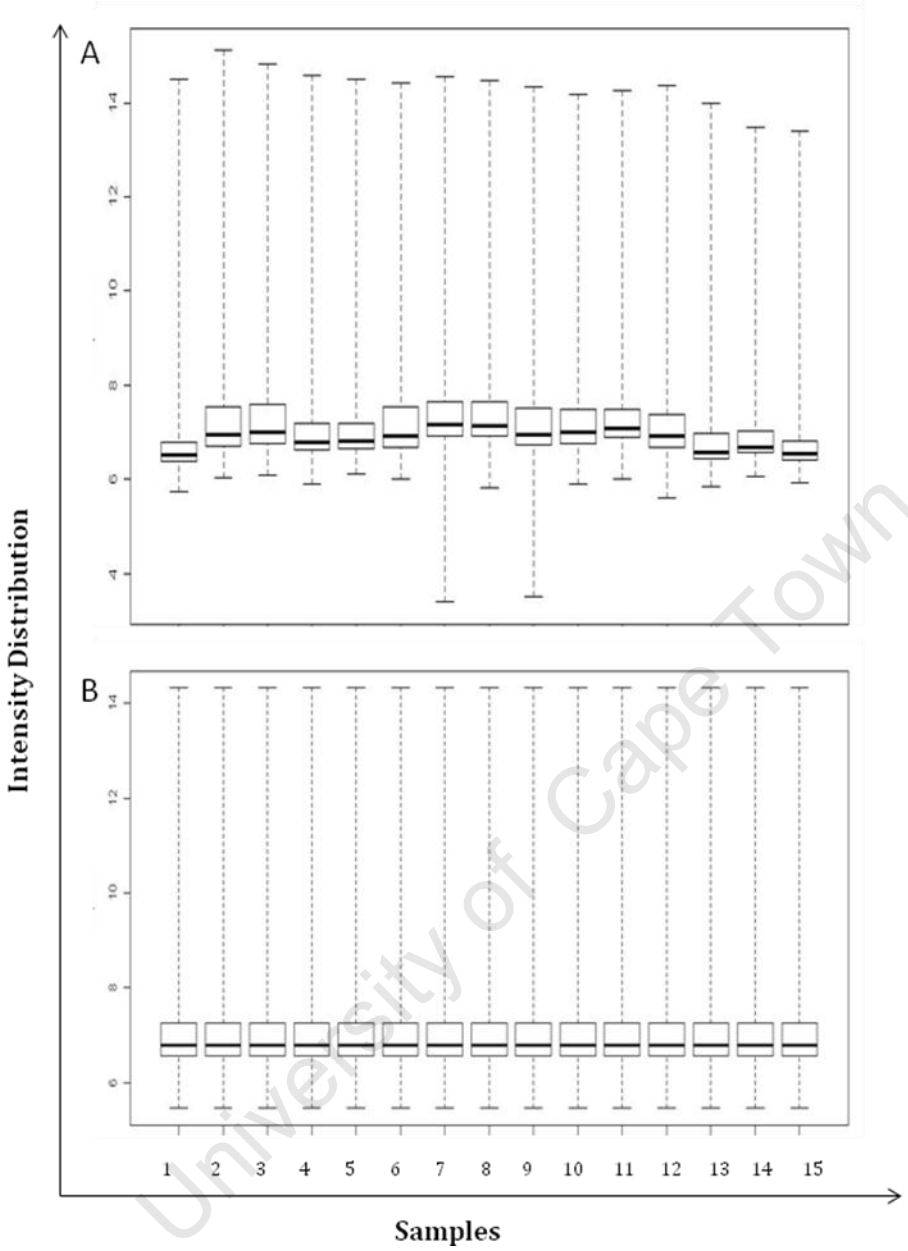

Figure 26 Quantile normalization of gene expression data from regulatory T cells isolated from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 mice at 3 weeks post infection with *L. major*. Samples 1 – 15 represent each of the samples from the above mouse strains from 3 independent biological experiments. For each experiment, 6 mice were used per strain. Raw intensity values **(A)** were adjusted by quantile normalization so that intensity distributions were equivalent among all samples **(B)** as indicated in the box plots. The black line in the middle of each

box represents the median gene expression value and the upper and lower limit of the box represents the 0.75 and 0.25 quantile respectively.

3.3.2 Identification of differentially expressed genes

As previously discussed, one aim of this study was to identify genes and pathways associated with IL-4Rα-induced TH1 and TH2 differentiation and how these genes may contribute to susceptibility or resistance during *L. major* infection at three weeks post infection. Thus far the pre-microarray quantitative RT-PCR has shown that the susceptible BALB/c produces more IL-4 (Figure 18) and the resistant C57BL/6 produces more IFN-γ (Figure 19) as compared to all other mouse groups, which produced intermediate levels of these classical TH1 and TH2 cytokines.

$CD4^+CD25^+$ Regulatory T cells are a major source of IL-10 and play an important role in the regulation of immune responses. Regulatory T cells are essential for the suppression of detrimental pathogenic responses especially to self-antigens, however, they may also lead to the suppression of beneficial responses. In C57BL/6 mice infected with *L. major*, the regulatory T cells are recruited to the site of infection, where they function in a way to suppress the effector cells to eliminate the parasites (Belkaid *et al.*, 2000). The depletion of $CD25^+$ cells in this mouse strain enhances the production of IFN-γ by $CD4^+$ T cells in the lesions. This resulted in clearance of the parasites. Similarly, in a susceptible BALB/c mouse strain, treatment with anti-CD25 increases resistance to *L. major* infection (Heinzel *et al.*, 1993). By regulating the biased TH2 response in susceptible BALB/c mice infected with *L. major*, the $CD4^+CD25^+$ regulatory T cells play a significant disease controlling role (Xu *et al.*, 2003). Other studies demonstrated that the transfer of naïve $CD4^+CD25^+$T cells in SCID mice reconstituted with $CD4^+CD25^-$ T cells, suppressed *L. major* disease development. This suggests that $CD4^+CD25^+$ T cells also inhibit TH2 responses and susceptibility to *L. major* infection (Xu *et al.*, 2003). Therefore, this study is aimed at identifying the role of regulatory T cells in *Leishmania* infection as current evidence suggests that the balance between regulatory T cells and effector cells is critical for immunity to *Leishmania*.

Differentially expressed genes in regulatory T cells were identified by comparative analyses between C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c, amongst others, using the same comparative analyses and criteria (fold change cut-off of ≥1.2 and a p-value < 0.05) as for the activated T cell microarray (Table 18, A3 and A4 in appendix). A total of 485 genes were differentially expressed in regulatory T cells from C57BL/6 mice as compared to BALB/c mice, with 53% being up-regulated and 47% being down-regulated in the C57BL/6 regulatory T cells. In contrast, 103 genes were differentially expressed in regulatory T cells from iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to BALB/c mice, with 52% being up-regulated and 48% being down-regulated in the iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells. Interestingly, seven genes (A130002I06RIK, C530038F07RIK, FAM115C, GDPD3, HIP1, IL4, PSG19) were differentially expressed with the same up- or down-regulatory patterns in both the C57BL/6 and the iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells as compared to BALB/c regulatory T cells (Figure 27).

Table 18 Summary of differentially expressed genes from regulatory T cell microarray.

		Regulatory T cell microarray	
		iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c	**C57BL/6 vs. BALB/c**
DE genes p > 0.05	**Total DE genes**	1054	1135
DE genes p > 0.05 fold change > ± 1.2	**Total DE genes**	103	485
	# DE genes up-regulated	53	256
	# DE genes down-regulated	50	229
	# DE genes with p<0.05	93	361
	# DE genes with *p* <0.01	10	111
	# DE genes with *p* <0.05	0	13
	# DE genes located in *Lmr* loci	32	147

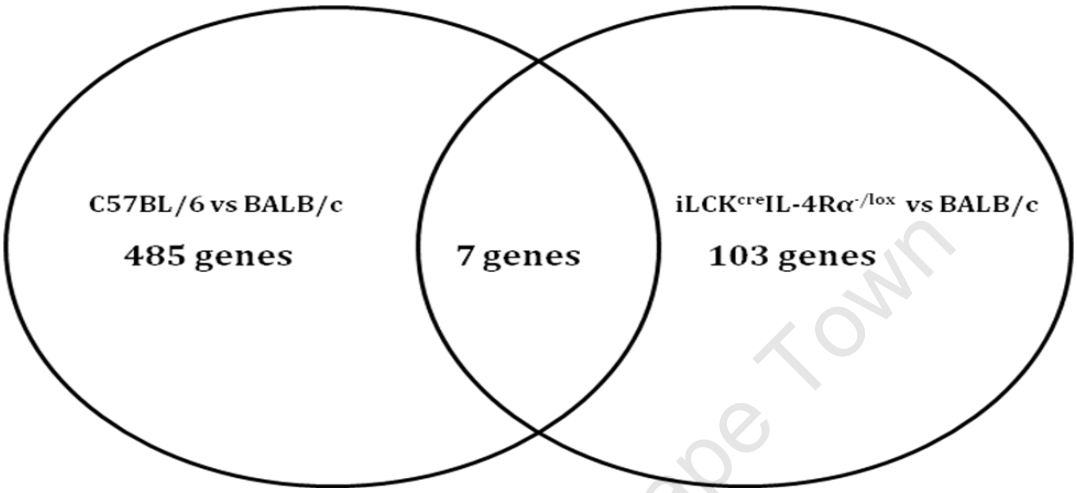

Figure 27 Regulatory T cells p value <0.05 Fc, 1.2

Overlapping differentially expressed genes in dataset comparisons comprising the BALB/c control group. Differentially expressed genes in common in both C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{-/lox}$ vs. BALB/c dataset comparisons from regulatory T cell microarray (p-value ≤ 0.05 and Fc < 1.2). Expression data from 3 independent biological replicates were used to generate the gene lists.

3.3.3 Biological relevance and interpretation of microarray data

After having generated the lists of differentially expressed genes, the next step was to determine if the microarray data was biologically relevant, and to interpret the results biologically within the context of the experiment.

3.3.3.1 Differential expression of IL-4Rα

Given that regulatory T cells from *L. major*-infected from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 were used in this microarray study, the expression of the IL-4Rα gene for each dataset was analysed for conformity to expected profiles. This analysis is shown in Figure 28A. Unexpectedly, no significant differences in IL-4Rα expression were obtained among the strains for the regulatory T cells. This result was further investigated by performing a BLAST analysis (Altschul *et al*., 1997) to determine the region of hybridization of the microarray probe to the IL-4Rα transcript (Figure 28B). This revealed that the probed used in the Illumina array mapped to exon 11 of IL-4Rα (Mohrs *et al*., 1999), an exon that is unaffected by the IL-4Rα deletion process. Based on this, the lack of significant differential IL-4Rα expression in the microarray among the strains was not a cause for concern as it could be reliably explained.

3.3.3.2 Mapping of differentially expressed genes to *Leishmania major* response (*Lmr*) Loci

Furthermore, 30% of the differentially expressed genes in C57BL/6 and 31% in iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells mapped to *Leishmania major* response (*Lmr*) loci (Tables A3 and A4 in appendix). This enrichment was found to highly statistically significant in C57BL/6 vs. BALB/c comparative analysis (Table 19), but not for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c analysis (Table 20).

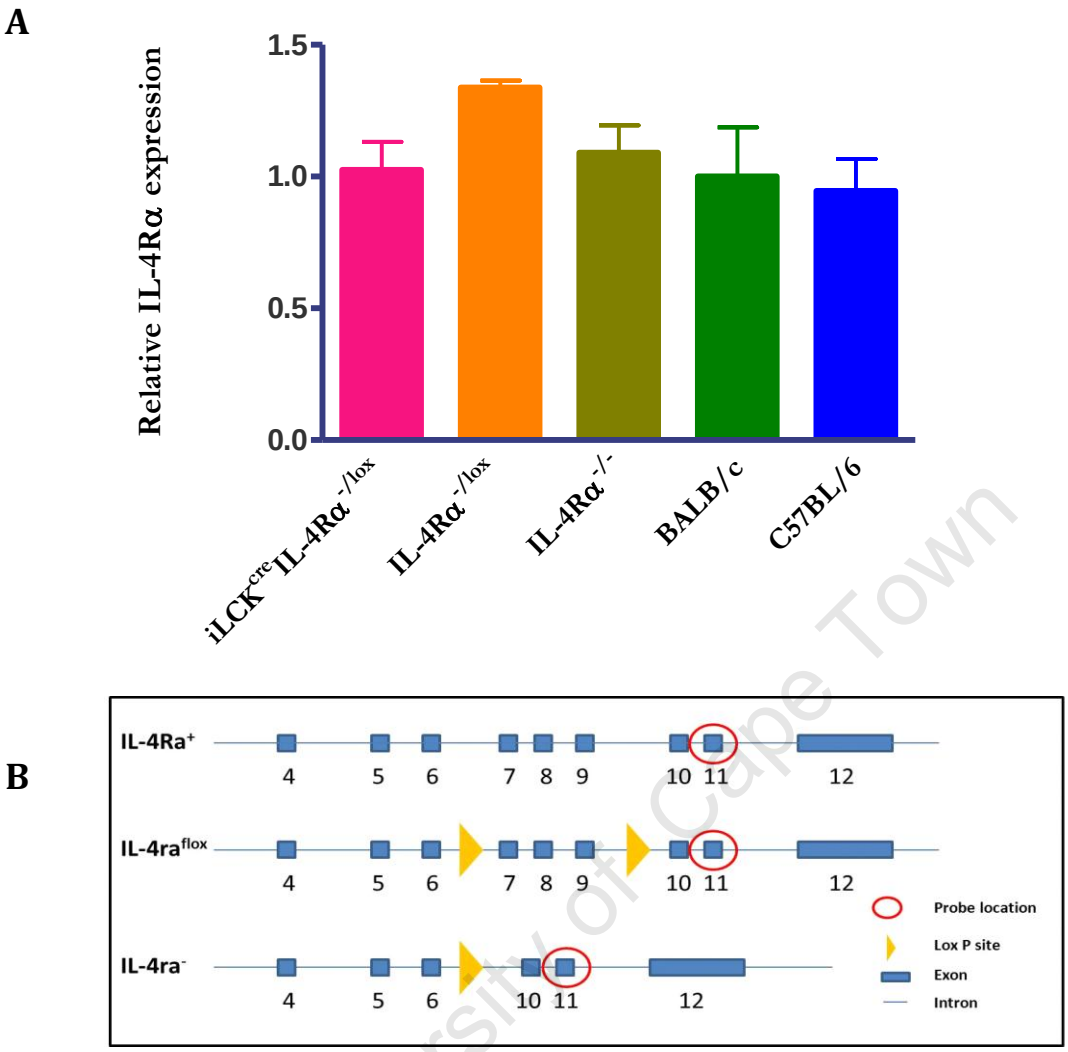

Figure 28 Analysis of IL-4Rα expression levels by microarray

A. Regulatory T cells from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 *L. major*-infected mice were subject to transcriptional profiling by microarray at three weeks post infection. Relative IL-4Rα expression levels post-normalisation of microarray data. All groups were compared to the BALB/c control group which was set to 1. Data are representative of three independent biological experiments. **B.** Location of probe used to detect IL-4Rα expression in the microarray. BLAST analysis revealed that the probe used for determining IL-4Rα expression levels mapped to exon 11 which is unaffected during the IL-4Rα deletion process. (Figure 26B illustration taken from Ms Umeshree Govender, MSc, 2012)

Table 19 Regulatory T cell microarray of C57BL/6 vs. BALB/c comparison showing linkage to *Lmr* loci. A two-tailed Chi-square test with Yates correction was used to analyze the 2x2 contingency table below.

	Rest of genome	DE genes	Total
# genes in *Lmr*	11278	147	**11425**
# gene not in *Lmr*	34003	338	**34341**
Total	**45281**	**485**	**45766**
Result:	χ^2= 7.191 with 1 degrees of freedom $p < 0.0073$		

Table 20 Regulatory T cell microarray of iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparison showing linkage to *Lmr* loci. A two-tailed Chi-square test with Yates correction was used to analyze the 2x2 contingency table below.

	Rest of genome	DE genes	Total
# genes in *Lmr*	11278	32	**11310**
# genes not in *Lmr*	34003	71	**34074**
Total	**45281**	**103**	**45384**
Result:	χ^2 = 1.769 with 1 degrees of freedom. $p < 0.1835$		

3.3.3.3 Functional clustering of the differentially expressed genes

Functional clustering of the differentially expressed genes into common signaling pathways, functional networks and molecular functions was done using Babelomics (http://babelomics.bioinfo.cipf.es) and Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com).

The top IPA functional networks that were most significant in both the C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparative analysis were cellular growth and proliferation, cell-mediated immune response, cell signaling, inflammatory response, cell-to-cell signaling and interaction, and cell death (Table 21 and Figures 29 and 30). Functional clustering using Babelomics (refer to 3.2.3.3) revealed that the differentially expressed genes from the regulatory T cell microarray was not as significantly enriched as compared to the analysis from the activated T cell microarray. When comparing the C57BL/6 vs. BALB/c gene IDs, two biological processes were significantly enriched. These biological processes were cell activation and immune response. Similar enrichment analysis used for the activated T cell microarray, using Babelomics was applied.

Table 21 Summary of the top networks from the Regulatory T cell microarray

C57BL/6 vs. BALB/c		iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c	
Associated network	**Score**	**Associated network**	**Score**
Gene Expression, Antimicrobial Response, Inflammatory Response 37	37	Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance	40
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	35	Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance	17
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cell Death	31	Cellular Response to Therapeutics, Cell Cycle, Nervous System Development and Function	16
Cell Death, Cellular Movement, Immune Cell Trafficking	27	Cell Death, Cellular Function and Maintenance, Reproductive System Development and Function	3
Gene Expression, Cellular Growth and Proliferation, Cell Cycle	24		

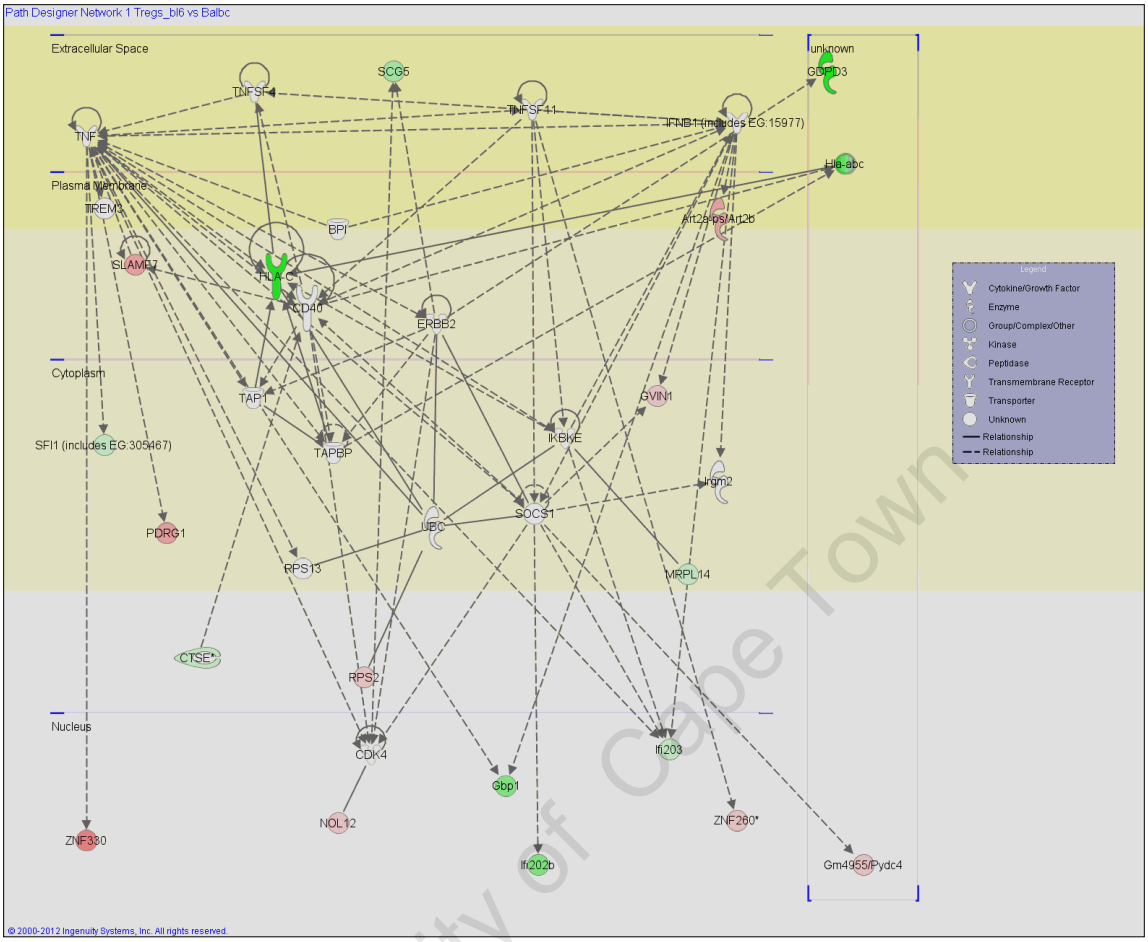

Figure 29 Network analysis of differentially expressed genes in C57BL/6 vs. BALB/c dataset comparisons (FC > 1.2, p < 0.05) from regulatory T cell microarray.

Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to identify known biological relationships among the differentially expressed genes. Relationships represented may be activation, expression, inhibition, localization, phosphorylation, protein-DNA interaction, protein-protein interaction, proteolysis, regulation of binding, RNA-RNA interactions, transcription or translocation.

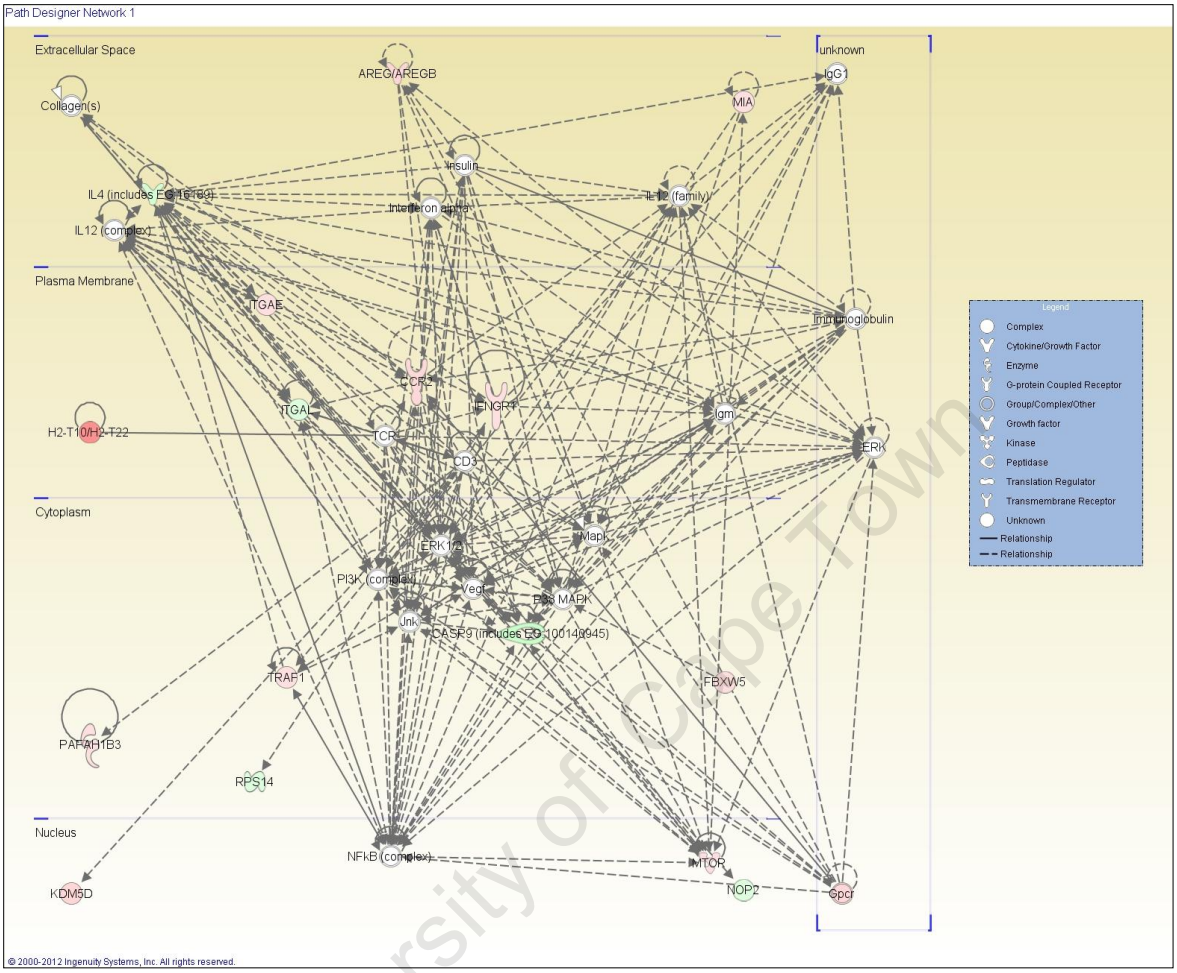

Figure 30 Network analysis of differentially expressed genes in iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c dataset comparisons (FC > 1.2, p < 0.05) from regulatory T cell microarray.

Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to identify known biological relationships among the differentially expressed genes. Relationships represented may be activation, expression, inhibition, localization, phosphorylation, protein-DNA interaction, protein-protein interaction, proteolysis, regulation of binding, RNA-RNA interactions, transcription or translocation.

