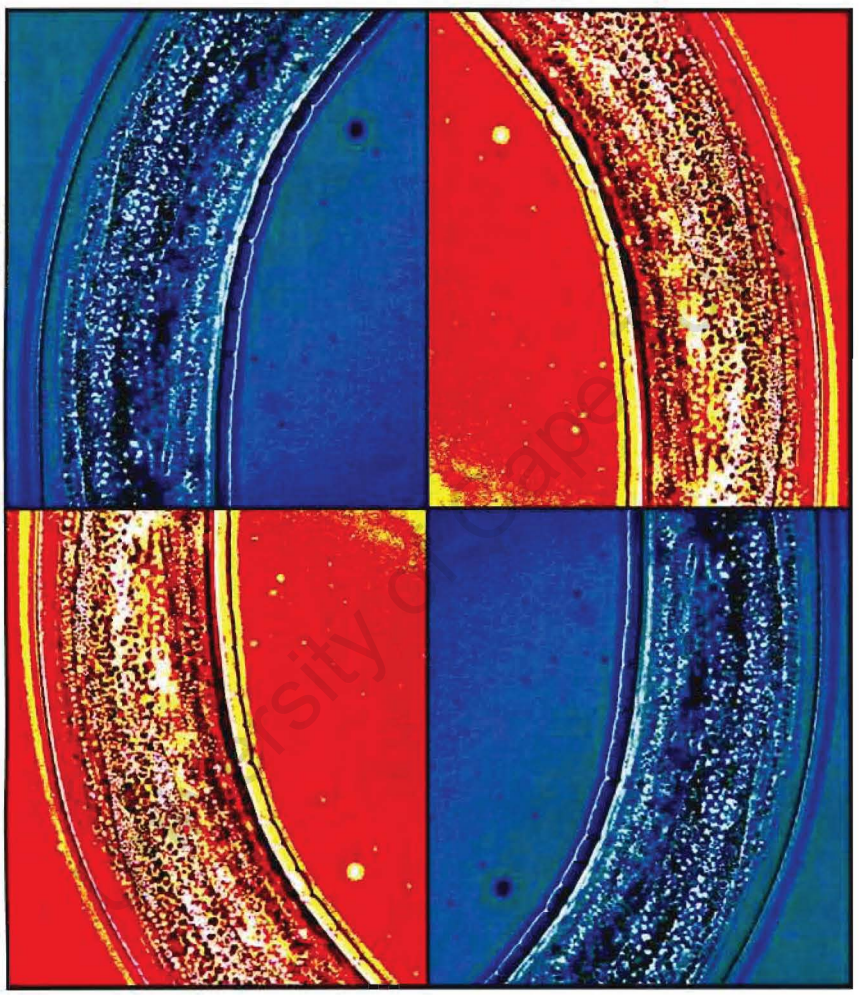
Characterisation of novel T cell specific IL-4 Receptor-alpha knockout mice:

Investigating the role of T cell responses to IL-4 in *Leishmania major* and *Nippostrongylus brasiliensis* infections.



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DECLARATION

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Cover page: Nematodes as art

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ABBREVIATIONS

AP	- Alkaline phosphatase
APC^f	- Allophycocyanin
APCs	- Antigen presenting cells
BAL	- Broncho-alveolar lavage
BCA	- Bicinchoninic Acid Protein Estimation
BSA	- Bovine serum albumin
Cre	- Cyclization recombinase
CRP	- C reactive protein
DCs	- Dendritic cells
DN	- Double negative
DP	- Double positive
DTH	- Delayed Type Hypersensitivity
ELISA	- Enzyme-linked Immunosorbent Assay
FACS	- Fluorescence-activated cell sorting
FCS	- Fetal Calf Serum
FITC	- Fluorescein
HRP	- Horse-radish peroxidase
IMDM	- Iseove's Modified Dulbeco's Medium
IFN-γ	- Interferon gamma
iNOS	- inducible Nitric Oxide synthase
i.p.	- intraperitoneal
Ig	- Immunoglobulin
KO	- Knockout
IL	- Interleukin
IL-4Rα	- Interleukin-4 Receptor alpha
IPTG	- Isopropyl-thiogalactoside
KO	- Knock out
LB	- Luria-Bertani medium
Lck	- Lck protein tyrosine
L. major	- *Leishmania major*
mAb	- Monoclonal antibody
MHC	- Major Histocompatibility Complex
MsLN	- Messenteric Lymph Node
MdLN	- Mediastinal Lymph Node
N. brasiliensis	- Nippostrongylus brasiliensis
NO	- Nitric Oxide
OCT	- Optimal cutting temperature compound
PAMPS	- Pathogen associated molecular patterns
PE	- Phycoerythrin
PLN	- Popliteal lymph node
PMA	- Phorbol 12-myristate 13-acetate
PNP	- 4-Nitrophenylphosphate
RT°	- Room temperature
SLA	- Soluble *L. major* antigen
TCR	- T cell receptor
T_H	- T-helper
TLRs	- Toll like receptors
Treg	- Regulatory T cell
WT	- Wild type (hemizygous IL-4R$\alpha^{-/flox}$)
X-gal	- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

T cell-specific IL-4 receptor-alpha (IL-4Rα) deficient mice (Lck^{cre}IL-$4R\alpha^{-/flox}$, $iLck^{cre}$IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$) were generated to study the importance of IL-4 responsive T cells in *Leishmania major* and *Nippostrongylus brasiliensis* infection models. By comparing the three T cell-specific IL-4Rα-deficient mouse strains (1 previously characterised and 2 novel strains), novel research tools enabling the investigation of IL-4 responsive T cell subpopulations in infection and hypersensitivity were developed. We described the characterisation and functional analysis of mice lacking the IL-4Rα chain selectively on T lymphocytes. The cellular specificity of IL-4Rα deletion was confirmed by quantitative PCR and FACS analysis and IL-4Rα expression compared on T cell subpopulations (CD4+, CD8+, γδ and NK T cells) between the three strains.

L. major infection represents a highly characterised model of T-helper (T_H) 1 immunity with IFN-γ activating infected macrophages for intracellular killing. All T cell specific IL-4Rα-deficient mouse strains became resistant to *L. major* infection dependent on an impaired T_H2 response, while total abrogation of IL-4 / IL-13 responsiveness was associated with chronic disease progression. T_H1 polarisation was demonstrated by increased IFN-γ production by CD4+ draining lymph node cells and the development of a protective Delayed Type Hypersensitivity. Abrogating IL-4Rα responsiveness on T cell subpopulations and retaining IL-4 / IL-13 mediated function on non-CD4+ T cells conferred immunity comparable with the C57/BL6 strain. *N. brasiliensis* infection represents a highly characterised model of T_H2 immunity and demonstrates an immune response to a complex array of diverse parasite antigens. All T cell specific IL-4Rα-deficient mouse strains were able to expel *N. brasiliensis* as Wild Type controls despite an impaired T_H2 response. Reduced IL-4 and IgE, fewer cells infiltrating the lungs and a decrease in airway goblet cell hyperplasia, demonstrated impaired T_H2 polarisation. Therefore, IL-4 mediated T_H2 cellular responses were not crucial for worm expulsion but were responsible for increased lung pathology in *N. brasiliensis* infection.

T cell responses to IL-4 are responsible for susceptibility to *L. major* infection and are the cause of hypersensitivity to *N. brasiliensis*. By targeting IL-4Rα signalling specific to T cells, a better understanding of the immune mechanisms involved in diseases will assist in developing effective cytokine therapy to achieve immunity.

1. Introduction

1.1 Innate and Adaptive Immunity

The mammalian immune system has evolved to allow recognition and elimination of pathogens and foreign material (antigens) whilst maintaining tolerance towards 'self' components (where tolerance is broken, autoimmunity is observed) (Davidson 1985; Roitt *et al.* 2001).

The innate immune response precedes adaptive immunity following infection. A number of cell types and serum proteins provide immediate protective responses, following contact with pathogens. The responses generally triggered by pattern recognition provide stimuli that shape the adaptive immune system. Important to the innate response are phagocytic macrophages and neutrophils. These cells bind to micro-organisms, internalise and kill them (Solomon *et al.* 1990). Eosinophils are triggered to release components such as eosinophil protease (EPO), which target parasites too large to be phagocytosed. They also release enzymes that inactivate mast cell products, and therefore decrease inflammatory responses (Solomon *et al.* 1990). Natural Killer (NK) cells are large granular lymphocytes involved in recognising and killing cells infected by intracellular pathogens. They have been shown to be the predominant source of IFN-γ produced early in the immune response (Scharton and Scott 1993). While NK T cells have been identified as major IL-4 producers in innate immunity (Yoshimoto and Paul 1994). Furthermore, $\gamma\delta$ T cells respond directly to pathogen-associated molecular patterns (PAMPS) independent of Major Histocompatibility Complex (MHC) presentation (Hedges *et al.* 2005).

A number of plasma proteins termed 'acute phase proteins' including complement and C-reactive protein (CRP) show a dramatic increase early in infection (Roitt *et al.* 2001). Complement is an important serum protein as it can be non-specific and assist innate immunity or target pathogens for phagocytosis, killing and presentation to the adaptive immune system.

Although innate immunity has been regarded as a non-specific system, investigations have shown a higher degree of specificity for this immune response (Akira and Takeda 2004). Toll-like Receptors (TLRs), described for their role in insect innate immunity against fungal infections (Lemaitre *et al.* 1996), have been shown to play a crucial role in early host defence against pathogens. TLRs expressed primarily on

macrophages and dendritic cells (DCs), recognise (PAMPS) and control the activation of these cells. Therefore, they have been described as linking innate recognition of non-self with the induction of adaptive immunity (Akira and Takeda 2004).

The adaptive immune response is organised around T and B lymphocytes. Since each lymphocyte displays a single kind of antigen receptor (generated through DNA rearrangement), the lymphocyte population is extremely diverse with an increased probability of recognising antigen (Akira and Takeda 2004). Antigen recognition is a key to the development of an effective adaptive response. Antigen presenting cells (APCs) are seen as the interface between the innate and adaptive immune systems. They carry MHC class II molecules, which are important in presenting antigens to CD4+ T helper (T_H) cells. Antigens in association with MHC I (found on all nucleated cells) alert CD8+ cytotoxic T cells to kill the infected cell (Roitt *et al.* 2001). Following interaction with APCs, CD4+ cells are activated and stimulate mononuclear phagocytes to increase their killing activity, and B cells to synthesise antigen specific antibodies. Appropriate immunological systems are effective against different types of infection. Intracellular pathogens induce a cellular immune response characterised by the T_H1 subset of CD4+ T cells and cytotoxic CD8+ T cells. In contrast, extracellular pathogens and allergens induce a humoral immune response, characterised by the T_H2 subset of CD4+ T cells (Roitt *et al.* 2001).

1.1.1 Cytokines

Cytokines are regulatory proteins secreted by a variety of immune system cells. Most cytokines are low molecular weight polypeptides or glycoproteins, which can be induced or constitutive homeostatic. Cytokine production is transient and the action radius is short (Vilcek 1998). They have an autocrine function, acting on cells that produce them; or a paracrine function, acting on surrounding cells (Roitt 1994). The actions of cytokines are pleiotropic; which stimulate or inhibit the differentiation, proliferation or function of immune cells and modulate inflammatory responses (Vilcek 1998). One cytokine can be produced by a variety of cell types and may often have overlapping functions on effector cells, whereas a cytokine cascade may result in the increase / decrease of other cytokines (Vilcek 1998; Brombacher 2000). Furthermore, Gene knockout mouse strains provide an essential tool in the analysis of cytokine function. However, compensatory mechanisms not normally present, may be activated in the absence of a gene (Vilcek 1998).

1.1.2 T cell differentiation

The differentiation of T helper cells into T_H1 or T_H2 subsets in response to infection and allergens plays an important role either in conferring immunity or mediating tissue damage. Which T cell subset gains predominance in an immune response depends on; (i) the type of APC, (ii) co-stimulatory molecules, (iii) the nature and dose of parasite and (iv) the immediate cytokine environment experienced at the time of antigen presentation (Onah and Nawa 2000). The cytokine IL-12 drives CD4+ T cell differentiation into T_H1 cells and induces IFN-γ and IL-2 cytokine release. In general, T_H1 responses are generated against intracellular pathogens (e.g. *L. major* shown in Figure 1.1) driving protective IgG2a and complement-fixing antibodies, macrophage activation, antibody dependent cell-mediated cytotoxicity and delayed-type hypersensitivity. T_H1 differentiation is initiated by signalling through; the T cell receptor (TCR), STAT-1 associated cytokine receptors and IL-12 (Weaver *et al.* 2006). STAT-1 signalling upregulates the transcription factor T-bet (Mullen *et al.* 2001), which stimulates IFN-γ gene expression and upregulates the IL-12 receptor (IL-12Rβ2). Signalling through STAT-4 via the IL-12Rβ2 stimulates expression of IFN-γ and the IL-18 receptor, thus creating a positive feedback.

A T_H2 response driven by IL-4 is necessary for the elimination of helminth infections (Urban *et al.* 1991; Kopf *et al.* 1993; Svetic *et al.* 1993) such as *Nippostrongylus brasiliensis* (*N. brasiliensis*) reviewed by (Finkelman *et al.* 1997). In allergic reactions on the other hand, the body develops an exaggerated T_H2 response to seemingly harmless antigens in genetically predisposed individuals which could lead to anaphylactic shock and death (Holgate 1999) (Figure 1.1). T_H2 differentiation is driven by TCR and IL-4 receptor alpha (IL-4Rα) signalling via the STAT-6 pathway. Together these signals upregulate expression of GATA-3 (Ferber *et al.* 1999). T_H1 and T_H2 responses counter regulate each other. STAT-1 downregulates T_H2 associated GATA-3; similarly STAT-6 suppresses T_H1 development by blocking IL-12Rβ2 expression (Weaver *et al.* 2006). Furthermore, IL-10 downregulates T_H1 polarisation, while IL-12 suppresses T_H2 polarisation.

Although the T_H1 / T_H2 paradigm is widely accepted, it is clear that it does not cover all inflammatory responses or autoimmune diseases. Recent publications (Bettelli *et al.* 2006; Mangan *et al.* 2006), have shown a subset of CD4+ IL-17-producing cells (T_H17), which are stimulated by IL-6, TGF-β1 and IL-23 and distinct from T_H1 or T_H2 cells. These T_H17 cells are involved in immunity to extracellular bacteria but also mediate autoimmune disease (Figure 1.1). In contrast, IL-6 inhibits the generation of

TGF-β1stimulated CD4+CD25+FoxP3+ regulatory T cells. The T cell paradigm, as illustrated in Figure 1.1, can therefore be summarised as; differentiation into T_H1 by IFN-γ, T_H2 by IL-4, T_H17 by IL-23/TGF-β1/IL-6 and Treg by TGF-β1. Another recent publication has identified an IL-17E (IL-25) dependent, non-B / non-T cell population that provides IL-4, IL-5 and IL-13 in *N. brasiliensis* infection. By knocking out IL-17E, mice are unable to control infection (Fallon *et al.* 2006). A role has also been described for IL-33; signalling via the ST2 pathway, which induces T_H2 associated cytokines has also been described (Schmitz *et al.* 2005). These results illustrate another exciting layer of complexity to the cytokine network induced by infection, opening new possibilities for the development of treatments.

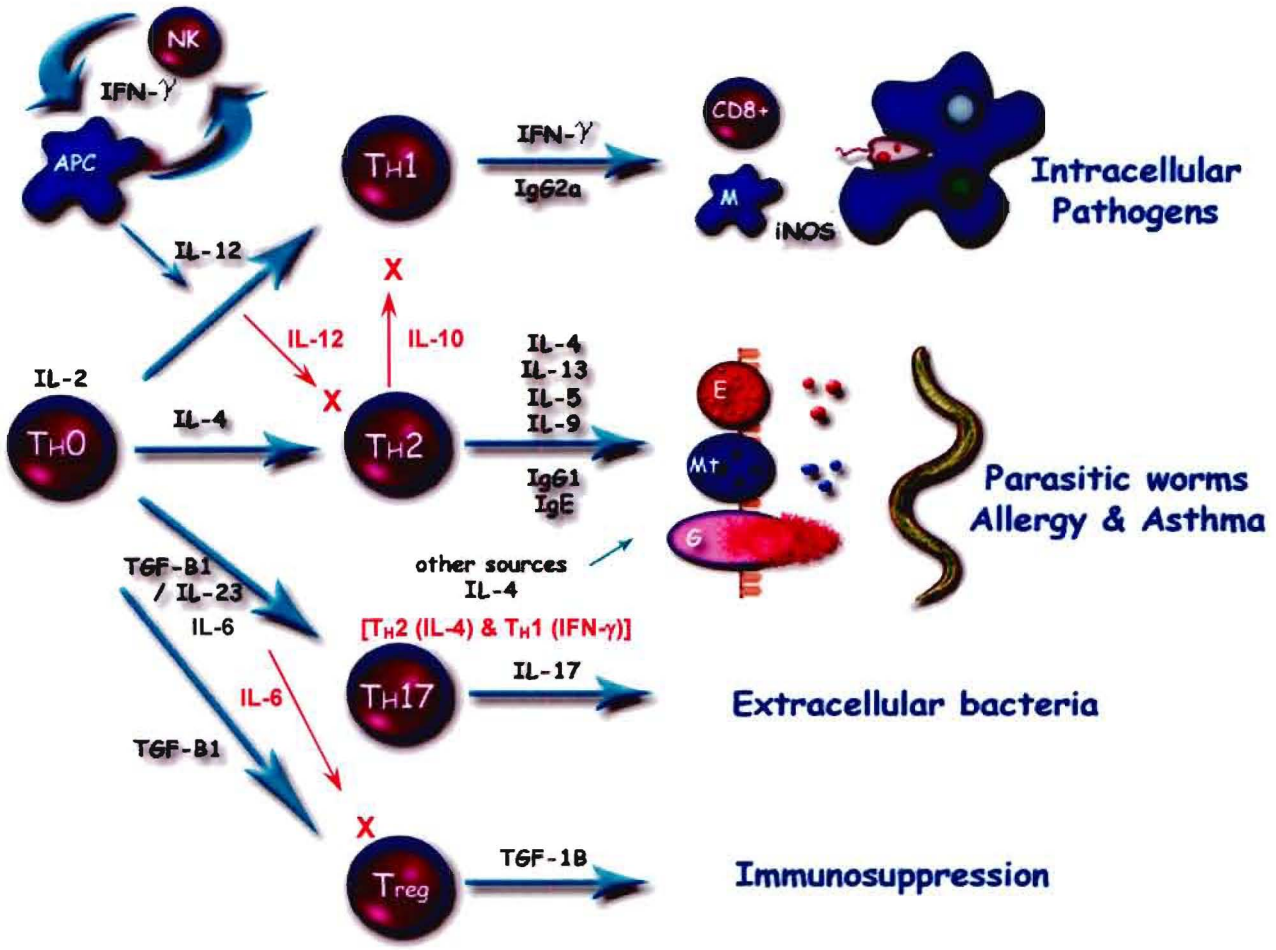

Figure 1.1: T-helper cell differentiation. T_H1 and T_H2 cells are derived from naïve T_H0 cells. T_H1 differentiation is prompted by IL-12 and IL-18 (from dendritic cells) and T_H2 prompted by IL-4 (from NK, B cells, basophils and γδ cells). An inflammatory response mediated by TGF-β1 induces T_H17 differentiation and a regulatory T cell response. Products of T_H1 and T_H2 cells inhibit T_H17 differentiation. Red arrows represent cross regulatory effects. (Illustrated according to Brombacher 2000; Roitt *et al.* 2001; Tato and O'Shea 2006).

1.2 Leishmania major

1.2.1 Background

It is considered by the WHO, that an estimated 12 million people are presently infected by *Leishmania,* with cutaneous Leishmaniasis infecting 1.5 million people annually. Leishmaniasis is endemic in 88 countries on 3 continents, yet routine control currently depends on early detection and prompt treatment. First-line drugs are available in treating the disease but, are expensive, have serious side effects and treatment takes a long time which results in resistance. A breakthrough for visceral Leishmaniasis was the registration of the first oral treatment in India. The drug (Milefosine) is relatively safe and highly effective, achieving cure rates of up to 98%. Although this is a breakthrough for visceral disease, there is currently no long-term effective treatment for cutaneous disease. Furthermore, concern has risen in persons infected with HIV; Leishmaniasis accelerates the onset of AIDS by immunosuppression and stimulating replication of the virus (www.who.int /leishmaniasis/research).

Infection begins with metacyclic promastigote parasites being transmitted to the host during a blood meal of the sandfly vector (*Phlebotomus papatasi*) (Figure 1.2). The parasites are phagocytosed by macrophages and survive in phagolysosomes, where they develop into amastigotes and are ingested by the sandfly. The *Leishmania major* (*L. major*) mouse model has been used extensively to unravel mechanisms underlying T cell differentiation into T_H1 or T_H2 cells. Essentially, most laboratory mouse strains (such as C57/BL6 mice) are able to control *L. major* infection, raising a polarised T_H1 response following infection (driven by IL-12) (Sacks and Noben-Trauth 2002; Sacks and Anderson 2004). Due to a genetic predisposition, BALB/c mice develop a T_H2 response 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 T_H1 polarisation in resistance to the disease, but also in identifying the processes involved in controlling T_H1 differentiation *in vivo* (Sacks and Anderson 2004).