In order to identify which DE genes were associated either with TH1 or TH2 differentiation during Leishmaniasis, intensive literature mining for each DE gene was done using the IPA knowledgebase (Ingenuity ® Systems, www.ingenuity.com) and the automated literature search tool, MILANO (Rubinstein & Simon, 2005). The molecular interaction networks of the differentially expressed genes with TH1- or TH2-associated molecules were assessed using IPA (Ingenuity ® Systems, www.ingenuity.com). The TH2 molecules assessed were IL-4, IL-13 and the TH2-associated transcription factors, STAT6 and GATA3 (Paul, 2010) and the DE genes having molecular interactions with these TH2 genes are summarized in Table 22. Similarly DE genes having molecular interactions with TH1-assosiated molecules such as IFN-γ, TNF, T-bet and STAT4 were identified and are summarized in Table 22. In Table 22, these interactions are depicted by grey shadowing.

Functional annotations such as 'differentiation', 'proliferation', 'activation', 'presentation,' 'recruitment' and 'homeostasis' are regularly used to describe the genes that are important in host defense and immunity against *L. major*. Differentially expressed genes which shared the same or similar combination of functional annotations were identified and are shown in Table 23 along with their molecular interaction with other molecules already known to be associated with TH1/TH2 response.

The above literature mining strategies present only a limited analysis of the gene lists, but were sufficient to identify genes in regulatory T cells that may be contributing to disease protection or progression during *L. major* infection.

Table 22 Genes associated with known interactions (grey) with TH2, TH1 and regulatory molecules in regulatory T cells (Ingenuity® Systems)

Gene	Gene ID	Regulatory Molecules			TH2 molecules					TH1 molecules				
		IL-10	TGF-β	FoxP3	IL-4	IL-13	IL-6	IL-2	STAT6	IFN-γ	TNF	IL-12	IL-18	STAT4
HLA-C	ILMN_196741									■				
CTSE	ILMN_253611	■											■	
Ifi202b	ILMN_210704						■							
H2-Q5	ILMN_196752	■								■				
SCG5	ILMN_209144		■											■
SFI1	ILMN_220674										■			
ADI1	ILMN_223121		■											
HLA-B	ILMN_247624	■								■	■			
TLR4	ILMN_217521	■					■			■	■	■		
GPR83	ILMN_189915			■										
IL4	ILMN_218517				■			■	■	■	■			
ZFP36L1	ILMN_185448										■			
CTSC	ILMN_214875		■											
B2M	ILMN_209318	■					■			■				
STX11	ILMN_215582									■				
PDRG1	ILMN_210839										■			
GPNMB	ILMN_211323						■					■		
ODC1	ILMN_219842									■	■			
BHLHE40	ILMN_211922		■											
Art2a-ps/Art2b	ILMN_221345									■				

CCL4	ILMN_222651													
ZNF330	ILMN_187056													
B2M	ILMN_209318													
PDRG1	ILMN_210839													
STX11	ILMN_215582													
XPO7	ILMN_204705													
ODC1	ILMN_219842													
Gbp1	ILMN_216856													
RNASE4	ILMN_210173													
ITGAE	ILMN_216214													
AIM2	ILMN_235623													
ITGAE	ILMN_212653													
CASP9	ILMN_212974													
IL4	ILMN_218517													
ITGAL	ILMN_218502													
RTP4	ILMN_253388													
NOP2	ILMN_210691													
CCR2	ILMN_213602													
FBXW5	ILMN_208949													
MIA	ILMN_194804													
RGS1	ILMN_191801													
IFNGR1	ILMN_214754													
TRAF1	ILMN_207421													

Table 23 Candidate genes from regulatory T cell Microarray with relevant biological function (Ingenuity® Systems)

Gene	Gene ID	Relationship with relevant molecules			Role in cell
		Regulates	Regulated by	Binds	
Differentiation					
CTSE	ILMN_253611	HTT, IL18, IL1B	IL10, EBI3, Bleomycin	BAG6	number, degradation and differentiation
TLR4	ILMN_217521	TNF, NFkB, IL6, Il12, IL8, Cytokine, IFNB1, IL10, IL1B, IL12B, P38 MAPK, CD86, MYD88, CXCL10, IRF3	Lipopolysaccharide, TNF, IFNG , LY96 , MYD88, IL1B, CpG oligonucleotide, HSP90B1	LY96 , MYD88, CD14	activation, production, maturation, apoptosis, induction, upregulation, response, recruitment
INPP4B	ILMN_212686	Akt		unknown	differentiation, proliferation
IL4	ILMN_218517	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression
ZFP36L1	ILMN_185448	TNF, HBB, CSF2	IRF4, macrophages,CD40LG, IL15	TNF	translation in, proliferation
GPNMB	ILMN_211323	IL6, IL12B , TAC1	IRF7, DNASE2, bone marrow-derived macrophages, monocyte-derived macrophages, neutrophils	PLA2G4A, TAC1, SMAD4, fucoidin, heparin	adhesion, proliferation
ODC1	ILMN_219842	ODC1, SERPINE1, CDK2, TLR2,	phorbol myristate acetate, EGF, nitric oxide, IFNG, lipopolysaccharide, ODC1	MYC, CDK9, ZNF148, ZNF281, KLF4, TNFRSF1A, SMAD3	growth, proliferation, transformation, cell death, apoptosis, morphology, survival, invasiveness, differentiation, expression
B2M	ILMN_209318	IFNG, MHC class I, Igg, HLA-C, Klra4, HLA-DQA1, STAT3, TFRC, IGF1R, HLA-E, Cd8, CD8A, IL6	IFNG, lipopolysaccharide, TNF, NEF, IFNB1, IL10, Interferon Alpha, CD40LG, GP, CD40, dendritic cells, IFNA2, STAT1, IFNAR1	HLA-C, HLA-B, HLA-A, HFE, CD1D, MHC Class I, TFRC, FCGRT, HLA-E, HLA-G, Mhc Class1 Heavy Chain, CD8A, H2-ld, CALR, TAP1	development, quantity, differentiation, proliferation, activation, apoptosis, activity, cytotoxicity, survival, accumulation
GPNMB	ILMN_211323	IL6, IL12B , TAC1	IRF7, DNASE2, bone marrow-derived macrophages, Enterotoxin B, PRL, FGF2, monocyte-derived macrophages, neutrophils, FAS	PLA2G4A, TAC1, SMAD4, fucoidin, heparin	adhesion, proliferation
ATP2C1	ILMN_211584	elemental calcium, Ca2+, heavy metal, TG, Calpain, TP73	Sos, FOS, HTT, guanidinopropionic acid, megakaryocytes, celecoxib	Atp2c2, ORAI1	homeostasis in, apoptosis, adhesion, quantity, morphology, reorganization, differentiation, expression in, activation in, replication in

MTOR	ILMN_204279	STAT3, MTOR		MTOR,STAT3	proliferation, growth, phosphorylation in, autophagy in, autophagy, differentiation, cell cycle progression, size, apoptosis, macroautophagy in
RPS14	ILMN_219718	RNA polymerase II	IL3, polyunsaturated fatty acids, arachidonic acid, CD28, Cd3	TAF9, MAPK3, DLG4, MAP3K1, LYAR, Ns3/4a, EIF1B, RBBP6, RIPK2, ANXA2, NFKB2, SMYD2, TSC22D1, MEPCE, RELB	translation in, differentiation, replication in, processing in
AREG/AREGB	ILMN_217903		AREG/AREGB, Fsh, beta-estradiol, phorbol myristate acetate	EGFR, WT1, Creb, BRCA1	proliferation, growth, maturation, apoptosis, differentiation, mitogenesis, invasion, recycling, migration, activation
CCR2	ILMN_213602	IFNG , CCL13, TNF, IL6, CD40, CXCL3, TIMP1, CCL5, CCL4, CXCL10, IL10, Il12, elemental calcium, Ca2+, CCL11	lipopolysaccharide, IL10, IL2, TNF, CCL2, CCL13, Tcr, IL15, IL1B, LDL, Immunoglobulin, MOG, dendritic cells, Cd3	CCL2, CCL13, CCL7, CCL8, CCR5, CCL, STAT3, G protein alpha I, CCL16, JAK2, CD4, CXCR4, ADRBK1, CX3CL1, Chemokine	recruitment, quantity, chemotaxis, migration, infiltration, accumulation, response, activation, entrance, proliferation
IFNGR1	ILMN_214754	STAT1, TNF, CXCL10, JAK2, IRF1 , NOS2, CCL13, MMP9, CCL5, IL1B, nitric oxide, JAK1, CXCL9, SOCS1, IL6	IFNG, Il12, TNF, IL2, Tcr, Cd3, phorbol myristate acetate, PBMCs, IL4 , IL13, CD2, PAEP, T lymphocytes	IFNG, STAT1, JAK1, JAK2, IFNGR1, IFNGR2, V ANTIGEN, Jak, Ifn gamma	activation, production, expression, quantity, synthesis, apoptosis, proliferation, activity, growth, killing
Proliferation					
HLA-B	ILMN_247624	STAT3, NFkB, HLA-B,LCK, VAV1, CD3E, CBL, GRB2, IFNG Igg1	IFNG NEF, Interferon Alpha, IFNB1 , lipopolysaccharide, TNF, GP, IL10, CD40, Interferon Beta, Ins1, CD40LG, Insulin, dendritic cells	B2M, VCP,CD8A, Tap complex (Antigen-processing transporter), Tcr, HLA-B	activation, proliferation, recognition by, protection, negative selection, positive selection, priming, engagement, presentation in, lysis
TLR4	ILMN_217521	TNF, NFkB, IL6, Il12, IL8, Cytokine, IFNB1 , IL10, IL1B, IL12B, P38 MAPK, CD86, MYD88, CXCL10, IRF3	lipopolysaccharide, TNF, IFNG, poly rI:rC-RNA, LY96, MYD88, E. coli B5 lipopolysaccharide, IL1B, CD14, MIF, CSF2, hyaluronic acid, CpG oligonucleotide, HSP90B1	LY96, MYD88, TIRAP, lipopolysaccharide, CD14, TICAM2, HMGB1, TICAM1, F, HSPD1, Env, TOLLIP, Hsp70, Hsp, HSP90B1	activation in, expression in, activation, production in, maturation, apoptosis, induction in, upregulation in, response, recruitment
INPP4B	ILMN_212686	Akt	LY294002, MITF, beta-estradiol, (+)-MK-801, methylselenic acid, AR	unknown	differentiation, proliferation
IL4	ILMN_218517	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression

ZFP36L1	ILMN_185448	TNF, HBB, CSF2	butyric acid, PDGF-BB, ARNT2, SIM1 (includes EG:20464), camptothecin, SU6656, methotrexate, MS4A1, Igm, IRF4, WR 1065, macrophages, THP-1 cells, CD40LG, IL15	PCBD1, MAPK14, PAFAH1B2, YWHAZ, TBP, ZFP36, NSD1, TNF	translation, proliferation
B2M	ILMN_209318	IFNG , MHC class I, FCGRT, Igg, HLA-C, Klra4, HLA-DQA1, STAT3, TFRC, heavy metal, IGF1R, HLA-E, Cd8, CD8A, IL6	IFNG, lipopolysaccharide, poly rI:rC-RNA, TNF, NEF, IFNB1, IL10, Interferon Alpha, CD40LG, GP, CD40, dendritic cells, IFNA2, STAT1, IFNAR1	HLA-C, HLA-B, HLA-A, HFE, CD1D, MHC Class I, TFRC, FCGRT, HLA-E, HLA-G, Mhc Class1 Heavy Chain, CD8A, H2-ld, CALR, TAP1	development, quantity, differentiation, proliferation, activation, apoptosis, activity, cytotoxicity, survival, accumulation
SLAMF7	ILMN_207150	FLT3LG, TXLNA, LTA	Bcr, tretinoin, TGM2	SLAMF7, TRIB2, MAK	cytolysis, proliferation, killing
GPNMB	ILMN_211323	IL6, IL12B , TAC1	MITF, IRF7, DNASE2, bone marrow-derived macrophages, Enterotoxin B, PRL, FGF2, monocyte-derived macrophages, neutrophils, FAS	PLA2G4A, TAC1, SMAD4, fucoidin, heparin	adhesion, proliferation
Rpl29	ILMN_221019	PAPPA	ARNT2, SIM1, IL3	UBC, NEDD8, ANXA2	proliferation, cell cycle progression
UBE2I	ILMN_184358	SMAD4,STAT1, NFKBIA, SREBF1, ZNF146, MYB, MDM2, TP53			sumoylation in, binding in, proliferation, expression in, response, contact growth inhibition, ligation in, cell death, transactivation in, migration
ODC1	ILMN_219842	ODC1, Cdc2, TLR2	phorbol myristate acetate, nitric oxide, IFNG , lipopolysaccharide, cyclosporin A, tretinoin, ODC1	SMAD3	growth, proliferation, transformation, cell death, apoptosis, morphology, survival, invasiveness, differentiation, expression in
BHLHE40	ILMN_211922	DNA endogenous promoter, DNA promoter, RNA polymerase II	TGFB1, CLOCK, beta-estradiol	UBC, SUMO2, BHLHE40	expression in, number, proliferation
MTOR	ILMN_204279	EIF4EBP1, RPS6KB1, p70 s6k, Akt, STAT3, S6K1, HIF1A, MTOR, ULK1, EIF4E, IRS1, Pp2a, AKT1, Pkc, EIF4EBP2	sirolimus, Akt, amino acids, RHEB, TSC2, AKT1, Insulin, INS, Ins1, LY294002, D-glucose, everolimus, RPTOR, IGF1, pi3k	RPTOR, RICTOR, RPS6KB1, FKBP1A, MLST8, EIF4EBP1, MAPKAP1, RHEB, AKT1, MTOR, AKT1S1, STAT3, RHEBP1, Akt, TRRAP	proliferation, growth, phosphorylation, autophagy, autophagy, differentiation, cell cycle progression, size, apoptosis, macroautophagy
INPP4B	ILMN_212686	Akt	LY294002, MITF, beta-estradiol, (+)-MK-801, methylselenic acid, AR	unknown	differentiation, proliferation

ITGAL	ILMN_218502	ICAM1, FN1, THBS1, ITGAL, ITGB2, Pkc, APP, ITGB3, PTK2B MAPK1, JUN, Alpha tubulin,	ITGB2, ITGAL, Tcr, Lmp1, TNF, ICAM1, heavy metal, Pertussis toxin, phorbol myristate acetate, protein-protein complex, IL8, MYC, IFNG,glucocorticoid	ICAM1, ITGB2, ICAM3, ICAM2, CAV1, Integrin beta, CD226, Cd3, ITGB7, C3, ITGAM	adhesion, binding, migration, proliferation, cell-cell adhesion, activation, aggregation, attachment, cell spreading, chemotaxis
NOP2	ILMN_210691	unknown	IL2,	CCNB1, MAP3K1, TAT, MYBBP1A, MCRS1, SUMO2, RNU11	proliferation
Activation					
HLA-C	ILMN_196741	HLA-B, IFNG, STAT3	IFNG, NEF	B2M, KIR2DL3, Klra4	cytotoxicity, activation, number
H2-Q5	ILMN_196752	ZAP70, FYN, LAT, LCK, Plc gamma, VAV1, CD3E, CBL, GRB2, elemental calcium, Ca2+, 1,4,5-IP3, peptide, Nfat, Itpr	lipopolysaccharide, Ifn type 1, dendritic cells, IFNG , IL10, Langerhans cells, RELB, Interferon Alpha, phosphatidylserine, IFNB1 , CD40, Ifn alpha/beta	unknown	activation in, priming
HLA-B	ILMN_247624	STAT3, NFkB, HLA-B, ZAP70, FYN, LAT, LCK, Plc gamma, VAV1, CD3E, CBL, GRB2, IFNG (includes EG:15978), Lilrb3 (includes others), Igg1	IFNG , NEF, Interferon Alpha, IFNB1, lipopolysaccharide, TNF, GP, poly rI:rC-RNA, IL10, CD40, Interferon Beta, Ins1, CD40LG, Insulin, dendritic cells	B2M, LILRB1, LILRB2, KIR3DL1, VCP, Kir, CD8A, Tap, Tap complex (Antigen-processing transporter), CANX, PDIA3, TAPBP, NP, Tcr, HLA-B	activation, proliferation, recognition, protection, negative selection, positive selection, priming, engagement, presentation, lysis
TLR4	ILMN_217521	TNF, NFkB, IL6, Il12, IL8, Cytokine, IFNB1 , IL10, IL1B, IL12B, P38 MAPK, CD86, MYD88, CXCL10, IRF3	lipopolysaccharide, TNF, IFNG, poly rI:rC-RNA, LY96, MYD88, E. coli B5 lipopolysaccharide, IL1B, CD14, MIF, CSF2, hyaluronic acid, CpG oligonucleotide, HSP90B1	LY96, MYD88, TIRAP, lipopolysaccharide, CD14, TICAM2, HMGB1, TICAM1, F, HSPD1, Env, TOLLIP, Hsp70, Hsp, HSP90B1	activation,production in, maturation, apoptosis, induction in, upregulation in, response, recruitment
GPR83	ILMN_189915	EGFR, GSK3B, FYN, ITGB2, Erk1/2, AREG/AREGB, ITGAM, ICAM1, Akt	CBFB, FOXP3, SMARCA4, amphetamine	LY86, INSR	activation in, tyrosine phosphorylation in, release in, molecular cleavage in, inhibition in, calcium response in, contact growth inhibition, apoptosis
IL4	ILMN_218517	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression

B2M	ILMN_209318	IFNG, MHC class I, Igg, HLA-C, Klra4, HLA-DQA1, STAT3, TFRC, IGF1R, HLA-E, Cd8, CD8A, IL6	IFNG, lipopolysaccharide, TNF, NEF, IFNB1, IL10, Interferon Alpha, CD40LG, GP, CD40, dendritic cells, IFNA2, STAT1, IFNAR1	HLA-C, HLA-B, HLA-A, HFE, CD1D, MHC Class I, TFRC, FCGRT, HLA-E, HLA-G, Mhc Class1 Heavy Chain, CD8A, H2-ld, CALR, TAP1	development, quantity, differentiation, proliferation, activation, apoptosis, activity, cytotoxicity, survival, accumulation
SLAMF7	ILMN_207150	FLT3LG, TXLNA, LTA		SLAMF7, TRIB2, MAK	cytolysis, proliferation, killing
UBE2I	ILMN_184358	SMAD4, DHX9, MAPK7, SUMO1, SREBF2, CHADL, **STAT1**, NFKBIA, SREBF1, ZNF146, MYB, MDM2, TP53	MAPK1, KRAS, lipopolysaccharide, norepinephrine, UBE2I, 3C	SMAD4, SUMO3, DAXX, MITF, SP100, UBE2I, UBA2	sumoylation in, binding in, proliferation, expression in, response, contact growth inhibition, ligation in, cell death, transactivation in, migration
CASP9	ILMN_212974	CASP3, CASP7, HTT, PARP1, CASP9 CASP6, BID, CASP3/6/7, BCL2, MCL1, RB1, Caspase, STK4, MAP3K1, PAK2	TNFSF10, FAS, TNF, APAF1, CYCS, Cytochrome C, BCL2, CASP9, doxorubicin, BCL2L1, HTT, dATP	APAF1, XIAP, CYCS, BIRC2, BCL2L1, IAP, HTT, BIRC7, BIRC6, DCC, BIRC3, BIRC5, NLRP1, CASP8, PARP1	apoptosis, cell death, molecular cleavage in, activation in, transmembrane potential, blebbing, DNA damage response, quantity, morphology, cleavage in
UBE2I	ILMN_184358	SMAD4, DHX9, MAPK7, SUMO1, SREBF2, CHADL, **STAT1**, NFKBIA, SREBF1, ZNF146, MYB, MDM2, TP53	APP, CEBPA, ERG, trovafloxacin, ETV1, FLI1, EWSR1, Yersinia pestis, E. coli B5 lipopolysaccharide, MAPK1, KRAS, lipopolysaccharide, norepinephrine, UBE2I, 3C	SMAD4, SUMO3, DAXX, MITF, SP100, UBE2I, UBA2	binding, proliferation, expression, response, contact growth inhibition, ligation, cell death, transactivation, migration
ATP2C1	ILMN_211584				homeostasis, apoptosis, adhesion, quantity, morphology, reorganization, differentiation, expression, activation, replication
RNASE4	ILMN_210173	GAG, Interferon Alpha, Cytokine, Chemokine, IL6,	HNF1A, NKX2-1, HOXA10, ERG, FLI1, ETV1, EWSR1, PRL, SUMO2, SUMO3, KRAS, progesterone, IFNB1	RNASE4, sorbitol, AMP, HNF1B, HNF1A, HNF4A	binding, dissociation, cell viability, binding in, production in, activation
AIM2	ILMN_235623	IL1B, IL18, CASP1	IFNG, decitabine, Influenza A virus	Ifi202b	activation in, cell death, maturation
MTOR	ILMN_204279	EIF4EBP1, RPS6KB1, p70 s6k, Akt, STAT3, S6K1, HIF1A, MTOR, ULK1, EIF4E, IRS1, Pp2a, AKT1, Pkc,	sirolimus, Akt, amino acids, RHEB, TSC2, AKT1, Insulin, INS, Ins1, LY294002, D-glucose, everolimus, RPTOR, IGF1, pi3k	RPTOR, RICTOR, RPS6KB1, FKBP1A, MLST8, EIF4EBP1, MAPKAP1, RHEB, AKT1, MTOR, AKT1S1, STAT3, RHEBP1, Akt,	proliferation, growth, phosphorylation in, autophagy in, autophagy, differentiation, cell cycle progression, size, apoptosis, macroautophagy in

		EIF4EBP2		TRRAP	
ITGAL	ILMN_218502	ICAM1, FN1, THBS1, ITGAL, ITGB2, Pkc, APP, ITGB3, PTK2B MAPK1, JUN, Alpha tubulin,	ITGB2, ITGAL, Tcr, Lmp1, TNF, ICAM1, heavy metal, Pertussis toxin, phorbol myristate acetate, protein-protein complex, IL8, MYC, IFNG,glucocorticoid	ICAM1, ITGB2, ICAM3, ICAM2, CAV1, Integrin beta, CD226, Cd3, ITGB7, C3, ITGAM	adhesion, binding, migration, proliferation, cell-cell adhesion, activation, aggregation, attachment, cell spreading, chemotaxis
AREG/AREGB	ILMN_217903		AREG/AREGB, Fsh, beta-estradiol, phorbol myristate acetate	EGFR, WT1, Creb, BRCA1	proliferation, growth, maturation, apoptosis, differentiation, mitogenesis in, invasion, recycling in, migration, activation in
CCR2	ILMN_213602	IFNG , CCL13, TNF, IL6, CD40, CXCL3, TIMP1, CCL5, CCL4, CXCL10, IL10, Il12, elemental calcium, Ca2+, CCL11	lipopolysaccharide, IL10, IL2, TNF, CCL2, CCL13, Tcr, IL15, IL1B, LDL, Immunoglobulin, MOG, dendritic cells, Cd3	CCL2, CCL13, CCL7, CCL8, CCR5, CCL, STAT3, G protein alpha I, CCL16, JAK2, CD4, CXCR4, ADRBK1, CX3CL1, Chemokine	recruitment, quantity, chemotaxis, migration, infiltration by, accumulation, response, activation, entrance, proliferation
IFNGR1	ILMN_214754	STAT1, TNF, CXCL10, JAK2, IRF1 , NOS2, CCL13, MMP9, CCL5, IL1B, nitric oxide, JAK1, CXCL9, SOCS1, IL6	IFNG, Il12, TNF, IL2, Tcr, Cd3, phorbol myristate acetate, PBMCs, IL4 , IL13, CD2, PAEP, T lymphocytes	IFNG, STAT1, JAK1, JAK2, IFNGR1, IFNGR2, V ANTIGEN, Jak, Ifn gamma	activation, production in, expression in, quantity, synthesis in, apoptosis, proliferation, activity, growth in, killing
Presentation					
HLA-C	ILMN_196741	HLA-B, IFNG, STAT3	IFNG, NEF	B2M, KIR2DL3, Klra4	cytotoxicity, activation, number
CTSE	ILMN_253611	HTT, IL18, IL1B	IL10, EBI3, Bleomycin	BAG6	number, degradation and differentiation
HLA-B	ILMN_247624	STAT3, NFkB, HLA-B, ZAP70, FYN, LAT, LCK, Plc gamma, VAV1, CD3E, CBL, GRB2, IFNG (includes EG:15978), Lilrb3 (includes others), Igg1	IFNG , NEF, Interferon Alpha, IFNB1, lipopolysaccharide, TNF, GP, poly rI:rC-RNA, IL10, CD40, Interferon Beta, Ins1, CD40LG, Insulin, dendritic cells	B2M, LILRB1, LILRB2, KIR3DL1, VCP, Kir, CD8A, Tap, Tap complex (Antigen-processing transporter), CANX, PDIA3, TAPBP, NP, Tcr, HLA-B	activation, proliferation, recognition by, protection, negative selection, positive selection, priming, engagement, presentation in, lysis

B2M	ILMN_209318	IFNG, MHC class I, Igg, HLA-C, Klra4, HLA-DQA1, STAT3, TFRC, IGF1R, HLA-E, Cd8, CD8A, IL6	IFNG, lipopolysaccharide, TNF, NEF, IFNB1, IL10, Interferon Alpha, CD40LG, GP, CD40, dendritic cells, IFNA2, STAT1, IFNAR1	HLA-C, HLA-B, HLA-A, HFE, CD1D, MHC Class I, TFRC, FCGRT, HLA-E, HLA-G, Mhc Class1 Heavy Chain, CD8A, H2-ld, CALR, TAP1	development, quantity, differentiation, proliferation, activation, apoptosis, activity, cytotoxicity, survival, accumulation
H2-T10/H2-T22	ILMN_215923	FYN, LCK, VAV1	lipopolysaccharide, dendritic cells, ifn type 1	TCR gamma delta, B2M	priming
Recruitment					
TLR4	ILMN_217521	TNF, NFkB, IL6, Il12, IL8, Cytokine, IFNB1 , IL10, IL1B, IL12B, P38 MAPK, CD86, MYD88, CXCL10, IRF3	lipopolysaccharide, TNF, IFNG, LY96, MYD88, IL1B, CD14, MIF, CSF2, hyaluronic acid, CpG oligonucleotide	LY96, MYD88, TIRAP, lipopolysaccharide, CD14, TICAM2, HMGB1, TICAM1, F, HSPD1, Env, TOLLIP, Hsp70, Hsp, HSP90B1	activation in, expression in, activation, production in, maturation, apoptosis, induction in, upregulation in, response, recruitment
CTSC	ILMN_214875	ELANE, PRTN3, CTSG	TGFB1, HRAS	CTSC, UBC, YPO0870	recruitment, accumulation, function
CCL4	ILMN_222651	CCR5, Ca2+, P38 MAPK	lipopolysaccharide, TNF, IFNG	CCR5, CCR1, CCBP2	chemotaxis, migration , recruitment
CCR2	ILMN_213602	IFNG , CCL13, TNF, IL6, CD40, CXCL3, TIMP1, CCL5, CCL4, CXCL10, IL10, Il12, elemental calcium, Ca2+, CCL11	lipopolysaccharide, IL10, IL2, TNF, CCL2, CCL13, Tcr, IL15, IL1B, LDL, Immunoglobulin, MOG, dendritic cells, Cd3	CCL2, CCL13, CCL7, CCL8, CCR5, CCL, STAT3, G protein alpha I, CCL16, JAK2, CD4, CXCR4, ADRBK1, CX3CL1, Chemokine	recruitment, quantity, chemotaxis, migration, infiltration by, accumulation, response, activation, entrance, proliferation
Homeostasis					
ATP2C1	ILMN_211584			Atp2c2, ORAI1	homeostasis in, apoptosis, adhesion, quantity, morphology, reorganization, differentiation, expression in, activation in, replication in
CCR2	ILMN_213602	IFNG , CCL13, TNF, IL6, CD40, CXCL3, TIMP1, CCL5, CCL4, CXCL10, IL10, Il12, elemental calcium, Ca2+, CCL11	lipopolysaccharide, IL10, IL2, TNF, CCL2, CCL13, Tcr, IL15, IL1B, LDL, Immunoglobulin, MOG, dendritic cells, Cd3	CCL2, CCL13, CCL7, CCL8, CCR5, CCL, STAT3, G protein alpha I, CCL16, JAK2, CD4, CXCR4, ADRBK1, CX3CL1, Chemokine	recruitment, quantity, chemotaxis, migration, infiltration by, accumulation, response, activation, entrance, proliferation

3.3.4 Identification of candidate genes in regulatory T cells that may confer protection or susceptibility to *L. major*

A similar mining strategy used for the activated T cell microarray to identify which differentially expressed genes in the regulatory T cells may be conferring protection or susceptibility to *L. major* (Figure 25). As expected, the resulting list of candidate genes that intersected with two or more functional groups were found to be enriched with genes previously described in the literature to play a role in the outcome of Leishmaniasis (Table A5) and18 genes were selected for further analysis.

3.4 Validation of differential expression of candidate genes

Following identification of candidate genes, standard procedure requires confirmation of the differential expression of the genes using quantitative RT-PCR on the original RNA sample. If indeed the microarray and qPCR results are in agreement, the differential expression of the genes can then be validated .Based on the focused functional clustering strategy (Figure 25), the differential expression of IL-18r1, STAT4, Rnf130, Rapgef4, CCR5 and CCL5 in the microarray and quantitative real-time RT-PCR experiments were compared (Figures 31 – 36).

IL-18r1 is a transmembrane receptor located on the plasma membrane that binds IL-18, IL-18RAP and IL-37. It is selectively expressed on murine TH1 cells but not TH2 cells (Xu *et al*., 1998) and can thus be used as a surface marker to distinguish TH1 cells from TH2 cells. IL-18r1 is involved in the T helper cell differentiation pathway. IL-18r1 has primary roles in cell inflammation, function and activation as well as immune response and signal transduction. This receptor regulates NFk-β, IFN-γ and Jnk and it is regulated by IL-12, STAT4 and IL-4 (Smeltz, Chen, Hu-Li, & Shevach, 2001). In the activated T cell microarray, IL18r1 was significantly up regulated in C57BL/6 mice as compared to the BALB/c mice; whereas similar levels of IL-18r1 expression was observed amongst the various knockout groups (iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$) and the BALB/c control. Quantitative real-time RT-PCR showed that the level of IL-18r1 expression was slightly elevated in activated T cells of the resistant C57BL/6 mice as compared to the susceptible BALB/c mice, however this elevation was not statistically significantly (Figure 31). In contrast, no differential expression was observed for IL-18r1 in the regulatory T cell microarray data amongst all the mouse groups, which was confirmed by quantitative RT-PCR (Figure 31).

STAT4 (Signal transducer and activator of transcription 4) is a transcription factor that binds IFN-γ and also plays a role in the T helper cell differentiation pathway. Its biological function is cell proliferation, differentiation and cell development (Hoey *et al*., 2003). This transcription regulator regulates IFN-γ, TNF, IL-18r1, IL-10 and is regulated by IL-12, IL-2, IL-12rb2, IFN-γ and IL-6 (Kaplan, 2005). Pathways that are associated with STAT4 includes, IL-12 signaling and production in macrophages, JAK/Stat signaling, dendritic cell maturation and T helper cell differentiation. In the microarray data, STAT4 is significantly up regulated in C57BL/6 activated T cells as compared to the BALB/c activated T cells, whereas similar levels of STAT4 was observed amongst the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ and BALB/c control activated T cells (Figure 32). However, this up regulation was not confirmed by quantitative real-time RT-PCR. No differential expression was observed for STAT4 in the regulatory T cell microarray data amongst all the mouse groups, which was confirmed by quantitative RT-PCR (Figure 32).

Rnf130 (ring finger protein 130) is a protein located on the cellular membrane and forms part of the cytoplasm and nucleus. It is regulated by STAT4 and its biological functions include apoptosis, programmed cell death and protein ubiquitination (Stelzl *et al*., 2005). A significantly decreased level of Rnf130 was observed in the activated T cells of resistant C57BL/6 mice as compared to the susceptible BALB/c control mice, whereas levels of Rnf130 was equivalent in the activated T cells of among the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ and BALB/c control mouse groups (Figure 33). No differential expression was observed for Rnf130 in the regulatory T cell microarray data amongst all the mouse groups, which was confirmed by quantitative RT-PCR (Figure 33). The regulatory T cell microarray shows a significant down regulation of this gene in the T cell specific iLCK^{cre}IL-4Rα$^{lox/-}$ mice when compared to the susceptible BALB/c and the other mouse groups.

Rapgef4 (Rap guanine nucleotide exchange factor) also known as Epac2 (exchange protein directly impacted by cAMP) is located in the cytoplasm and its biological activities include regulation of protein phosphorylation and energy reserve metabolic process. Rapgef4 forms part of the G-protein coupled receptor protein signaling pathway (Oldenburger *et al*., 2012). Rapgef4 was significantly up regulated in activated T cells of C57BL/6 mice as compared to the BALB/c mice; but was expressed at similar levels amongst the iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ and the BALB/c control activated T cells (Figure 34). Quantitative real-time RT-PCR showed that the level of Rapgef4 expression was slightly elevated in activated T cells of the resistant C57BL/6 mice as compared to the susceptible BALB/c mice, but this elevation was not statistically significantly (Figure 34). No differential expression was observed for Rapgef4 in the regulatory T cell microarray data amongst all the mouse groups, which was confirmed by qPCR (Figure 34). Although not statistically significant, the levels of Rapgef4 tended to be higher in the C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ in both the activated and regulatory T cell populations, suggesting that Rapgef4 may provide resistance to *L.major,* and that the absence of IL-4Rα might play a role in the switch from a susceptible TH2 response to a resistant TH1 response.

Chemokines function in the recruitment, polarization, activation and differentiation of T cells (Murphy 1994, Baggiolini *et al*., 1997, Yoshie *et al*., 1997 and Rossi 2000).CCR5 (Chemokine receptor type 5), also known as CD195, is a protein found on the surface of white blood cells and acts as a receptor for chemokines. This receptor is predominantly expressed on T cells, macrophages, dendritic cells and microglia. It is known that TH1 and TH2 cells have different chemokine receptors. TH1 cells express CCR5, whereas TH2 cells are known to express CCR3 (also found on eosinophils and basophils) (Wysocki *et al*., 2005). CCR5 was significantly up-regulated in both the activated T cells of C57BL/6 and iLCK^{cre}IL-4Rα$^{-/lox}$ mice as compared to susceptible BALB/c mice. CCR5 was found to be expressed at 4.73 fold higher levels in activated T cells and 2.17 fold higher in regulatory T cells from resistant C57BL/6 mice infected with *L. major* as compared to susceptible BALB/c. Similarly, in iLCK^{cre}IL-4Rα$^{-/lox}$ mice infected with *L major* expressed a 2 fold higher level of CCR5 in both activated T cell and regulatory T cell populations as compared to

BALB/c (Figure 35). Although not statistically significant, quantitative real-time RT-PCR showed that the levels of CCR5 were much higher in in both the activated and regulatory T cell populations of C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to BALB/c controls, suggesting that CCR5 is important for providing resistance to *L. major.* In the activated T cell microarray, we observe significance of all the mouse groups when compared to the susceptible BALB/c group.

CCL5 (Rantes) is the ligand for CCR5 (Santiago *et al*., 2004) and was expressed at 2.42 fold higher levels in activated T cells and 2.73 fold higher in regulatory T cells from resistant C57BL/6 mice infected with *L. major* as compared to BALB/c. CD4^{+} T cell specific IL-4Rα deficient (iLCK^{cre}IL-4Rα-$^{/lox}$) mice infected with *L major* expressed a 1.3 fold higher level of CCL5 in activated T cell populations as compared to BALB/c (Figure 36). Once again, although not statistically significant, quantitative real-time RT-PCR showed that the levels of CCL5 were much higher in the C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ activated and regulatory T cell populations as compared to BALB/c controls, suggesting that CCL5 is important for providing resistance to *L. major.*

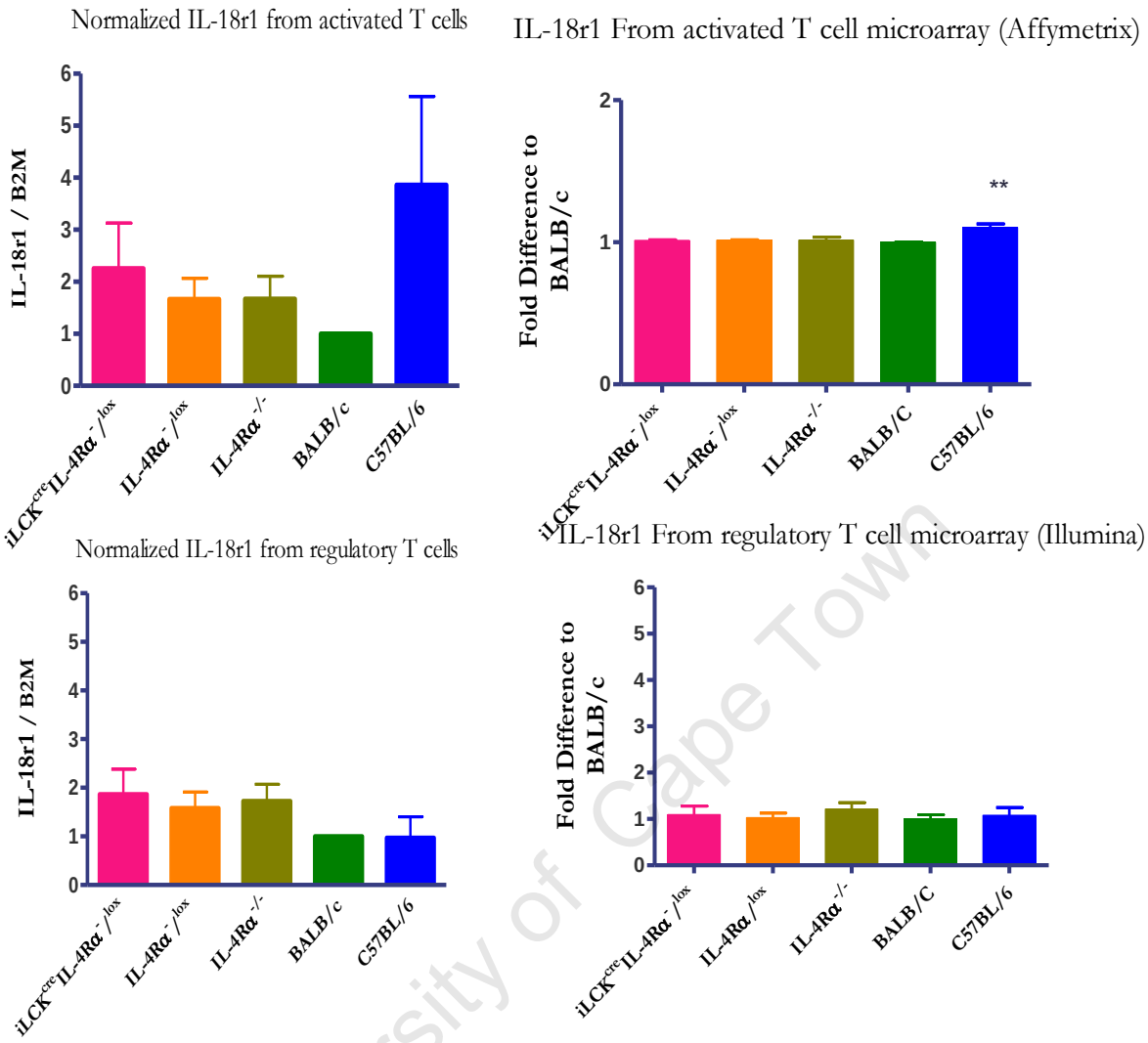

Figure 31 IL-18r1 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative IL-18r1 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. ** P<0.01

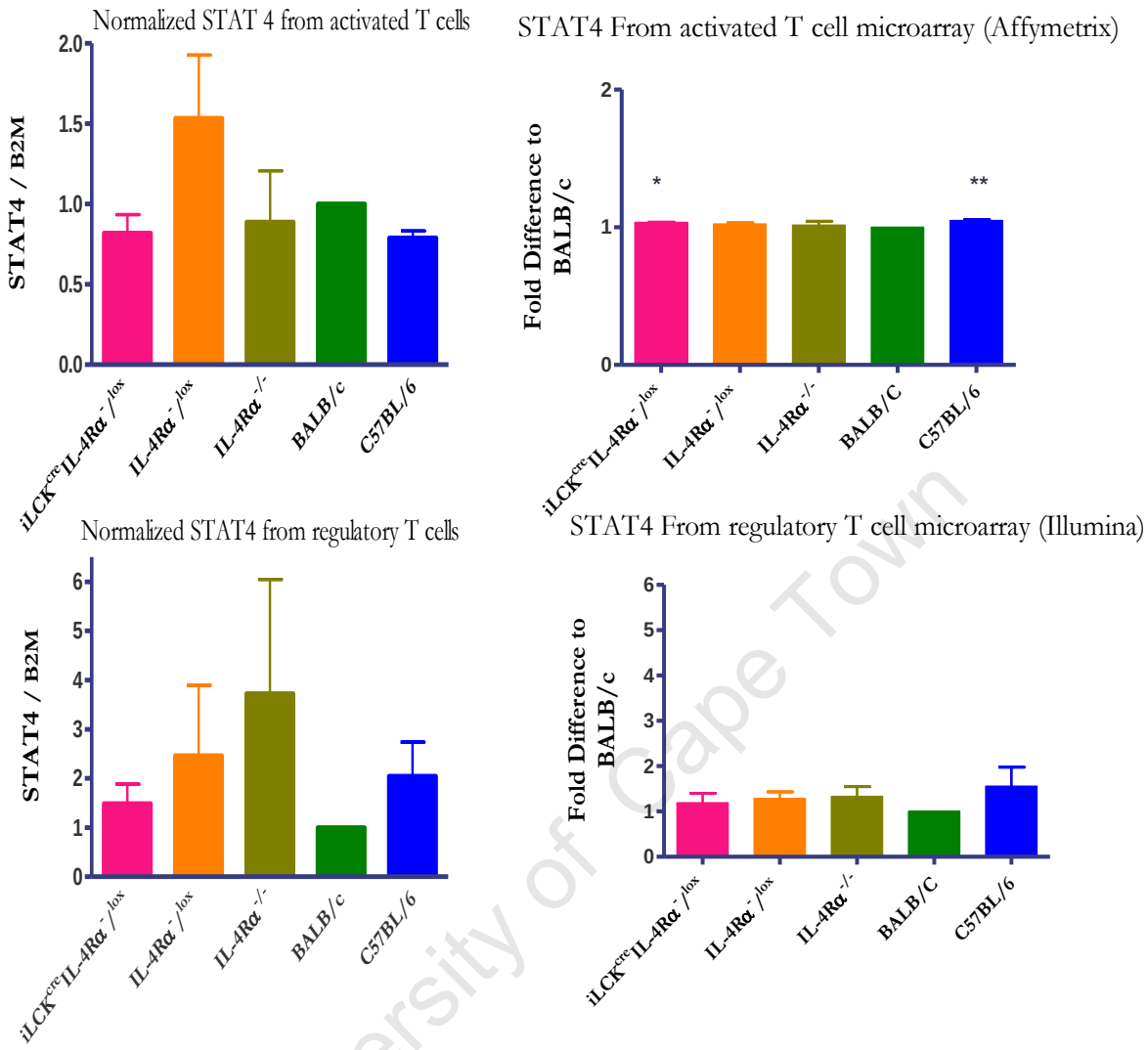

Figure 32 STAT4 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative STAT4 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. * P<0.05, ** P<0.01

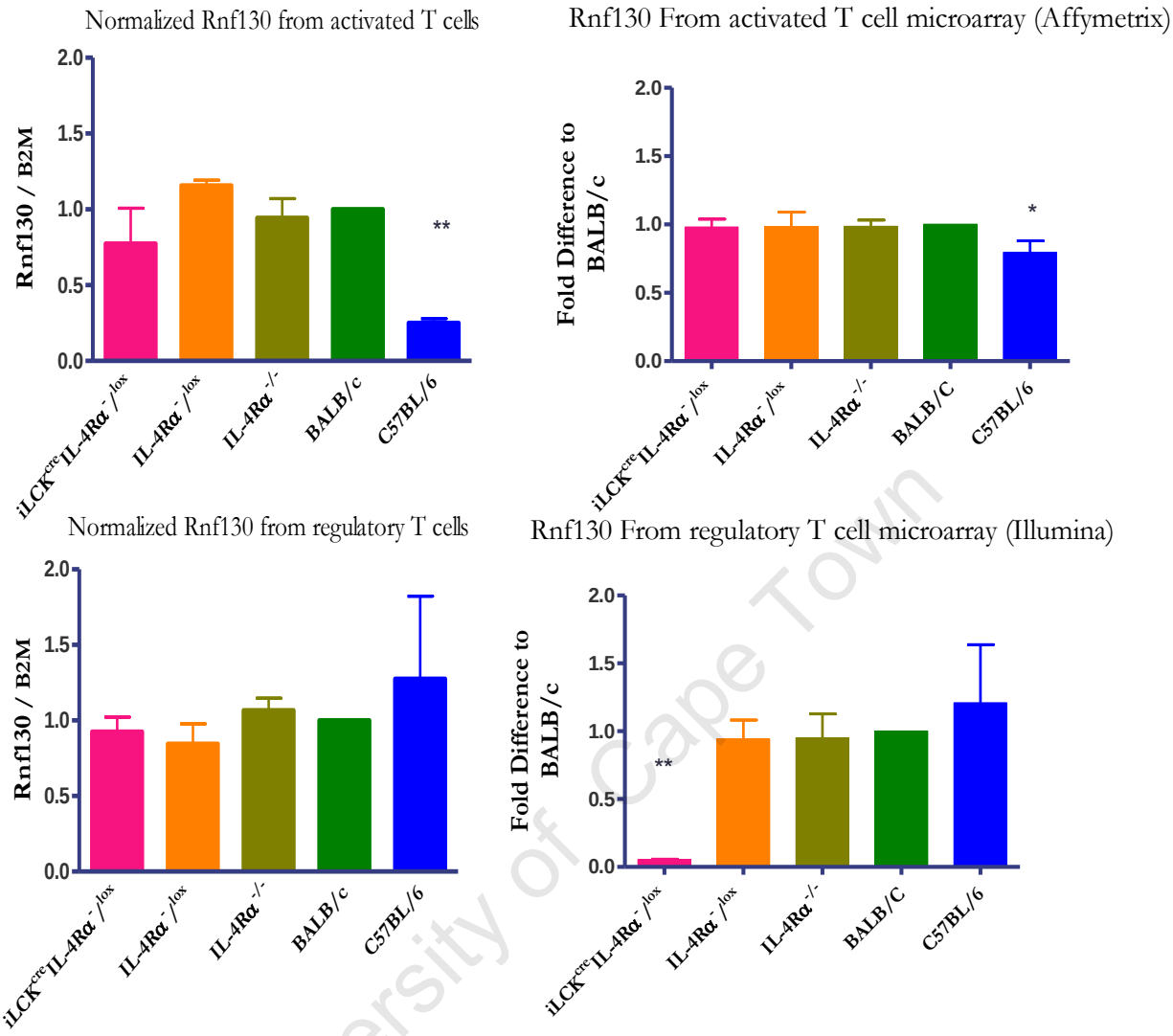

Figure 33 Rnf130 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative Rnf130 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. * P<0.05, ** P<0.01

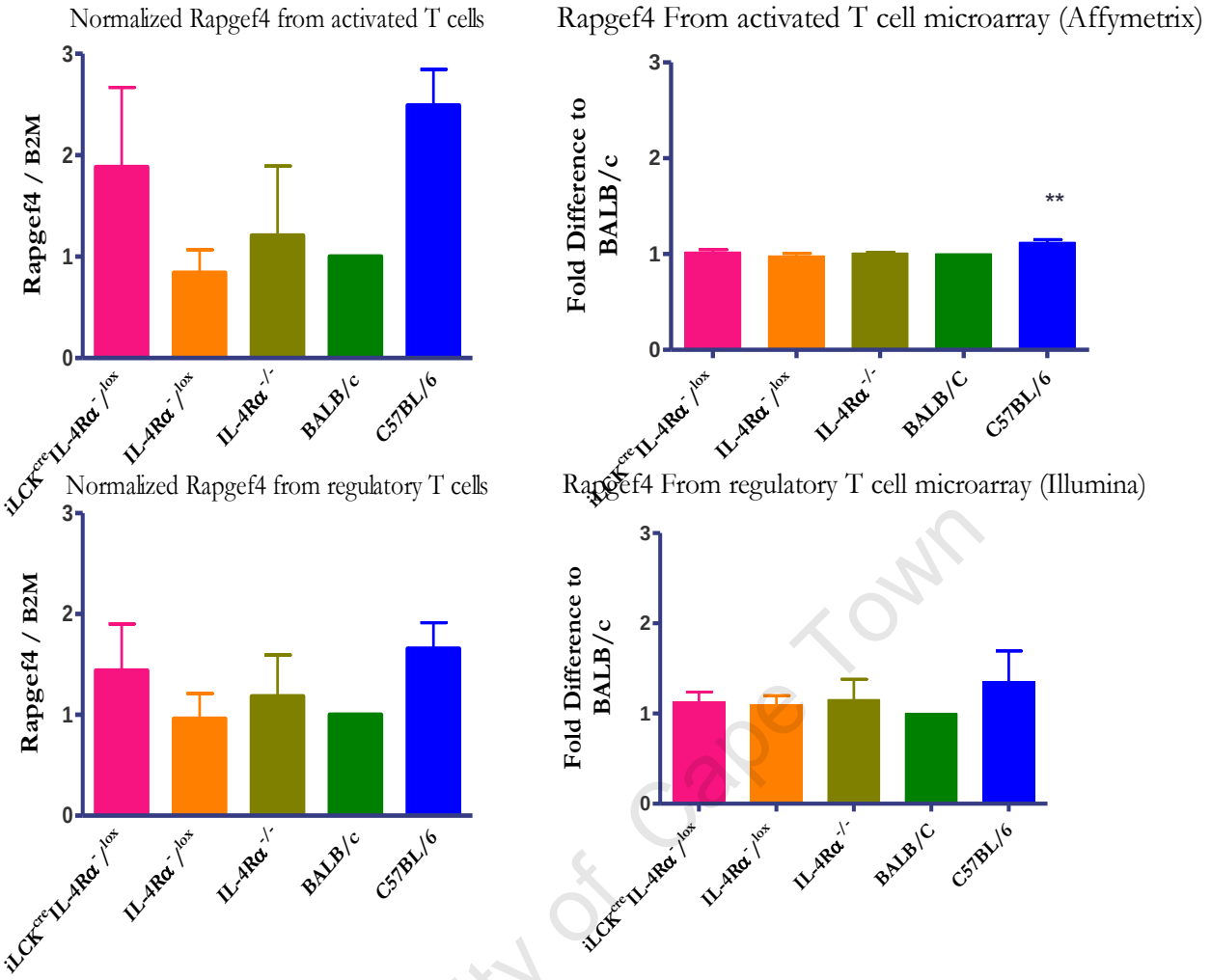

Figure 34 Rapgef4 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative Rapgef4 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. ** P<0.01