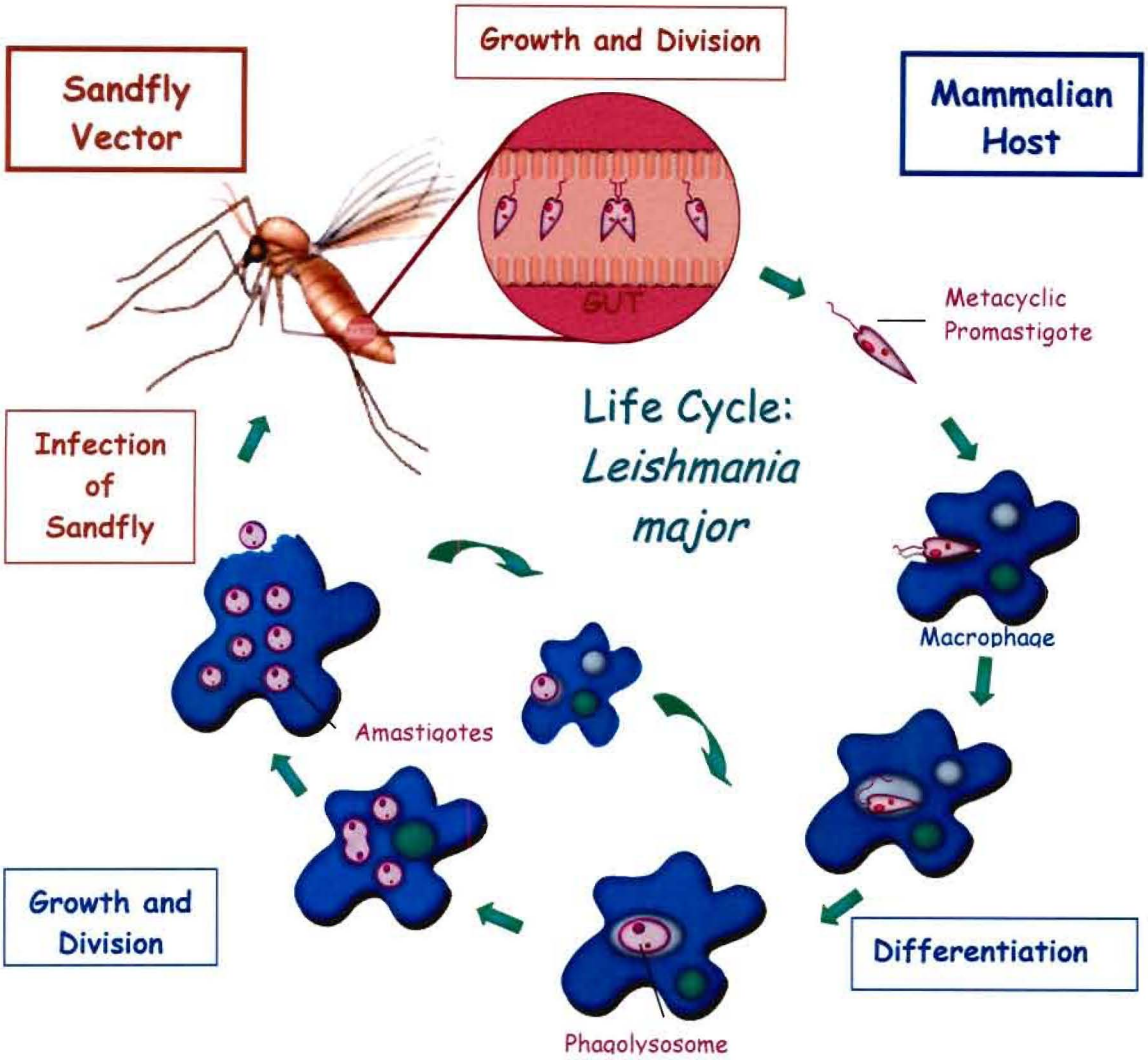

Figure 1.2: Life cycle of *Leishmania major*. Metacyclic promastigote *L. major* parasites are injected into the skin of a mammalian host by the bite of an infected sandfly. The parasites are phagocytosed by macrophages and survive in phagolysosomes, where they develop into amastigotes. Amastigotes infect other macrophages, or infected macrophages are ingested by the sandfly once again. They then develop into flagellate promastigotes in the gut of the sandfly and await the next blood meal (adapted from (Brombacher 2000; Roitt *et al.* 2001; Sacks and Noben-Trauth 2002).

1.2.2 The Immune Response to L. major

The current understanding of Leishmaniasis in mice is that T_H1 cells are protective, while T_H2 cells promote infection. T_H1 effector cells produce high levels of IFN-γ and tumour-necrosis factor (TNF), which upregulate the expression of inducible nitric oxide synthase (iNOS) and activate infected macrophages for intracellular killing of *L. major* amastigotes / promastigotes (Sypek *et al.* 1993; Wei *et al.* 1995; Guler *et al.* 1996). Nonhealing disease is associated with a T_H2 response (driven by IL-4), including; the secretion of IL-4, IL-5, IL-9 and IL-13 cytokines, high anti-*Leishmania* antibody titres and alternatively activated macrophages (Gordon 2002). Macrophages, important host cells for *Leishmania,* induce Arginase I in response to T_H2 cytokines. Arginase was demonstrated to be essential for *Leishmania* survival

and promoted intracellular parasitic growth (Roberts *et al.* 2004; Iniesta *et al.* 2005). Supporting this data was a study, in which the impairment of alternative macrophage activation delayed *L. major* disease progression in non-healing BALB/c mice (Holscher *et al.* 2006). Furthermore, Regulatory T cells (CD4+CD25+) have been shown to suppress the ability of CD4+ effector T cells to eliminate *L. major* from the site of infection in C57/BL6 mice (Belkaid *et al.* 2002). Therefore, C57/BL6 mice maintain immunity to reinfection. In contrast, IL-10 production by regulatory T cells has been suggested to be responsible for the inability of BALB/c mice to control parasite growth (Radwanska *et al.* 2006, submitted).

The hypothesis stated in a review by (Sacks and Noben-Trauth 2002) is that *L. major* initiates early IL-4 production (Belkaid *et al.* 2000), which is successfully redirected by IL-12-dependant mechanisms to a T_H1 response in resistant mice. In susceptible mice IL-4 production is maintained and drives a T_H2 response which leads to disease progression (Reiner *et al.* 1994). This was confirmed by the early neutralisation of IL-12, which abrogates resistance in C57/BL6 mice (Scharton-Kersten *et al.* 1995) and the administration of IL-12 clearing the parasite in susceptible BALB/c mice (Sypek *et al.* 1993). Furthermore, BALB/c mice treated with anti-IL-4 Abs before infection were able to contain the infection and establish immunity against reinfection (Sadick *et al.* 1990). The role of IL-4 in susceptibility to *Leishmania* remains controversial, with *L. major* substrains influencing the outcome of disease in IL-4 deficient mice (Noben-Trauth *et al.* 1999). Some studies have shown IL-4 to be responsible for susceptibility to *L. major* (Kopf *et al.* 1996), while others have shown IL-4 independent mechanisms responsible for susceptibility (Noben-Trauth *et al.* 1996). Further studies in IL-4R$\alpha^{-/-}$ BALB/c mice demonstrated that IL-13 played a protective role in maintaining host immunity to chronic *L. major* infection (Mohrs *et al.* 1999). However, IL-13 has been implicated as a susceptibility factor in *L.* major infection (Matthews *et al.* 2000).

Recent investigations in our lab have dissected the importance IL-4Rα signalling in the absence of IL-4 responsive CD4+ T Lymphocytes (Radwanska, *et al.* 2006 submitted). By deleting the IL-4Rα (and cellular response to IL-4) specifically from CD4+T cells (Lck^{cre}IL-4R$\alpha^{-/flox}$), BALB/c mice developed a healing disease phenotype and control infection similar to resistant C57/BL6 mice. Therefore, the complete abrogation of IL-4Rα from all cells is not appropriate to confer immunity to BALB/c mice.

1.3 Nippostrongylus brasiliensis

1.3.1 Background

Intestinal nematodes (roundworms) are prevalent in humans and are thought to infect approximately one billion people worldwide (Finkelman *et al.* 1997). Belonging to this family are Strongylata, which can be subdivided into three superfamilies; the Strongyloidea predominantly in man (e.g. *Ancylostoma duodenale* and *Necator americanus*), Trichostrongyloidea in rodents (e.g. *Nippostrongylus brasiliensis*) and Metastrongyloidea lung worms in mammals (Schmidt and Roberts 1989). The infection and migration pattern of *Nippostrongylus brasiliensis* (*N. brasiliensis*) is similar to that of several human intestinal round worm infections; including *Ancylostoma duodenale* and *Necator americanus* and can be used to investigate the immune response to these parasites (Gause *et al.* 2003).

The most cost effective approach at controlling hookworm infections (besides the sanitary disposal of faeces) is through population-wide treatment with Albendazole or Mebendazole. As reinfection occurs within a few months, repeated use and eventual drug resistance is probable. As stated by the WHO, "A safe and cost-effective vaccine would provide an important new tool for the control of hookworm infection". Successful immunisation against these worms requires understanding of the immune mechanisms that are involved in their expulsion (www.who.int/vaccine_research).

The life cycle of *N. brasiliensis* in the host (Figure 1.3), begins with subcutaneous infection by third stage (L3) larvae. Upon initial infection localised T_H2 driven responses occur in draining lymph nodes. L3 larvae migrate to the lung via the circulatory system causing considerable lung damage (demonstrated by perforated lungs 24 hrs post infection). L3 moult to L4 larvae and migrate via the trachea to the small intestine 3 days after infection. The immune reaction stimulated by this parasite is both, systemic and mucosal and most studies have focused on the mucosal response in the lung and small intestine (Gause *et al.* 2003). T_H2 mediated responses are responsible for the expulsion of the worm within 14 days post infection, with IL-13 being the essential cytokine (Gause *et al.* 2003). Important to note: a study demonstrated that secretory products from infective *N. brasiliensis*, induced a rapid allergic airway inflammatory response within 11 days post sensitisation (Marsland *et al.* 2005). This demonstrates a possible systemic reason for the lung pathology observed days after the worms have cleared. Therefore, of interest in this project are the mucosal and systemic immune responses.

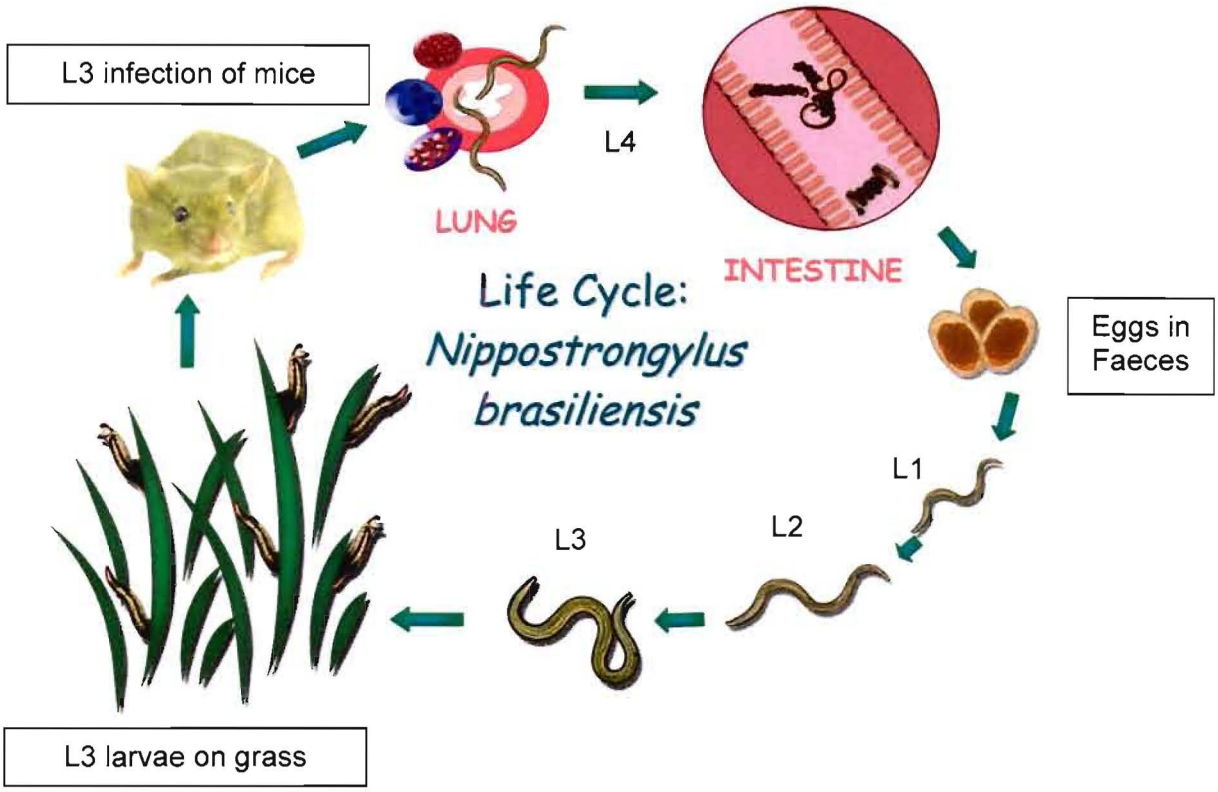

Figure 1.3: Life cycle of *Nippostrongylus brasiliensis*. Third stage larvae (L3) infect mice and migrate in the circulation to the lungs where they elicit a highly polarised T_H2 response. They then moult to L4 larvae and migrate to the intestine (< 50 hrs post infection) where eggs are produced. Worms are usually expelled by 14 days post-infection (adapted from Gause *et al.* 2003).

1.3.2 Immune Response to N. brasiliensis

The correct immune response is critical to the outcome of infection. T_H2 responses are important in the resistance of infection. A central role for CD4+ T cells in *N. brasiliensis* has been demonstrated by depletion of these cells with specific rat anti-mouse mAb's prior to infection (Katona *et al.* 1988).

Well-established T_H2 induced effector mechanisms following *N. brasiliensis* infection are; (i) Eosinophilia (Yamaguchi *et al.* 1988; Coffman *et al.* 1989), (ii) Mucosal mastocytosis (Madden *et al.* 1991), (iii) Synthesis of pathogen specific antibodies such as IgE and IgG1 (or IgG4 in humans) (Vitetta *et al.* 1985; Coffman *et al.* 1986), (iv) Goblet cell hyperplasia (McKenzie *et al.* 1998) and (v) Promotion of T_H2 cytokine responses such as IL-5, IL-10 and IL-9 (Coffman *et al.* 1989).

Despite the close association between nematode infections; and eosinophilia, mastocytosis, antibody production and goblet cell hyperplasia; it is still difficult to demonstrate a role for these effector mechanisms in resistance to primary *N. brasiliensis* infection. Although significant eosinoplilia is observed (associated with an increase in IL-5), blocking the recruitment of eosinophils with anti-IL-5 mAbs does not prevent the expulsion of *N. brasiliensis* (Coffman *et al.* 1989; Else and Finkelman 1998). Supporting this data were studies in which the eosinophil lineage was completely deleted and the worms were expelled (Yu *et al.* 2002), or IL-5 transgenic mice trapped the worms at the site of infection (Daly *et al.* 1999). Likewise, treatment of mice with anti-IL-3 and anti-IL-4 mAbs (results in 85% decrease in mast cells) did not prevent worm expulsion (Madden *et al.* 1991; Else and Finkelman 1998). Furthermore, a non-essential role for antibodies was demonstrated in STAT-6 knockout mice, which developed parasite specific T_H2 antibodies but were unable to expel the worm (Else and Finkelman 1998; Urban *et al.* 1998). Evidence, suggesting that goblet cell hyperplasia is important in worm expulsion, was shown in a correlation with peak mucin production observed in *T. spiralis* (Else and Finkelman 1998) and IL-13 driven goblet cell hyperplasia (McKenzie *et al.* 1998; Herbert *et al.* 2004). It has been shown that CD4+ T cells play an essential role in secretion of T_H2 cytokines correlated with worm expulsion (Finkelman *et al.* 2004). Furthermore, IL-4Rα (Barner *et al.* 1998; Finkelman *et al.* 1999; Urban *et al.* 2001) and STAT-6 (Takeda *et al.* 1996; Urban *et al.* 1998) were required for host protection against *N. brasiliensis*. Moreover, IL-4Rα responsiveness on smooth muscle cells was shown to be beneficial for *N. brasiliensis* expulsion by augmenting T_H2 cytokine responses and goblet hyperplasia (Horsnell *et al.* 2006, submitted).

It is well established that IL-4 and IL-13 have key roles in the development of a T_H2 cell phenotype. However, protection against *N. brasiliensis* is IL-4 independent. IL-4 deficient mice expel worms as wild-type mice (reviewed by Brombacher 2000). An essential role for IL-13 in worm expulsion, was highlighted in studies involving IL-4Rα-deficient (Noben-Trauth *et al.* 1997; Brombacher 2000), STAT-6 (Shimoda *et al.* 1996; Brombacher 2000) or IL-4 / IL-13 double deficient mice (Brombacher 2000; Ritz *et al.* 2002) which were unable to expel the worms. Furthermore, blocking IL-13 in BALB/c mice inhibited the expulsion of *N. brasiliensis* (Urban *et al.* 1998; Brombacher 2000). It is important to mention that a recent publication by Voehringer *et al.* 2006, has suggested a role for IL-4 / IL-13 expression by haematopoietic cells of the innate immune system in *N. brasiliensis* control. Their findings reveal a partitioning of IL-4 / IL-13-mediated effector function between adaptive T_H2 cells and innate immune cells. They suggested that tissue infiltration by effector cells was dependent on IL-4 / IL-13

from innate immune cells such as basophils. In order to investigate the role of IL-4 specifically on T cell function in *N. brasiliensis* infection, mice with a *lox*P-flanked IL-4Rα allele and Cre-recombinase expression were generated (described below). Thus, restricting Cre-mediated *lox*P recombination to T cells only.

1.4 IL-4 and the IL-4 Receptor-alpha (IL-4Rα)

Murine IL-4 is a glycoprotein with a molecular weight of 14-19 kDa (Yokota *et al.* 1986), localised on chromosome 11 together with genes of IL-5 and IL-13 (Morgan *et al.* 1992). The human IL-4 gene consists of, four exons and three introns spanning 10kb (Arai *et al.* 1989). IL-4 binds to its target cells through the IL-4Rα, which is localised to chromosome 7 (Mosley *et al.* 1989). Innate sources of IL-4, such as basophils (Min *et al.* 2004), mast cells (Plaut *et al.* 1989), γδ T cells (Ferrick *et al.* 1995), NK1.1$^+$ T cells (Yoshimoto and Paul 1994), eosinophils (Sabin *et al.* 1996) and conventional T cells (Launois *et al.* 1995; Noben-Trauth *et al.* 2000) have been shown to initiate T_H2 differentiation. However, IL-4-independent T_H2 differentiation has also been described (Noben-Trauth *et al.* 1997; Brombacher 2000; Jankovic *et al.* 2000; Mohrs *et al.* 2000; Ritz *et al.* 2002; Cunningham *et al.* 2004). Furthermore, IL-4 / IL-13 have been shown not to be essential for T_H2 differentiation, as demonstrated in IL-4Rα and STAT-6-deficient mice that are able to generate sufficient numbers or T_H2 cells in response to nematodes (Noben-Trauth *et al.* 1997; Finkelman *et al.* 2000). In order to respond to cytokine signals, cells require the appropriate cytokine receptor.

The IL-4 receptor consists of a 140-kDa IL-4Rα chain which, in association with the gamma common (γc) chain, binds IL-4 with high affinity. The IL-4Rα chain also functions as a component of the IL-13 receptor by association with IL-13Rα1 (Nelms *et al.* 1999). Although IL-13 has no functional receptor on murine lymphocytes, IL-4 and IL-13 share a number of functional characteristics (Brombacher 2000). They both signal through the IL-4Rα pathway and their activity is dependent on STAT-6 activation. They also have key roles in the maintenance of T_H2 responses. The importance of STAT-6 activation in IL-4Rα signalling was shown in STAT-6 knockout mice infected with *N. brasiliensis*. The mice failed to develop CD4+ T_H2 cells and could not expel the worm (Urban *et al.* 1998) similar to that of IL-4Rα global knockout mice (Nelms *et al.* 1999).

1.5 Cell-specific Gene Targeting

The IL-4 Receptor-alpha (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 and is accomplished by using Cre / *loxP* recombination. Cyclization recombinase (Cre) inserted downstream of the promoter (Lck or CD4) recognises a pair of *loxP* binding sites flanking the gene of interest (Exon7 through 9 of IL-4Rα). Cre-recombinase brings the two *loxP* sites together, removing the intervening DNA (Nagy 2000). To specifically target IL-4Rα deletion on T cells, Cre-recombinase expression was driven using T cell specific promoters (Figure 1.4). Three T cell specific IL-4Rα-deficient BALB/c mice were created: (i) Lck proximal promoter driven deletion = $Lck^{cre}IL4R\alpha^{-/flox}$ mouse – characterised by Radwanska, *et al.* 2006 submitted, (ii) Improved Lck proximal promoter driven deletion = novel $Lck^{cre}IL4R\alpha^{-/flox}$ mouse and (iii) CD4 promoter driven deletion =novel $CD4^{cre}IL4R\alpha^{-/flox}$ mouse. IL-4Rα hemizygosity (mice bearing one floxed and one disrupted IL-4Rα allele: -/flox) increases the probability of Cre-mediated deletion of the floxed allele without affecting the function of the gene.

1.6 T cell development (CD4 and Lck expression)

In order to understand which T cell subsets IL-4Rα would be deleted on, it is necessary to determine where lck and CD4 driven Cre deletion would occur. T lymphocytes make cell development choices: T cell rather than B cell, TCRαβ or TCRγδ, CD4 (helper) or CD8 (killer) and T_H1 or T_H2 (see Figure 1.5). These decisions require a change in gene expression and with the exception of the T cell receptor (TCR) occur without change in DNA sequence (Nagy 2000; Lee *et al.* 2001).

CD4 is a cell surface glycoprotein, usually on helper T-cells, which recognises Major Histocompatibility Complex class II molecules on target cells (Roitt 1994). The point of initial CD4 expression in T cell development has been suggested to be after the $CD4^-CD8^-$ double-negative (DN) stage (Lee *et al.* 2001). However, CD4 has also been shown to be expressed on the earliest T-lineage precursor cells, thought to extend to the earliest thymocytes, after which it is lost at the DN precursor stage and then regained at the $CD4^+CD8^+$ double-positive (DP) stage (Wu *et al.* 1991). These results suggest that the IL-4Rα be deleted from all T cells (including αβ and γδ T cells). However, if the former is true the IL-4Rα would only be deleted from DP T cells, including $CD4^+$, $CD8^+$ and possibly NKT cells (Godfrey *et al.* 2004). Due to

discrepancies in the literature as to when CD4 is first expressed in T cell development, the characterisation of the novel CD4^{cre}IL-4Rα$^{-/flox}$ mouse is essential to establish the specificity of the deleted IL4Rα.