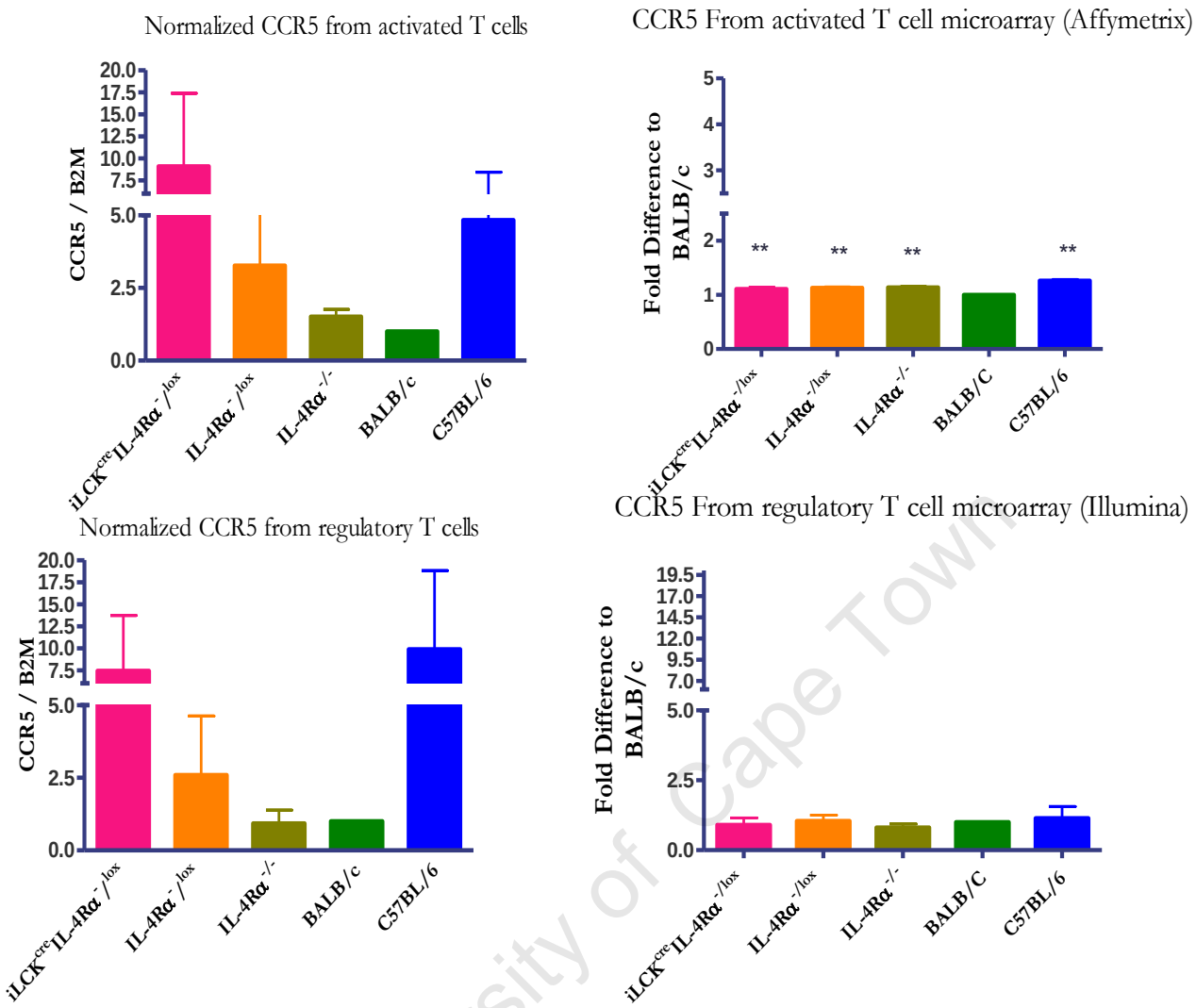

Figure 35 CCR5 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative CCR5 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. ** P<0.01

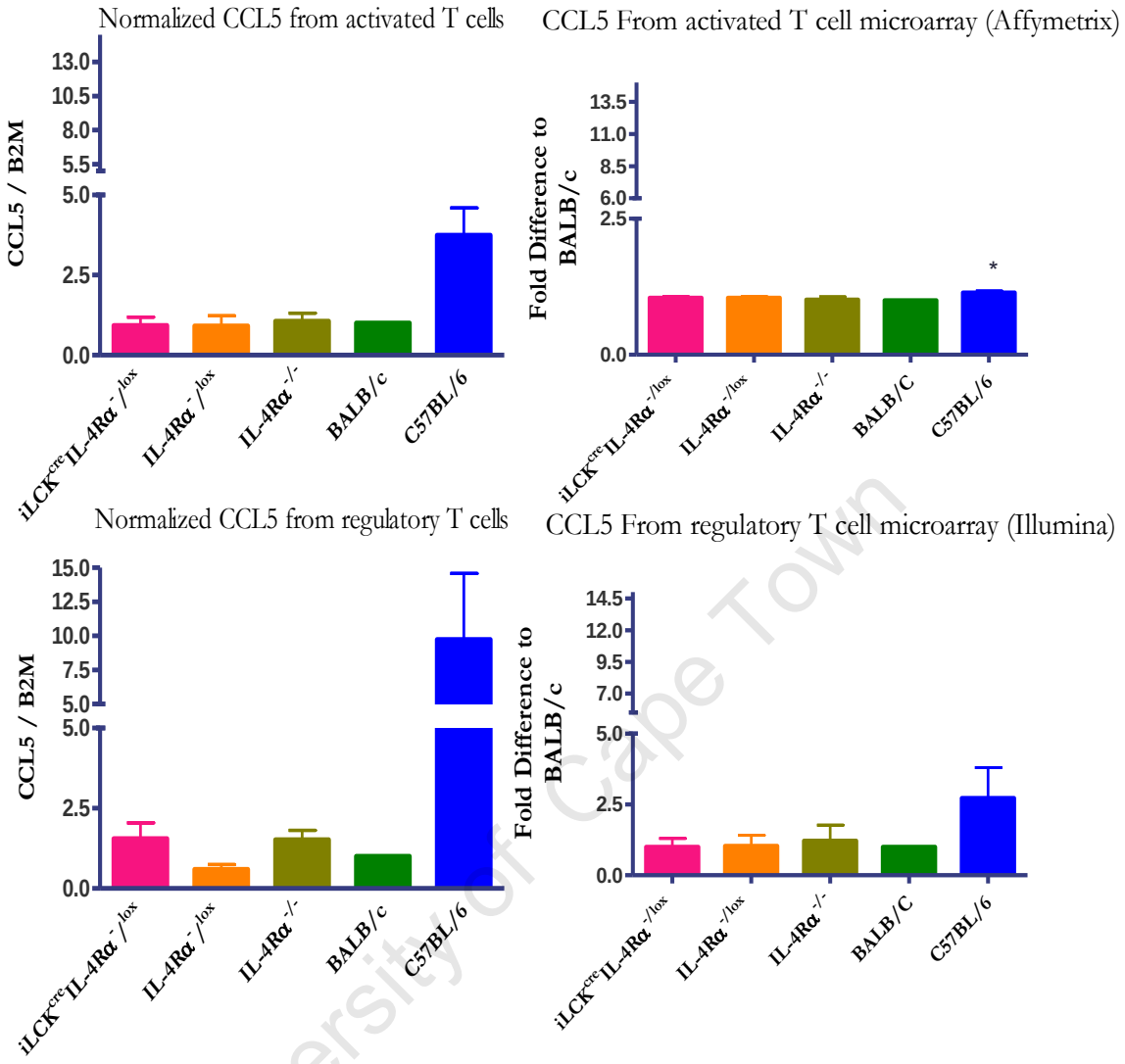

Figure 36 CCL5 expression by quantitative PCR and microarray for activated T cells and regulatory T cells.

A. Activated and regulatory T cells were sorted to greater than 99% purity, the RNA was extracted, converted to cDNA and linearly amplified prior to analysis of expression levels by real time quantitative PCR. Data was normalized to expression levels of the B2M housekeeping genes and values are shown as fold change relative to the BALB/c group. For each strain T cells were isolated from pooled popliteal lymph nodes of 6 mice. **B.** Activated T cells and regulatory T cells from all strains *L. major*-infected mice were subject to transcriptional profiling by microarray at 3 weeks post infection. Relative CCL5 expression levels post-normalization of microarray data. All groups were compared to the BALB/c control group which was set to 1. Each graph represents the average values for three independent biological experiments. * P<0.05

3.5 CCR5 expression using FACS for validation

CCR5 was selected as a lead candidate gene for further study in order to determine if the differential expression of CCR5 mRNA correlated to differences in protein expression. Therefore iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$mice together with the susceptible BALB/c and resistant C57BL/6 controls, were infected subcutaneously with 2x10^{6} promastigote stage *L. major* into the left hind footpad. After three weeks post-infection the expression of CCR5 in activated T cells and regulatory T cells isolated from infected lymph nodes, was measured by FACS analysis (Figure 37) using the gating strategy shown in Figures 14 and 15. The expression levels of CCR5 protein on the surface of activated T cells was significantly up-regulated in the C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to the susceptible BALB/c mice, thereby supporting the differential mRNA expression observed in the microarray and quantitative real time PCR data. This data strongly suggested that in the susceptible BALB/c mice, absence of IL-4Rα on CD4^{+} T cells results in bias towards a resistant TH1 response, and that CCR5 is involved in this TH1 polarization.

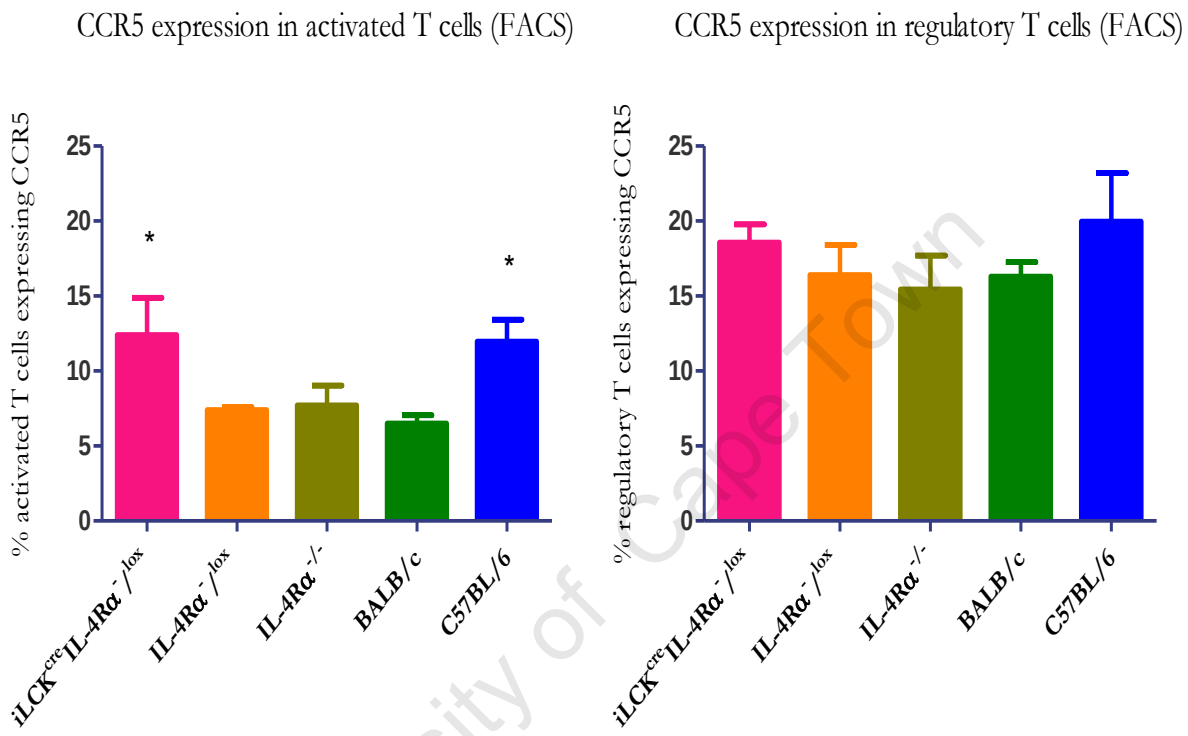

Figure 37 CCR5 expression in activated and regulatory T cells by FACS.

FACS analysis of Activated (A) and regulatory (B) T cells of popliteal lymph nodes cells from mice infected with 2x10^6 promastigote stage *L. major*. The graph represents the average values for five mice per group. For statistical significance, all groups were compared to the susceptible BALB/c group. * P<0.05

In summary, the aim of this study was to identify the gene expression patterns in $CD4^+$ T cells that are associated with resistance or susceptibility to *L. major*, using a transcriptomics approach. Firstly, knockout mice, $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$, together with their controls, susceptible BALB/c and resistant C57BL/6, were infected subcutaneously with $2x10^6$ promastigote stage *L. major* into the left hind footpad. Disease progression was monitored for three weeks. At the time of euthanasia, activated and regulatory T cells were sorted, RNA extracted and linearly amplified to generate cDNA. Pre-validation quantitative real time PCR was performed to phenotypically characterize the known markers for TH1 (resistance) and TH2 (susceptibility). Transcriptional profiling by microarray was used to assess the differences in activated and regulatory T cells isolated from the various mouse groups ($iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$, BALB/c and C57BL/6). DE genes were identified by comparative analyses between C57BL/6 vs. BALB/c and $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$ vs. BALB/c, amongst others, using a fold change cut-off of ≥ 1.2 and a p-value < 0.05. The lists of differentially expressed genes were then analysed using IPA and Babelomics microarray analysis software, and assigned functional annotations. Intensive literature mining combined with a focused functional clustering strategy (Figure 25), identified differentially expressed genes that belonged to more than one "focus" group. Candidate genes belonging to two or more of the focus groups were then selected for further analysis. The rationale for selecting genes which intersected with two or more (ie >50%) of the focus functional groups, was that this strategy may increase the probability of selecting candidate genes which have a significant biological impact on the resistance of *L. major* infections. Indeed differentially expressed genes that intersected with two or more functional groups were found to be enriched for these genes which have already been described in the literature to play a significant role in resistance to *L. major* (Table A5 in appendix).

A few genes were selected for further study, and the differential expression for some of the candidate genes was confirmed by quantitative RT-PCR on the original RNA sample. Good correlation was observed between the quantitative PCR data and the microarray data. The strong overlap of the microarray data and the validation quantitative PCR data supports

the strength of the microarray experiments and the potential use of the data to identify genes important during *L. major* infection. CCR5 was selected as a possible candidate gene for confirmation of the observations seen in the respective microarrays and quantitative real time PCR.

Chapter 4

Discussion and Conclusions

Discussion

Experimental *Leishmania* infections in mice have been widely used to study the control of TH1/TH2 differentiation, and to elucidate mechanisms underlying susceptibility/resistance to intracellular microbial infection (Radwanska *et al*., 2007, Locksley and Scott, 1991). Resolution of leishmaniasis is dependent on the coordinated interactions between components of cell mediated immune response, specifically the activation of targeted T-cell populations to secrete appropriate cytokines which in turn activate macrophages (Sacks & Noben-Trauth, 2002). Typically in murine models of *Leishmania* infection, resistant C57BL/6 mice are able to control and heal dermal lesions, while susceptible BALB/c mice develop severe pathology, manifested by progressive lesion development, necrosis, and death (Reiner and Locksley, 1995). Resistance to *L. major* infection is dependent on a sustained IL-12-driven type-1 response mainly characterized by the production of IFN-γ by $CD4^+$ TH1 cells (Gorak, Engwerda, & Kaye, 1998; Walker *et al*., 1999), which in turn mediates protection by inducing NOS-2 expression and nitric oxide production in classically activated macrophages (Holscher *et al*., 2006, Guler *et al*., 1996, Park *et al*., 2002, Liew *et al*., 1993).

In contrast, non-healing disease in BALB/c mice is associated with a TH2 response characterized by secretion of mainly IL-4, IL-5, IL-9, and IL-13 (Kopf *et al*., 1996; Matthews *et al*., 2000; Mohrs *et al*., 1999, 2000), high anti-Leishmania antibody titres, arginase-1 production by macrophages (Holscher *et al*., 2006, Iniesta *et al*., 2002) and visceral dissemination of parasites (Laskay *et al*., 1995). Conventional opinion and early studies with *L. major* (Heinzel *et al*., 1989, Leal *et al*., 1993) suggested than an IL-4 driven TH2 response and associated cytokines like IL-13 counter-regulated TH1 responses. Consequently, it was expected that a TH2 response would be detrimental to the outcome of disease. Indeed, susceptible BALB/c mice deficient for IL-4 (Kopf *et al*., 1996), IL-13 (Matthews *et al*., 2000), the IL-4Rα (Mohrs *et al*., 1999), or STAT6 (Hoffmann *et al*., 2000) were able to contain infection with *L. major* and TH2 cytokines like IL-4, IL-9, IL-10 and IL-13 were shown to be susceptible factors. However, more recently, new evidence has come to light that suggests that the development of effective type-1 immunity can involve the

significant involvement of the TH2 cytokines IL-4 and IL-13 (King *et al*., 2005, Minty *et al*., 1997). Both cytokines, IL-4 and IL-13 share a common signaling pathway through the IL-4Rα chain. A functional IL-4R (type I) requires assembly of IL-4Rα with a γc chain, while interaction of IL-4Rα with an IL-13Rα1 subunit leads to formation of a functional IL-13 receptor (type II) (Kelly-Welch *et al*., 2003). Mice with a global deletion of the IL-4Rα on all cells (IL-4Rα$^{-/-}$) are unable to respond to IL-4 and IL-13. During *L. major* infection these IL-4Rα$^{-/-}$ mice still developed a TH2 response and were able to control infection during the acute stage of disease (although with a higher parasite burden). However, during the later chronic stages of disease, the IL-4Rα$^{-/-}$ mice were unable to maintain resistance and developed full blown leishmaniasis (Radwanska *et al*., 2007). This suggested that IL-4Rα-mediated mechanisms, other than TH2 responses are responsible for disease progression in *L. major*-infected BALB/c mice. Indeed, the data demonstrated that IL-4Rα responsiveness on macrophages promoted alternative activation of macrophages, which in turn contributed to the susceptibility of BALB/c mice. In the absence of alternative macrophage activation, protective classical macrophage activation was found to be enhanced resulting in increased microbiocidal activity and protective TH1 responses (Holscher *et al*., 2006).

Similarly, studies using CD4^{+} T cell specific IL-4Rα (iLCK^{cre}IL-4Rα$^{lox/-}$) deficient mice on a susceptible BALB/c background, demonstrated that loss of IL-4Rα responsiveness on CD4^{+} T cells together with the presence of IL-4Rα responsiveness on other non-CD4^{+} cell types, lead to clinical immunity in "non-healer" BALB/c mice infected with *L. major* (Radwanska *et al*., 2007). This suggests that during *L. major* infection, signaling via the IL-4Rα on CD4^{+} T cells induces a cascade of events that ultimately results in susceptibility and disease progression. In this study, the aim was to identify genes in CD4^{+} T cells (activated and regulatory T cells) that are associated with resistance or susceptibility to *L. major*, with the long term goal that these genes (and their encoding protein products) can serve as targets for rational drug design. This was investigated using DNA microarrays, GeneChip Mouse Exon 1.0 ST Array on an Affymetrix platform and Illumina ® MouseWG-6 v2.0 Expression

BeadChips on an Illumina Bead array to analyze the gene expression profiles of IL-4Rα responsive and non-responsive $CD4^+$ T cells isolated from the draining lymph nodes of susceptible WT BALB/c mice and resistant mice $CD4^+$ T cell-specific ($iLCK^{cre}$IL-$4R\alpha^{lox/-}$), global IL-$4R\alpha^{-/-}$, littermate control IL-$4R\alpha^{lox/-}$, and resistant C57BL/6 mice respectively.

4.1 Comparative *L. major* infection

Activated T cells ($CD4^+CD44^{+med\text{-}hi}CD62L^{+lo}$) and regulatory T cells ($CD4^+CD25^+$) were isolated from $CD4^+$ T cell specific ($iLCK^{cre}$IL-$4R\alpha^{lox/-}$), littermate control (IL-$4R\alpha^{lox/-}$), global knockout (IL-$4R\alpha^{-/-}$), susceptible BALB/c control and resistant C57BL/6 mice. The activated and regulatory T cells were isolated and the phenotype of the infected mice were assessed to confirm that the infection was indeed successful, the biological replicates we all reproducible across all experiments and that the infection phenotype was in accordance with published data (Mohrs *et al*., 2000; Radwanska *et al*., 2007). The C57BL/6 mice showed resistance with a healing TH1 phenotype and the BALB/c mice showed susceptibility with a non-healing TH2 phenotype at three weeks post infection.

It is known that the early events that lead to the initial development of TH1 or TH2 response take place within the first two days after infection (Sacks & Noben-Trauth, 2002). At three weeks post infection, the acute stage of the disease, there is no distinctive TH1 or TH2 phenotype evident, although the total IgE titres in the C57BL/6, IL-$4R\alpha^{-/-}$, and $iLCK^{cre}$IL-$4R\alpha^{lox/-}$ were lower than in the BALB/c, which suggests a predominant type 1 immune response, the SLA-specific IgG2b responses were equivalent between all the mouse groups.

The use of *L. major* infection is used widely to investigate T helper subset development (Radwanska *et al*., 2007). During *L. major* infection, BALB/c mice produce type 2 antibodies which are significantly reduced in mice that lack IL-4Rα (Mohrs *et al.*, 1999 and Mohrs *et al*, 2000). Furthermore, a deletion of IL-4Rα results in a shift towards the production of type 1 antibodies IgG2a and IgG2b. Production of IgE is dependent on IL-4 signaling (Shimoda *et al*, 1996). In order to assess IL-4Rα-responsiveness in T cells during acute *L. major*

infection, serum antibody levels of IgE and soluble Leishmania antigen (SLA)-specific IgG2b were analyzed in the various IL-4Rα-knockout mice, iLCK^{cre}IL-4Rα $^{lox/-}$, IL-4Rα $^{lox/-}$, IL-4Rα$^{-/-}$, and control BALB/c and C57BL/6. IL-4Rα responsiveness of T cells in the iLCK^{cre} IL-4Rα$^{lox/-}$ mice, during the acute phase of the disease was demonstrated by assessing the levels of total IgE. The levels of total IgE were equivalent between the iLCK^{cre}IL-4Rα$^{lox/-}$ and C57BL/6 mice, whereas in the IL-4Rα$^{-/-}$ mice the total IgE level was significantly impaired and was at the detection level of the ELISA (Figure 12). This is as previously reported, as the presence of IL-4Rα on B cells is needed to produce IgE (Radwanska *et al.*, 2007). Since IgE production is strictly dependent on IL-4 signaling (Shimoda *et al*., 1996) this data indicates very strong signaling via the IL-4Rα in the BALB/c and the IL-4Rα $^{lox/-}$ mice during the acute phase of *L. major* infection, which is associated with TH2 associated type 2 immune responses. Similarly, since C57BL/6 mice are known to mount a TH1 associated immune response to *L. major* infection and that the SLA-specific IgE levels were equivalent between the iLCK^{cre}IL-4Rα$^{lox/-}$ and C57BL/6 mice, this data suggested that the iLCK^{cre}IL-4Rα$^{lox/-}$ may also have developed TH1 associated immune response despite its susceptible BALB/c genetic background. High IgG2a levels are indicative of a type 1 response (Mohrs *et al*., 1999). During the acute phase of *L. major* infection, it is not clear whether it is a definite type 1 or type 2 response, as the various knockout groups have similar levels of expression for the various TH1 and TH2 markers. However, in experiment C we observe higher levels of the antibody for the resistant C57BL/6 when compared to the susceptible BALB/c group. This observation from the IgG2a indicates a possible shift towards a type 1 response.

During the acute phase of disease all the mouse groups appeared phenotypically similar, eg. footpad swelling, parasite burden and antibody responses etc. We observe that the results for the biological experiments are reproducible and it corresponds to previously published data (Radwanska *et al.*, 2007, Mohrs *et al*., 1999). However, a clear phenotype is not evident at this selected time point for the experiment. A more powerful experimental design would have been to include several time points after infection to detect the very early events involved in the induction of TH1 / TH2 differentiation and the establishment of type 1 or type 2 immune responses. These time points could include 1, 2, 7, 14, 21 and

28 days post infection. However, this experiment was not possible due to economic constraints.

4.2 Activated and regulatory T cell isolation

In order to assess the differential gene expression of activated T cells and regulatory T cells in the draining popliteal lymph node during acute murine *L. major* infection, both cell populations needed to be isolated at both high purity and yield. For the isolation of the activated T cells, a yield and purity of above 99% was obtained. It is important to note that one limitation of this study was that it was not possible to isolate a >99% pure regulatory T cell population. This would require that the isolated $CD4^+$ T cells be stained for both FoxP3 in addition to CD25 and FoxP3 would require intracellular staining, which would result in the loss of RNA from the cells. However, it was confirmed by intracellular FACS that the yield and purity of 99% for the regulatory T cells contained on average above 80% $FoxP3^+$ regulatory T cells. Not having a 100% pure $FoxP3^+$ regulatory T cell population could have its pitfalls, in that the regulatory T cell population would contain a 20% contamination of activated T cells. This mixed regulatory T cell population may lead to some of the activated T cell genes to be differentially expressed in the regulatory T cell microarray.