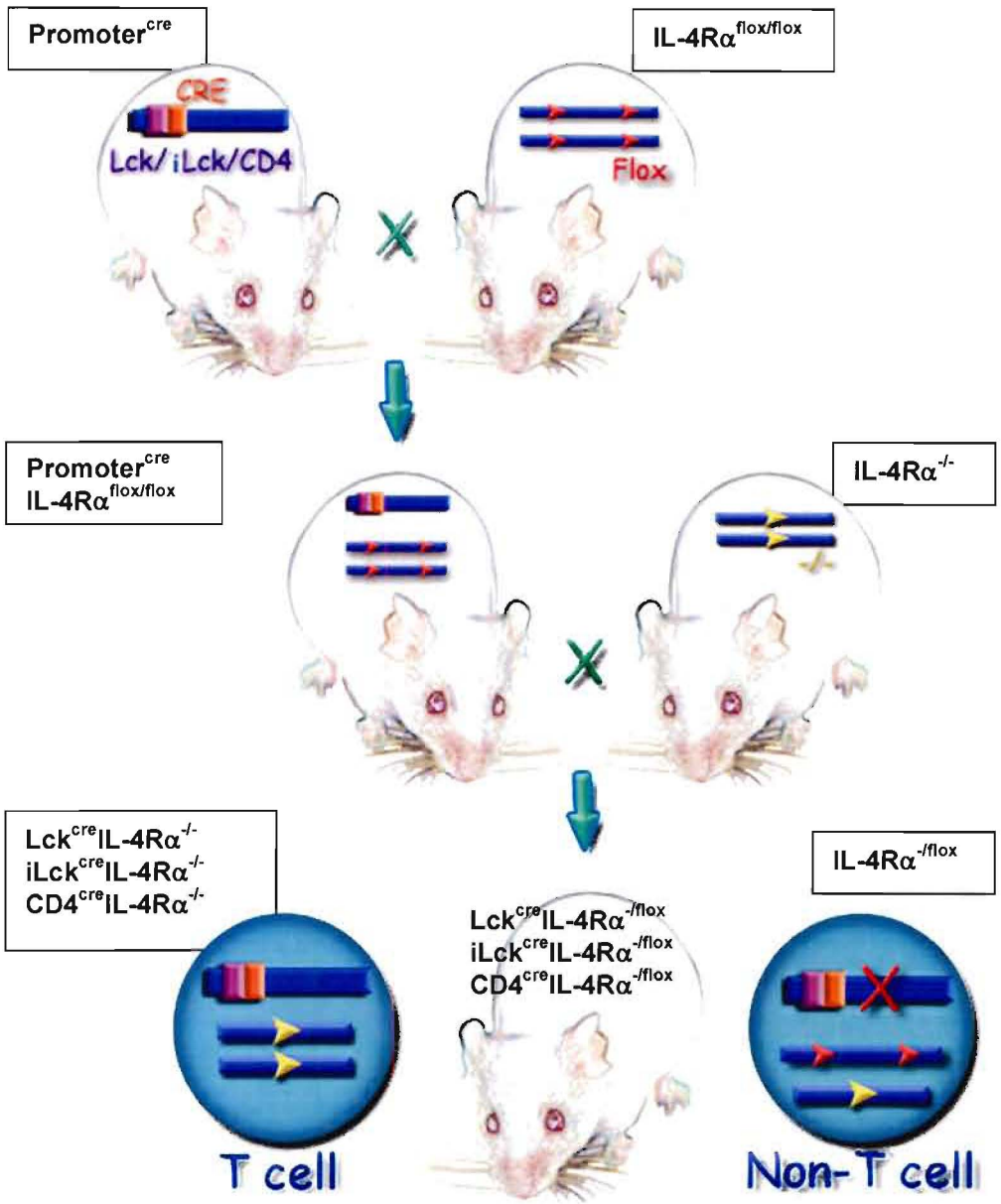

Figure 1.4: Generation of T cell specific IL-4Rα deficient BALB/c mice by the Cre/*loxP* recombination system. Cre-recombinase was inserted after the Lck, iLck and CD4 promoters and 2 *loxP* sites were inserted into the IL-4Rα gene. Crossing the flox / flox strain with Lck^{cre}, iLck^{cre} and CD4^{cre} generated flox mice expressing Cre. These mice were crossed with a global IL-4Rα$^{-/-}$ mouse to generate Lck^{cre}IL4Rα$^{-/flox}$, novel iLck^{cre}IL4Rα$^{-/flox}$ and CD4^{cre}IL4Rα$^{-/flox}$ mice (illustrated according to Mohrs *et al.* 1999; Brombacher 2000; Herbert *et al.* 2004; Radwanska *et al.* 2006 submitted).

Lck protein tyrosine is a kinase involved in the T cell signal transduction pathway (Janeway and Travers 1996). In T cell differentiation, Lck is necessary to halt β chain rearrangement and induce cell proliferation; therefore its absence stops the development before the DN stage (Janeway and Travers 1996). The precise divergence of the γδ T cells remains unclear (Tanigaki *et al.* 2004) and has been suggested to be after the initial expression of Lck (Lee *et al.* 2001). This would result in the deletion of the IL-4Rα from γδ T cells. If γδ T cells diverge from the αβ TCR lineage prior to Lck expression they would retain the IL-4Rα. A recent publication in our lab (Radwanska *et al.* 2006, submitted) has demonstrated the partial deletion of IL-4Rα from CD8 T cells using Lck^{cre} driven deletion, for this reason an improved $iLck^{cre}$ mouse was acquired. To determine the nature of IL-4Rα deletion it is essential to characterise the expression of the receptor on T cell subpopulations (CD4, CD8, γδ and NK T cells).

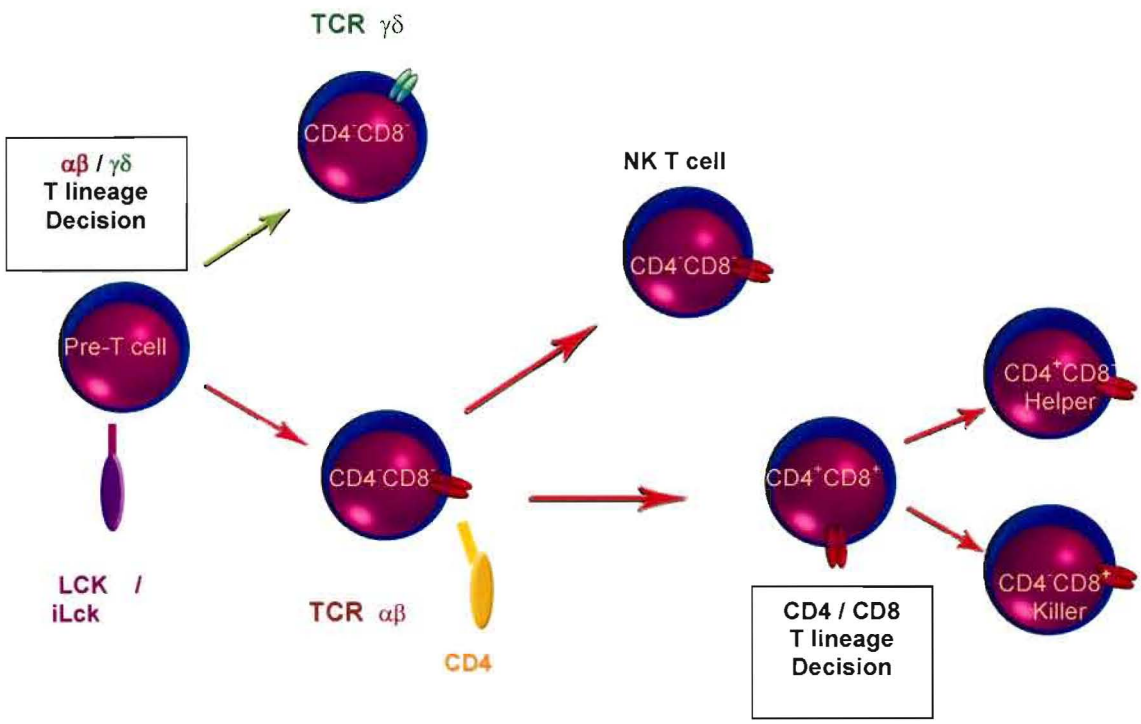

Figure 1.5: T cell development. Stages of T cell development at which Lck^{cre} (and i Lck^{cre}) or $CD4^{cre}$ transgenes are first expressed. T cell lineage decisions are between, TCRαβ or TCRγδ, CD4 (helper) or CD8 (killer) and TH1 or TH2. Abbreviations: TCR-T cell Receptor, Lck- Lck protein tyrosine (illustrated according to the above literature).

In conclusion, by deleting the IL-4Rα at sequential stages of T cell development, the role of T cell responses to IL-4 in infection and allergy may be determined. As IL-4 has been shown to be an important mediator in promoting a polarised T_H2 response against various infectious diseases and allergens, it is important to understand its potential role in shaping the adaptive immune response by means of innate T cell responses. Our results demonstrate that the deletion of IL-4Rα is restricted to T cells with normal expression on non-T cells. The expression of IL-4Rα is shown to be only partially deleted from T cell subpopulations. T cell-specific IL-4Rα-deficient BALB/c mice infected with *L. major* develop a healing disease phenotype and clinical immunity similar to genetically resistant C57/BL6 mice. This suggests that polarised T_H2 cellular responses may be responsible for susceptibility to *L. major.* We show that T cell-specific IL-4Rα-deficient mice can successfully clear *N. brasiliensis* despite an impaired T_H2 response, indicating that IL-4 mediated cellular responses are not crucial for *N. brasiliensis* expulsion and are the cause of increased lung pathology.

2. Materials and Methods

2.1 Characterisation of T-cell specific IL-4Rα knockout mice

2.1.1 Generation of T-cell specific IL-4Rα knock out mice

As previously described Lck^{cre}IL-4Rα$^{-/flox}$ (Radwanska *et al.* 2006, submitted), novel iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice were generated by mating floxed IL-4Rα$^{flox/flox}$ mice with transgenic Lck^{cre} (Gu *et al.* 1994) iLck^{cre} and CD4^{cre} mice (Lee *et al.* 2001). These mice were back-crossed to the BALB/c strain for 9 generations and then with global IL-4Rα$^{-/-}$ knockout (KO) mice (Mohrs *et al.* 1999) to generate Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice. Transgene negative IL-4Rα$^{-/flox}$ (WT) littermates and global IL-4Rα$^{-/-}$ mice were used as controls in all experiments, while C57/BL6 mice were used as resistant controls in the *L. major* infection model. The mice were all bred and housed in the Animal Facility, Chris Barnard Building, University of Cape Town and maintained in specific pathogen free (SPF) conditions and handled as per care of laboratory animals protocol (Coligan *et al.* 1994). In addition, the animal facility was routinely checked for pinworm and all mice were genotyped (to confirm deletion) in the Dept. of Immunology. All experiments complied with the South African Code of Practice, and were approved by the University of Cape Town's Animal Ethics Committee.

2.1.2 Genotyping of T-cell specific IL-4Rα knockout mice

The genotype of Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice was confirmed by the following PCR reactions in Table 2.1 (Mohrs *et al.* 1999):

Table 2.1: Primers for genotyping T cell specific IL-4Rα mice.

Target	Primer
WT forward	5'-TGACCTACAAGGAACCCAGGC-3'
WT reverse	5'-CTCG GCGCACTGACCCATCT- 3'
KO forward	5'-GGCTGCTGACCTGGA ATAACC- 3'
KO reverse	5'-CCTTTGAGAACTGCGGGCT-3'
loxP forward	5'-CCC TTCCTGGCCCTGAATTT-3'
loxP reverse	5'-GTTTCCTCCTACCGCTGATT-3'
Cre forward	5'-GGTGAACGTG CAAAACAGGCTCTA-3'
Cre reverse	5'-ACCTGTGCATCCTGAATGAT- 3'

PCR conditions were as follows: 94°C/1min, 94°C/30sec, 55°C-60°C/20sec (primer dependant), 72°C/1min for 40 cycles and 72°C/5min using a MJ thermocycler (Biozym, Hessissch, Oldendorf, Germany).

2.1.3 Analysis of surface IL-4Rα expression by FACS

IL-4Rα surface expression was analysed in T-lymphocytes including CD4 cells ($CD4^+$ / $CD8^-$), CD8 cells ($CD4^-$ / $CD8^+$), γδ T cells (γ-$chain^+$ / $CD3^+$/ β-$chain^-$) and NK T cells (β-$chain^+$ / $DX5^+$). Furthermore, the surface expression of IL-4Rα was analysed on NK cells (β-$chain^-$ / $DX5^+$) and B cells ($CD19^+$ / $CD3^-$). Spleens or lymph nodes were isolated and single cell suspensions were prepared as described in appendix D. All monoclonal antibody incubation steps were performed in FACS buffer (see Appendix A for all solutions). The cells were incubated with 1% heat inactivated rat serum to prevent non-specific binding and stained with fluorescent labelled anti-mouse mAbs for IL-4Rα, CD3, CD4, CD8, DX5, CD19, β-chain or γ-chain. All antibodies were purchased from BD pharmigen, unless otherwise stated (see appendix B). Acquisition was performed using FACS Calibur (Beckton-Dickinson, South Africa) and analysed by Cellquest.

2.1.4 Efficiency of IL-4Rα deletion by Real-Time PCR

To establish the efficiency of IL-4Rα deletion at the genomic level from FACS sorted cells, Real-Time quantitative PCR reactions were designed. Primers amplifying genomic regions of the IL-4Rα gene affected or unaffected by the deletion were used (see table 2.2). The PCR products were successfully cloned into the pGEM-t Easy vector to prepare standards, allowing for comparative results.

Table 2.2: Primers for determining the efficiency of IL-4Rα deletion

Target	Primer
Intron 5 forward	5'-AACCTGGGAAGTTGTG-3'
Exon 5 reverse	5'-CACAGTTCCATCTGGTAT-3'
Intron-exon 8 forward	5'-GTACAGCGCACATTGTTTTT-3'
Exon 8 reverse	5'-CTCGGCGCACTGACCCATCT-3'

*Intron-exon 5 remains constant and Intron-exon 8 is deleted.

2.1.4.1 Real-Time PCR standards

A conventional PCR reaction was used to produce PCR product for each primer pair described above. The PCR product was purified by (i) Agarose gel extraction with the QIAquick Gel Extraction Kit (Qiagen, Germany) or (ii) PCR product purification with QIAquick PCR Purification Kit according to the manufacturer's instructions. The purified PCR products were ligated into the pGEM-T Easy vector (Promega, USA) using the Rapid DNA Ligation Kit (Roche, Germany) (shown in Figure 2.1). The Taq-Polymerase used in the PCR reaction, added an extra adenosine (A) at the end of the product. The vector containing a Thymidine (T) overhang facilitated the ligation

via A/T overlay. Ligation mix was added to calcium chloride competent Top 10 *E. coli* cells (Invitrogen, Gibco BRL) and incubated on ice for 30 min. The cells were heat-shocked at 42°C for 45 sec, placed on ice for 2 min and room temperature (RT°) SOC medium was added. After incubation with shaking (160 rpm) at 37°C for 1 hr, the cells were plated on LB agar plates containing 50 µg/ml ampicillin (pGEM-T Easy expresses the gene for ampicillin resistance), 40 mg/mL X-gal and 100 mM IPTG for blue / white colony screening. Non-recombinant cells synthesize β-galactosidase, which breaks down X-gal and produces a blue colour, white cells are therefore recombinant and were selected. Mini-preps were prepared using QIAprep® Spin Miniprep Kit (Qiagen, Germany) and positive constructs were identified by restriction enzyme screening as shown in Table 2.3:

Table 2.3: Restriction enzymes used for double digestion

PCR Product	Enzyme
Intron-exon 5	*EcoR* I, *Pst* I
Intron-exon 8	*Sph* I, *Eco*1CRI

The digested bands were visualised by electrophoresis on a 1-2% agarose gels containing ethidium bromide (which intercalates between nucleic acid bases and fluoresces red-orange under ultra violet light). Plasmids containing the correct inserts were sequenced by automated nucleotide sequencing, midi-preps were prepared using Wizard®Plus Midipreps DNA Purification Systems (Promega, Madison, USA) and glycerol stocks were stored at -80 °C.

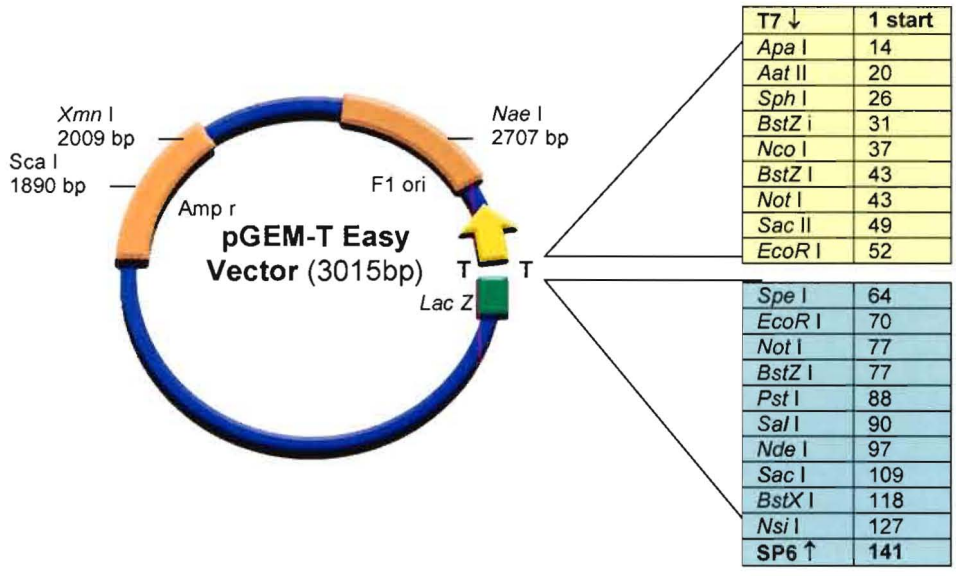

Figure 2.1: pGEM-T Easy Vector circle map and sequence reference points. PCR products (Intron-exon5 and Intron-exon8) were cloned into the pGEM-T Easy vector using T overhangs, compatible with the product from the thermostable polymerase.

2.1.4.2 Fluorescence-activated cell sorting (FACS)

Cells (1 X 10^7 – 5 X 10^7) were treated as described above and labelled with rat anti-mouse CD4, CD8 or CD19 mAbs. Cell sorting was performed on a FACS Vantage (Beckton-Dickinson, South Africa) in FACS buffer without Sodium Azide (NaN_3) as previously described (Dai *et al.* 1997). The sorted cells (1 X 10^5 – 1 X 10^6) were collected in 1% BSA with the resulting $CD4^+$, $CD8^+$ or $CD19^+$ population at a purity of >99%. The cells were centrifuged at 1200 rpm for 5 min at 4°C and placed in 1 ml of digestion buffer for genomic DNA extraction or Tri-reagent (Molecular Research, USA) / 1 X 10^6 cells for mRNA extraction.

2.1.4.3 DNA / mRNA extraction and Real Time PCR

DNA was extracted from FACS sorted cells by overnight incubation at 55°C in digestion buffer and 0.75 mg/ml proteinase K (Roche, Germany). Tris equilibrated phenol (0.5 ml, pH8) was added and after centrifugation (10 000 rpm for 3 min at 4°C) the aqueous phase was collected. This step was repeated with 0.5 ml phenol/chloroform (1:1); the DNA was precipitated with 100% ice cold ethanol and 3M sodium acetate and stored at -20°C overnight. The pelleted DNA was washed in 70 % ethanol, dissolved in ddH_2O and the absorbance determined at 260 nm.

The presence (genomic DNA) of IL-4Rα was analysed by Real Time PCR on a Lightcycler (Roche, Germany), by the "Standard Curve Method" using β-actin as a housekeeping gene. The relative copy number of Intron-exon 8 vs Exon 5 were expressed.

2.1.5 Functional analysis of cellular IL-4Rα responsiveness

2.1.5.1 T-cell proliferation

FACs sorted $CD4^+$, $CD8^+$, $\gamma\delta^+$ and NK T cells (5 X 10^4 / well) in triplicate were restimulated with serial dilutions of IL-2 or IL-4 (BD Pharmingen) in complete IMDM containing 5 ng/ml Phorbol 12-myristate 13-acetate (PMA). T cells obtained from mouse spleens or lymph nodes demonstrate low levels of cytokine expression. PMA is a protein kinase C activator, which enhances cytokine expression (Coligan *et al.* 1994). Following culture for 48 hrs in 5% CO_2 at 37°C, cells were pulsed with 1 µCi [^{3}H] thymidine (AEC Amersham, Sweden) for 18 hrs. Cells were harvested and thymidine incorporation was determined by a liquid scintillation counter. Values represent the mean ± SEM from triplicate cultures.

2.1.5.2 In Vitro T_H2 differentiation

$CD4^+$ lymph node cells were purified using the magnet cell sorting (MACS) bead system as instructed by the manufacturer (Miltenyi Biotec, Germany). Positively selected cells were stained and acquired by FACS Calibur revealing > 90% purity. The cells were cultured at 1 X 10^6 / ml in flat bottom microwells (2 X 10^5 / well) pre-coated with anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml) and cultured in RPMI supplemented with IL-2 (20 U/ml). T helper development was driven by IL-12 (5 ng/ml), IL-4 (50 ng/ml) or IL-13 (100 ng/ml) and neutralising antibody for IL-4 (11B11, 10 ug/ml) or IFN-γ (50 μg/ml) as indicated. After 72 hrs, the cells were washed and new media was added containing the above supplements. After 48 hrs the cells were washed extensively, transferred to fresh anti-CD3 coated microwell plates and cultured in IL-2 (20 U/ml). Cell free supernatants were collected after 48 hrs of culture without additional supplements. Values represent means and standard deviations of triplicate cultures.

2.1.5.3 Induction of IgE response

Mice were sensitised by intraperitoneal (i.p.) inoculation of 50 μg Ovalbumin (Sigma, Germany) in 1.5% aluminum hydroxide adjuvant (Sigma, Germany) and boosted at 7 and 14 days. Alum is the most frequently used adjuvant in commercial vaccines and is associated with the induction of a T_H2 response in animal models (Rimaniol *et al.* 2004) Blood was collected at days 0, 7 and 14 by warming the tail with a heat lamp and incising across the lateral vein. The blood was collected in a microtainer gel separation tube (BD, USA) and centrifuged at 4500 rpm for 20 min at 4°C to separate the serum from the cells and then stored at 4 °C or -20°C until used.

2.2 *Leishmania major* Infection Studies

L. major LV 39 (MRHO/Sv/59/P-strain) (Mattner *et al.* 1996) was maintained by continuous passage in susceptible BALB/c mice. The mice were infected subcutaneously in one hind footpad with 2 x 10^6 stationary phase metacyclic promastigote parasites/ 50ul. Footpad swelling was monitored weekly using a pocket thickness gauge (Mitutoyo, Japan). The infected footpad (swelling lesions) or popliteal lymph node was removed and placed in 5 ml Schneiders medium (Sigma) supplemented with 20% Fetal Calf Serum (FCS) in a tissue culture flask. After 4 days in a humidified chamber at RT°, the parasites were counted in 0.1% glutaraldehyde daily. Once the stationary growth phase was reached (day 6 – 7) with ~ 10^8 parasites / ml the parasites were prepared for infection. Measurements were taken at weekly

intervals until sacrifice at 3, 6, 8 or 12 weeks post infection or a swelling of ~5mm was recorded.

2.2.1 Quantification of parasite burden

Parasite burden was determined by the limiting dilution method. Mice were killed by CO_2 asphyxiation and sterilised with 70% ethanol. The infected footpad was collected in Schneider's media and popliteal lymph node in IMDM with 10% FCS. The footpad was homogenised and a single cell suspension was prepared from the lymph node to a final volume of 6.4 ml (100 µl = 2^6 parasites). Two-fold serial dilutions were prepared in Schneider's media with 20% FCS and the cultures were examined for parasites after ~ 7 days at RT°.