Extraction of good quality RNA from the sorted activated and regulatory T cells was imperative to be able to assess differential gene expression as accurately as possible. Only twenty seven percent of the samples displayed RNA Integrity (RIN) values below 7 – the recommended minimum for microarray analysis. However, the method for cDNA synthesis from the RNA template used both oligo-dT and random internal primers, ensuring that even partially degraded transcripts would be amplified (Tariq *et al*., 2011). This method thus allowed for the samples with RIN values between 6 and 7. The electropherograms obtained indicated pure cDNA with no genomic DNA contamination. Despite having samples with lower RIN values, all samples were successfully amplified and the cDNA quality and yield obtained were sufficient for both the microarray and quantitative RT-PCR confirmation studies.

To assess whether the cDNA was indeed a reflection of the RNA content and that the phenotype of the activated and regulatory T cells was as expected given the genotype, quantitative real time PCR was performed on IFN-γ and IL-4, marker genes associated with either TH1 or TH2 differentiation respectively (Alexander, Satoskar, & Russell, 1999). The IL-4 expression profile for the susceptible BALB/c and C57BL/6 was consistent with what is known, where resistant C57BL/6 mice displayed reduced levels of IL-4 when compared to susceptible BALB/c mice. This is observed for both activated and regulatory T cells. The IFN-γ expression profiles for the controls were in line with what has previously been published, where susceptible BALB/c mice have reduced levels of IFN-γ when compared to the resistant C57BL/6 mice for both activated and regulatory T cells. The knockout mice expressed similar reduced levels of IFN-γ for both the activated and regulatory T cells, indicating a bias towards TH2 response, and intermediate levels of IL-4 for the two cell populations.

The quality control analysis of the cDNA therefore indicated the successful preservation of the differential expression of good quality mRNA, from the stage of cell isolation to cDNA synthesis and amplification, and therefore provided a solid foundation onto which to base the microarray analysis.

4.3 Gene expression profiling of activated T cells

The global transcriptional profile of the activated T cells obtained from draining lymph nodes of iLCK^{cre}IL-4Rα $^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 mice. This was done in order to elucidate the mechanisms by which signaling via the IL-4Rα on CD4^{+} T cells causes susceptibility to *L. major*.

Gene expression patterns were compared early during infection in T cells in the absence or presence of IL-4Rα signaling, with the aim to identify genes and pathways associated with IL-4Rα-induced TH1 and TH2 differentiation and how these genes may contribute to susceptibility or resistance during *L. major* infection. The knockout groups, iLCK^{cre}IL-

4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$ and IL-4Rα$^{-/-}$ all produced intermediate levels of these classical TH1 and TH2 cytokines. Three weeks post infection good correlation amongst the controls from the quantitative real time PCR data was observed. The time point chosen for the microarray analysis was three weeks post infection. At this time point all the mouse groups appeared phenotypically similar, e.g. footpad swelling, parasite burden etc. A more powerful experimental design would have been to include several time points after infection to detect the very early events involved in the induction of TH1 / TH2 differentiation and the establishment of type 1 or type 2 immune responses. These time points could include 1, 2, 7, 14, 21 and 28 days post infection. However, this experiment was not possible due to economic constraints. Thus we further proceeded to investigate the differentially expressed genes amongst the various experimental groups at three weeks post infection.

This was achieved by identifying genes differentially expressed in activated T cells and various comparisons were performed. We sought to investigate how similar the transcriptional response of the iLCK^{cre}IL-4Rα$^{lox/-}$ is to the C57BL/6. By comparing the mRNA expression profiles of C57BL/6 to BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ to BALB/c, these comparisons will identify genes important for mediating protection during *L. major* infection.

For the comparison involving the iLCK^{cre}IL-4Rα$^{lox/-}$ dataset, a fold change and p-value cut-off of 1.2 and 0.05 was chosen. This resulted in a sizeable number of genes to work with and minimal noise. It was decided to use this set of criteria for both comparisons to obtain the lists of differentially expressed genes.

This study focused on IL-4/IL-13 driven TH1 /TH2 function during *L. major* infection. Thus, genes that are differentially expressed in the presence or absence of IL-4Rα-signaling were of interest. The C57BL/6 vs. BALB/c comparison was also of interest as these groups represent the resistant and susceptible control groups. The genes in this comparison may contribute to the differing susceptibility to *Leishmania* infection that is seen in the two mouse strains. In the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparison, both these mouse strains have a susceptible background. The iLCK^{cre}IL-4Rα$^{lox/-}$ mouse lacks the IL-4Rα on CD4^{+} T

cells, whereas the BALB/c have an active receptor present. The genes from this comparison could possibly play a role in TH1 differentiation and or resistance. In addition to these comparisons, when overlaying these two comparisons and obtaining the common genes from both datasets, these genes adds to the evidence that the microarray data is biologically relevant.

A total of 456 genes were differentially expressed in activated T cells from C57BL/6 mice as compared to BALB/c mice, with 46% being up-regulated and 54% being down-regulated in the C57BL/6 activated T cells. In contrast, only 17 genes were differentially expressed in activated T cells from iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to BALB/c mice, with 70% being up-regulated and 30% being down-regulated in the iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells. Interestingly, four genes (Gdpd3, CCR5, Otud6b and H2-T10) were differentially expressed with the same up- or down-regulatory patterns in the C57BL/6 and the iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells as compared to BALB/c activated T cells. Of these genes, it is known that CCR5 plays an important role in the differentiation process, in that it aids in the formation of the immunological synapse. The integrity of the immunological synapse is strengthened by the presence of CCR5 and aids in the differentiation process.

An important step in the biological interpretation of the data was to confirm that the IL-4Rα expression profile. Activated T cells from three week infected lymph nodes were subjected to transcriptional profiling. It was observed that littermate control IL-4Rα$^{lox/-}$, global knockout IL-4Rα$^{-/-}$ and iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells have significantly reduced expression of IL-4Rα when compared to activated T cells from susceptible BALB/c mice. These results confirms the dosage effect of the IL-4Rα alleles, in that BALB/c mice have 2 functioning alleles of the receptor compared to the knockouts that have only one (IL-4Rα$^{lox/-}$). In the global knockout IL-4Rα$^{-/-}$ mRNA expression of the IL-4Rα receptor was still observed, even though the gene is not making a functional IL-4Rα protein. A possible hypothesis for this observation is the detection algorithm used for Affymetrix exon array image analysis, which calculated overall gene expression by taking an average across all 12 exons of the IL-4Rα, even though exons 7, 8 and 9 were deleted.

It was determined that 41% of the differentially expressed genes in the C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ activated T cells mapped to *Leishmania major* response (*Lmr*) loci. This enrichment was found to highly statistically significant in C57BL/6 vs. BALB/c comparative analysis, but not for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c analysis.

The strength of the microarray data was confirmed by the appearance of many genes that formed networks and were involved in pathways. These known networks / pathways included cellular growth and proliferation; cell-mediated immune response; cell signaling; inflammatory response; cell-to-cell signaling and interaction and cell death. The top associated networks for the two comparisons, C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c, shows a trend in that similar networks have high scores amongst the comparisons and play a role in resistance during *L. major* infections. In addition, we also observed that the molecules from the network analysis have a number of interactions with other genes. For the C57BL/6 vs. BALB/c network analysis, we observed various interactions including interactions between IL-4, STAT5, TNF, JAK, SlamF7 and Ephx1. These genes are in particular of interest as they are in some way associated to T helper differentiation. The network analysis for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparison also yielded interactions between IL-4, CCR5, H2-T10 and STAT5. These genes from the networks mentioned above all have roles in the T helper differentiation which relates to some of the objectives of the thesis.

The investigation of how these molecules from the network analysis associated with TH2 and TH1 molecules followed next. The TH1 molecules selected to show association to was, IFN-γ, TNF, IL-12, IL-18 and STAT4; for the TH2 molecules association to IL-4, IL-13, and IL-6, IL-2, STAT6 and GATA3 was shown. A number of genes have associations with both TH1 and TH2 molecules, with a few genes interacting with the regulatory molecules, IL-10 and TGF-β. The last strategy was to identify candidate genes that utilized the gene ontology terms associated with known marker genes (proliferation, activation, presentation and recruitment). Candidate genes were identified as putative markers of TH1 / TH2 and/or resistance vs. susceptibility during *L. major* infection during the acute phase of the disease. It was hypothesized that differentially expressed genes (fold change cut-off of 1.2 and a p

value of < 0.05) that were identified should display a number a qualities. These included, the genes should be located on known Leishmania resistance or susceptibility loci; genes should be involved in pathways associated to T cell receptor signaling, ie. be involved in TH1 / TH2 differentiation; the genes should be involved in the formation of the immunological synapse, which aids in the differentiation process or these genes should be involved in processes like cell-to-cell signaling and interaction; cellular growth and proliferation etc. A mining strategy was employed to extract and assign functional data to the differentially expressed genes and to cluster these genes into focus groups. The lead candidate genes that belonged to more than 50% of the focus functional groups were found to be enriched with genes that play significant roles in resistance to *L. major.*

4.4 Gene expression profiling of regulatory T cells

The global transcriptional profile of the regulatory T cells obtained from draining lymph nodes of $iLCK^{cre}IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{lox/-}$, $IL\text{-}4R\alpha^{-/-}$, BALB/c and C57BL/6 mice was assessed via microarray. This was performed to elucidate the mechanisms by which signaling via the IL-4Rα on $CD4^+$T cells causes susceptibility to *L. major* in a population of regulatory T cells ($CD4^+CD25^+$). For the regulatory T cells, it was not possible to obtain a 100% pure regulatory T cell population as this population of cells contains a mixed population of cells, including activated T cells. To obtain a pure population of regulatory T cells, it would be required that the isolated $CD4^+CD25^+$ cells be stained intracellularly with FoxP3 and this staining procedure would result in the loss of RNA from the cells. Considering that the regulatory T cells are a mixed population, it is observed that the differentially expressed genes from the regulatory T cell microarray contained genes from the activated T cell microarray.

As discussed previously, the aim of this study was to identify genes and pathways associated with IL-4Rα-induced TH1 and TH2 differentiation and how these genes may contribute to susceptibility or resistance during *L. major* infection at three weeks post infection. Pre-microarray quantitative RT-PCR data compared favorably with a previous ,

publication from our group (Radwanska *et al.*, 2007). We proceeded to investigate the differentially expressed genes amongst the various experimental groups.

This was achieved by identifying genes differentially expressed in regulatory T cells and various comparisons performed. These included C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c, amongst others. The same criteria used for obtaining the fold change and p-values in the activated T cell microarray were implemented for the regulatory T cell microarray. Given the few number of differentially expressed genes obtained in the comparisons involving the iLCK^{cre}IL-4Rα$^{lox/-}$ dataset from the regulatory T cell microarray, a fold change and p-value cutoff of 1.2 and 0.05 was chosen. This allowed for a reasonable number of genes to be used for downstream pathway analyses. Furthermore, a quick scan of these gene lists showed that the genes obtained were biologically relevant. Lowering the fold change cut-off did come at the expense of noise/false positives and this was taken into account in the further analyses.

A total of 485 genes were differentially expressed in regulatory T cells from C57BL/6 mice as compared to BALB/c mice, with 53% being up-regulated and 47% being down-regulated in the C57BL/6 regulatory T cells. In contrast, 103 genes were differentially expressed in regulatory T cells from iLCK^{cre}IL-4Rα$^{lox/-}$ mice as compared to BALB/c mice, with 52% being up-regulated and 48% being down-regulated in the iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells. Interestingly, seven genes (A130002I06RIK, C530038F07RIK, FAM115C, GDPD3, HIP1, IL4, PSG19) were differentially expressed with the same up- or down-regulatory patterns in both the C57BL/6 and the iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells as compared to BALB/c regulatory T cells.

As previously done for the activated T cells, we next investigated the IL-4Rα expression in the microarray and whether it was in accordance with the genotype of each strain. The expected result is that compared to IL-4Rα$^{lox/-}$ T cells, IL-4Rα$^{-/-}$ and iLCK^{cre}IL-4Rα$^{lox/-}$ T cells, should have significantly reduced expression and BALB/c T cells should shower higher expression as result of two IL-4Rα alleles as compared to one. This is observed for the activated T cells discussed earlier. However, no significant differences in IL-4Rα

expression were obtained among the strains for the regulatory T cells. This result was further investigated by performing a BLAST analysis (Altschul *et al*., 1997) to determine the region of hybridization of the microarray probe to the IL-4Rα transcript. This revealed that the probed used, mapped to exon 11 of IL-4Rα (Mohrs *et al*., 1999), an exon that is unaffected by the IL-4Rα deletion process. Taking this into account, the lack of significant differential IL-4Rα expression in the microarray among the strains was not a cause for concern.

It was determined that 30% of the differentially expressed genes in C57BL/6 and 31% in iLCK^{cre}IL-4Rα$^{lox/-}$ regulatory T cells mapped to *Leishmania major* response (*Lmr*) loci. This enrichment was found to highly statistically significant in C57BL/6 vs. BALB/c comparative analysis however not for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c analysis.

The strength of the microarray data was confirmed by the appearance of many genes that formed networks and were involved in pathways. These known networks / pathways included inflammatory response; cell-to-cell signaling and interaction; development and function, immune cell trafficking; cellular development, gene expression and cellular growth and proliferation. The network with the highest score across the three comparisons was cell-to-cell signaling and interaction. Unlike in the activated T cell microarray, the networks did not show similar trends.

In addition, it was observed that the molecules from the network analysis have a number of interactions with other genes. For the C57BL/6 vs. BALB/c network analysis, various interactions including interactions between TNF, IFN-β, TNFSF, ifi202b, CD40, SOCS1 and SlamF7 was observed. Many of these genes have been published previously to be associated with known *Lmr* loci (Kurey *et al.,* 2009). The network analysis for the iLCK^{cre}IL-4Rα$^{lox/-}$ vs. BALB/c comparison also yielded interactions between NFK-β, IgG1, Erk, Traf1, IL-4 and IL-12. These genes from the networks mentioned above all have roles in the T helper differentiation.

Next to be investigated was how these molecules from the network analysis associated with regulatory, TH2 and TH1 molecules. The regulatory molecules selected to show

association to was, IL-10, TGF-β and FoxP3. A number of genes were found to have associations with the regulatory, TH1 and TH2 molecules. The last strategy was to identify candidate genes that utilized the gene ontology terms associated with known marker genes (proliferation, activation, presentation, recruitment and homeostasis). In total, sixty one genes were identified as putative markers of TH1 / TH2 and/or resistance vs. susceptibility during *L. major* infection during the acute phase of the disease.

A similar mining strategy was employed used for the activated T cell microarray, The lead candidate genes that belonged to more than 50% of the focus functional groups were found to be enriched with genes that play significant roles in resistance to *L. major.*

4.5 Identification of candidate genes that may confer protection or susceptibility to *L. major*

Based on the mining strategies applied for both activated T cell and regulatory T cell microarrays, a number of genes were selected as lead candidate genes that may confer protection or susceptibility to *L. major*. One of these genes was CCR5. The significant biological impact for a number of the DE genes during *L. major* infection has been demonstrated in gene deficient mouse models of cell lines. For example, mice deficient for CCR5 displayed skewing towards TH2 differentiation (Wong *et al*., 2003). Therefore, co-clustering of DE genes with these "high biological impact" genes may therefore highlight which of the DE genes should be selected for further analysis. Of the intersection of DE genes, 9 genes were initially selected for further analysis. The standard procedure requires confirmation of the differential expression of these genes using quantitative RT-PCR on the original RNA sample. If indeed the microarray and PCR results are in agreement, genes can then be validated biologically.

From the comparisons that were performed, C57BL/6 vs. BALB/c and iLCK^{cre}IL-4Rα$^{-/lox}$ vs. BALB/c and applying the mining strategy, a number of genes were identified. The study

investigated IL-4 driven TH1 / TH2 function during *L. major* infection. Thus, genes that are DE in the presence or absence of IL4Rα signaling were of interest. To home in on these genes, the number of overlapping genes in the dataset for the abovementioned comparisons was identified. Common genes from the comparisons would be of interest, as it would identify how similar the responses are as both C57BL/6 and iLCK^{cre}IL-4Rα$^{-/lox}$ mice display a resistant phenotype during *L. major* infection. The following genes were common from the two primary comparisons in the activated T cell microarray, CCR5, Mt2, Kdm5d, H2-T10, Gdpd3 and Otud6b. Common genes from the two primary comparisons in the regulatory T cell microarray included, A130002I06RIK, C530038F07RIK, FAM115C, GDPD3, HIP1, IL4 and PSG19. The following three genes were selected to be investigated further, CCR5, H2-T10 and Gdpd3. CCR5 and H2-T10 from the activated T cell microarray, where both these genes were up-regulated in C57BL/6 and iLCK^{cre}IL-4Rα$^{-/lox}$ mice and GDPD3, as this gene was common to both microarrays and down-regulated in both C57BL/6 and iLCK^{cre}IL-4Rα$^{-/lox}$ mice.

Chemokines function in the recruitment, polarization, activation and differentiation of T cells (Murphy 1994, Baggiolini *et al*., 1997, Yoshie *et al*., 1997 and Rossi 2000). CCR5 (Chemokine receptor type 5), also known as CD195, is a protein found on the surface of white blood cells and acts as a receptor for chemokines. This receptor is predominantly expressed on T cells, macrophages, dendritic cells and microglia. It is known that TH1 and TH2 cells have different chemokine receptors. TH1 cells express CCR5, whereas TH2 cells are known to express CCR3 (also found on eosinophils and basophils).

CCR5 was selected as a lead candidate gene to confirm differential expression as observed in the respective microarrays and quantitative RT-PCR. This gene was one of the DE genes that intersected with two or more of the focus functional groups from the activated T cell microarray. CCR5 have multiple roles in the immune response (Kawai *et al*., 1999 and Rabin *et al*., 1999). Firstly, in the recruitment of cells to the site of inflammation and subsequently determines the appropriate immune response by facilitating the immunological synapse formation. The formation of the immunological synapse, which is an organized structure that forms at the interface between an antigen presenting cell (APC)

and a T cell, is facilitated by the initial polarization of a T cell (Wong *et al*, 2003). A prerequisite for signaling through the T cell receptor is the aggregation of lipid rafts at the synapse. These lipid rafts are enriched with signaling molecules that serve as platforms for signaling pathways. Chemokine receptor function depends on lipid rafts. Once CCR5 is activated by CCL4, it will localize to lipid rafts. CCR5 activation is dependent on the integrity of the lipid rafts. Wong *et al* demonstrates when treated with cholesterol extracting agents, disruption of the ligand binding and signaling from CCR5 occurs.

CCR5 also serves as a receptor for a number of inflammatory chemokines, including CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (Rantes). CCL5 is the ligand for CCR5. Induction of TH1 responses by bone marrow derived macrophage and dendritic cells can occur through the interactions between CCR5 and its ligand, independent of IL12. Thus, chemokines can affect T cell differentiation (Rissoan *et al*., 1999). A previous study has shown that $CCR5^{-}$ mice, gave rise to a skewed TH2 cytokine profile (Wong *et al*., 2003). Therefore, it appears that CCR5 is involved in determining the fate of T helper cells. We hypothesize that the interaction between CCR5 and CCL5 aids in the initial recruitment of T cells to an inflamed popliteal lymph node (during infection with an intracellular parasite *L. major*) and also aids in the differentiation of the TH1 cells.

Consistent with this hypothesis is the observation that IL-4Rα-deficient T cells from *L. major* infected $iLCK^{cre}IL\text{-}4R\alpha^{-/lox}$ and T cells from WT C57BL/6 mice had a 2-fold higher expression of CCR5 and CCL5 when compared to T cells from WT control mice (BALB/c). CCR5 was one of the differentially expressed genes observed in the microarray and this was confirmed by quantitative RT-PCR. CCR5 was found to be expressed at 4.73 fold higher levels in activated T cells and 2.17 fold higher in regulatory T cells from resistant C57BL/6 mice infected with *L. major* as compared to WT (BALB/c). $CD4^{+}$ T cell specific IL-4Rα deficient ($iLCK^{cre}IL\text{-}4R\alpha^{-/lox}$) mice infected with *L major* expressed a 2 fold higher level of CCR5 in both activated T cell and regulatory T cell populations as compared to WT susceptible BALB/c. CCL5 was also differentially expressed in microarray and quantitative RT-PCR demonstrated that CCL5 was expressed at 2.42 fold higher levels in activated T cells and 2.73 fold higher in regulatory T cells from resistant C57BL/6 mice infected with *L.*

major as compared to WT susceptible BALB/c mice. $CD4^+$ T cell specific IL-4Rα deficient ($iLCK^{cre}IL\text{-}4R\alpha^{-/lox}$) mice infected with *L major* expressed a 1.3 fold higher level of CCR5 in activated T cell populations as compared to BALB/c mice.

H2-T10 (Histocompatibility 2-T region locus 10) was the next candidate gene to investigate further as it was one of the DE genes that intersected with two or more of the focus functional groups. This gene is a protein coding gene that maps to chromosome 17, and forms an integral part of the plasma membrane. H2-T10 was differentially expressed and was significantly up regulated in both the resistant C57BL/6 and $iLCK^{cre}IL\text{-}4R\alpha^{-/lox}$ groups when infected with *L. major*. Interestingly, H2-T10 binds the T cell receptor and is regulated by IFN-γ type 1 and dendritic cells. H2-T10 is involved in the regulation of three genes, FYN, LCK and Vav1. Functional characteristics of these genes are listed in Table A5 in the appendix. Of interest to this study, FYN, LCK and Vav1 are lipid raft associated signaling components that form part of the T cell receptor complex (Dykstra *et al*., 2003). The biological processes associated with H2-T10 include antigen processing and presentation as well as immune response. H2-T10 may not be directly linked to the formation of the immunological synapse, however, it regulates three genes that have a direct interaction with the T cell receptor and the lipid raft associated signaling components. Currently there are no gene deficient mouse models available.

The cellular and subcellular location of FYN is the immunological synapse, membrane rafts, and plasma membrane. It is located on chromosome 10 and is involved in G-protein coupled receptor signaling and T cell receptor signaling. Its biological processes include, T cell co-stimulation and activated T cell proliferation. FYN is known to regulate CD3 TCR and is regulated by TCR, CD3 and FYN.

The cellular and subcellular location of LCK is immunological synapses, membrane compartments, membrane rafts, and plasma membrane. It is located on chromosome 4 and is involved in T cell receptor signaling, IL-2 signaling and NFkβ signaling. Its biological processes include positive regulation of T cell receptor signaling, T cell co-stimulation,

proliferation, apoptosis and T cell differentiation. LCK is known to be regulated by CD3, TCR and binds CD4 and CD3.

The cellular and subcellular location of VAV1 is the membrane rafts, cytoplasmic fraction and plasma membrane. It is located on chromosome 17 and is involved in CD28 signaling in T helper cells, T cell receptor signaling and PKCθ signaling in T lymphocytes. Its biological processes include proliferation, cell development, immune response regulating cell surface receptor signaling, regulation of cell adhesion, T cell activation, T cell co-stimulation and T cell differentiation. VAV1 is known to regulate RAC1, CDC42 and is regulated by TCR, CD3 and CD28.

Gdpd3 (Glycerophosphodiester phosphodiesterase domain containing 3) is a protein coding gene which maps to chromosome 7 and is located in lmr21, near the Dice 2 locus. This locus is involved in glycerophospholipid metabolism. Gdpd3 forms an integral part of the membrane and its molecular function is glycerophosphodiester phosphodiesterase activity. It is known that Gdpd3 interacts with cullin2 (CUL2) (Bennet *et al.*, 2010). Cullin2 is involved in the adaptive immune system and antigen processing, ubiquitination and proteosome degradation. Currently there are no gene deficient mouse models available for cullin2 only cell lines. In addition, cullin2 is also involved in the negative regulation of the Jak-Stat (Janus tyrosine kinase-signal transducer and activator of transcription) signaling pathway. This signaling pathway aids in mediating the transduction of information between cells that are essential for development and its primary role is cell proliferation. Functional characteristics of Gdpd3 are listed in Table A5 in the appendix. Gdpd3 was selected as a candidate gene as it intersected with two or more of the focus functional groups. This gene was observed to be significantly down regulated in the resistant C57BL/6 and iLCK^{cre}IL-4Rα$^{-/lox}$ groups when infected with *L. major* for three weeks.

CCR5 was selected as one of the lead candidate genes. From the results obtained in this study, we observed an up regulation of CCR5 in C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$, CD4^{+} T cell knockout mice when compared to the susceptible BALB/c mice. It was observed that even on a susceptible background, when IL-4Rα signaling is omitted on CD4^{+} T cells only, CCR5

is up regulated at mRNA level (quantitative RT PCR) similar to that of resistant C57BL/6 mice. It is also known that CCR5 plays an important role in the formation of the immunological synapse, hence aiding in the TH1 / TH2 differentiation process. We therefore hypothesize that in C57BL/6 and iLCK^{cre}IL-4Rα$^{lox/-}$ (BALB/c mice with a deletion of IL-4Rα signaling on CD4^{+} T cells only), there is a possible difference in the composition and integrity of the immunological synapse, resulting in the differential altered induction of TH1 / TH2 differentiation. The gens that support this hypothesis are Gdpd3, H2-T10 and CCR5. These genes are involved in pathways such as lipid metabolism, raft formation, antigen presentation and T helper cell differentiation. These genes are described in detail above and cited by literature to support this hypothesis.