2.2.2 Ex vivo cytokine production

2.2.2.1 Individual Lymph Node restimulation

Individual popliteal lymph node single cell suspensions were prepared (as Appendix E) and 2 x 105 cells / well were restimulated in a 96 well, round bottom culture plate (Costar, Cambridge, USA) with 50 µg/ml Soluble L. major antigen (SLA) or IMDM / 10% FCS media (control). The cells were incubated for 48-72 hrs at 37°C, 5% CO_2 and the supernatant was stored at -80°C until use. SLA was prepared from stationary phase metacyclic promastigotes by sonication 3 times for 30 sec at 4°C in the presence of protease inhibitors (Sigma) and frozen at -80°C until needed. The protein concentration was measured using BCA protein kit (Pierce, Rockford, IL) as described in Appendix E.

2.2.2.2 CD4+ restimulation with Antigen Presenting Cells (APCs)

Popliteal lymph node single cell suspensions were pooled and purified (by negative selection) for $CD4^+$ cells using the Biomag® bead system (Qiagen), as per manufacturer's instructions. APCs were T cell depleted by FACs sorting of non-Thy1.2 labeled splenocytes or incubation with rabbit serun. T cell depleted APC were fixed with Mitomycin C (50 µg/ml, 20 min at 37°C) and washed 3X in complete IMDM. SLA at 50 µg/ml was added to 2 x 10^5 purified CD4+ T cells and 1 x 10^5 APCs and incubated for 48-72 hrs at 37°C, 5% CO_2 and the supernatant was stored at -80°C until used.

2.2.3 *Delayed Type Hypersensitivity (DTH) reaction*

DTH is a hypersensitivity reaction occurring within 48-72 hours and mediated by T_H1 cytokine release from *L. major* sensitised T-cells (Roitt 1994). Mice were anaesthetised and inoculated with 10 μg/ml SLA in the right hind footpad. Footpad swelling was measured using the pocket thickness gauge at 24 hr intervals for 4 days.

2.2.4 *Enzyme-linked Immunosorbent assays (ELISA)*

2.2.4.1 Cytokine ELISAs

The concentration of cytokines; IFN-γ (T_H1), IL-4, IL-13 and IL-10 (T_H2) were determined by sandwich ELISA. Ninety-six well plates (Nunc-immuno maxisorb, Denmark) were coated with primary 'capture' antibody (see appendix C for all antibodies and standards) and incubated overnight (o/n) at 4°C. The plates were washed 4X (between each step) with washing buffer and blocked with 2% milk powder o/n 4°C. Neat samples or standards (diluted 3-fold in dilution buffer) were added and incubated at 4°C o/n. The appropriate biotinylated secondary antibody was added for 3 hrs at 37°C followed by Streptavidin coupled to Horseradish peroxidase (HRP) for 1 hr at 37°C. The activity of HRP was measured using the TMB Microwell Peroxidase Substrate System and stopped with 1M H_3PO_3. The optical density (OD) was measured at 450 nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany).

2.2.4.2 Antigen Specific Antibody ELISAs

An indirect ELISA was used to detect specific antibodies (IgE, IgG1 and IgG2a) against SLA. A 96 well plate was coated with 5 μg/ml of SLA and the protocol was followed as above. Individual sera samples were added at 10-fold dilutions. The plates were incubated with anti-mouse polyclonal alkaline phosphatase (AP) labelled antibodies for 1 hr at 37°C and developed with 4-Nitrophenylphosphate (PNP) (Fluka, Steinheim, Switzerland) diluted in substrate buffer to a concentration of 1 mg/ml. The OD was measured at 405 nm (492 nm reference) as above.

2.3 *Nippostrongylus brasiliensis* Infection Studies

N. brasiliensis was maintained by passage through 6-8 Wistar rats. Live L_3 larvae (~5000 worms / rat in 0.9% saline) were injected subcutaneously in the nape of the neck using a 20G needle (Fine-Ject, Germany). The faeces were collected from Day 5 - Day 8 post infection in fungizone (Gibco) treated dH_2O. The emulsified faeces were mixed into a paste with equal volumes of activated charcoal (Sigma, Germany) allowing the eggs to hatch and the larvae to migrate to the edge of the Whattman filter paper.

Infective *N. brasiliensis* L3 third-stage larvae were isolated from filter paper 7 days later. Worms were washed in 0.65% NaCl and 750 larvae/mouse in saline were injected subcutaneously into experimental mice. The mice were killed at 1, 3, 7, 10 or 14 days post infection. Two sections of the intestine were removed and subdivided in 4% formalin for histology or OCT (Sakura, Japan) for immunohistochemistry. The remainder placed in 0.65% NaCl to determine parasite burden. After Broncho-alveolar lavage (BAL) was performed, the lungs were removed for parasite burden or to prepare single-cell suspensions for FACs analysis of cellular infiltrates. Blood was collected to determine antibody (IgE, IgG1 and IgG2a) levels.

2.3.1 Quantification of parasite burden

2.3.1.1 Worm burden in Intestine and Lung

Each intestine was incised longitudinally or lung was cut finely, wrapped in gauze and incubated in a 50 ml Falcon tube (containing 0.65 % NaCl) for 24 hrs at 37°C. After allowing the worms to migrate out they were counted in a marked petri dish (Sterilin, UK) to determine parasite load.

2.3.1.2 Egg counts

The number of eggs present (relative to weight of faeces) was recorded daily and used as an indication of parasite burden. Faeces from individual mice were collected from day 5 to day 14 and emulsified in 1-2 ml 0.65% NaCl. Saturated NaCl (at least 5M) was added to 10ml and after centrifugation for 5 min at 1300 rpm the top layer (2 ml) was removed and counted in a McMaster egg Chamber.

2.3.2 Ex vivo cytokine production

CD4+ T cells were negatively selected from pooled mesenteric (MsLN) or mediastinal (MdLN) lymph nodes at days 1, 3, 7, 10 or 14 post infection using the Biomag® bead

system (Qiagen), as per manufacturer's instructions. Cells were restimulated for 72h with anti-CD3 (clone 145-2C11; 20μg/ml) and the supernatants were collected and stored at -80°C until use.

2.3.3 Broncho-alveolar lavage (BAL)

Mice were killed by i.p. injection of a lethal dose of anaesthetic. The trachea was cannulated and BAL was performed by flushing the lungs and airways 3 times with 1 ml IMDM / 5% FCS. The BAL cells were counted and 1 X 10^5 cells were spun onto glass slides. Slides were stained with Diff-quik (Clinical Science Diagnostics, South Africa) according to the manufacturer's instructions. The percentage neutrophils, eosinophils, lymphocytes and macrophages were determined using standard histology criteria.

2.3.4 Lung tissue single cell suspensions

Individual lungs were cut into small pieces using a scalpel blade and incubated in DMEM (Gibco) containing 50 U/ml Collagenase I (Gibco-Invitogen) and 13 μg/ml DNase I (Roche, Germany) for 90 min at 37°C. The tissue was pressed through a 70 μm sieve and Red Blood Cells (RBC) were lysed with RBC lysis buffer for 5 min on ice. The remaining cells were collected by centrifugation (1200 rpm for 5 min at 4°C) and stained with GR-1, CD11b, CD11c, CD3 and CD19 mAbs for FACS analysis.

2.3.5 Enzyme-linked Immunosorbant assays (ELISA)

2.3.5.1 Cytokine ELISAs

The concentration of cytokines; IFN-γ (T_H1), IL-4, IL-5 and IL-13 (T_H2) were determined by sandwich ELISA (as described above; *L. major* infection studies). TMB substrate was used to detect HRP activity and the OD was measured at 450 nm.

2.3.5.2 Total Antibody ELISAs

A sandwich ELISA was used to detect total antibodies (IgE, IgG1 and IgG2a) in individual mouse serum. A commercial 'capture' antibody (opposed to soluble parasite antigen as for *L. major*) was used. An AP labelled secondary antibody was developed with PNP and the OD was measured at 405 nm (492 nm reference).

2.3.6 Histology Techniques

All the histology techniques were performed by the Dept. of Histology, Groote Schuur Hospital, OMB and a detailed description of the protocol can be seen in Appendix E. The intestine and lungs were processed and stained with Haematoxilin & Eosin (H & E) and the Periodic Acid Schiffs (PAS) stain (for mucs producing goblet cells).

2.3.7 Periodic Acid Schiff's (PAS) Assay for Glycoproteins in mucin

Glycoproteins in mucin of lung and intestine tissue were quantified according to (Mantle and Allen 1978). Tissue samples were collected in PBS and homogenised. After 20 min centrifugation at 10 000 rpm the supernatants were collected and stored at -20°C until needed. A 1:10 dilution was made of the supernatant, to which periodic acid solution was added. A standard was prepared from commercially available stomach porcine mucin (Sigma). The samples were incubated for 1 hr at 37°C and decolourised Schiff's reagent added. After incubation at room temperature for 30min, the absorbance was determined on a spectrophotometer at 555nm.

2.4 Statistics

Values are given as mean ± SD or SEM and significant differences were determined using the unpaired one or two-tailed Student's t-test. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ (GraphpadPrism™).

3. Results

3.1 Characterisation of T-cell specific IL-4Rα-deficient mice

3.1.1 Generation and genotyping of T cell specific IL-4Rα-deficient BALB/c mice

Conditional cell-specific IL-4Rα-deficient BALB/c mice were generated as previously described by Herbert *et al.* 2004 and Radwanska *et al.* 2006, submitted (Figure 1.6 and 3.1.1A). IL-4Rα$^{flox/flox}$ mice were crossed with global IL-4Rα$^{-/-}$ mice to obtain hemizygous IL-4Rα$^{-/flox}$ (WT) mice (bearing one floxed and one disrupted allele) (Figure 3.1.1A). Cell-specific gene disruption in T cells was achieved through an intercross between hemizygous IL-4Rα$^{-/flox}$ mice and transgenic Lck^{cre} (Gu *et al.* 1994), improved Lck^{cre} and CD4^{cre} mice (Lee *et al.* 2001) on an IL-4Rα$^{-/-}$ background. To distinguish between Lck^{cre} (Gu *et al.* 1994) and improved Lck^{cre} (Lee *et al.* 2001), the later were termed iLck^{cre}. The use of IL-4Rα hemizygous (IL-4Rα$^{-/flox}$) mice increased the efficiency of Cre-mediated deletion of the floxed allele. Insertion of *lox*P sites had no influence on IL-4 receptor function, demonstrated by a dose-dependent proliferation of IL-4-stimulated lymphocytes in IL-4Rα$^{flox/flox}$ mice (Herbert *et al.* 2004).

Transgene-bearing hemizygous Lck^{cre}IL-4Rα$^{-/flox}$ (Radwanska *et al.* 2006 submitted), iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ (unpublished) mice (referred to collectively as T cell-specific IL-4Rα-deficient mice) were identified by PCR genotyping (Figure 3.1.1B-representative gel). Primers targeting Intron 6 and Intron 10 amplified product (471 bp) in IL-4Rα-deleted mice (Deletion). In BALB/c mice, the targeted region was too large for PCR amplification hence no PCR product was present (lane 5). As expected, T cell-specific IL-4Rα-deficient mice (lanes 1-3), WT (hemizygous IL-4Rα$^{-/flox}$) mice (lane 4- due to one disrupted and one floxed allele) and global IL-4Rα$^{-/-}$ (lane-6) were positive for IL-4Rα deletion. Primers designed to flank the *lox*P insertion site amplified a 188 bp product (floxed lanes 1-4) or a 94 bp product (BALB/c lane 5). The inserted Cre-transgene was amplified (450 bp) in all T cell-specific IL-4Rα-deficient mice (lanes 1-3) and not in WT (IL-4Rα$^{-/flox}$), BALB/c and global IL-4Rα$^{-/-}$ mice (lanes 4-6). These data confirm the successful generation of Cre transgenic, *lox*P flanked, IL-4Rα disrupted mice.

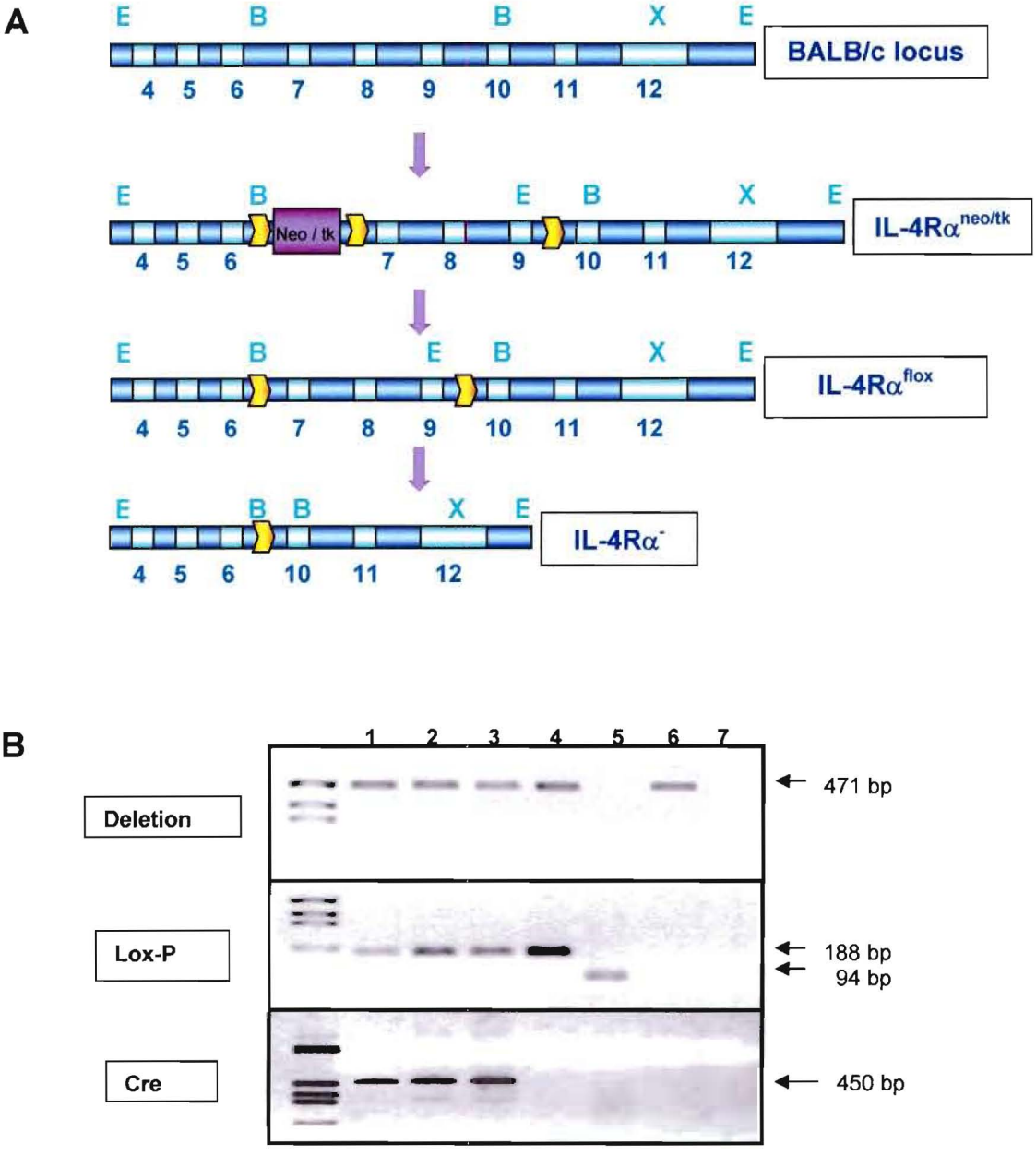

Figure 3.1.1: Generation of T cell specific IL-4Rα deficient BALB/c mice.
(A) IL-4Rα gene locus and targeted deletion. Introduction of (neo/tk) selection cassette flanked by tow *lox*P sites (arrowhead), and one *lox*P 5' of exon 10 resulted in the generation of the conditional "floxed" mouse after Cre-mediated recombination. An intercross between the floxed strain and Lck^{cre}, $iLck^{cre}$ and $CD4^{cre}$ knockin 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 (described by Herbert *et al.* 2004). **(B) Genotyping of transgene bearing T cell specific IL-4Rα-deficient mice*** Identification of IL-4Rα deletion and Lck^{cre}, $iLck^{cre}$ and $CD4^{cre}$ transgenes was achieved by three PCR reactions. IL-4Rα deletion (471 bp), *lox*-P (188 bp), or BALB/c (94 bp) and Cre-recombinase (450 bp) specific PCR. PCR products were visualised by electrophoresis on 1.6% agarose gel. 1- Lck^{cre}IL-4Rα$^{-/flox}$, 2- $iLck^{cre}$IL-4Rα$^{-/flox}$, 3- $CD4^{cre}$IL-4Rα$^{-/flox}$, 4- WT (hemizygous IL-4Rα$^{-/flox}$), 5- BALB/c, 6- IL-4Rα$^{-/-}$ and 7- negative control.

* Routine genotyping was performed by Erica Smit and Wendy Green, Dept of Immunology

3.1.2 Analysis of IL-4Rα surface expression by FACS

IL-4Rα surface expression was determined by flow cytometry and compared on various cell types to confirm specificity of the deletion. As expected, IL-4Rα was expressed on the surface of CD3+/CD4+, CD3+/CD8+, and CD19+ cells in WT mice and completely abrogated from all cell types in the global IL-4Rα$^{-/-}$ mice (Figure 3.1.2A). Surface expression of IL-4Rα on CD3+/CD4+ T cell subsets was completely abrogated in all T cell-specific IL-4Rα-deficient mice. IL-4Rα was efficiently deleted from CD3+/CD8+ cells of the iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ but only partially deleted from this subset in Lck^{cre}IL-4Rα$^{-/flox}$ mice. Cellular specificity of the deletion was confirmed by normal expression of IL-4Rα on B lymphocytes and NK cells. Surface expression of IL-4Rα was also determined on γδ (Figure 3.1.2B) and NK T (Figure 3.1.2C) cell subpopulations. The geometric mean fluorescence of IL-4Rα expression between all groups was compared: (i) γδ T cells, WT = 5.11, IL-4Rα$^{-/-}$ = 2.54, Lck^{cre}IL-4Rα$^{-/flox}$ = 5.19, iLck^{cre}IL-4Rα$^{-/flox}$ = 3.74 and CD4^{cre}IL-4Rα$^{-/flox}$ = 5.54. (ii) NK T cells, WT = 9.03, IL-4Rα$^{-/-}$ = 5.25, Lck^{cre}IL-4Rα$^{-/flox}$ = 7.28, iLck^{cre}IL-4Rα$^{-/flox}$ = 6.54 and CD4^{cre}IL-4Rα$^{-/flox}$ = 6.04. Table 3.1 summarises the surface expression of IL-4Rα on T cell subpopulations of WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice.

Table 3.1 Summary of IL-4Rα surface expression on T cell subpopulations

	CD4+	CD8+	γδ T cell	NK T cell
WT	Expressed	Expressed	Expressed	Expressed
IL-4Rα$^{-/-}$	Deleted	Deleted	Deleted	Deleted
Lck^{cre}IL-4Rα$^{-/flox}$	Deleted	Partial	Expressed	Partial
iLck^{cre}IL-4Rα$^{-/flox}$	Deleted	Deleted	Deleted	Deleted
CD4 ^{cre}IL-4Rα$^{-/flox}$	Deleted	Deleted	Expressed	Deleted

These results demonstrate successful T cell-specific, IL-4Rα deletion, with variation in T cell subpopulations between Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice.

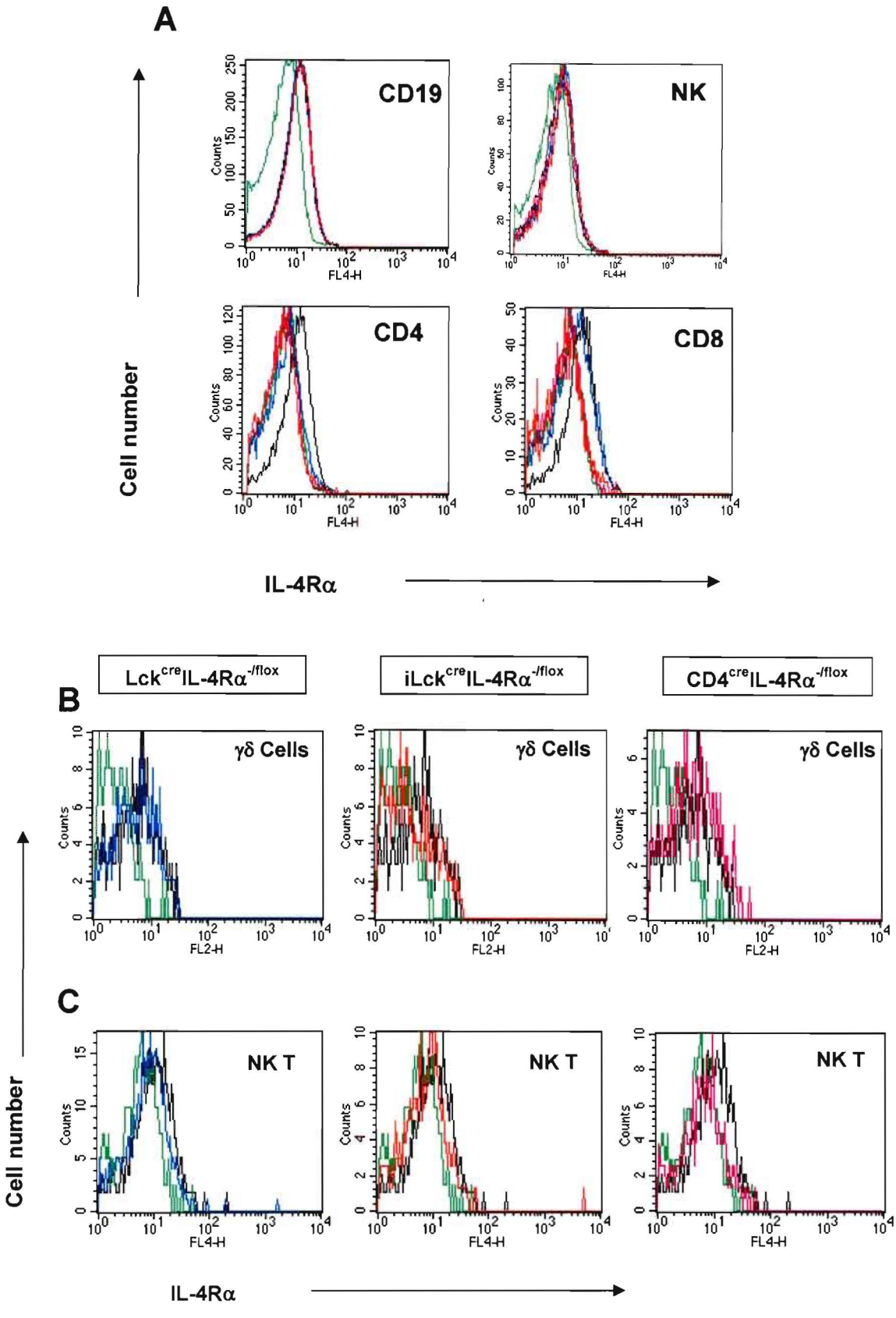

Figure 3.1.2: Cell type-specific IL-4Rα surface expression (FACS).
Spleen cells from naïve mice were co-stained using mAbs against IL-4Rα and various cell surface markers. **(A)** CD19 for B cells, NK+ / β-chain- for NK cells and CD4 and CD8 for T cell subpopulations. **(B) γδ T cells** were γδ TCR+ / β-chain TCR-. **(C) NK T cells** were DX5+ / β chain+ / CD3+ (■ WT, ■ IL-4Rα$^{-/-}$, ■ Lck^{cre}IL-4Rα$^{-/flox}$, ■ iLck^{cre}IL-4Rα$^{-/flox}$ and ■ CD4^{cre}IL-4Rα$^{-/flox}$).

3.1.3 *Efficiency of IL-4Rα deletion by Real-Time PCR*

To establish the efficiency of IL-4Rα deletion at the genomic level from FACS sorted cells, Real-Time quantitative PCR reactions were designed. Primers amplifying genomic regions of the IL-4Rα gene affected or unaffected by the deletion were used. The PCR products were successfully cloned into the pGEM-t Easy vector to prepare standards, allowing for comparative results. Figure 3.1.3A illustrates the successful excision of Intron-exon 8 and Intron-exon 5 from the vector using restriction digestion enzymes. PCR products from each 'insert positive' clone were sequenced and aligned with the genomic sequence of BALB/c or C57/BL6 mice (Figure 3.1.3B). Genomic DNA used for PCR reactions was isolated from >99% purified lymphocyte subpopulations. Figure 3.1.3C demonstrates the FACS analysis of unsorted cells (CD19 and CD4) and sorted CD19, CD8 and CD4 lymphocytes.

The number of intron-exon 8 alleles (deleted in IL-4Rα$^{-/-}$ and 1 copy in WT [IL-4Rα$^{-/flox}$] mice) relative to the number of intron-exon 5 alleles (both present in all cells) of the IL-4Rα were determined in CD4, CD8 and CD19 positive cells. As expected, no copies of intron-exon 8 were detected in IL-4Rα$^{-/-}$ deficient mice in all three cell types (Figure 3.1.4). Efficient deletion of IL-4Rα (intron-exon 8) from CD4+ cells was determined in all mouse strains following 75-80 PCR cycles (Figure 3.1.4A). PCR products were electrophoresed on agarose gel to confirm the product size and absence of product in negative samples. Supporting FACS data shown above, CD8+ cells from Lck^{cre}IL-4Rα$^{-/flox}$ mice yielded feint intron-exon 8 PCR product suggesting a partial deletion in this cell population (Figure 3.1.4B). No intron-exon 8 PCR product was detected in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice. An equivalent ratio of intron-exon 8 verses intron-exon 5 were maintained in CD19+ cells in Lck^{cre}IL-4Rα$^{-/flox}$ and WT mice, but the ratio for iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice was lower (Figure 3.1.4C).

The number of intron-exon 8 copies (from real time PCR) was calculated as a percentage relative to the number of intron-exon 5 copies. CD4+ T cells in Lck^{cre}IL-4Rα$^{-/flox}$ mice = 94.26%, iLck^{cre}IL-4Rα$^{-/flox}$ mice = 93.64% and CD4^{cre}IL-4Rα$^{-/flox}$ mice = 90.91% deletion (Figure 3.1.4A). CD8+ T cells in Lck^{cre}IL-4Rα$^{-/flox}$ mice = 82.17%, iLck^{cre}IL-4Rα$^{-/flox}$ mice = 99.72% and CD4^{cre}IL-4Rα$^{-/flox}$ mice = 95.96% deletion (Figure 3.1.4B). These data demonstrates an efficient and specific deletion of IL-4Rα in CD4+ T cells from all T cell-specific IL-4Rα-deficient mice. IL-4Rα was efficiently deleted from CD8+ T cells in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice and partially in Lck^{cre}IL-4Rα$^{-/flox}$ mice.

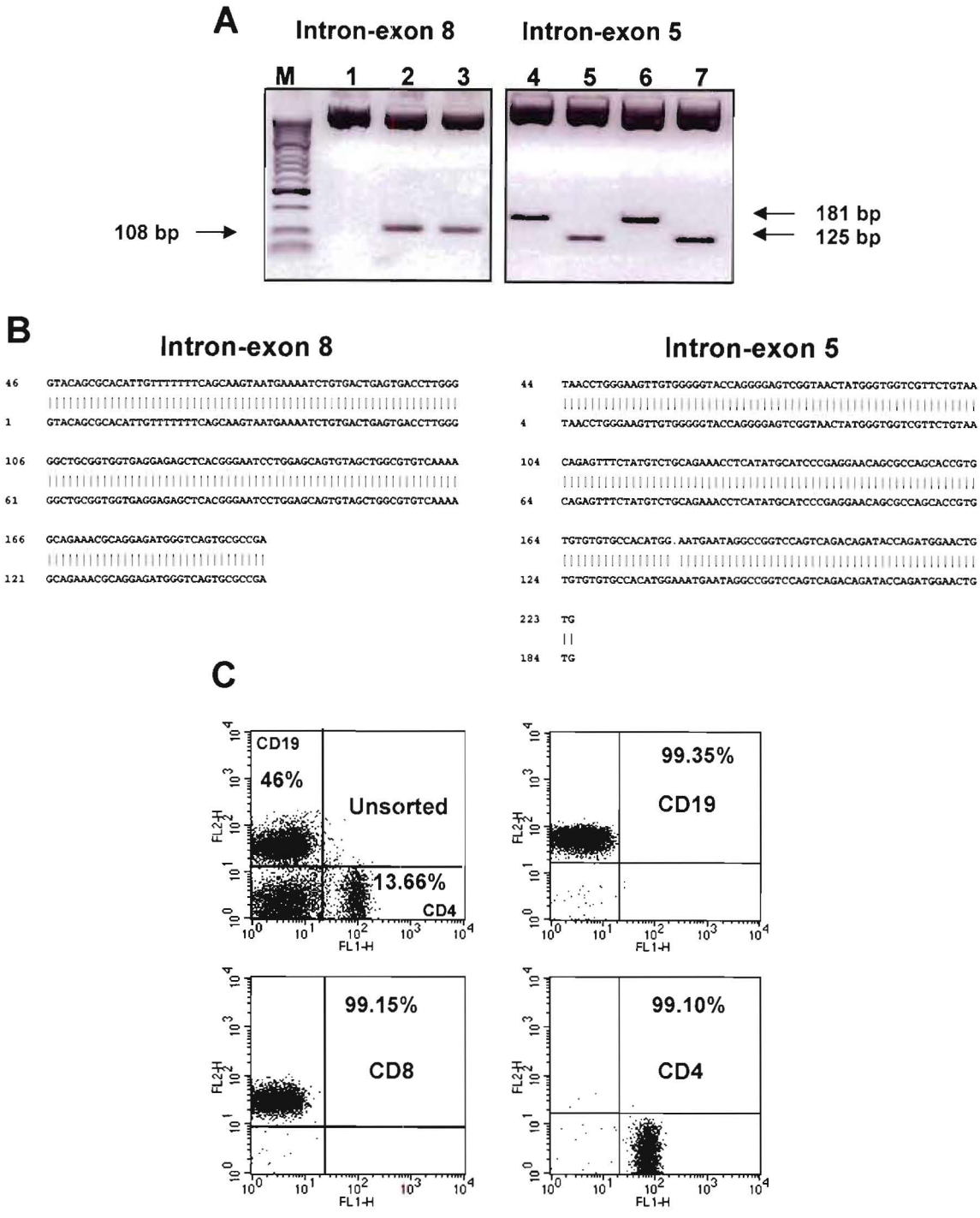

Figure 3.1.3: Real Time PCR standards and pure lymphocyte populations
(A) Real Time PCR Standards. Intron-exon 5 and intron-exon 8 PCR products were cloned into PGEM-t Easy. Positive clones (2 for each insert) were identified by digestion with restriction enzymes. Intron-exon 8, *SpH*1+*Eco*1cR1 (108 bp: lanes 2 and 3) and intron-exon 5, *Pst*1 (125bp: lanes 5 and 7) and *Eco*R1 (181bp: lanes 4 and 6). Lane 1 = digested, insert negative vector. **(B) Aligned sequences.** The PGEM-t Easy / PCR product construct were sequenced and aligned with the murine IL-4Rα gene sequence (bottom). **(C) FACS sorting of Lymphocyte populations.** Spleens or lymph nodes were isolated and single cell suspensions were prepared. Cells were stained with CD4, CD8 or CD19 and sorted on a FACS Vantage (purity = > 99%). Unsorted cells were stained with CD4 and CD19.

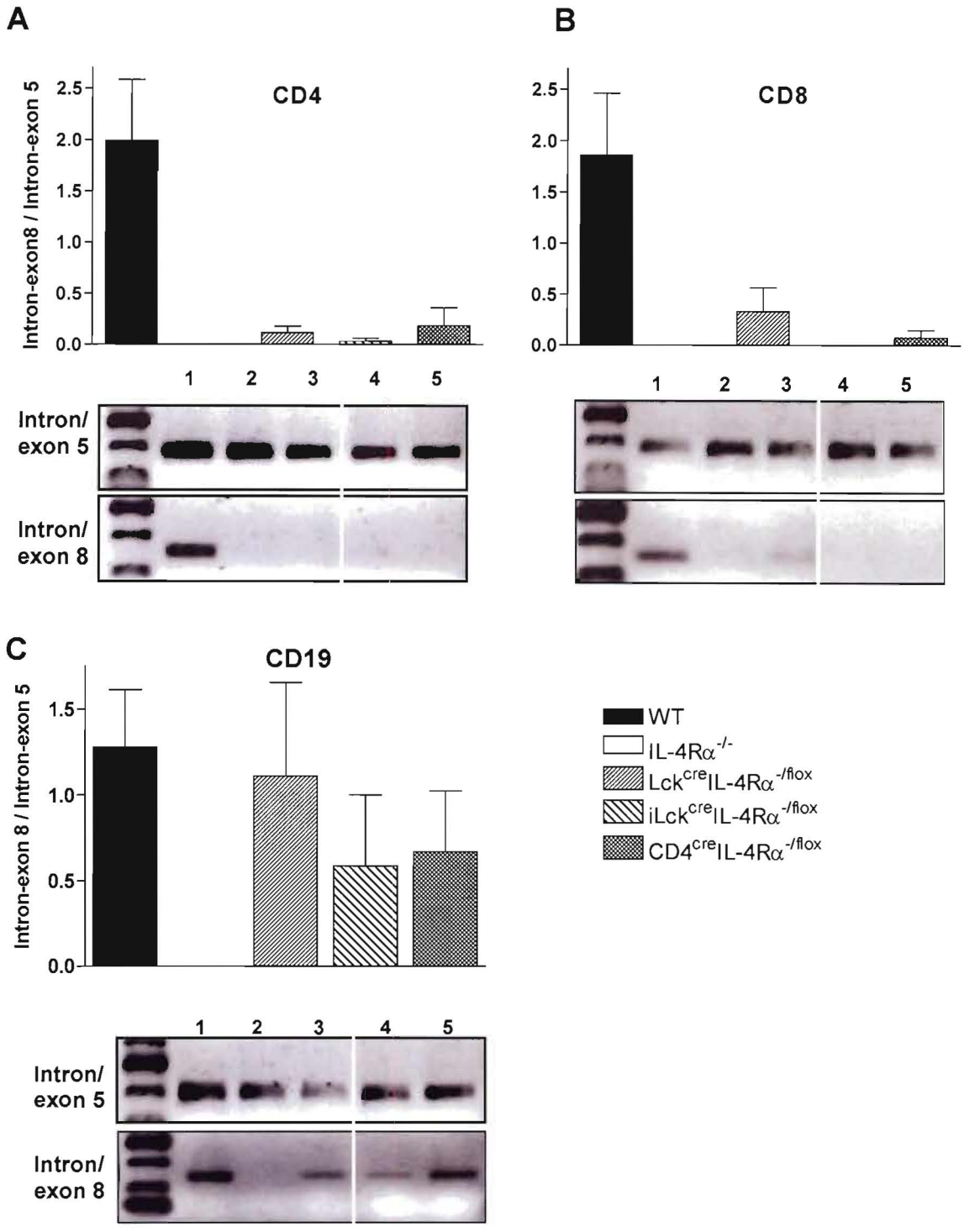

Figure 3.1.4: Efficiency of the deletion of IL-4Rα from CD4+ and CD8+ cells. Two specific Real Time PCR reactions were designed to quantify the amount of IL-4Rα present on purified CD4+ (**A**), CD8+ (**B**) and CD19+ (**C**) cells. Primers for intron-exon 5 (not effected by the deletion -181bp) and intron-exon 8 (absent after Cre/*lox*P deletion – 153bp) were used. Copies of intron-exon 8 are shown relative to copies of intron-exon 5. PCR products were visualised on a 2% agarose gel. Results represent two experiments and mean= ± SEM. **1-** WT, **2-** IL-4Rα$^{-/-}$ **3-** Lck^{cre}IL-4Rα$^{-/flox}$, **4-** iLck^{cre}IL-4Rα$^{-/flox}$ and **5-** CD4^{cre}IL-4Rα$^{-/flox}$

3.1.4 Selective impairment of IL-4Rα function in T lymphocytes

IL-4 is known to induce proliferation of naïve CD4+ T cells. To determine if the deletion of IL-4Rα would impair IL-4 function on CD4+ T cells, they were isolated from whole lymph nodes. CD4+ T cells from naïve WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice were stimulated with increasing doses of IL-2 or IL-4 and PMA. DNA synthesis was measured by [^{3}H] thymidine incorporation. The proliferative response to IL-4 of all IL-4Rα knockout stains was abrogated (Figure 3.1.5A). In contrast, lymphocytes from control WT mice showed dose-dependent proliferation to IL-4. When stimulated with IL-2 all the BALB/c strains demonstrated a dose-dependent proliferative response. The variability of this response between the strains was due to variation in the assay and not strain differences (when compared with previous experiments). These results confirm an IL-4Rα specific impairment of IL-4 signalling.

IL-4 also induces the differentiation of naïve T_H cells into T_H2 cells *in vitro* (Le Gros *et al.* 1990), while IL-12 drives T_H1 development (Seder *et al.* 1993). CD4+ T cells were stimulated with anti-CD3/CD28 and treated with IL-12/anti-IL-4 to induce T_H1 development or IL-4/anti-IFN-γ to induce T_H2 development. Figure 3.1.5B demonstrates that T_H1 polarisation was achieved in all five strains following IL-12/anti-IL-4 treatment. In contrast, IL-4 was only able to drive T_H2 polarisation in WT mice and confirms IL-4Rα abrogation in the three T cell-specific IL-4Rα-deficient and IL-4Rα$^{-/-}$ mice. Furthermore, iLck^{cre}IL-4Rα$^{-/flox}$ mice had the lowest response to IL-4 and IFN-γ and closely resembled the global IL-4Rα$^{-/-}$ mice.

IgE antibody secretion is strictly dependent on IL-4 signalling on B lymphocytes. To demonstrate normal function of the IL-4Rα on B lymphocytes, mice were sensitised with OVA-alum at weekly intervals for three weeks. Total serum IgE titres were determined at week 3 by ELISA. T cell-specific IL-4Rα-deficient mice were able to elicit an antigen induced antibody response, while global IL-4Rα$^{-/-}$ mice could not produce antibodies (Figure 3.1.5C). CD4^{cre}IL-4Rα$^{-/flox}$ mice produce higher levels of IgE compared with iLck^{cre}IL-4Rα$^{-/flox}$ and WT. In summary, these results show the effective impairment of IL-4Rα-mediated functions on T cells but not B cells of Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$.

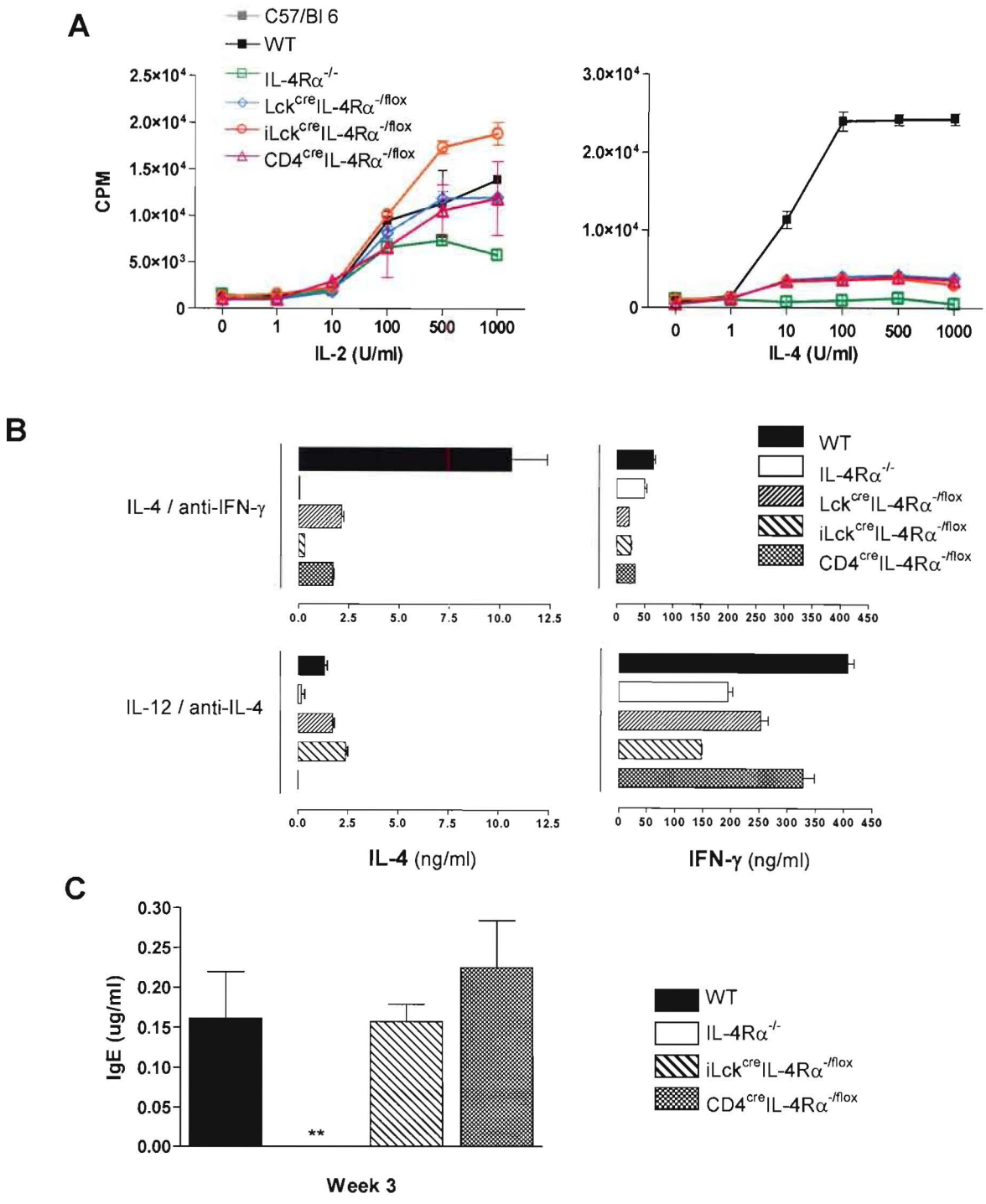

Figure 3.1.5: Functional analysis of IL-4Rα responsiveness.
(A) Impaired T cell proliferation. Lymph node cells from WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ were stimulated with serial dilutions of IL-2 (control) and IL-4. DNA synthesis was measured by (^{3}H) Thymidine incorporation and Mean ± SD from triplicate cultures (one experiment). **(B) Impaired *In Vitro* T_H2 differentiation.** T_H1 versus T_H2 differentiation was promoted with IL-4/anti-IFN-γ or IL-12/anti-IL-4 mAbs. IFN-γ or IL-4 secretion was determined. Results represent single, separate experiments for T_H1 and T_H2. Mean ± SEM from triplicate cultures. **(C) Unimpaired induction of IgE response.** Mice were immunised and boosted with ovalbumin. Serum was harvested at week 3 post-immunisation and total IgE levels were analysed by ELISA. IgE levels of week 3 were subtracted from naïve IgE levels. Mean = ± SEM and p = ** = < 0.01 (* = WT vs all groups). Results are from one experiment.

In summary, these data describes the generation, characterisation and functional analysis of mice lacking the IL-4Rα chain selectively on T lymphocytes. Efficient T cell specific IL-4Rα deletion was confirmed by loss of IL-4Rα expression on T cell subpopulations, with variation between Lck^{cre}IL-4R$\alpha^{-/flox}$, iLck^{cre}IL-4R$\alpha^{-/flox}$ and $CD4^{cre}$IL-4R$\alpha^{-/flox}$ mice. The effective impairment of IL-4Rα-mediated functions on T cells was demonstrated by an impaired IL-4-induced CD4+ T cell proliferation and T_H2 differentiation. Normal IL-4 responsiveness on non-T cell populations was demonstrated by IgE production in response to antigens. T cell-specific IL-4Rα-deficient mice were generated to elucidate the importance of IL-4Rα signalling during *Leishmania major* and *Nippostrongylus brasiliensis* infection. By comparing Lck^{cre}IL-4R$\alpha^{-/flox}$ with two novel (iLck^{cre}IL-4R$\alpha^{-/flox}$ and $CD4^{cre}$IL-4R$\alpha^{-/flox}$) mouse strains a role for IL-4Rα responsive T cell subpopulations in disease could be studied.

3.2 IL-4/IL-13 responsive non-T cell populations are essential to transform *Leishmania major* infected BALB/c mice to a healer phenotype

Global abrogation of IL-4/IL-13 signalling is not sufficient to confer a fully resistant phenotype in BALB/c mice (Mohrs *et al.* 1999; Radwanska *et al.* 2006, submitted). Rather, resistance to *L. major* infection requires the presence of bone marrow derived IL-4/IL-13 responsive non-CD4+T cells (Radwanska *et al.* 2006, submitted). In order to investigate the role of IL-4-responsive subpopulations of T cells in *L. major* susceptibility, we compared three T cell-specific IL-4Rα-deficient mice (Lck^{cre}IL-$4R\alpha^{-/flox}$, iLck^{cre}IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$ BALB/c mice). C57/BL6 and transgene negative WT (IL-$4R\alpha^{-/flox}$) mice were used as resistant and susceptible controls. A comparison was made with global IL-$4R\alpha^{-/-}$ BALB/c mice.