4.6 Validation of candidate genes

In addition to following the mining strategy to obtain DE genes, a few other gens were selected to for quantitative RT-PCR validation. Following identification of candidate genes, the standard procedure is to confirm the differential expression of the genes by quantitative RT-PCR on the original RNA sample. If the microarray and PCR results are in agreement, genes are validated biologically. The following genes were evaluated to check conformity, IL-18r1, STAT4, Rnf130, Rapgef4, CCR5 and CCL5. These genes were selected based on its relevance to TH1 / TH2 differentiation and being part of the main focus groups obtained when implementing the mining strategy.

IL-18r1 expression by quantitative real time PCR and the differential expression of the receptor by microarray, similar trends of expression amongst the various knockout groups (iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$) and its controls (BALB/c and C57BL/6) were observed. A slightly elevated level of IL-18r1 expression in the resistant control, C57BL/6 in the activated T cell population was observed when compared to the other BALB/c mice. From the PCR data the elevation is not significantly higher, although in its microarray of the activated T cells, significance is shown. This receptor is selectively expressed on murine

TH1 cells but not TH2 cells (Xu *et al*., 1998), is involved in the T helper differentiation pathway and can thus be used as a surface marker to distinguish TH1 cells from TH2 cells.

STAT4 expression by quantitative real time PCR and the differential expression of the transcription factor by microarray showed similar trends of expression in the quantitative real time PCR data from the activated T cell population amongst the various knockout groups, however we observe a significantly higher expression of STAT4 was observed in the resistant C57BL/6 group than in the activated T cell microarray. STAT4 expression appears to be elevated in the global IL-4Rα$^{-/-}$ mice from the regulatory T cell PCR data, however, this is not seen in the microarray. STAT4 binds IFN-γ and also plays a role in the T helper cell differentiation pathway (Hoey *et al*., 2003). Ramp3 expression by quantitative real time PCR and the differential expression of the receptor by microarray showed similar trends of expression amongst the various knockout groups (iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$) and its controls (BALB/c and C57BL/6). In the activated T cell microarray a significantly higher expression of this gene in the global IL-4Rα$^{-/-}$ mice was observed when compared to the susceptible BALB/c control. In the regulatory T cell population similar expression of Ramp3 across all the groups for both the quantitative real time PCR and the microarray was observed. Ramp3 is primarily involved intracellular protein transport, receptor-mediated endocytosis, regulation of G-protein coupled receptor protein signaling pathway and protein transport (Kuwasako, *et al*., 2008).

A significantly decreased level of Rnf130 in resistant C57BL/6 mice was observed when compared to the susceptible BALB/c control in the activated T cell population. This observation was seen in both the quantitative real time PCR data as well as in the microarray data. The knockout groups, iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$ expressed equivalent levels of Rnf130 when compared to the BALB/c control in the activated T cell data. Equivalent expression levels of Rnf130 were observed for all the groups in the regulatory T cell population from the quantitative real time PCR data as well as the microarray data. This gene is regulated by STAT4 which is a transcription factor for TH1 differentiation.

For Rapgef4 expression, the resistant C57BL/6 mice consistently expressed elevated levels of Rapgef4 when compared to the susceptible BALB/c mice. This was observed in the quantitative PCR data as well as in the microarray data in both activated and regulatory T cells. Both iLCK^{cre}IL-4Rα$^{lox/-}$ and global knockout IL-4Rα$^{-/-}$ expressed higher levels of this gene in the quantitative PCR data for both cell populations when compared to the susceptible BALB/c control. It can thus be deduced that Rapgef4 has a possible role in resistance to *L. major* and that the absence of IL-4Rα may play a role in the switch from a susceptible TH2 response to a resistant TH1 response.

IL-18r1, STAT4, Rnf130, Rapgef4 were selected to perform validation quantitative RT PCR amongst the other genes, CCR5 and CCL5 on because of its possible role TH1 / TH2 differentiation.

4.7 Conclusion and future work

The study aimed to identify the gene expression patterns in CD4$^+$ T cells that are associated with resistance or susceptibility to *L. major*, using a transcriptomics approach.

The specific objectives of this study were to (1) perform a minimum of three comparative *L. major* infections comprising from iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6 mice at 3 weeks post infection; (2) isolate activated and regulatory T cells at three weeks post infection; (3) purify total RNA isolated from activated and regulatory T cells and profile the gene expression of these by DNA microarray; (4) analyze the microarray using bioinformatics to identify genes and pathways important for host protection or disease progression and (5) validate the microarray results by quantitative RT- PCR.

For this study, all of the specific objectives were met. In total, three independent biological experiments were completed and the activated and regulatory T cells were isolated to both high purity and yield. The RNA was successfully extracted from the T cells and was linearly amplified to generate cDNA. Pre-validation quantitative real time PCR and phenotypic data indicated that the biology of the experiments were of a high standard. Transcriptional

profiling by microarray was used to assess the differences in activated and regulatory T cells isolated from the various groups (iLCK^{cre}IL-4Rα$^{lox/-}$, IL-4Rα$^{lox/-}$, IL-4Rα$^{-/-}$, BALB/c and C57BL/6).

Various comparisons from both the activated T cell and regulatory T cell microarrays were explored and in addition a mining strategy was developed to generate a list of differentially expressed genes. Functional data of the DE genes that were clustered in the "focus" groups based on their gene ontology associations, Babelomics / IPA analysis and previously published functional roles was reviewed. Candidate genes belonging to two or more of the focus groups was then selected for further analysis. The rationale for selecting genes which intersected with two or more (ie >50%) of the focus functional groups, was that this strategy may increase the probability of selecting candidate genes which have a significant biological impact on the resistance of *L. major* infections. Differentially expressed genes that intersected with two or more functional groups were found to be enriched for these genes which have already been described in the literature to play a significant role in resistance to *L. major.*

Following identification of candidate genes, standard procedure is to confirm the differential expression of the genes by quantitative RT-PCR on the original RNA sample. A few genes where selected to be validated using quantitative RT-PCR. Good correlation was observed between the quantitative PCR data and the microarray data. The strong overlap of the microarray data and the validation quantitative PCR data supports the strength of the microarray experiments and the potential use of the data to identify novel genes important during *L. major* infection.

The study hypothesized that the integrity of the immunological synapse and pathways related to it, including lipid metabolism and raft formation aid in the T helper cell differentiation. A number of pathways and networks for this hypothesis were enriched in our datasets. Differentially expressed genes were also found to be linked to known *Lmr* loci which further confirmed that the microarrays were of biological relevance. We also further

hypothesize that signaling via the IL-4Rα may change the integrity and composition of the immunological synapse resulting in T helper cell differentiation.

Future experiments would include *in vitro* siRNA knock-down experiments. This entails knocking down the candidate gene with siRNA in either bone marrow derived macrophages or thioglycollate-elicited peritoneal macrophages infected with *L. major* and then stimulating the cells with either IL-4 and IL-13 (TH2) or IFN-γ and LPS (TH2). The expression of known TH1 and TH2 markers will be assessed to determine whether the absence of the candidate gene results in an altered expression profile.

Lead candidate genes that induce an altered TH1 or TH2 phenotype in the *in vitro* siRNA experiments will be validated *in vivo* using gene deficient mice. This entails infecting mice deficient for the candidate gene with *L. major* and then assessing the disease phenotype. Mice lacking the candidate genes associated with TH2 are expected to be resistant to infection as TH2 differentiation are associated with enhanced disease severity (Radwanska *et al*., 2007). In contrast, mice lacking the candidate genes associated with TH1 are expected to be susceptible to infection as TH1 are known to play a protective role during infection (Radwanska *et al*., 2007).

In conclusion, this study serves as a basis for the identification of novel genes associated with susceptibility and or resistance to *L. major* infection when IL-4Rα is not present of $CD4^+$ T cells. Differentially expressed genes were hypothesized to play an important role in the formation and integrity of the immunological synapse, thereby aiding in the differentiation process. The ultimate aim of this work may be extrapolated to humans and mechanisms of controlling *L. major* infection may be identified so as to improve the quality of life of the patients suffering from the disease.

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Appendix

Appendix A

General reagents

Anaesthetic 1.2 ml Anaket-V (100 mg/ml) (Centaur labs, Isando) 0.8 ml Rompun (2 %) (Bayer, Germany) 8.0 ml PBS (1X) **FACS Buffer** 0.1% BSA (Roche) 0.05% NaN_3 (Merck) Made up in 1X PBS **Phosphate Buffered Saline (PBS 10X)** 80g NaCl (1.37M) 2g KCl (0.03M) 14.4g H_2PO_4 (0.01 M) 2.4g KH_2PO_4 Dissolve in 1 L ddH2O **Red cell lysis buffer** 5 mM EDTA 150 mM NaCl 10 % glycerol 25 mM Tris-Cl pH 7.5 0.1 % SDS 1 % Triton –X 100 0.5 % Non idet P-40 0.5 % Deoxycholate 5 mM PMSF Make to 1L with ddH_2O	**Blocking Buffer** 20g Milk powder (spar instant) (2 %) Make up to 1 L with 1X PBS **Coating Buffer** 10 ml 10x PBS 8g BSA (Merck) Make to 1L in ddH_2O and pH 9.5 **Dilution Buffer** 10g BSA (1 %) (Roche) 0.2g NaN_3 (0.02 %) (Merck) Make up to 1L with 1X PBS **Substrate Buffer** 0.2g NaN3 (0.02 %) 97 ml di-ethanolamine 0.8g $MgCl_2.6H_2O$ 700 ml ddH_2O Adjust the pH to 9.8 and make up to 1 L with ddH_2O **Washing buffer** 20g KCL 20g KH_2PO_4 144g $Na_2HPO_4.H_2O$ 800g NaCl (Merck-BDH) 50 ml Tween 20 (Sigma) 100 ml 10 % NaN_3 (Merck) Make up to 5 L with ddH_2O

Appendix B: Antibody details

The gating strategies of cellular quantifications in the popliteal lymph node in Chapter 3 are given below.

Table 23 List of FACS antibodies

Antibody	Conjugation	Dilution factor
CD4	PerCP	1:640
CD44	FITC	1:320
CD62L	APC	1:1280
CD4	FITC	1:1280
CD25	Biotin	1:320
CCR5	PE	1:100
FoxP3	PE	1:50
7AAD		1:1000
Rat serum		1%
FcγRII/III		1:325

Table A.1. Differentially expressed genes in C57BL/6 vs BALB/c, (FC >1.2, p < 0.05) in activated T cell microarray (Number of genes = 456).

Probe_IDs	Gene_ID	Regulation in C57BL/6 vs. BALB/c	*p*-value	*Lmr* locus
6964245	Gdpd3	-63.44	6.5923E-05	*Lmr21*
6855022	H2-Ea	-58.65	0.01658462	
6753067	Ctse	-24.96	0.00158577	*Lmr20*
6901952	Gbp1	-14.09	0.00507624	
6889893	Scg5	-7.63	0.00097295	*Lmr14*
6788290	Il4	-7.15	0.00452007	*Lmr15*
6764289	Ifi202b	-7.11	0.00632603	*Lmr8**
6813887	Ctsl	-6.99	0.00281704	
6980101	Cd209d	-6.86	0.02486814	
6755378	Kmo	-6.39	0.00733372	*Lmr8*
6935701	Alox5ap	-6.16	0.01825812	
6960834	Siglech	-6.03	0.0039643	*Lmr21*
6993138	Ccr9	-5.34	0.0005895	
6905419	F630111L10Rik	-5.31	0.03591767	*Lmr11*
6763706	Atp1b1	-5.13	0.00033475	*Lmr8*
6844387	Olfr164	-5.12	0.00931687	*Lmr23*
6905408	P2ry14	-5.07	0.04572455	*Lmr11*
6818958	Rnase6	-4.95	0.04265478	
6876735	Kynu	-4.61	0.02071858	
6939130	Ociad2	-4.59	0.01928861	
6751535	Ramp1	-4.43	0.00914693	*Lmr20*
6861850	Tcf4	-4.37	0.03128071	*Lmr13*
6991264	Ctsh	-4.15	0.01504898	
6780570	Havcr1	-3.89	0.02588761	*Lmr15*
6849761	Dnahc8	-3.76	0.01815916	*Lmr1*
6780777	Rnf130	-3.66	0.04996613	*Lmr15*
7015521	Cybb	-3.61	0.02243947	
6798285	Pld4	-3.58	0.0206608	
6837008	Csf2rb2	-3.53	0.00906494	
6890312	Ttbk2	-3.43	0.00224024	*Lmr14*
6988855	Cadm1	-3.31	0.0144072	*Lmr2**
6959584	Tyrobp	-3.30	0.01386917	*Lmr21*
6809655	Cd180	-3.27	0.01598122	
6911337	Lyn	-3.16	0.04123993	*Lmr9**
6980091	Cd209a	-3.02	0.04885126	
6760642	Col6a3	-2.89	0.02927433	*Lmr20*
6887854	Chn1	-2.79	0.00034702	

6907714	Sycp1	-2.71	0.00445272	*Lmr11*
6892964	Pltp	-2.70	0.04218833	*Lmr16*
6789325	Cd68	-2.70	0.01782912	*Lmr15*
6876238	Angptl2	-2.60	0.0049717	
6990952	Bckdhb	-2.60	0.03129954	
6908745	Rwdd3	-2.54	0.00569991	*Lmr11*
6849968	H2-Eb1	-2.54	0.04733397	*Lmr1*
6880718	Sqrdl	-2.53	0.00920881	*Lmr14*
6784345	Grn	-2.50	0.01547731	
6969874	Art2a	-2.50	0.04605417	
6854393	Fahd1	-2.47	0.01324762	*Lmr1*
6998900	Tdgf1	-2.43	0.03240203	
6804405	Grhl1	-2.39	0.00198439	*Lmr22*
6755630	Sccpdh	-2.38	0.04776039	*Lmr8*
6871874	Gcnt1	-2.37	0.02454751	
6907652	Slc22a15	-2.37	0.00162074	*Lmr11*
6870697	Atrnl1	-2.37	0.00878645	
6868041	Cybasc3	-2.36	0.01036561	
6865551	Ppic	-2.32	0.01483366	*Lmr13*
6762804	Rgs18	-2.31	0.02247044	*Lmr8*
6782919	Ccl8	-2.28	0.01361971	*Lmr15*
6905422	P2ry13	-2.23	0.02555837	*Lmr11*
6983115	Zfp868	-2.22	0.00415558	*Lmr10*
6865902	2010002N04Rik	-2.21	0.02912583	*Lmr13*
6782105	Mgl1	-2.18	0.02506773	
6954269	Hpgds	-2.16	0.02895954	*Lmr4*
6873111	Blnk	-2.14	0.01919636	
6800913	Polr2h	-2.14	0.02482039	*Lmr23*
6790655	Scpep1	-2.14	0.04942001	
6851103	Kat2b	-2.13	0.01181798	*Lmr1*
6870104	As3mt	-2.12	0.01481824	
6878810	Olfr1094	-2.12	0.04049741	
6791914	Cd79b	-2.11	0.02859897	
6850017	H2-DMb1	-2.10	0.00488079	*Lmr1*
6927227	2810405K02Rik	-2.07	0.00578993	
6914190	Tlr4	-2.07	0.03831536	
6817396	Adk	-2.03	0.04470448	
6946785	Gng12	-2.00	0.04521871	*Lmr4*
6961991	Hddc3	-1.99	0.02278186	*Lmr21*
6758027	Tbc1d8	-1.99	0.04980325	*Lmr20*
6918015	Pla2g2d	-1.97	0.0261803	
6995526	1110032A03Rik	-1.97	0.04089204	*Lmr2*

6955698	Lrig1	-1.97	0.03099483	
6820323	5031414D18Rik	-1.95	0.0193377	
6978923	Cdh1	-1.94	0.02713129	
6903088	Hey1	-1.93	0.00129306	
6855202	Ppp1r11	-1.93	0.02711894	*Lmr1*
6958059	Ldhb	-1.91	0.01779387	
6912577	Polr1d	-1.90	0.04378076	
6968126	Arrdc4	-1.90	0.00465251	*Lmr21*
6805108	Otud6b	-1.89	0.02503586	*Lmr9*
7017699	Pls3	-1.89	0.03064641	
6941685	Aldh2	-1.89	0.01761513	
6766063	Adat2	-1.88	0.03333431	
6874631	Phyh	-1.86	0.01362147	
6965901	Atp1a3	-1.85	0.02059886	*Lmr21*
6966145	Zfp36	-1.84	0.0409055	*Lmr21*
6815111	Jmy	-1.84	0.01340618	
6792649	Timp2	-1.83	0.0076297	
6848572	Fgfr1op	-1.82	0.02431158	*Lmr7*
6882611	Phf20	-1.82	0.00081202	*Lmr16*
6759549	Ikzf2	-1.80	0.02637114	*Lmr20*
6803161	Lgmn	-1.79	0.03119906	
6956765	Rassf4	-1.77	0.01963004	
6831800	Csf2rb	-1.77	0.01117184	
6960578	Nav2	-1.77	0.03073576	*Lmr21*
6789339	Amac1	-1.76	0.01737601	*Lmr15*
6861751	D18Ertd653e	-1.75	0.0366812	*Lmr13*
6977589	Inpp4b	-1.74	0.04629778	
6980598	2410022L05Rik	-1.72	0.01847857	
6924366	Scp2	-1.72	0.02248478	
6848553	Tagap	-1.71	0.04648494	*Lmr7*
6812214	Tubb2a	-1.71	0.0354927	
6958269	Itpr2	-1.70	0.00341098	
6919417	Tox	-1.68	0.04876886	*Lmr9*
6954385	Gadd45a	-1.68	0.00215183	*Lmr4*
6977846	Phkb	-1.67	0.04377779	
6754138	Rgs16	-1.67	0.00376459	*Lmr8*
7017604	Renbp	-1.66	0.01429746	
6996191	Glce	-1.63	0.03817523	*Lmr2*
6867566	Cpt1a	-1.62	0.00232489	
6835984	Mtss1	-1.61	0.00299817	
6971656	Chst15	-1.61	0.0261638	*Lmr21*
6980107	Cd209c	-1.61	0.00617314	

6957272	D130058E03	-1.61	0.04692001	
6919320	Penk	-1.61	0.02431637	*Lmr9*
6900254	Dennd2d	-1.61	0.04807682	*Lmr11*
6779432	Bcl11a	-1.60	0.04357257	*Lmr6*
7011377	Ddx26b	-1.60	0.04949251	
6933014	Ptpn13	-1.60	0.00268147	
6785762	Nudcd3	-1.58	0.02497472	*Lmr6*
6807074	Syk	-1.57	0.043697	
6774719	Arid5b	-1.57	0.016905	*Lmr19*
6849327	Nme3	-1.57	0.00496098	*Lmr1*
6958078	St8sia1	-1.56	0.03236801	
6843384	Ttc3	-1.56	0.03983991	*Lmr18*
6922026	Ptgr1	-1.55	0.04448142	
6935094	Zcwpw1	-1.55	0.03197834	
6956926	Erc1	-1.55	0.01509309	
6994705	Spa17	-1.55	0.0256844	*Lmr2*
6922241	Alad	-1.54	0.02551281	
6913133	1700055D18Rik	-1.54	0.00701961	
6860430	LOC100042767	-1.54	0.00316635	
6788728	4930412M03Rik	-1.54	0.03322737	
6860780	Tnfaip8	-1.53	0.01937005	*Lmr13*
6757120	Stau2	-1.53	0.01469778	*Lmr20*
6784829	Map2k6	-1.52	0.03913024	
6963422	Swap70	-1.51	0.02156892	
6876219	Ptrh1	-1.50	0.01388439	
6991461	Slc9a9	-1.50	0.0064597	
6968643	Ntrk3	-1.50	0.0312964	*Lmr21*
6873476	Calhm2	-1.49	0.01854893	
6874085	4930506M07Rik	-1.49	0.04709669	
6849515	Pacsin1	-1.49	0.0075557	*Lmr1*
6806219	Fars2	-1.48	0.02197318	
6769204	Ndufs7	-1.47	0.04799513	*Lmr19*
6968018	Mef2a	-1.46	0.0487577	*Lmr21*
6899743	Sv2a	-1.46	0.03546496	*Lmr11*
6901737	Adh1	-1.46	0.01272266	
6929260	Klhl7	-1.45	0.03069464	
6979439	Plcg2	-1.45	0.03998227	
6827944	Gpr183	-1.44	0.00313854	
6752571	Ccdc93	-1.44	0.02379693	*Lmr20*
6769634	Gnptab	-1.43	0.00883444	*Lmr19*
6776152	4932415G12Rik	-1.43	0.01740469	*Lmr19*
6960287	Napsa	-1.42	0.04542982	*Lmr21*

6964382	Itgax	-1.42	0.00868636	*Lmr21*
6789741	Serpinf1	-1.42	0.01737644	*Lmr15*
6831927	Cby1	-1.41	0.04816391	
6796339	Rdh12	-1.41	0.01254551	*Lmr22*
6946931	Vps24	-1.40	0.03604998	*Lmr4*
6770013	Eea1	-1.40	0.01541705	*Lmr19*
6800060	Ahr	-1.40	0.02449899	*Lmr22*
6890193	Ccdc32	-1.39	0.00408642	*Lmr14*
6950397	8430419L09Rik	-1.39	0.01696055	
6840199	Lpp	-1.38	0.01271137	*Lmr12*
6909792	Dapp1	-1.38	0.02904414	
6843184	Ifngr2	-1.37	0.02228764	*Lmr18**
6784236	Atp6v0a1	-1.36	1.6143E-05	
6838754	Calcoco1	-1.36	0.03439407	
6767402	Sesn1	-1.36	0.0178282	
6892384	Edem2	-1.36	0.03287585	*Lmr16*
6847824	4932438H23Rik	-1.36	0.01130555	*Lmr18*
6887179	Dpp4	-1.36	0.03912838	
6847748	Tiam1	-1.35	0.017937	*Lmr18*
6882499	a	-1.34	0.01621417	Lmr13*
6975064	Fut10	-1.34	0.0178798	*Lmr10*
6765460	Cr2	-1.34	0.04972229	*Lmr20*
6840629	Heg1	-1.34	0.0485809	*Lmr12*
6834975	Rnf19a	-1.34	0.02107743	
6792650	RP23-394O9.3	-1.33	0.03040018	
6869027	Jak2	-1.33	0.03823321	
6754776	Brp44	-1.32	0.04305995	*Lmr8*
6983608	Smad1	-1.32	0.01569378	
6992490	Epm2aip1	-1.32	0.04386657	
6910088	Hs2st1	-1.32	0.01992169	
6989553	Hexa	-1.32	0.00428176	*Lmr2*
6899016	Arhgef11	-1.31	0.00314213	*Lmr11*
6957144	Lag3	-1.31	0.0428661	
6993465	Endod1	-1.31	0.03284209	*Lmr17*
6831157	Phf20l1	-1.30	0.04118845	
6780443	Ebf1	-1.30	0.00193787	*Lmr15*
6929655	Khk	-1.30	0.02175911	
6884277	Tpd52l2	-1.30	0.00395644	
6794059	Rnaseh1	-1.30	0.03558572	*Lmr22*
6938678	Tlr1	-1.30	0.0179491	
6804033	Wdr60	-1.29	0.02515024	
6949473	Fbxl14	-1.29	0.03827057	

6783330	Rnf43	-1.29	0.02758997	*Lmr15*
6810333	Esm1	-1.29	0.02181983	
6783626	Mbtd1	-1.29	0.04412346	
6875961	Mrps2	-1.29	0.00908533	
6823742	Hacl1	-1.28	0.04367946	
6954638	Tgoln1	-1.28	0.0119932	*Lmr4*
6849992	Wdr46	-1.27	0.02327175	*Lmr1*
6934662	0610007L01Rik	-1.27	0.02883146	
7015255	Mid1	-1.27	0.04315216	
6857431	Strn	-1.26	0.01562701	
6876943	Kif5c	-1.26	0.01426185	
6906433	D930015E06Rik	-1.26	0.04360848	*Lmr11*
6774336	Sgpl1	-1.26	0.01659704	
6885070	Pip4k2a	-1.26	0.00773225	
6976609	Ddx60	-1.26	0.00477551	*Lmr10*
6838349	Senp1	-1.26	0.01173002	
6772763	Taar7a	-1.25	0.01469976	
6861711	Slmo1	-1.25	0.04893485	*Lmr13*
6796331	Arg2	-1.25	0.03292034	*Lmr22*
6931790	Srd5a3	-1.25	0.03587391	
6871499	Dak	-1.25	0.0334434	
6868055	A430093F15Rik	-1.24	0.00500853	
6926502	Fhad1	-1.24	0.03466988	
6859781	Wdr33	-1.24	0.003683	*Lmr13*
6863417	6030446N20Rik	-1.23	0.00523553	
6771259	D930020B18Rik	-1.23	0.04349905	*Lmr5*
6752848	Mgat5	-1.23	0.01930707	*Lmr20*
6798601	Ncoa1	-1.23	0.02461384	*Lmr22*
6899967	Trim45	-1.22	0.02302552	*Lmr11*
6790621	Msi2	-1.22	0.04944914	
6917069	Gnl2	-1.22	0.02969357	
6765186	Rps6kc1	-1.22	0.00666749	*Lmr20*
6837105	Csnk1e	-1.22	0.04659457	
6868371	Gna14	-1.21	0.02738894	
6992887	Exog	-1.21	0.04574609	
6914007	Orm1	-1.20	0.00331422	
6823653	Il17rb	-1.20	0.04861021	
6788862	Ulk2	-1.20	0.04080512	*Lmr15*
6882397	Dnmt3b	-1.20	0.04275656	*Lmr14*
6839008	Cluap1	-1.20	0.02654342	*Lmr23*
6991777	Cep70	-1.20	0.02283281	
6767631	Hace1	-1.20	0.02091835	

6791881	Limd2	1.20	0.03539809	
6971359	Zfp689	1.20	0.0228968	*Lmr21*
6934892	Stx1a	1.20	0.0372897	
6918125	Padi2	1.20	0.0102547	
6844291	Med15	1.21	0.01790483	*Lmr23*
6946018	AI854703	1.21	0.0369642	
6950257	Tas2r117	1.21	0.04993896	
6931181	Pgm1	1.21	0.04371763	
6788595	Olfr30	1.21	0.02641861	*Lmr15*
6863323	Esco1	1.22	0.02036344	
6764089	Ly9	1.23	0.01675059	*Lmr8*
6976975	Tm6sf2	1.23	0.04117926	*Lmr10*
6753033	Lgtn	1.24	0.02806524	
6933512	Tpst2	1.24	0.03163461	
6787741	Nipal4	1.25	0.04377136	*Lmr15*
6933675	Pxn	1.25	0.01921934	
6836341	Sla	1.25	0.01222784	
6791171	Tbx21	1.25	0.0377593	
6983172	Armc6	1.25	0.04338844	*Lmr10*
6798153	Rcor1	1.26	0.04960617	
6785463	Npb	1.26	0.00233673	
6901084	Tram1l1	1.27	0.0209641	*Lmr11*
6874947	Itih5	1.27	0.04200181	
6832256	Bik	1.27	0.04788735	
6856365	A930025A13Rik	1.27	0.02786781	
6803895	Akt1	1.27	0.00868045	
6871903	Ostf1	1.27	0.03951073	
6813094	Susd3	1.28	0.02561303	
6984071	Snx20	1.28	0.02237282	
6887089	Rbms1	1.28	0.01839074	
6892380	Trpc4ap	1.29	0.04093896	
6934902	Tbl2	1.29	0.0293296	
6963898	2610020H08Rik	1.30	0.02873141	
6891898	Napb	1.30	0.01708198	*Lmr14*
6848179	Dscam	1.30	0.01806987	*Lmr18*
6893556	Zbp1	1.30	0.00922888	
6925590	Txlna	1.30	0.0059244	
6802507	Pomt2	1.31	0.01442624	*Lmr22*
6790581	Olfr462	1.32	0.02214632	*Lmr15*
6880776	Sema6d	1.32	0.03081683	*Lmr14*
6905421	Gpr87	1.32	0.00236886	*Lmr11*
6915559	Fggy	1.33	0.0153838	

6852389	2410091C18Rik	1.34	0.02472063	
6942441	Hip1	1.34	0.00856514	
6991942	9630041A04Rik	1.34	0.02906521	
6963558	Arntl	1.35	0.00597454	
6996127	Lrrc49	1.35	0.04661955	*Lmr2*
6931321	Nsun7	1.36	0.01004226	
6887079	Cd302	1.36	0.01519311	
6893279	Nfatc2	1.37	0.0389197	*Lmr16**
6992172	Dusp7	1.37	0.01127009	
6884267	Lime1	1.38	0.00532311	
6798392	Vipr2	1.38	0.04682953	
6768853	Adora2a	1.39	0.03268465	*Lmr19*
6966328	Usf2	1.40	0.02724905	*Lmr21*
6953800	Nod1	1.41	0.04516387	*Lmr4*
6962107	Sh3gl3	1.41	0.04095174	*Lmr21*
6977692	Lphn1	1.41	0.04398454	
6917063	9930104L06Rik	1.41	0.02005684	
6883125	Mmp9	1.41	0.03582836	
6945786	Zyx	1.41	0.04431665	*Lmr4*
6774020	Man1a	1.41	0.04041668	
6749392	Stat4	1.42	0.01936232	*Lmr20*
6868110	Ms4a4d	1.42	0.00574496	
6790928	Wfikkn2	1.43	0.02793603	
6987446	Zfp809	1.43	0.03514745	*Lmr2*
6917656	Paqr7	1.43	0.04575547	
6893186	Ube2v1	1.43	0.03408674	
6758588	Obfc2a	1.44	0.03515568	
6992878	Acaa1a	1.44	0.03055147	
6981573	Wrn	1.45	0.04127672	*Lmr10*
6849241	Dcpp1	1.45	0.03906687	*Lmr1*
6862448	Pard6g	1.45	0.00117398	
6850786	Ccnd3	1.45	0.01200664	*Lmr1*
6885431	Tmem141	1.45	0.03993847	
6769366	Chst11	1.45	0.04328637	*Lmr19*
6791921	Ern1	1.46	0.01863611	
6919885	Otud6b	1.46	0.03193141	*Lmr9*
6913975	Bspry	1.46	0.03766598	
6852849	Cript	1.46	0.00234591	
6953929	Vopp1	1.46	0.03848534	*Lmr4*
6775472	Gna15	1.47	0.01729143	*Lmr19*
6892285	Cdk5rap1	1.47	0.03742276	*Lmr16*
6817616	4931406H21Rik	1.47	0.03664528	

6844407	Olfr170	1.48	0.04161195	*Lmr23*
6919003	Tnfrsf25	1.48	0.04864592	
6806893	A830005F24Rik	1.48	0.04067765	
6854384	Gfer	1.48	0.03765385	*Lmr1*
6978736	Cmtm3	1.49	0.04763099	
6893057	Sulf2	1.49	0.02793558	
6994883	Crtam	1.50	0.02214225	*Lmr2*
6875038	Il2ra	1.50	0.04352504	
6998584	Abhd14a	1.51	0.00850314	
6976956	Lpar2	1.51	0.04966683	*Lmr10*
6954982	Hk2	1.51	0.00157301	*Lmr4*
6956965	Cecr5	1.54	0.00467121	
6958974	Pglyrp1	1.55	0.00619044	*Lmr21*
6907945	Chi3l3	1.55	0.0212381	*Lmr11*
6932336	Afp	1.55	0.01156338	*Lmr3*
6972990	Ube2m	1.55	0.0326654	*Lmr21*
6975307	Tmem66	1.56	0.00253581	*Lmr10*
6860710	Eno1	1.57	0.02514762	
6968836	Fsd2	1.57	0.0093477	*Lmr21*
6810682	Calml3	1.58	0.04816957	
6958905	Slc1a5	1.59	0.00273613	*Lmr21*
6881123	Slc20a1	1.59	0.02939269	*Lmr14*
6868216	Olfr1469	1.60	0.04894665	
6959557	Zfp260	1.61	0.04204331	*Lmr21*
6750519	Il8rb	1.61	0.03039108	
6844400	Olfr168	1.61	0.02375756	*Lmr23*
6945837	Olfr435	1.62	0.0076167	*Lmr4*
6760042	Fam124b	1.62	0.03609505	*Lmr20*
6872250	Tjp2	1.63	0.04661669	
6858871	Cables1	1.63	0.02399719	
6959674	Gm6725	1.65	0.04515151	
6766839	Themis	1.66	0.04995736	
6918177	B830004H01Rik	1.66	0.01104493	
6907137	Tnfaip8l2	1.66	0.04972191	*Lmr11*
6990167	Rora	1.66	0.02735123	
6963608	Pde3b	1.66	0.00954652	
6754537	Tnfsf18	1.67	0.006713	*Lmr8*
6953443	Igf2bp3	1.67	0.01916275	*Lmr4*
6848721	D17Ertd663e	1.68	0.01322297	
6957428	Klri2	1.68	0.03230592	
6878038	Rapgef4	1.69	0.00644244	
6888569	Olfr1197	1.69	0.03123225	

6779827	Nsg2	1.71	0.02860555	*Lmr6*
7013153	Cypt2	1.71	0.03034799	
6771052	Ifng	1.72	0.0473049	*Lmr5**
6807609	2010111I01Rik	1.72	0.02133265	
6814043	Zfp85-rs1	1.73	0.02001141	
6932118	Stap1	1.74	0.02568874	*Lmr3*
6858910	Ttc39c	1.74	0.00947665	
6769481	Btbd11	1.76	0.02181908	*Lmr19*
6981182	Hgsnat	1.78	0.02052178	*Lmr10*
6852438	Cyp1b1	1.78	0.02248427	
6970164	Gvin1	1.78	0.02180314	
6854341	Dnase1l2	1.79	0.00212067	*Lmr1*
6878794	Olfr1036	1.80	0.00262062	
6888668	Olfr1259	1.82	0.02966071	
6792107	Fam20a	1.82	0.00617188	
6868511	BC016495	1.85	0.01720217	
6921381	Coro2a	1.86	0.00683686	
6868243	Olfr1505	1.86	0.04501316	
6890699	Kcnip3	1.86	0.04194681	*Lmr14*
6963114	Olfr618	1.87	0.02262087	
6888413	Olfr1040	1.87	0.03072553	
6926021	Sepn1	1.89	0.01078634	
6855087	Tnf	1.89	0.00556036	*Lmr1**
6950115	Klrb1f	1.89	0.02842606	
6769357	Txnrd1	1.90	0.01484215	*Lmr19*
6871117	Ctsw	1.92	0.03562476	
6754027	Fam129a	1.93	0.02879366	*Lmr8*
6868176	Fam111a	1.93	0.01922008	
6873060	Cyp2c40	1.95	0.0373028	
6888898	Cd82	1.95	0.01203409	
6814055	Zfp87	1.96	0.01992911	
6822297	Clybl	1.97	0.02862278	
6845444	Slc15a2	1.97	0.03229823	*Lmr12*
6845435	Cd86	1.99	0.03660526	*Lmr12*
6868091	Ms4a4c	2.00	0.01958345	
6820472	Epsti1	2.00	0.00120701	
6814044	Zfp874	2.00	0.03118594	
6913042	Glipr2	2.02	0.03575521	
6748893	Il18rap	2.04	0.02706447	*Lmr20*
6763572	Fasl	2.04	0.02340819	*Lmr8*
6957025	Klrg1	2.08	0.01842798	
6850345	Olfr130	2.10	0.00351156	*Lmr1*

6748889	Il18r1	2.12	0.01945442	*Lmr20*
6968781	Furin	2.12	0.01724665	*Lmr21*
7000764	9330169L03Rik	2.17	0.04907309	
6849091	Zfp52	2.23	0.01596919	*Lmr1*
6882538	Acss2	2.24	0.00460851	*Lmr16*
6977108	Zfp617	2.25	0.00381266	*Lmr10*
6780551	Havcr2	2.26	0.00260582	*Lmr15*
6761691	Dbi	2.26	0.0422967	*Lmr20*
6836829	Dgat1	2.28	0.02975661	
6836723	Ly6i	2.30	0.04980991	
6797585	Serpina3f	2.36	0.00641612	
6954415	Il12rb2	2.37	0.00985311	*Lmr4*
6764093	Slamf7	2.39	0.00391687	*Lmr8*
6970166	Gm4759	2.39	0.00898416	
6790288	Ccl5	2.43	0.02554016	*Lmr15*
6940658	Abcg3	2.46	0.00204207	
7015995	Zfp300	2.49	0.04242324	
6857435	Eif2ak2	2.56	0.00351408	
6901957	Gbp2	2.57	0.00074727	
6831538	Ly6e	2.65	0.00904188	
6806034	Serpinb6b	2.71	0.03184738	
6825872	Cysltr2	2.72	0.02299229	
6815542	Marveld2	2.72	0.01591547	
6954418	Il23r	2.90	0.0159262	*Lmr4*
6939338	Hopx	3.30	0.02732588	
6957421	Klrc2	3.31	0.01114157	
6968871	Hdgfrp3	3.33	0.03857217	*Lmr21*
6826179	Dnajc15	3.63	0.0199817	
6823429	D14Ertd449e	3.63	0.00171368	
6963907	Tmem159	3.65	0.01373536	
6905746	Lxn	3.68	0.00478389	*Lmr11*
6764246	Ifi204	3.93	0.03175784	*Lmr8**
6831959	Apobec3	4.06	0.01570741	
6993154	Ccr5	4.73	0.00109599	
6878045	Rapgef4	5.45	0.00780361	
6899171	Gon4l	5.45	0.01420384	*Lmr11*
6970066	AI451617	8.03	0.03235411	
6957423	Klrc1	11.76	0.00069355	
6855155	**H2-T10**	16.61	0.0075139	*Lmr1*
6970053	Trim12	37.84	0.00166596	
6992174	Rpl29	47.05	0.01240538	

*** published candidate gene in *Lmr* locus**

Table A.2. Differentially expressed genes in iLCK^{cre}IL-4Rα$^{-/lox}$ vs BALB/c, (FC >1.2, p < 0.05) in activated T cell microarray (Number of genes = 17).

Probe_IDs	Gene_ID	Regulation in iLCK vs. BALB/c	*p*-value	*Lmr* locus
6964245	Gdpd3	-76.25	0.000021	*Lmr21*
6957472	Tas2r104	-2.40	0.033647	
6964160	Il4ra	-2.38	0.006111	*Lmr21**
6889440	A130004G07Rik	-2.06	0.028503	
6805108	Otud6b	-2.02	0.024273	*Lmr9*
6993154	Ccr5	1.93	0.045993	
6890837	Il1a	1.99	0.009904	*Lmr14*
6791347	Krtap1-5	2.02	0.036811	
6780732	Olfr10	2.03	0.023253	*Lmr15*
6923155	Mrpl48	2.07	0.032981	
6828417	Rpl37	2.14	0.032996	
6978290	Mt2	2.37	0.033796	
6855166	H2-T3	2.79	0.004422	*Lmr1*
7023069	Kdm5d	12.23	0.002277	
6855155	H2-T10	33.32	0.000137	*Lmr1*
7023079	Ddx3y	38.22	0.000899	
7023072	Eif2s3y	44.52	0.000680	

*** published candidate gene in *Lmr* locus**

Table A.3. Differentially expressed genes in C57BL/6 vs BALB/c (FC >1.2, p < 0.05) in regulatory T cell microarray (Number of genes = 485).

Probe_IDs	Gene_ID	Regulation in C57BL/6 vs. BALB/c	*p*-value	*Lmr* locus
5090722	GDPD3	-10.90	0.024798618	*Lmr21*
4390451	H2-D1	-9.63	0.019308612	*Lmr1*
2140639	CTSE	-5.91	0.035493231	*Lmr20*
650601	IFI202B	-5.61	0.037002387	*Lmr8**
5690246	GBP1	-4.99	0.009930821	
1170400	C530038F07RIK	-2.78	0.00147442	
7380725	MRPL14	-2.74	2.98713E-05	*Lmr1*
3710678	1110032A13RIK	-2.74	0.04384901	
3400504	8430419L09RIK	-2.71	0.048482916	
2940544	LOC672474	-2.70	0.000700868	
5820767	4930477M19	-2.68	0.001540044	
1570095	LOC242459	-2.31	0.033473978	
6110278	LOC333685	-2.24	0.000186438	
5870367	LOC666403	-2.21	0.00801494	
5290546	CTSE	-2.21	0.007293356	*Lmr20*
6420561	ADI1	-2.20	0.017263467	*Lmr22*
1710196	LOC100048583	-2.08	0.034520971	
1090671	INPP4B	-2.04	0.010573464	
4560626	GLO1	-2.00	0.02252529	*Lmr1*
6290338	TLR4	-1.95	0.046564514	
4860035	CRIPT	-1.93	0.003017595	
7040195	GPR83	-1.93	0.039411024	*Lmr17*
1300445	IL4	-1.92	0.016736936	*Lmr15*
7400739	ACSL5	-1.90	0.010929393	
5910181	LOC638034	-1.88	0.030928467	
2940064	INPP4B	-1.88	0.002609918	
3130646	ZFP36L1	-1.81	0.00066302	*Lmr22*
670014	TRAPPC2L	-1.79	0.0224206	
7610750	LOC381157	-1.78	0.041431587	
6590451	CC2D2A	-1.75	0.003731041	
6980274	HMGA1	-1.74	0.024133771	*Lmr1*
2470220	POLR2F	-1.74	0.048306506	
6620446	IL27RA	-1.73	0.04211852	
4890100	LOC100047674	-1.70	0.035618938	
4900475	LOC100046025	-1.70	0.014855695	

4040424	TOR1AIP1	-1.70	0.014993998	*Lmr8*
1580468	5730481H23RIK	-1.69	0.02915888	
4010243	RAMP1	-1.64	0.005159457	
3290605	SLC2A9	-1.64	0.037058254	
6100132	2610024H22RIK	-1.61	0.022219483	
6510445	PPP1R11	-1.61	0.028245279	*Lmr1*
4290739	PLEKHK1	-1.60	0.027212147	
610274	FADS1	-1.59	0.004807787	
4390286	LOC386513	-1.59	0.012638243	
6860598	AI449175	-1.59	0.009843749	
3830601	ENO3	-1.58	0.030989417	*Lmr15*
1770458	SLAMF1	-1.58	0.016677494	*Lmr8*
6450438	STK38L	-1.58	0.034521683	
2340121	AP1B1	-1.57	0.041230675	*Lmr6*
2000228	FUT10	-1.57	0.001973673	*Lmr10*
6220603	RWDD3	-1.57	0.047272654	*Lmr11*
3710086	LOC544988	-1.56	0.009385961	
3170435	CAMK4	-1.55	0.021686593	*Lmr13*
6270327	GRHL1	-1.55	0.01119788	*Lmr22*
1740626	MRPS10	-1.54	0.02542475	*Lmr1*
6580373	LOC280487	-1.54	0.020891857	
2140368	1500001E21RIK	-1.54	0.010420728	
1090767	ENO2	-1.53	0.032946981	
2190086	PDZK8	-1.52	0.011499518	
540131	RCN1	-1.52	0.033452958	*Lmr14*
2000121	VDAC2	-1.52	0.001817328	
4860133	C230096C10RIK	-1.52	0.010470679	
7330189	ADCY7	-1.52	0.041610608	
4570670	STAU2	-1.52	0.019121112	*Lmr20*
5090400	CUGBP1	-1.51	0.036168354	
3850112	DNAHC8	-1.51	0.005987084	*Lmr1*
6400706	RABGAP1L	-1.51	0.024656866	*Lmr8*
2900441	5133400G04RIK	-1.50	0.043019916	
5690139	MANBA	-1.50	0.008523954	
770162	E2F2	-1.50	0.029303533	
6940136	MLLT4	-1.49	0.009243437	
4890634	TAF5L	-1.49	0.013085506	
5810398	RNASE4	-1.49	0.031790411	
6900044	LAT	-1.49	0.045714583	*Lmr21*
4490082	LOC385289	-1.49	0.044087386	

3390020	PCCB	-1.49	0.02400995	
360204	SLC35A4	-1.48	0.018541575	*Lmr13*
110630	EBPL	-1.47	0.007603217	
2350669	2810046L04RIK	-1.46	0.043783703	
5420170	GBP6	-1.46	0.039948889	
5080132	CUGBP1	-1.46	0.011829162	
2710639	H2-T24	-1.45	0.004500366	
3060224	PDZD8	-1.45	0.049874362	
1570544	C730026O12RIK	-1.45	0.038956773	
2070243	ALDH5A1	-1.45	0.018901103	
4850142	PHF20	-1.45	0.018139278	*Lmr16*
2810369	0710001D07RIK	-1.45	0.049608301	
7050397	NTN4	-1.44	0.048365914	*Lmr19*
7160079	SCL000408.1_6	-1.44	0.049909786	
3870370	D8ERTD738E	-1.44	0.03884528	
1300079	LOC381471	-1.44	0.019445636	
1980343	COL6A3	-1.43	0.018212434	*Lmr20*
5360647	RNASEN	-1.43	0.04382817	
5270164	FUT10	-1.43	0.004294804	*Lmr10*
4830156	9630041B11RIK	-1.43	0.004080547	
5700722	ECE1	-1.43	0.009928606	
6020398	ETS1	-1.43	0.020326955	*Lmr2*
3940050	1500010G04RIK	-1.43	0.00927059	
4810056	H13	-1.42	0.01174552	*Lmr14*
4290593	FVT1	-1.42	0.006155602	
3290246	CRBN	-1.42	0.01665948	
5720021	4930540L03RIK	-1.41	0.02814004	
4560731	1110014O20RIK	-1.41	0.027723991	
6900133	MTSS1	-1.41	0.02288777	
6220669	4930505D03RIK	-1.41	0.019976874	
4730767	LOC436539	-1.40	0.037887311	
3940528	LOC386446	-1.40	0.015148263	
4560148	1810027O10RIK	-1.40	0.031155869	
5670128	FAM105A	-1.39	0.00704101	
2600424	SQRDL	-1.39	0.000364941	*Lmr14*
2600156	A630004K05RIK	-1.39	0.045205132	
6450403	2410005O16RIK	-1.39	0.045834941	
670707	TTC3	-1.38	0.027915306	*Lmr18*
6580768	2810416G20RIK	-1.38	0.007046061	
3840575	LOC677008	-1.38	0.020398964	

4210477	MAPBPIP-PENDING	-1.38	0.041666816	
5390019	PLA2G4B	-1.38	0.048352887	*Lmr14*
1580161	DNAJC10	-1.38	0.036176421	
6580347	LPHN1	-1.38	0.038750768	
5260382	FDXR	-1.37	0.044992019	
5820647	FDXR	-1.37	0.039286106	
4610246	OCIAD1	-1.37	0.025574963	
2190615	2310045K21RIK	-1.36	0.014134114	
3420242	LOC674611	-1.36	0.038478121	
4640070	FTO	-1.36	0.03289406	
3420544	PMEPA1	-1.36	0.009140799	*Lmr16*
1770669	TSSC1	-1.36	0.030062093	*Lmr22*
3170195	FUT10	-1.36	0.003348306	*Lmr10*
4640093	TCRB-V8.3	-1.36	0.032950374	
5260278	ETS1	-1.35	0.002282727	*Lmr2*
6520451	ANGPTL7	-1.35	0.01881548	
610086	LOC381904	-1.35	0.010117351	
1580088	CCND2	-1.35	0.01496842	
1030243	9430038I01RIK	-1.35	0.024037325	
5220639	EDEM2	-1.34	0.048744879	*Lmr16*
3400630	ANGPTL2	-1.34	0.007310436	
7000315	BC002216	-1.34	0.032163981	
5090739	CLEC16A	-1.33	0.041641748	
2750414	VAV3	-1.33	0.024013586	*Lmr11*
2450528	SAMD10	-1.32	0.025587028	
6220750	TOE1	-1.32	0.008732543	
1500364	STK35	-1.32	0.019104287	*Lmr14*
7320181	SLC39A11	-1.32	0.044444985	
5720674	1110002L01RIK	-1.32	0.037115676	
3990630	PDCD4	-1.32	0.046919629	
2230133	DDX24	-1.31	0.04851923	
6520360	CD3E	-1.31	0.036519391	*Lmr2*
1690189	E2F6	-1.31	0.025716108	*Lmr22*
160040	A930005G04RIK	-1.31	0.014164717	
6280753	9430028F23RIK	-1.31	0.001837776	
6280270	1700022C21RIK	-1.31	0.020763507	
4480333	PPIC	-1.31	0.04493856	*Lmr13*
1300437	FAM115C	-1.31	0.013639261	*Lmr4*
3830338	TCP11L2	-1.30	0.042799203	*Lmr19*
380438	TSHZ3	-1.30	0.006484261	*Lmr21*

1470202	ELK3	-1.30	0.046532803	*Lmr19*
1300619	A130065C03RIK	-1.30	0.02658408	
6110519	ELK3	-1.30	0.008409067	*Lmr19*
6200682	HEXIM2	-1.29	0.002015059	
2750079	AKAP1	-1.29	0.033674174	
7650086	TMEM136	-1.29	0.033063144	
7200048	1700054N08RIK	-1.29	0.023817552	
650626	RWDD3	-1.29	0.049359179	*Lmr11*
6350193	TBC1D4	-1.29	0.030333667	
6860102	KLHDC4	-1.29	0.039347912	
670646	1700052K11RIK	-1.29	0.028165941	
4670753	CD97	-1.28	0.015257937	
4480519	HERC4	-1.28	0.036303331	
1440079	6030413C11RIK	-1.28	0.030218924	
3870202	B3GALT4	-1.28	0.027408449	*Lmr1*
6200400	SNX27	-1.28	0.010391671	
6290332	PLEKHA8	-1.28	0.017299954	*Lmr4*
650706	9030025P20RIK	-1.28	0.0344097	
5960673	PHF20	-1.28	0.047878753	*Lmr16*
6450291	KCTD10	-1.27	0.019887025	
6370672	ADAM26A	-1.26	0.011277176	*Lmr10*
5720059	PAPOLA	-1.26	0.048800973	
7510315	TRIM33	-1.26	0.02479773	*Lmr11*
2370592	ABLIM1	-1.26	0.026356127	
7100300	TMC6	-1.26	0.030413505	
6510333	SFRS1	-1.26	0.016147285	
3170092	SCL0001233.1_26	-1.26	0.037647551	
870068	TRBV30_X16695_T_CELL_RECEPTOR_BETA_VARIABLE_30_160	-1.26	0.009105639	
6420722	NME3	-1.26	0.008349652	*Lmr1*
2630735	LOC100045737	-1.26	0.031891269	
6770368	LOC276878	-1.26	0.007470219	
990692	TMOD3	-1.25	0.026816201	
3850017	TGFBR1	-1.25	0.01486183	
5340598	INTS10	-1.25	0.022433746	*Lmr10*
7040750	LRBA	-1.25	0.032166043	
1010703	ATP6V0E2	-1.25	0.038668493	*Lmr4*
3850114	4930505D03RIK	-1.25	0.046976285	
4220091	NRADD	-1.24	0.024908899	
3400471	JAK1	-1.24	0.033133559	