3.2.1 Control of acute *L. major* infection in T cell specific IL-4Rα-deficient mice

C57/BL6, WT, IL-$4R\alpha^{-/-}$, Lck^{cre}IL-$4R\alpha^{-/flox}$, iLck^{cre}IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$ BALB/c mice were infected subcutaneously with 2 x 10^6 stationary phase metacyclic promastigote *L. major* (LV39) in the hind footpad. Footpad swelling in the infected left hind footpad (shown in Figure 3.2.1A with an uninfected right hind footpad) was measured weekly to deterrnine disease progression. As expected, the nonhealer WT BALB/c strain developed uncontrolled footpad swelling, often accompanied by ulceration and necrosis within 7 weeks of infection. Due to these symptoms, the mice were killed at week 7; and no results were available at week 12 post infection. All T cell-specific IL-4Rα-deficient BALB/c mice were able to resolve infection with similar kinetics as resistant C57/BL6 mice (Figure 3.2.1B). IL-$4R\alpha^{-/-}$ mice were able to control infection compared with WT mice but showed significantly larger footpad swelling than T cell-specific IL-4Rα-deficient and resistant C57/BL6 mice.

To more accurately assess disease progression and control of infection, parasite numbers were quantified from footpad lesions and draining popliteal lymph nodes (PLN). Parasite numbers were measured by limiting dilution cultures. In the early stages of disease (week 3), T cell-specific IL-4Rα-deficient mice had significantly less number of parasites in the footpad compared with WT mice (similar to C57/BL6 mice), but no significance was observed in the PLN (Figure 3.2.2A). At week 6, a similar pattern was observed in the footpad and PLN with T cell-specific IL-4Rα-deficient mice having significantly less parasites than WT mice. T cell-specific IL-4Rα-deficient mice also had significantly less parasites than IL-$4R\alpha^{-/-}$ mice in the footpad, but not in the PLN (Figure 3.2.2B). By week 12 post infection, all T cell-specific IL-4Rα-deficient mice had significantly less parasites in the footpad than IL-$4R\alpha^{-/-}$ mice (comparable with C57/BL6 mice). Although the T cell-specific IL-4Rα-

deficient mice had significantly less parasites in the PLN than IL-4Rα$^{-/-}$ mice (Figure 3.2.2C), the parasite number remained significantly higher than C57/BL6 mice (significance not shown). When comparing the three T cell-specific IL-4Rα-deficient mice; iLck^{cre}IL-4Rα$^{-/flox}$ mice had fewer parasites at weeks 6 and 12 than Lck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice. However, this did not correspond with a previous experiment at week 6. These data demonstrate that T cell-specific IL-4Rα-deficient mice control acute *L. major* infection with no significant difference in disease progression and parasite burden between the three strains.

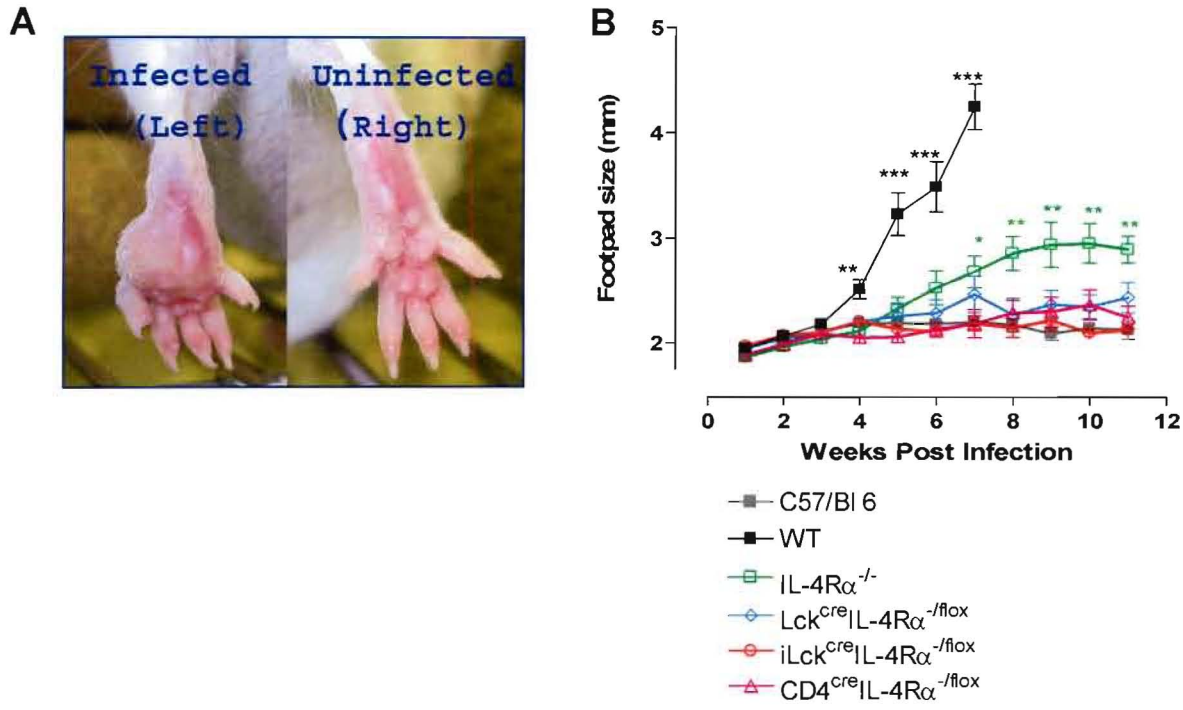

Figure 3.2.1: Control of acute *L. major* infection in T cell specific IL-4Rα-deficient mice (Footpad swelling). C57/BL6, WT (BALB/c), IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice (5-10 per group) were infected with 2 x 10^6 *L. major* LV39 parasites in the left hind footpad. **(A) Infected footpad.** Infected vs uninfected footpad in a susceptible BALB/c mouse. **(B) Disease progression.** T cell specific IL-4Rα-deficient mice controlled *L. major* infection, comparable with resistant C57/BL6 disease progression. Representative results of 3 experiments for weeks 3 and 6 and 1 experiment for week 12 is shown. Significance is compared with C57/BL6 mice. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Mean = ± SD.

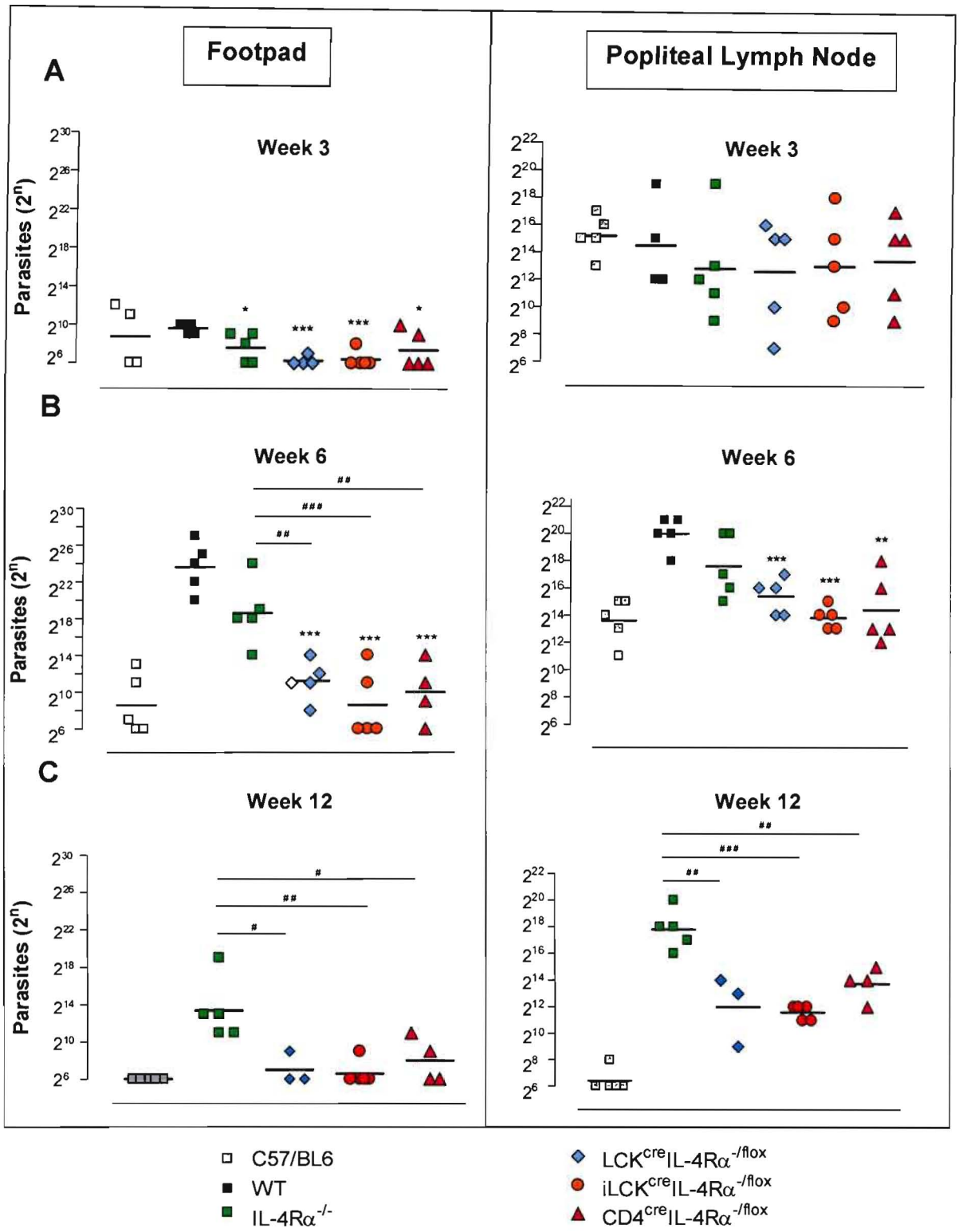

Figure 3.2.2: Control of acute *L. major* infection in T cell specific IL-4Rα-deficient mice (Parasite burden). At weeks 3 **(A)**, 6 **(B)** and 12 **(C)** post infection, 5 mice per group were killed and the parasite burden in footpad and draining popliteal lymph node determined. Limiting dilutions (2-fold) of individual samples were prepared in Schneider's insect media. Significance was compared between WT and all knockout groups (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$) or IL-4Rα$^{-/-}$ and T cell specific IL-4Rα-deficient mice. # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$. Results from weeks 3 and 12 are from 1 experiment and representative results from 2 experiments were chosen for week 6, mean = ± SEM.

3.2.2 *T cell-specific IL-4Rα-deficient mice exhibit an impaired T_H2 phenotype*

Cytokines have been shown to play an important role in determining resistance or susceptibility to *L. major*. IFN-γ stimulates the production of iNOS by macrophages as a control mechanism against *L. major* infection (Stenger *et al.* 1994) and an IL-4-dependent mechanism in susceptibility to *L. major* has been described (Mohrs *et al.* 1999). Due to the acquisition of a resistant phenotype by T cell-specific IL-4Rα mice (previously demonstrated in Lck^{cre}IL-4$R\alpha^{-/flox}$ by Radwanska *et al.* 2006, submitted), cytokines were detected to determine the nature of T-helper differentiation in all three T cell-specific IL-4Rα-deficient mice. Cytokine levels were measured in the supernatants after soluble *leishmania* Ag (SLA) stimulation of unsorted PLN cells. At weeks 6 and 12 post infection, all T cell-specific IL-4Rα-deficient mice showed a tendency for increased IFN-γ production (Figure 3.2.3). Significantly higher levels of IFN-γ (compared with IL-4$R\alpha^{-/-}$ mice) were detected at week 6 for all T cell-specific IL-4Rα-deficient mice and week 12 for Lck^{cre}IL-4$R\alpha^{-/flox}$ and $CD4^{cre}$IL-4$R\alpha^{-/flox}$ mice.

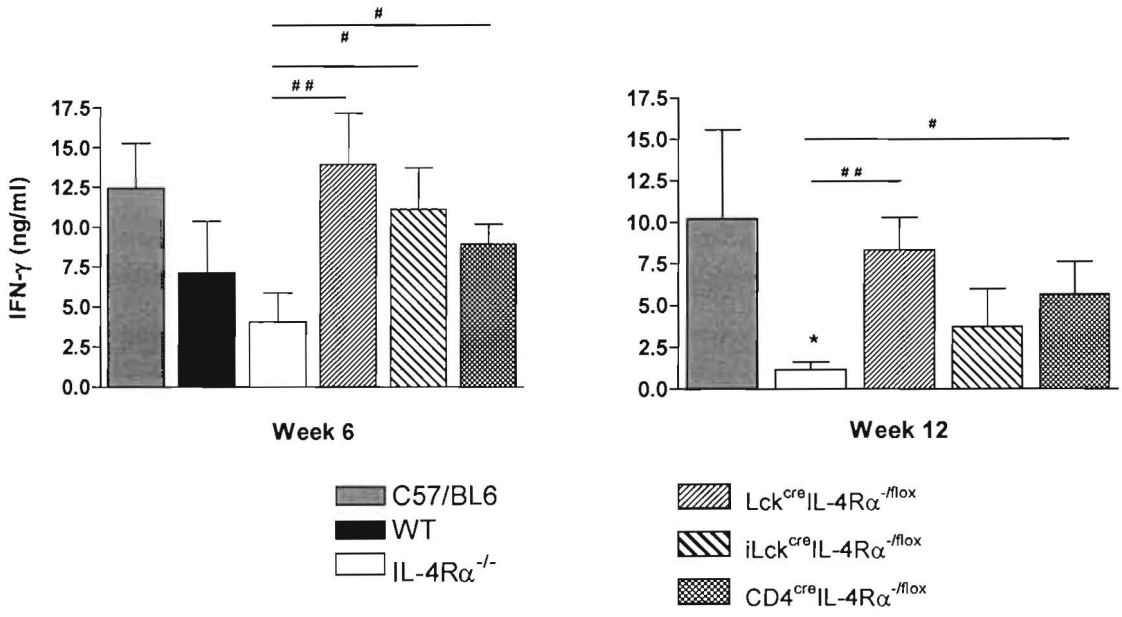

Figure 3.2.3: IFN-γ production in T cell specific IL-4Rα-deficient mice (whole PLN). Draining lymph node cells from 4-5 individual mice per group were isolated at weeks 3 (data not shown), 6 and 12. Cells were stimulated in complete IMDM with 50 ug/ml SLA. Supernatants were collected after 72 hrs and IFN-γ detected by ELISA. Results represent 1 experiment with means of triplicate values ± SEM from duplicates. The significance compares IL-4$R\alpha^{-/-}$ vs T cell specific IL-4Rα-deficient mice (#) and C57/BL6 vs IL-4$R\alpha^{-/-}$(*). *, # = $p < 0.05$, and **, ## = $p < 0.01$.

Whole lymph node restimulation indicates the cytokine environment of the lymph node where many cells play a role. Therefore, to identify cytokines produced by the CD4+ T cell population, CD4+ cells were sorted from pooled PLN per strain and restimulated with fixed T cell depleted splenocytes pulsed with SLA. Again, CD4+ T cells from T cell-specific IL-4Rα-deficient mice demonstrate a trend towards an increased IFN-γ secretion, with Lck^{cre}IL-4$R\alpha^{-/flox}$ significantly higher than WT mice (Figure 3.2.4A). Impaired T_H2 responses were demonstrated by lower IL-4 and IL-13 secretion (Figure 3.2.4B). All knockout strains showed significantly less IL-4 production than WT mice at weeks 3 and 6, but these levels remained higher than C57/BL6 mice at week 6. Furthermore, IL-13 production by all T cell-specific IL-4Rα-deficient mice was comparable with C57/BL6 mice at week 12. IL-10 is a T_H2 / regulatory T cell cytokine associated with T_H1 downregulation and a susceptibility factor in *L. major*. IL-10 secretion by CD4+ T cells showed a trend towards decreased secretion in all T cell-specific IL-4Rα-deficient mice with iLck^{cre}IL-4$R\alpha^{-/flox}$ demonstrating significantly lower IL-10 levels than IL-4$R\alpha^{-/-}$ mice (Figure 3.2.4C). However, the levels of IL-10 demonstrated, were not significantly lower than WT mice, and remained significantly higher than C57/BL6 mice. These data demonstrate a significantly impaired T_H2 cytokine response in T cell-specific IL-4Rα-deficient mice with a tendency for increased IFN-γ production.

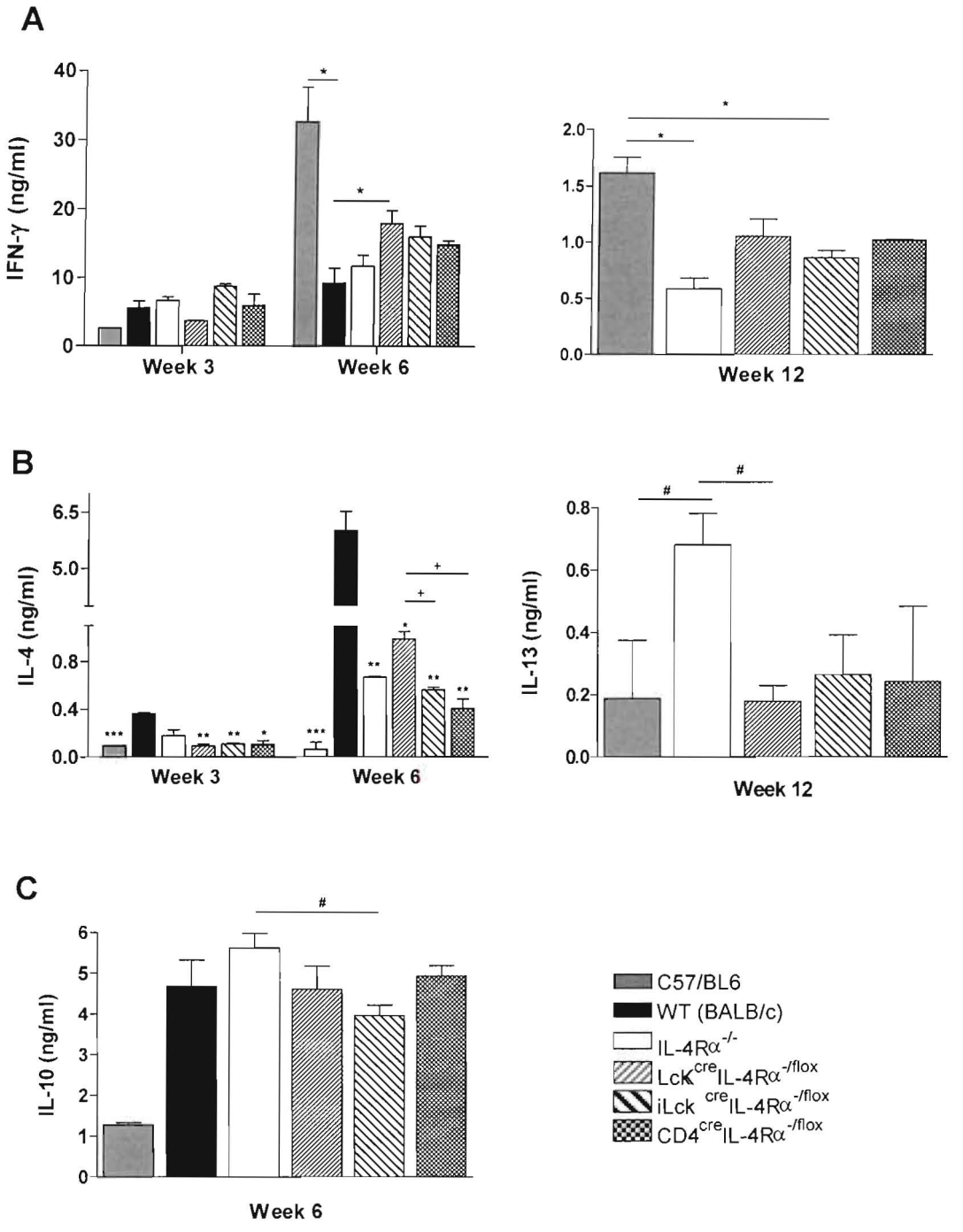

Figure 3.2.4: Type-2 immunity is impaired in CD4+ T cell specific IL-4Rα-deficient cells. Draining lymph node cells from 4-5 mice per group were pooled and CD4+ cells isolated at weeks 3, 6 and 12. Cells were cultured with fixed T cell depleted splenocytes (APCs) pulsed with 50 ug/ml SLA. Supernatants were collected after 72 hrs and IFN-γ **(A)**, IL-4, IL-13 **(B)** and IL-10 **(C)** were detected by ELISA. Results represent 1 experiment with means of duplicate values ± SEM from duplicates. Significance: * = all groups vs WT, # = all groups vs IL-4Rα$^{-/-}$ and + = significance between T cell specific IL-4Rα-deficient mice. *, #, + = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3.2.3 *Enhanced Delayed-Type Hypersensitivity (DTH) reaction in T cell-specific IL-4Rα mice*

Delayed-type hypersensitivity is a delayed transient response to previously encountered antigen, occurring within 24-72 hrs and mediated by cytokines released from sensitised T cells. A positive response indicates a T_H1 driven protective response to the antigen. *L. major* infected C57/BL6, WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice were inoculated subcutaneously in the right hind footpad with 10 ug/ml SLA at weeks 3 and 6. Footpad swelling was determined every 24 hrs for 4 days (Figure 3.2.5). T cell-specific IL-4Rα-deficient mice all developed DTH responses significantly higher than WT and IL-4Rα$^{-/-}$ mice (comparable with C57/BL6 mice). After 48 hrs the DTH response of the T cell-specific IL-4Rα-deficient mice had reduced considerably, compared to a prolonged DTH in C57/BL6 mice but remained significantly higher than WT and IL-4Rα$^{-/-}$ mice. These results suggest an early protective T cell response to *L. major* in T cell-specific IL-4Rα-deficient mice which was maintained at week 6 post infection.