5130523	B230399E16RIK	-1.24	0.001774212	
4670528	PPP2R4	-1.24	0.005651688	
7200491	1110007A13RIK	-1.24	0.028261492	
5870192	AOF1	-1.24	0.005355389	
4780050	CBLC	-1.24	0.025048325	
4830717	NSDHL	-1.24	0.029382761	
1230307	TRMT2B	-1.24	0.020298989	
3140543	GNA-RS1	-1.24	0.012779433	
1690292	BC094916	-1.23	0.040691424	
5670468	SH3MD4	-1.23	0.040028112	
2570220	VGLL1	-1.23	0.016045139	
2490333	EDEM2	-1.23	0.009839398	*Lmr16*
240343	TBC1D15	-1.23	0.041189792	*Lmr19*
4390037	SOX9	-1.23	0.036892602	
3140102	PAOX	-1.23	0.045331407	
3360349	ZMIZ2	-1.23	0.00541049	*Lmr6*
1010600	AIFM1	-1.22	0.019341269	
6480377	AOF1	-1.22	0.026123917	
2260594	OLFR1403	-1.22	0.004016447	
6350537	APOA2	-1.22	0.010781362	*Lmr8*
6250195	SFXN3	-1.22	0.028025785	
3370026	E430039K24RIK	-1.22	0.012790774	
4540561	CHGB	-1.22	0.031031417	*Lmr14*
270274	POLR1A	-1.22	0.032432816	*Lmr4*
70201	PRPF31	-1.22	0.023856648	
2600187	IQCF1	-1.21	0.024840048	
3940593	C630004H02RIK	-1.21	0.047714644	
7210392	COL6A3	-1.21	0.007551684	*Lmr20*
7550603	SORBS2	-1.21	0.015383469	*Lmr10*
4180767	3632411M23RIK	-1.21	0.007496129	
2710138	N4BP2	-1.21	0.009651222	
3870743	1810008A18RIK	-1.20	0.03501973	*Lmr19*
6130300	CTNNBL1	-1.20	0.027846661	*Lmr16*
7050524	D630016F07RIK	-1.20	0.006363115	
7400709	LOC381129	-1.20	0.015091714	
4850692	9930004G02RIK	1.20	0.002983825	
3140037	5930437C20RIK	1.20	0.019241074	
5340176	6720451B11RIK	1.20	0.033383514	
5360632	DOCK5	1.20	0.007116738	
6060554	CPEB4	1.20	0.035824136	*Lmr6*

6290411	PANK1	1.20	0.005339989	
4150767	LOC100041194	1.21	0.042664927	
50093	LOC238966	1.21	0.043510544	
7570768	PSG19	1.21	0.02765374	*Lmr21*
7100477	KCNJ4	1.21	0.008280216	
5310446	TRDMT1	1.21	0.046759978	
4220193	PGPEP1	1.21	0.023008928	*Lmr10*
2750746	SLC30A4	1.21	0.017398286	*Lmr14*
6130328	PLSCR1	1.21	0.047667773	
1990437	STAC3	1.21	0.046117174	
2490044	CRTAM	1.22	0.039322567	*Lmr2*
6650184	GSTT2	1.22	0.027776428	*Lmr19*
5340221	AU018091	1.22	0.020180455	
60358	LOC383062	1.22	0.032036643	
4210196	EDNRB	1.22	0.023332755	
7380075	LOC240871	1.22	0.024071424	
1340500	AW125391	1.22	0.019646487	
1710202	PSMG2	1.23	0.024528816	*Lmr13*
2680382	GP1BB	1.23	0.001604651	
1230608	TTC9C	1.23	0.019023829	
4540327	SYNGR3	1.23	0.004652134	*Lmr1*
4290253	GALNT10	1.24	0.041446472	*Lmr15*
7400707	EPS8L1	1.24	0.021115448	
3190020	4930579C12RIK	1.24	0.002588221	
70468	MOSC2	1.24	0.048665022	
3130100	PARG	1.25	0.041510856	
360706	BC117090	1.25	0.013963372	
7050139	TAAR8A	1.25	0.044076295	
3800722	KLF8	1.25	0.016456135	
1980682	ARV1	1.25	0.015971002	
6900600	H2AFJ	1.26	0.002570242	
3710152	SH3GL3	1.26	0.025345144	*Lmr21*
2070270	HIP1	1.26	0.046417147	
6550392	UNC119B	1.26	0.01901874	
3400521	9930108O06RIK	1.26	0.047645819	
5560167	DNAJB4	1.27	0.022221979	
4120093	LOC100045840	1.27	0.032550651	
60762	FBXO3	1.27	0.041090099	
7100681	LOC383466	1.28	0.013199973	
2100386	NDUFB10	1.28	0.024814651	*Lmr1*

5890437	SNX13	1.28	0.019126507	*Lmr22*
2100605	PCM1	1.28	0.047055812	*Lmr10*
520100	5031439A09RIK	1.28	0.020857168	
1690368	LOC547380	1.28	0.014571145	
6110114	TEX261	1.28	0.028117032	*Lmr4*
870288	4921513D23RIK	1.29	0.04623151	
1300040	ADH5	1.29	0.04919657	
6290768	CD52	1.29	0.038237705	
4900520	1500001L15RIK	1.29	0.034316996	
6250689	MT-ND4	1.30	0.026020681	
5090039	ACOX1	1.30	0.045455062	
7100674	LY96	1.30	0.015679792	*Lmr20*
4920382	DDX17	1.30	0.020549092	
1570446	MRPL19	1.31	0.033200661	*Lmr4*
2760484	ABCC1A	1.31	0.029167696	
4150593	MXD1	1.31	0.043109957	
6400347	LOC624610	1.31	0.001779167	
1580338	TXNRD1	1.31	0.039680398	*Lmr19*
6940672	RAD23B	1.31	0.034171486	
540402	GPN2	1.31	0.034722242	
5890056	MAN2B1	1.31	0.037214809	
6130075	NDRP	1.32	0.026042801	
5090754	DUS2L	1.32	0.025081553	
2900255	WSB2	1.32	0.035115085	
6060731	LOC100047840	1.32	0.043446927	
7210458	CDK5R1	1.32	0.047191861	*Lmr15*
5550035	RRAD	1.32	0.029033338	
1010008	NUDT7	1.32	0.006776351	
5820619	ZFP160	1.32	0.029018298	*Lmr1*
6330195	CAMK2N1	1.33	0.045786274	
2710735	FLYWCH2	1.33	0.006555428	*Lmr1*
6110138	2210410E06RIK	1.33	0.024750914	
5360474	PI4K2B	1.34	0.020318225	
5080632	NUP88	1.34	0.010152987	*Lmr15*
2000035	D530024B08RIK	1.34	0.036760533	
4640500	ART2A	1.34	0.02096064	
3120328	ITGB1BP1	1.34	0.037562707	
3130433	TTC27	1.34	0.020314903	
460528	2010015J01RIK	1.34	0.026535711	
4250280	NAPRT1	1.35	0.047657669	

3420528	4933407N01RIK	1.35	0.025657702	
1500301	TUBA8	1.35	0.032855854	
4040671	CHD9	1.35	0.028168438	
3420615	ASCC2	1.36	0.002006052	*Lmr6*
2490072	TLR11	1.36	0.021433063	
5810274	TMBIM4	1.37	0.033728631	*Lmr5*
4180471	NT5E	1.37	0.011403616	
3710672	2810436B12RIK	1.37	0.019603565	
4070019	SIDT2	1.37	0.044435618	*Lmr2*
3310176	USP21	1.38	0.035360067	*Lmr8*
4290180	CASP4	1.39	0.030818504	*Lmr2**
4290100	HPCAL1	1.39	0.046953803	
4280458	COPS2	1.39	0.006530682	*Lmr14*
3120020	PDXDC1	1.39	0.026921925	*Lmr23*
3870278	MCART1	1.39	0.005392499	
5810292	5830417I10RIK	1.40	0.047383896	
1660367	SMOX	1.41	0.011181575	*Lmr14*
3360068	5830417I10RIK	1.41	0.016646312	
5420739	FES	1.41	0.033390801	*Lmr21*
6380148	FBXO34	1.41	0.03972668	
4890192	LPIN1	1.42	0.018296436	*Lmr22*
6760286	EG668139	1.43	0.009699956	
6220170	RPGR	1.43	0.010581347	
4390576	AI451617	1.43	0.009262019	
290286	CHM	1.43	0.029111641	
3420600	CPM	1.44	0.003043826	
2570451	MRPS23	1.45	0.029122535	*Lmr15*
6480735	RBM13	1.45	0.047349991	
4590093	5830417I10RIK	1.46	0.009591583	
6110551	AHCY	1.46	0.032503065	*Lmr16*
5690603	BTBD11	1.46	0.048054463	*Lmr19*
1110138	ANKRD27	1.46	0.009871775	*Lmr21*
270341	EMB	1.46	0.039455086	
5360307	4732457N14	1.47	0.034424669	
4560504	MRPS7	1.47	0.016922055	
1980048	GIMAP5	1.47	0.041462774	*Lmr4*
5080286	NSG2	1.47	0.016557321	*Lmr6*
6620577	MS4A4C	1.48	0.04808842	
4640328	2610307O08RIK	1.48	0.007365826	
4610095	DNAJC3	1.49	0.003163988	

4780066	CSRP1	1.49	0.022191048	*Lmr8*
5690370	SCL0001032.1_178	1.49	0.010566723	
4050369	HSD11B1	1.50	0.024646992	*Lmr20*
1230241	4631422C13RIK	1.51	0.006152361	
4150370	E030040G24RIK	1.51	0.029972007	
540048	XPNPEP1	1.51	0.015932937	
1170343	MINA	1.51	0.011762041	*Lmr12*
4060270	2410078J06RIK	1.52	0.039265675	
7200750	PUSL1	1.53	0.001825142	
780504	NDE1	1.54	0.040856985	*Lmr23*
2970136	LNP	1.54	0.018242142	
6650440	LY6K	1.54	0.037047718	
4890113	LOC100045567	1.55	0.014356072	
6660039	MAD	1.55	0.03811996	
4810630	9430032E06RIK	1.55	0.013389065	
7150243	BDH1	1.55	0.004319045	*Lmr12*
6200386	LOC433801	1.56	0.033909651	
1990500	9930105H17RIK	1.56	0.036912428	
5550066	4933426K07RIK	1.57	0.001191276	
6590139	1700047I17RIK1	1.57	0.03502075	
2490068	FGFR1OP2	1.58	0.016927008	
5420685	NUDT7	1.58	0.012308105	
4220189	APOB48R	1.58	0.032933779	
1030682	SLC11A2	1.59	0.03049791	
5690148	TOR1AIP2	1.59	0.01576437	*Lmr8*
3990246	AHCY	1.60	0.01290993	*Lmr16*
20438	SCL0002022.1_2978	1.61	0.001511742	
7160202	ACADL	1.62	0.025688782	*Lmr20*
5130593	2310042D19RIK	1.62	0.002805166	
6200646	4930473A06RIK	1.62	0.015535425	
3060600	RAB6B	1.63	0.031977113	
6020445	1700047I17RIK1	1.63	0.046863752	
3440646	PRKCB	1.63	0.010168998	
2900373	1190002H23RIK	1.63	0.017258082	
4480148	BTBD11	1.64	0.017104964	*Lmr19*
5890458	PIK3R4	1.64	0.006750345	
3060326	3830402I07RIK	1.65	0.017607319	
940278	SCLY	1.65	0.046328078	*Lmr20*
5560465	MTDNA_ATP8	1.65	0.027583464	
1170446	MTMR6	1.65	0.00887721	

2480382	NOL10	1.66	0.040518108	*Lmr22*
2260301	CCDC46	1.67	0.00937484	
2850398	ASB2	1.67	0.003971032	
3990435	MGMT	1.67	0.009106333	*Lmr21*
6380379	PON2	1.68	0.000177964	
6900546	CCL25	1.69	0.012480945	
3890328	OAS1G	1.69	0.02280121	
1990639	APAF1	1.70	0.011028229	*Lmr19*
2000747	TNFRSF4	1.70	0.043583137	
1400204	ARHGAP26	1.71	0.029544345	*Lmr13*
4850082	ENPP5	1.71	0.005078858	*Lmr1*
2260427	A130002I06RIK	1.72	0.036483142	
6980672	BTLA	1.73	0.016309666	*Lmr12*
4780437	VTI1B	1.73	0.041324109	*Lmr22*
6110193	CHD4	1.73	0.03132145	
1090291	XRCC4	1.75	0.002487976	
3450167	SNX10	1.75	0.021092114	*Lmr4*
7610669	GMFB	1.76	0.017999382	
940639	CCNDBP1	1.77	0.008675382	*Lmr14*
5690301	GABARAPL1	1.78	0.032336904	
2030593	HSPA8	1.78	0.014663134	
1980255	ITPA	1.78	0.011349603	
270719	2510049I19RIK	1.79	0.034088354	
3130292	IDB2	1.79	0.045785157	
3940348	DNAJB13	1.80	0.006830445	
3520500	1700123O20RIK	1.82	0.016350353	
3370717	GADD45GIP1	1.85	0.013858221	
450735	GVIN1	1.85	0.002936564	
1580021	RPL29	1.87	0.000196768	
4490301	EG637748	1.87	0.025663106	
6060390	RPS2	1.88	0.028355466	
3890767	SV2C	1.90	0.011168715	
650717	LILRB4	1.90	0.045021478	
6940259	CCR2	1.91	0.015777769	
4880639	2810021O14RIK	1.94	0.026336696	
5310722	1200015F23RIK	1.95	0.005566011	
540543	TM7SF3	1.99	0.023425142	
4540082	SERPINA3F	2.00	0.002990713	
6510482	ADAM17	2.01	0.015504837	
5670040	CRYBG3	2.01	0.002354085	*Lmr12*

6370451	OTTMUSG00000016644	2.01	0.049409049	
7050148	G430022H21RIK	2.02	0.028504164	
3440056	RING1	2.03	0.017560926	*Lmr1*
450594	KLHL28	2.05	0.008598248	*Lmr22*
2630673	PCM1	2.07	0.001666612	*Lmr10*
5820709	LOC240921	2.12	0.037860146	
2070014	NFKB2	2.13	0.001936343	
2970088	LOC229810	2.18	0.000896507	
4390131	TMEM66	2.20	0.005448319	*Lmr10*
6940386	H2-Q7	2.21	0.01053833	
1070598	ZFP260	2.23	0.028005869	*Lmr21*
2940164	RBM13	2.24	0.00238316	
5900370	LOC381140	2.26	0.00031351	
3130630	CDKN1A	2.32	0.043310202	*Lmr1*
2690639	NSG2	2.32	0.045807137	*Lmr6*
5960102	A530055J02RIK	2.33	0.02743687	
5700754	B3GALT6	2.33	0.019472319	
870279	CABLES1	2.35	0.042016042	
2850082	1200003I07RIK	2.36	0.001536451	
1690753	PRDX2	2.37	0.015271833	
5820739	GP49A	2.39	0.011290857	
6860681	HOPX	2.41	0.01290554	
5670035	HGSNAT	2.42	0.001784441	*Lmr10*
4760255	TOR1AIP2	2.45	0.017810617	*Lmr8*
5550671	LY6C1	2.50	0.001802893	
3120397	DOCK8	2.52	0.007537909	
6550376	LY6E	2.52	0.035642311	
4780184	LY6E	2.53	0.02959187	
3130692	LOC385279	2.54	0.011645859	
2940367	ART2B	2.55	0.027727146	
5570646	CHCHD4	2.61	0.000398441	
1230341	BHLHB2	2.63	0.014692725	
3460463	MANBAL	2.76	0.008602689	*Lmr16*
3060687	RPL29	2.86	0.012504189	
4810747	FSD2	2.87	0.008676776	*Lmr21*
2710341	NSG2	2.98	0.025056594	*Lmr6*
7210349	APOBEC3	3.16	0.004459444	
7400519	PDRG1	3.28	0.001136237	*Lmr14*
4220162	STX11	3.35	0.004076645	
3170703	LOC545056	3.50	0.026846707	

3930093	LOC100043821	3.65	0.001057309	
5340328	ZFP330	3.72	0.000102911	
5720719	NSG2	4.61	8.76295E-05	*Lmr6*
2760445	FSD2	4.70	0.005741936	*Lmr21*
4120750	SLC15A2	4.78	0.010990516	*Lmr12*
3990427	0610010I05RIK	4.97	0.006394751	
3780736	H2-K1	5.57	0.004748943	*Lmr1*
6270682	SLC15A2	6.38	0.034703682	*Lmr12*
2060474	RBM28	6.64	0.001308864	
7570196	TRIM59	7.76	0.017881158	*Lmr11*
4070402	EG241041	23.31	0.018017417	

*** published candidate gene in *Lmr* locus**

Table A.4. Differentially expressed genes in iLCK^{cre} IL-4Rα$^{lox/-}$ vs BALB/c, (FC >1.2, p < 0.05) in regulatory T cell microarray (Number of genes =103).

Gene_ID	Regulation in iLCK vs. BALB/c	*p*-value	*Lmr* locus
GDPD3	-11.91	0.024618405	*Lmr21*
THUMPD1	-9.57	0.011774589	
XIST	-3.17	0.026669264	
XIST	-2.97	0.045401352	
PRCP	-2.02	0.001060697	
MINT-PENDING	-2.00	0.046669397	
IL4	-1.44	0.023974876	*Lmr15*
RUVBL1	-1.38	0.028969522	
GPR108	-1.34	0.049601816	*Lmr1*
FAM115C	-1.34	0.012431219	*Lmr4*
KLK1B21	-1.34	0.004303247	*Lmr21*
LOC100043322	-1.32	0.048385903	
SFTPC	-1.31	0.004762556	
LOC232787	-1.29	0.041890792	
RTP4	-1.29	0.047850894	*Lmr12*
6030413C11RIK	-1.29	0.029553113	
C530038F07RIK	-1.27	0.026484634	
NOL1	-1.27	0.039322253	
PDZK8	-1.27	0.006383045	
NOL5	-1.27	0.019697414	
MGC60742	-1.26	0.040994501	
GPRC5B	-1.26	0.021410928	
LOC385112	-1.25	0.041372333	
ATP6V1G1	-1.25	0.047525362	
CDC42SE1	-1.24	0.00587767	*Lmr11*
GLT8D2	-1.24	0.027983617	*Lmr19*
GTF2I	-1.24	0.014576493	
BCL9	-1.24	0.021921508	*Lmr11*
BAK1	-1.24	0.02441454	*Lmr1*
LOC382229	-1.24	0.040852124	
SYT11	-1.24	0.025059497	
SLC9A9	-1.23	0.037659302	
BC008163	-1.23	0.014208395	
2310001L23RIK	-1.22	0.048581967	
PSMC4	-1.22	0.02332712	*Lmr21*
SSX2IP	-1.22	0.047109794	
HOXC11	-1.22	0.031121486	

LOC434960	-1.21	0.004245102	
KLHL36	-1.21	0.042022136	
A530058L02RIK	-1.21	0.019124233	
PHLDA3	-1.21	0.020423322	*Lmr8*
LOC386468	-1.21	0.031091693	
LOC278666	-1.21	0.03342157	
A730011L01RIK	-1.21	0.041748141	
PSCD1	-1.21	0.016155441	
TMEM192	-1.20	0.02138284	*Lmr10*
5430405G05RIK	-1.20	0.044632704	
SOX13	-1.20	0.016689833	*Lmr8*
OLFR166	-1.20	0.032784445	*Lmr23*
NOTO	-1.20	0.031667968	
LOC100045551	1.20	0.044534953	
NFS1	1.20	0.03442322	
HIP1	1.20	0.040731047	
MAPK10	1.20	0.032763246	*Lmr3*
OLFR1265	1.20	0.016775536	
GM382	1.21	0.048012261	
RNF170	1.21	0.014097065	*Lmr10*
RRBP1	1.21	0.015055243	*Lmr14*
FAM13B	1.21	0.033388671	
2310004H21RIK	1.21	0.023051732	
FZD5	1.21	0.028733743	*Lmr20*
IFNGR1	1.21	0.026025191	
A230057G18RIK	1.21	0.047926419	
CDK10	1.21	0.037298392	
1700109H08RIK	1.21	0.023680244	
PNRC2	1.22	0.04875417	
ACSL3	1.22	0.023985667	*Lmr20*
PHXR5	1.22	0.026489412	
OLFR324	1.22	0.045263732	
FOXK1	1.22	0.037927825	
ZFP101	1.22	0.006661833	*Lmr1*
4631416M11RIK	1.23	0.012774489	
NRIP1	1.23	0.022783099	*Lmr18*
4930539E08RIK	1.23	0.004015415	*Lmr1*
EG226654	1.23	0.035326704	
4930527B16RIK	1.23	0.037231148	
1700109H08RIK	1.23	0.027231433	
LOC383110	1.23	0.043082262	
LOC381630	1.24	0.01465551	

PSG19	1.24	0.049276031	*Lmr21*
GEM	1.25	0.027616939	*Lmr9*
C530020B06RIK	1.26	0.016399936	
TAPBPL	1.26	0.018173748	
2610301B20RIK	1.26	0.048390908	*Lmr9*
C130054H24RIK	1.27	0.015147705	
TMBIM4	1.27	0.027018802	*Lmr5*
CHIC2	1.29	0.039012089	
LOC100043968	1.29	0.003333294	
RBBP9	1.30	0.03854793	*Lmr14*
BRAF	1.32	0.023358506	
TCF12	1.33	0.019801787	
SKIL	1.34	0.03471503	*Lmr11*
RGS10	1.35	0.041840761	*Lmr21*
TRAF1	1.39	0.021456964	
RGS1	1.46	0.001634603	*Lmr8*
SGIP1	1.46	0.039179197	
LOC436541	1.49	0.02906417	
A130002I06RIK	1.58	0.048894278	
LOC277146	1.65	0.0310446	
DDX3Y	1.79	0.01474494	
LOC634171	2.29	0.015808494	
MT-ATP6	2.88	0.014081228	
H2-T10	4.84	0.037054581	*Lmr1*

Table A5 Candidate genes that intersected with two or more functional groups (Ingenuity Systems).

Gene Symbol	Gene Name	Relationship with relevant molecules			Role in cell
		Regulates	Regulated by	Binds	
H2-T10	histocompatibility 2, T region locus 22	LCK, FYN and VAV1	lipopolysaccharide, dendritic cells and IFN type 1	TCR gamma delta, B2M	priming
LCK	lymphocyte-specific protein tyrosine kinase	No data available	TCR, CD3	CD4, CD8	differentiation and apoptosis
FYN	FYN oncogene related to SRC, FGR, YES	CD3-TCR	TCR, CD3 and FYN	CBL, KHDRBS 1, PTK2	Proliferation
VAV1	vav 1 guanine nucleotide exchange factor	Rac, RAC1, CDC42	TCR, CD3 and CD28	LCP2, GRB2, ZAP70	Proliferation
Gdpd3	Glycerophosphodiester phosphodiesterase domain containing 3	No data available	Chlorcyclizine, perhexiline, imipramine	No data available	No data available
Cul2	Cullin2	CUL2, VHL, TGFB1	CUL2, NEDD8,RBX1	VHL, TCEB1, RBX1	Cell cycle progression, cell proliferation and G1/S phase transition
CCR5	Chemokine receptor type 5	CCR5, Ca2+, CCL4	lipopolysaccharide, CCL5, TNF	CCL4, CCL5, CCL3	chemotaxis, migration, trafficking
CCL5	Chemokine ligand 5	CCR5, Ca2+, TNF	TNF, lipopolysaccharide, IFNG	CCR5, CCR1, CCR3	chemotaxis, migration, activation
IL-18r1	Interleukin-18 receptor 1	NFkB, IFNG, Jnk	Il12, IL4, STAT4	IL18, IL18RAP, IL37	function, binding, cytolysis
STAT4	Signal transducer and activator of transcription 4	IFNg, TNF, SOCS3	IL-12, Interferon alpha, IL-2	IFNg, IL12RB2, Cytokine receptor	Differentiation, Production and development
Rnf130	ring finger protein 130	unknown	STAT4	UBE2D3, UBE2D1, LYSU	apoptosis

Rapgef4	Rap guanine nucleotide exchange factor	Rap1, Ins1, Insulin	Cyclic AMP, sulfonylurea	RIMS2, PCLO, RAP1A	binding, synaptic transmission, exocytosis
HIP1	huntingtin interacting protein 1	HIP1, CASP3	HTT, HIP1, TGM2	HTT, IFT57, Clathrin	Apoptosis, cell death and activation
IL-4	Interleukin-4	STAT6, IFNG, TNF	Cd3, CD28, Th2 cells	IL4R, IL2RG, Nfat	differentiation, proliferation, expression
PSG19	pregnancy specific glycoprotein 19	No data available	RBPJ, NOTCH1	RBPJ	No data available
FAM115C	family with sequence similarity 115, member C	No data available	No data available	No data available	No data available
A130002106RIK	No data available	No data available	No data available	No data available	No data available
C530038F07RIK	No data available	No data available	No data available	No data available	No data available