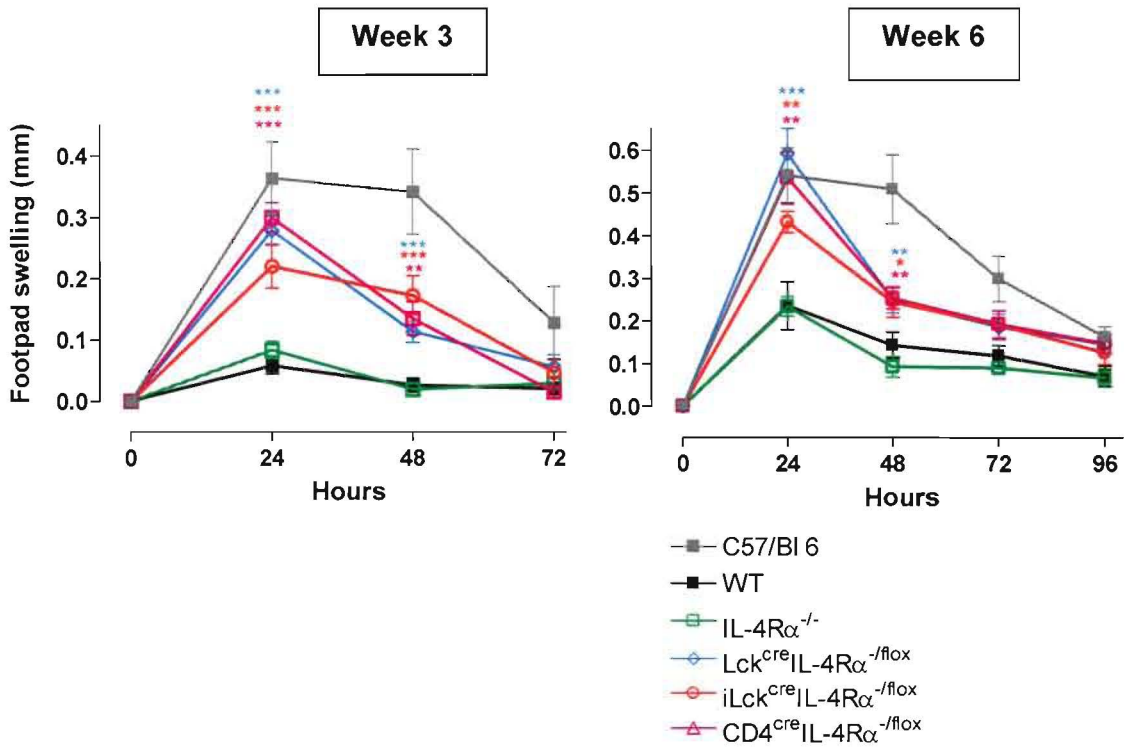

Figure 3.2.5: Development of *Leishmania* specific DTH. C57/BL6, WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice (3-6 weeks post-infection with *L. major*) were injected with 10µg of SLA into the contralateral hind footpad. Footpad swelling was monitored every 24 hours for 4 days. Significance between IL-4Rα$^{-/-}$ and T cell specific IL-4Rα-deficient mice, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Mean = ± SEM of 10 mice per group).

3.2.4 *L. major* Specific Antibodies

To determine the type of antibody response in *L. major* infected strains, serum was obtained at weeks 3 and 6 (IgG1/2a) and week 8 (IgE) post-infection. *L. major*-Ag-specific IgG1, IgG2a and total IgE titres were measured by ELISA. As known, *L. major* infected BALB/c mice develop a dominant T_H2 response while IL-4Rα$^{-/-}$ mice were unable to produce T_H2 antibodies (due to the lack of IL-4Rα on B cells). At an early stage of *L. major* infection, no differences we observed between all BALB/c strains. By week 6, T cell-specific IL-4Rα-deficient mice had significantly lower levels of IgG1. Lower levels of IgE were also determined at week 8 post-infection. C57/BL6 mice demonstrated very low antibody levels for all isotypes, this could be due to a predominant T_H1 response. These data support previous cytokine data of an impaired T_H2 response.

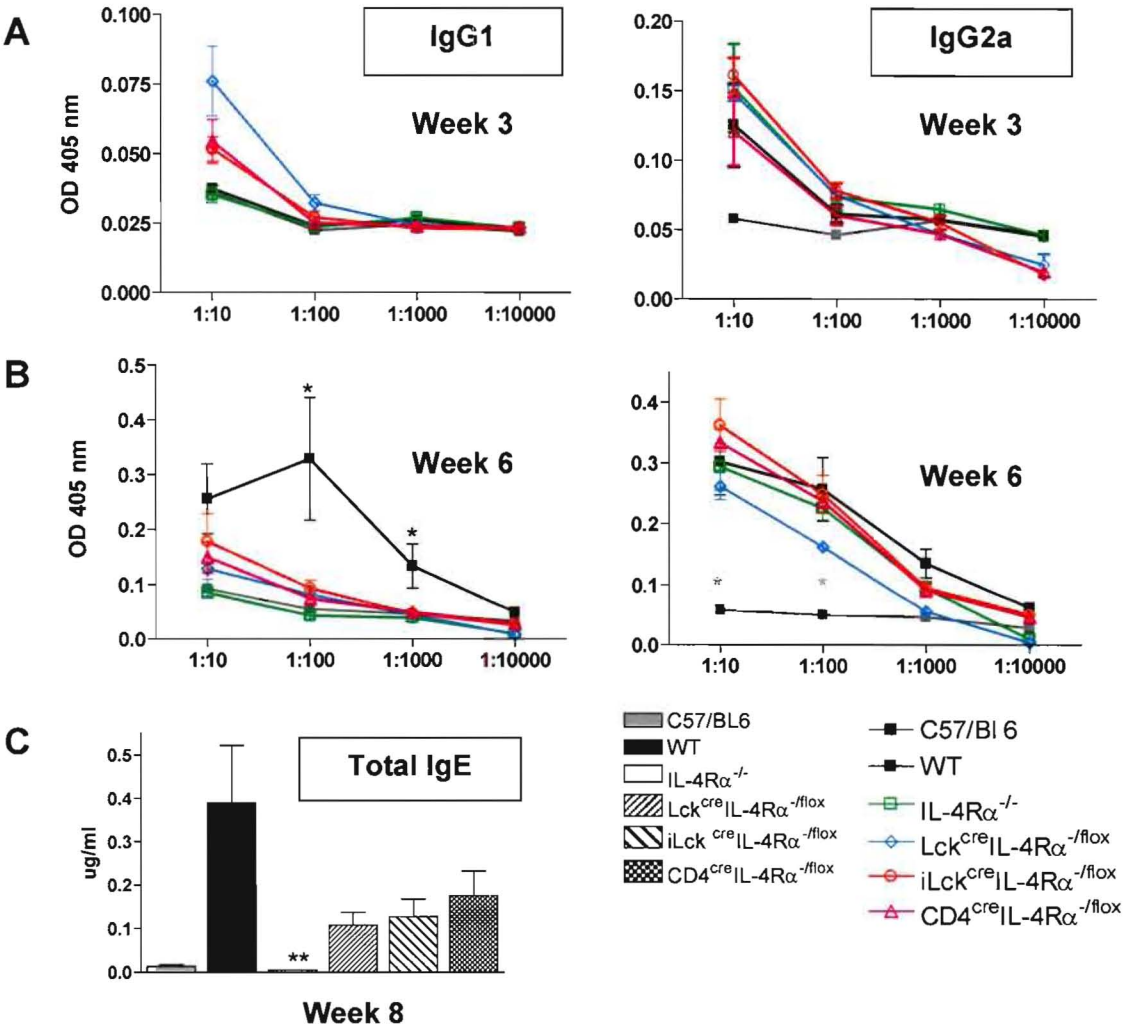

Figure 3.2.6: *L. major* specific antibodies. *L. major* serum was obtained from 4-5 mice per group at 3 weeks - 1 experiment **(A)**, 6 weeks - 2 experiments **(B)** and 8 weeks - 1 experiment **(C)** post infection. *L. major*-Ag-specific IgG1 and IgG2a and Total IgE titres were determined by ELISA (mean ± SEM). The significance is all groups vs WT, * = $p<0.05$ and ** = $p<0.001$

In summary, the mouse models used in this study allowed investigation of the role of IL-4 signalling specifically on T cell subpopulations in determining the control of *L. major* infection. All T cell-specific IL-4Rα-deficient mice could control acute *L. major* infection with no significant difference in disease progression and parasite burden between the three strains (supported by studies on *L. major* infection of Lck^{cre}IL-$4R\alpha^{-/flox}$ mice by Radwanska *et al.* 2006, submitted). The mice demonstrated similar kinetics of lesion development and resolution as those observed in genetically resistant C57/BL6 mice. In contrast, BALB/c and IL-$4R\alpha^{-/-}$ mice developed progressive lesion swelling with increased parasite numbers. T cell-specific IL-4Rα-deficient mouse strains demonstrated an impaired T_H2 cytokine response with a tendency for increased IFN-γ production. The suppression of IgG1 antibodies supported this data. An early protective T cell response to *L. major* was demonstrated in all T cell-specific IL-4Rα-deficient mice which was maintained at week 6 post infection, suggesting the early control of infection. Together this data demonstrates that clinical immunity can be achieved in mice on a susceptible BALB/c background.

3.3 IL-4 responsive T cells are not crucial for worm expulsion and increase lung pathology in *Nippostrongylus brasiliensis* infection.

In order to investigate the role of IL-4 responsive T cell subpopulations in helminthic infections, comparative infection studies with the gastrointestinal helminth *N. brasiliensis* were performed. Little is known about the immune response in the lung stage of *N. brasiliensis*, therefore we determined; (i) infection induced cellular infiltrates in the lungs and airways, (ii) cytokine secretion by CD4+ T cells from the draining lymph node and (iii) mucus production. We also studied the disease kinetic in the intestine and compared iLck^{cre}IL-4R$\alpha^{-/flox}$ and CD4^{cre}IL-4R$\alpha^{-/flox}$ T cell-specific IL-4Rα-deficient mice with transgene negative WT (IL-4R$\alpha^{-/flox}$) and global IL-4R$\alpha^{-/-}$ mice. Lck^{cre}IL-4R$\alpha^{-/flox}$ mice were investigated independently. Furthermore, as IL-4Rα is disrupted on all T cell subpopulations in iLck^{cre}IL-4R$\alpha^{-/flox}$ but not on $\gamma\delta$ T cells in CD4^{cre}IL-4R$\alpha^{-/flox}$ mice, a role for IL-4 responsive $\gamma\delta$ T cells in *N. brasiliensis* infection could be studied using these mouse strains. Infective L3 larvae migrate to the lung by day 1, leave tracts and severely damage the lungs by day 3 (Figure 3.3.1). The worms migrate to the intestine by day 7 and the lungs recover (Figure 3.3.1). The lung gross pathology was similar in all mouse strains (data for Lck^{cre}IL-4R$\alpha^{-/flox}$ not shown).

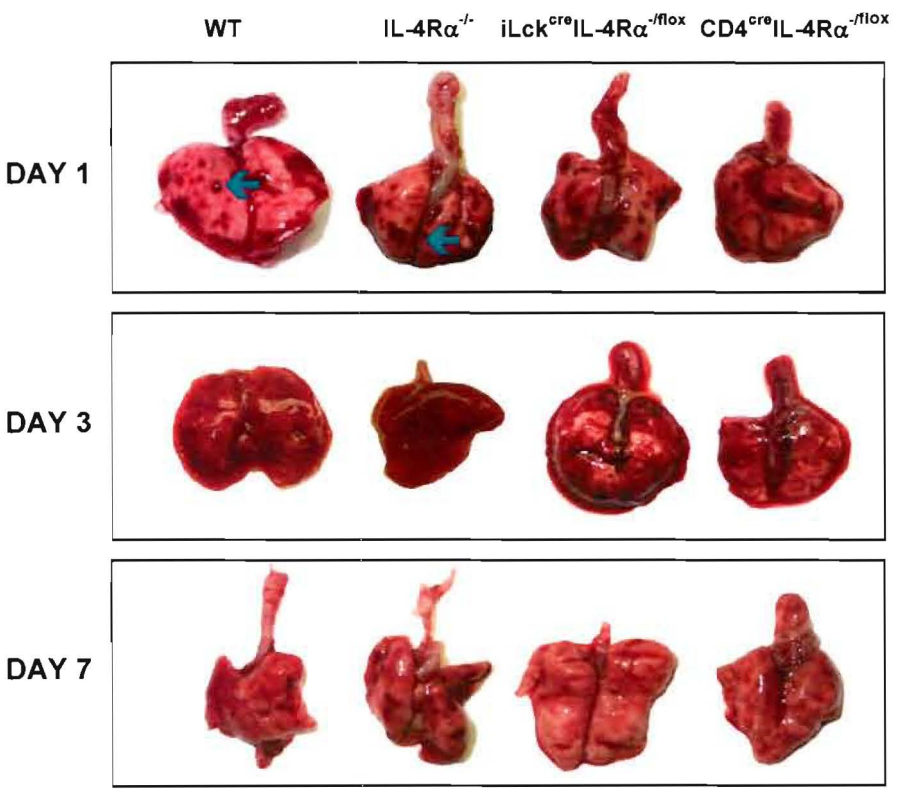

Figure 3.3.1: Lung gross pathologpy of *N. brasiliensis* infected BALB/c mice. WT, IL-4R$\alpha^{-/-}$, iLck^{cre}IL-4R$\alpha^{-/flox}$ and CD4^{cre}IL-4R$\alpha^{-/flox}$ BALB/c mice were infected with 750 L3 stage *N. brasiliensis* larvae. Mice were killed on days 1, 3 or 7 post infection. On day 1 post infection L3 larvae migrate into the lung leaving tracts (←). The damaged lungs (day 3) recover by day 7.

3.3.1 *Cellular infiltration in the lungs of T cell specific IL-4Rα-deficient mice (FACS).*

Previous histological results in our lab have shown infiltration of inflammatory cells into the lungs of *N. brasiliensis* infected WT mice. To determine the phenotype of these cellular infiltrates, FACS analysis was performed on single cell suspensions from the lungs of infected WT, IL-4Rα$^{-/-}$, Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice. Figure 3.3.2 demonstrates three of the cell populations identified. Highly granular cells (indicated as R2 on the forward and side scatter dot plot) were identified as GR-1+ / CD11b+ granulocytes. These cells resemble neutrolphils and eosinophils as basophils are GR-1- (Min *et al.* 2004). CD3+ and CD19+ lymphocytes are indicated as R3 and were less granular as shown in the forward and side scatter dot plot.

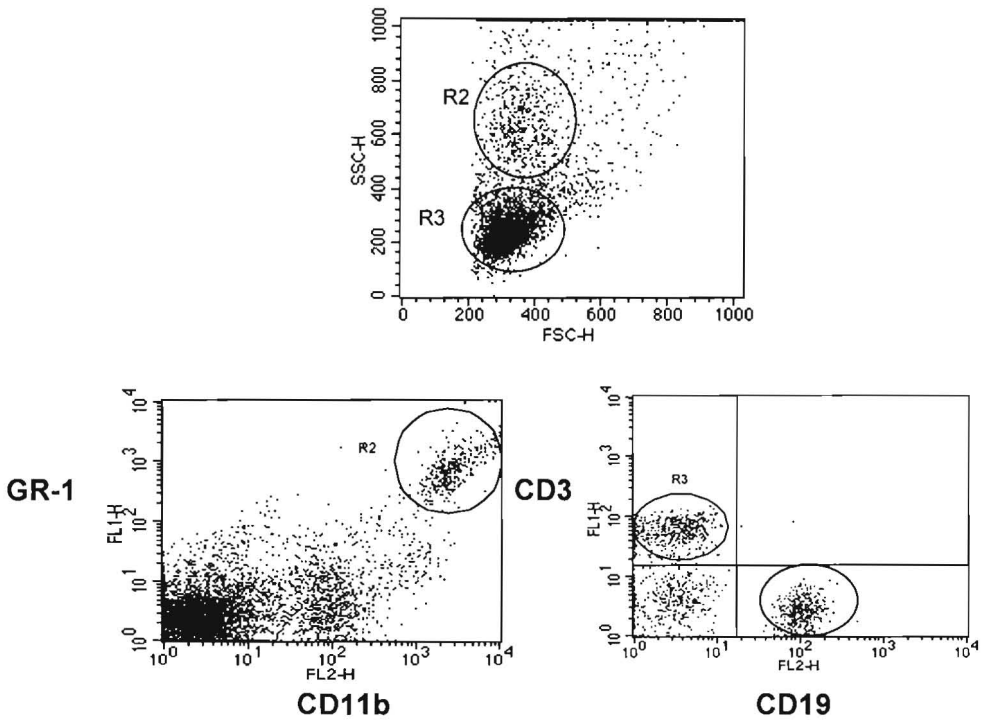

Figure 3.3.2: FACS analysis of lung single cell suspension. Single cell suspensions were prepared from lung by digesting the tissue in DMEM containing DNASE I and Collagenase I. Lung cells were stained with CD3/CD19 (for lymphocytes) and CD11b / GR-1 (for granulocytes).

Figure 3.3.3A demonstrates that significantly less CD3+ T cells were found in the lung tissue of iLck^{cre}IL-4Rα$^{-/flox}$ and Lck^{cre}IL-4Rα$^{-/flox}$ mice when compared with WT and were comparable to IL-4Rα$^{-/-}$ mice. Although CD4^{cre}IL-4Rα$^{-/flox}$ mice had significantly less CD3+ T cells when compared with WT controls, an increased number of cells was observed when compared with iLck^{cre}IL-4Rα$^{-/flox}$ and Lck^{cre}IL-

4Rα$^{-/flox}$ mice. In contrast, granulocytic infiltration (CD11b+ / GR-1+) was significantly increased in IL-4Rα$^{-/-}$ mice compared with all strains. These results may demonstrate an IL-4Rα dependent mechanism for CD3+ lung infiltration in T cell-specific IL-4Rα-deficient mice. This suggests IL-4 / IL-13, direct / indirect regulatory mechanisms for granulocytes.

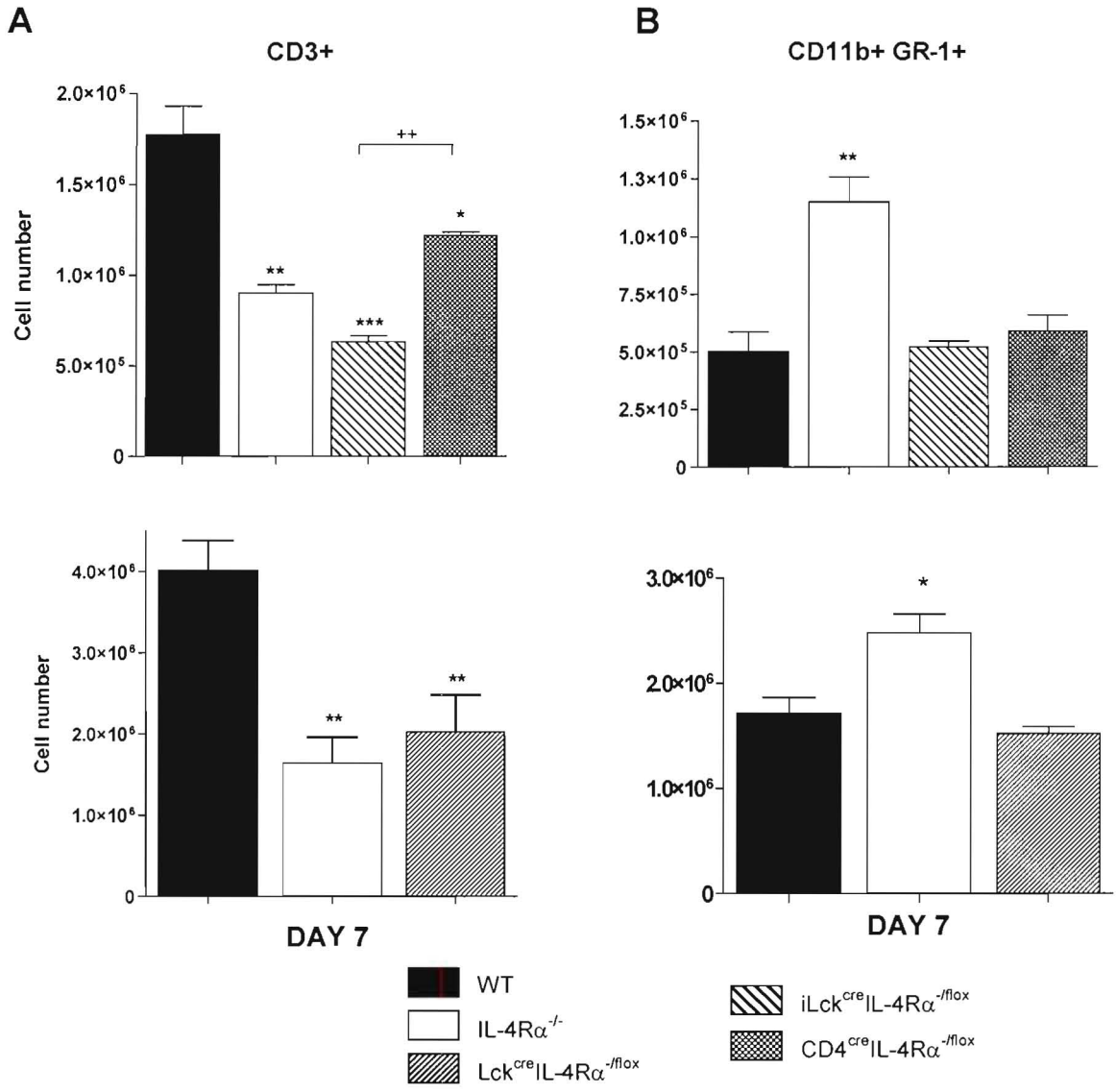

Figure 3.3.3: Cellular infiltration in lungs of T cell specific IL-4Rα-deficient mice (FACS). Lung single cells were stained for CD3+ lymphocytes **(A)** or CD11b+ / GR-1+ granulocytes **(B)**. Representative results are shown of 1 experiment for iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice (top graphs) and 2 experiments for Lck^{cre}IL-4Rα$^{-/flox}$ mice (bottom graphs). Individual mice, 3-5 per group were used, Mean = ± SEM and * = $p < 0.05$, **, ++ = $p < 0.01$ and *** = $p < 0.001$ (* = all groups vs WT and + = between T cell specific IL-4Rα-deficient mice).

3.3.2 *Cellular influx into lung airways of T cell specific IL-4Rα-deficient mice (BAL).*

During *N. brasiliensis* infection, an increased cellular response in the lungs drives the influx of activated cells into the airways. To determine the phenotype of activated cells in the airways of *N. brasiliensis* infected mice, the lungs were washed by broncho-alveolar lavage (BAL) and the cells were centrifuged by cytospin and the cellular infiltrates determined. Cells typically observed in the BAL of *N. brasiliensis* infected mice (macrophages, lymphocytes and neutrophils- X1000) are illustrated in Figure 3.3.4A and cells attached to L3 stage larvae were also observed (100X). The trend of cellular influx into the lung airways resembled cellular infiltration into the lung (FACS data shown in Figure 3.3.3). Decreased lymphocyte infiltration was seen when comparing iLck^{cre}IL-4Rα$^{-/flox}$ and IL-4Rα$^{-/-}$ mice with WT controls (no difference with CD4^{cre}IL-4Rα$^{-/flox}$ mice. Furthermore, granulocytic infiltration was higher in IL-4Rα$^{-/-}$ mice when compared with all mouse strains and confirmed FACS analysis (Figure 3.3.4B and C). The next step was to determine if impaired cellular infiltration was due to impaired T_H2 cytokine secretion by IL-4 responsive cells.

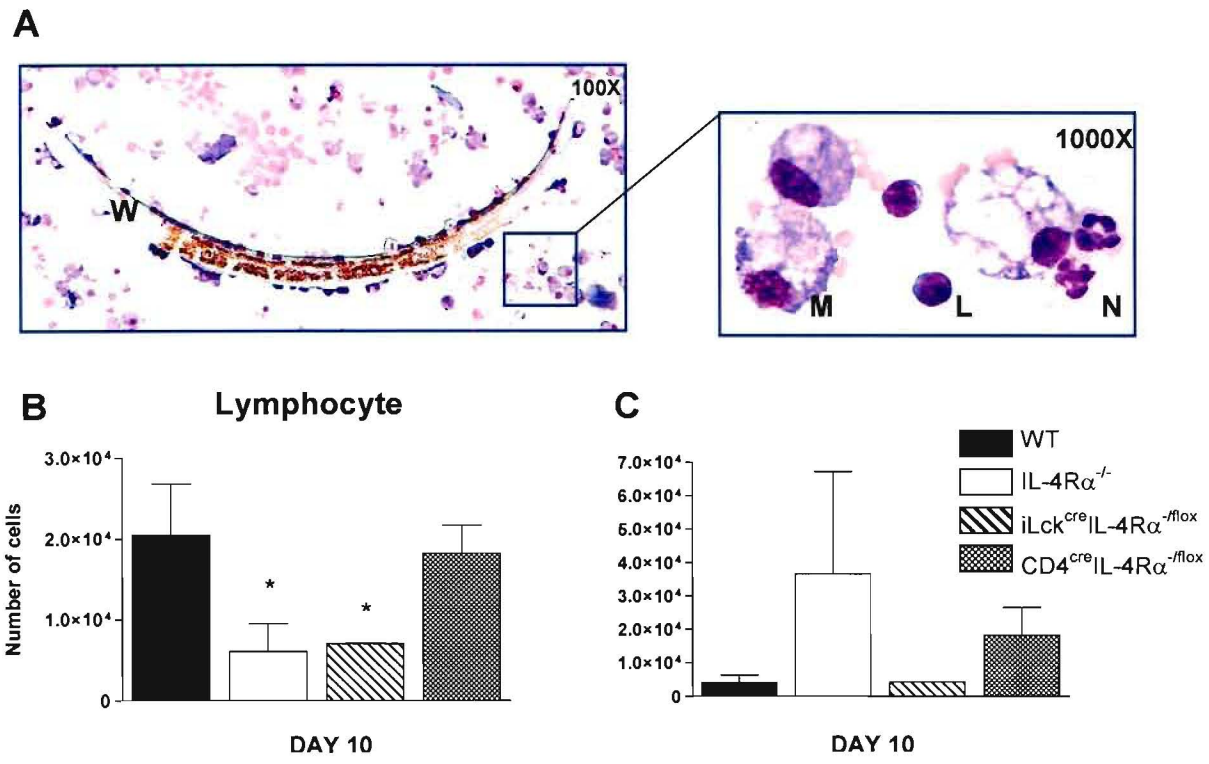

Figure 3.3.4: Cellular influx into lung airways of T cell specific IL-4Rα-deficient mice (BAL)*. Lungs were flushed with IMDM and cells were centrifuged onto glass slides and stained with Diff-quik. **(A)** Macrophages (M), lymphocytes (L), neutrophils (N) and *N. brasiliensis* (W) as viewed at 100X and 1000X. **(B) Lymphocytes (C) Neutrophils.** Results are from 1 experiment of individual mice, (3-5 per group) with mean = ± SEM and * = $p < 0.05$.

* Cytospin, staining and differential cell counts performed by Helen Mearns

3.3.3 An impaired T_H2 response in the lungs of T cell specific IL-4Rα-deficient mice

Immunity to *N. brasiliensis* is associated with a T_H2 polarised response with IL-4 and IL-13 activating goblet cell hyperplasia (Brombacher 2000). STAT-$6^{-/-}$ and IL-4R$\alpha^{-/-}$ mice have diminished T_H2 responses to *N. brasiliensis* and are unable to control infection (Kopf *et al.* 1993 and Noben-Trauth *et al.* 1997). Therefore, we determined the cytokine secretion profile of CD4+ T-helper cells from the draining lymph node of the lung (mediastinal lymph node). *N. brasiliensis* induced a strong T_H2 response in WT mice, demonstrated by high levels of IL-4 (Figure 3.3.5A). Interestingly, all T cell-specific IL-4Rα-deficient mice (Lck^{cre}IL-4R$\alpha^{-/flox}$ data not shown) had impaired IL-4 production which suggested an impaired T_H2 response. These results were comparable with IL-4R$\alpha^{-/-}$ mice. Although IL-13 plays a key protective role in the expulsion of helminths, IL-4 promotes T_H2 differentiation and was determined as an indicator of a T_H2 response. Interestingly, WT mice maintained high levels of IFN-γ compared with the IL-4R$\alpha^{-/-}$ mice. Despite an impaired T_H2 response, T cell-specific IL-4Rα-deficient mice maintained low levels of IFN-γ on day 10 (Figure 3.3.5B). These data demonstrate an impaired T_H2 response in the draining lymph node of the lungs in T cell-specific IL-4Rα-deficient mice.

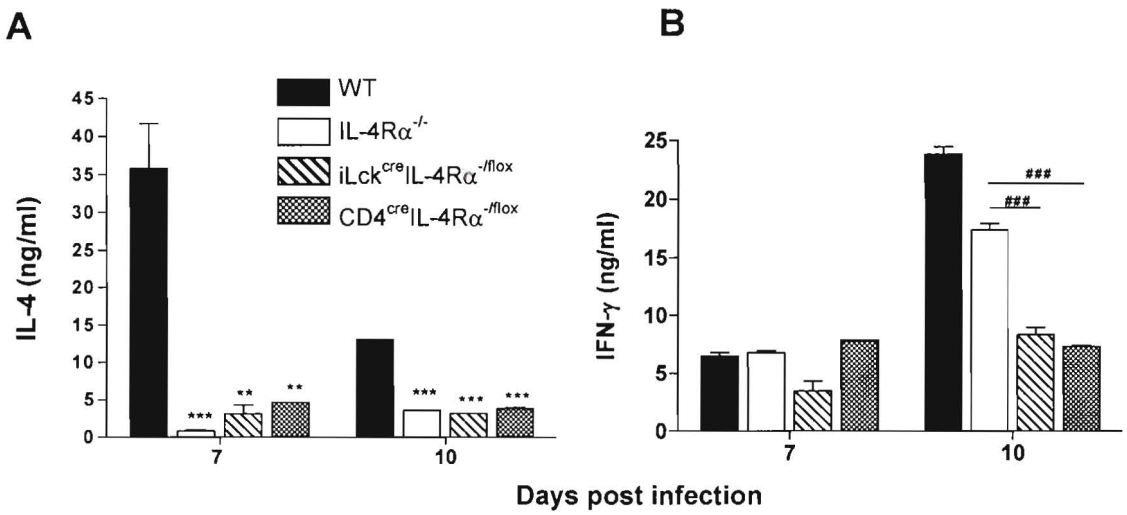

Figure 3.3.5: T cell specific IL-4Rα-deficient mice show an impaired T_H2 response. WT, IL-4R$\alpha^{-/-}$, $iLck^{cre}$IL-4R$\alpha^{-/flox}$ and $CD4^{cre}$IL-4R$\alpha^{-/flox}$ BALB/c mice were killed on day 7 and 10 post infection. CD4+ T cells were sorted from pooled mediastinal lymph nodes of 4-5 mice per group. Cells were restimulated with anti-CD3 and supernatants were collected after 72 hrs. IL-4 **(A)** and IFN-γ **(B)** were detected by ELISA. For all figures; Mean = ± SD. * = Significance of WT vs all knockout strains and # = IL-4R$\alpha^{-/-}$ vs T cell-specific IL-4Rα-deficient mice. *, # = $p < 0.05$, **, ## = $p < 0.01$ and *** = $p < 0.001$. Results are from 1 experiment.

3.3.4 *Lung goblet cell hyperplasia was transiently reduced in T cell specific IL-4Rα-deficient mice.*

Although *N. brasiliensis* emigrates from the lung, a T_H2 immune response characteristic of allergic airway inflammation is sustained for an additional 14 days accompanied by mucus production in the lung of WT mice (Marsland *et al.* 2004 and Marsland *et al.* 2005). *N. brasiliensis* induced goblet cell hyperplasia was previously shown to be impaired in IL-4Rα$^{-/-}$ mice (Barner *et al.* 1998). To determine whether the observed impaired T_H2 response affected induction of goblet cell hyperplasia in the lungs of T cell-specific IL-4Rα-deficient mice, lung histology sections were stained for mucus with PAS on days 3, 7 and 10 post infection. No mucus was observed on day 3 in all mouse strains (Figure 3.3.6). By day 7, mucus was observed in the lungs of WT mice but not in the lungs of all knockout mouse strains. Mucus production in the lungs of iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ were comparable with WT mice on day 10 post infection but remained impaired in IL-4Rα$^{-/-}$ mice.

A quantitative PAS assay for mucin glycoproteins confirmed the above observations (Figure 3.3.7). Mucus was demonstrated on day 3 with all mouse strains showing similar levels of mucin. By day 7, mucus production in WT control lungs was increased compared to all the other groups. As demonstrated in histology, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains, showed an increased mucus production compared to IL-4Rα$^{-/-}$ mice but lower than WT controls. Together, these results suggest delayed mucus production in the lung of T cell-specific IL-4Rα-deficient mice with no significant differences between iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains.

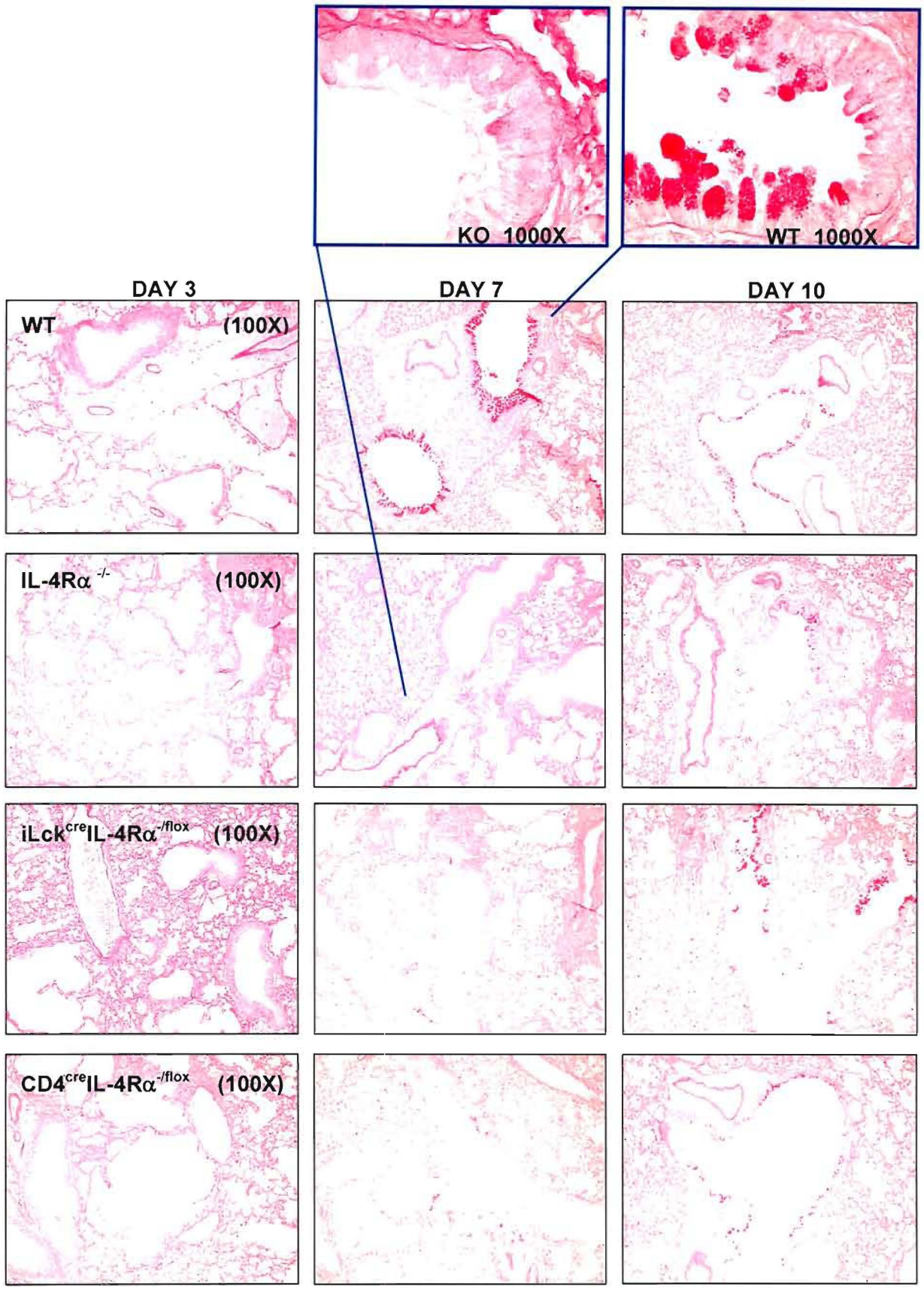

Figure 3.3.6: Lung goblet cell hyperplasia. Mucus producing goblet cells were visualised (100X magnification) using PAS staining at days 3, 7 and 10 post infection. A representative photograph was taken from individual mice of 3-5 mice per group

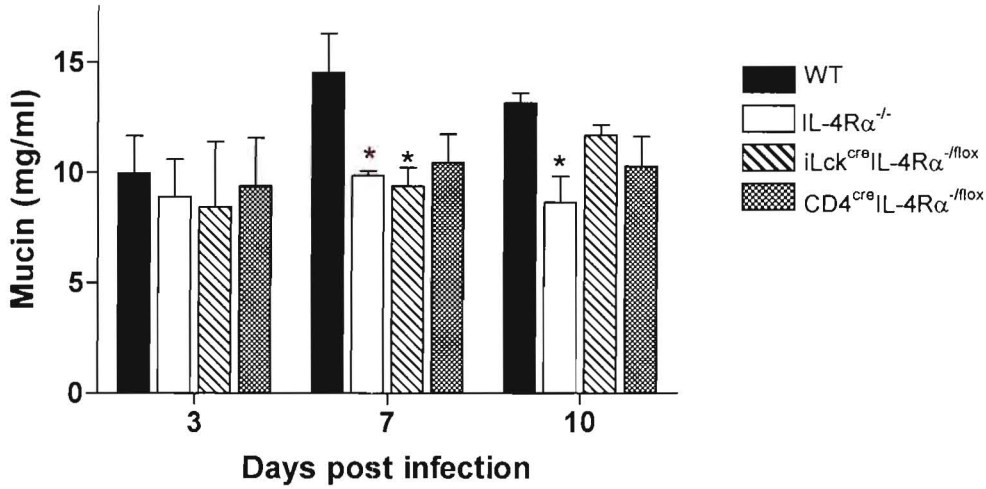

Figure 3.3.7: PAS assay for mucin. Mucus production by *N. brasiliensis* infected goblet cells was detected. Lung tissue was homogenised and centrifuged, glycoproteins were detected in the supernatant. Values indicate mean ± SEM with * = $p < 0.05$ and *** = $p < 0.001$ compared with WT. Data is representative of 1 experiment. Results are from individual mice (4-5 per group).

3.3.5 T cell-specific IL-4Rα-deficient mice control N. brasiliensis infection.

In immunocompetent mice, adult worms are expelled by days 9-12 after infection (Barner, *et al.* 1998) however IL-4Rα$^{-/-}$ mice are unable to expel *N. brasiliensis* and maintain a chronic infection. We wanted to determine whether the absence of IL-4Rα on T cells would affect the outcome of *N. brasiliensis* infection. Therefore, the number of eggs in the faeces of infected mice were determined from days 5-10 (or day 14, data not shown) post infection (Figure 3.3.8A). Egg production in T cell-specific IL-4Rα-deficient mice was similar to WT mice, which peaked at day 7 and declined thereafter due to protective immune responses of the host (Barner *et al.* 1998; Brombacher 2000). As previously shown, the IL-4Rα$^{-/-}$ mice demonstrated a significantly prolonged egg production with eggs present until day 14 post infection (Barner *et al.* 1998; Herbert *et al.* 2004). Interestingly, T cell-specific IL-4Rα-deficient mice were able to expel adult worms with kinetics observed in WT mice; while IL-4Rα$^{-/-}$ mice showed delayed expulsion (Figure 3.3.8B, Lck^{cre}IL-4Rα$^{-/flox}$ data not shown). Although, there were worms present on Day 14 in CD4^{cre}IL-4Rα$^{-/flox}$ mice, the results represent one (out of four) mice and not significantly different to WT. These data demonstrate that T cell-specific IL-4Rα-deficient mice (with no differences observed between T cell-specific IL-4Rα-deficient strains) control *N. brasiliensis* infection as WT mice.

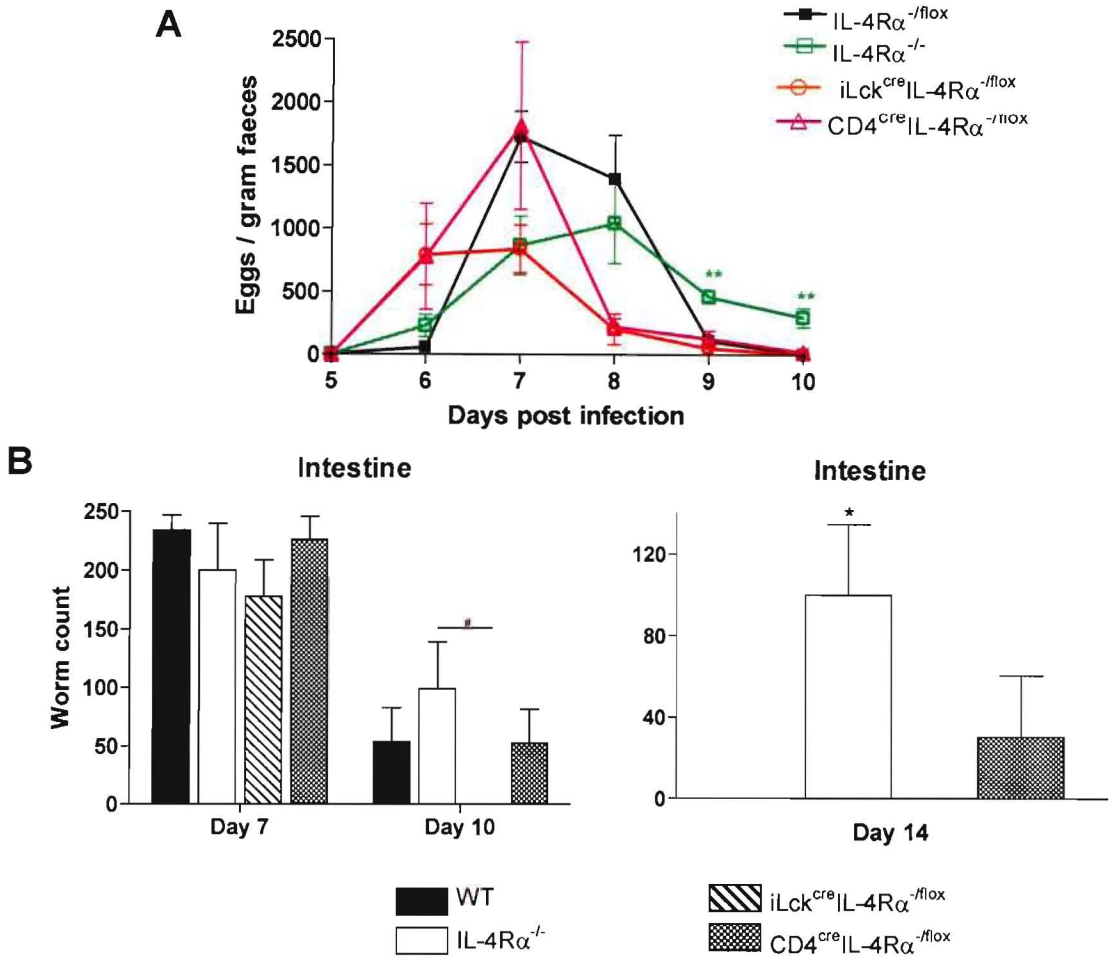

Figure 3.3.8: T cell-specific IL-4Rα-deficient mice control *N. brasiliensis* infection. WT, IL-4Rα$^{-/-}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice were infected with 750 L3 stage *N. brasiliensis* larvae. Mice were killed on days 1, 3, 7, 10 or 14 post infection. **(A)** Worm egg production was determined daily from day 5 in faeces from individual mice (4 mice per group). Representative results chosen from 2 experiments and mean = ± SEM. (** = $p < 0.01$). **(B)** Worms present in the intestine were determined 10 and 14 days post infection from 1 experiment. 3-5 mice per group were used with mean = ± SEM (* = $p < 0.05$ WT vs IL-4Rα$^{-/-}$ and # = $p < 0.05$ IL-4Rα$^{-/-}$ vs T cell-specific IL-4Rα-deficient mice).

3.3.6 T cell specific IL-4Rα-deficient mice control N. brasiliensis infection despite an impaired T_H2 response

As described in the lung draining lymph node, we determined the cytokine secretion profile of CD4+ T-helper cells from the draining lymph node of the intestine (mesenteric) of infected mice. *N. brasiliensis* induced a strong T_H2 response in WT mice, demonstrated by high levels of IL-4 (Figure 3.3.9A). Interestingly, as demonstrated in the lung, all T cell-specific IL-4Rα-deficient mice (Lck^{cre}IL-4Rα$^{-/flox}$ data not shown) had a striking reduction in IL-4 secretion, which confirmed an

impaired T_H2 response. These results were comparable with IL-4R$\alpha^{-/-}$ mice. In the draining lymph node of the intestine, IL-4R$\alpha^{-/-}$ mice demonstrated significantly increased IFN-γ levels compared with all mouse strains (Figure 3.3.9B), suggesting a T cell differentiation shift towards a T_H1 response. Despite an impaired T_H2 response, T cell-specific IL-4Rα-deficient mice maintained low levels of IFN-γ (Figure 3.3.9B). Although, IL-4 production was significantly impaired in all knockout strains, it was not completely abrogated. The levels of IL-4 produced in response to *N. brasiliensis*, although reduced, may be sufficient for IL-4 responsive non-T cells to expel the worms. Although IL-13 results were not available, IL-13 has been shown to be responsible for expulsion.

Another indication of an impaired T_H2 response was demonstrated by significantly lower IgE levels of both iLck^{cre}IL-4R$\alpha^{-/flox}$ and CD4^{cre}IL-4R$\alpha^{-/flox}$ compared with WT mice (Figure 3.3.9C). IgE production was reduced but not abrogated as with IL-4R$\alpha^{-/-}$ mice. IgG2a, a T_H1 associated antibody was significantly increased in IL-4R$\alpha^{-/-}$ mice but only slightly higher in iLck^{cre}IL-4R$\alpha^{-/flox}$ when compared to WT mice. These data demonstrate the successful clearance of *N. brasiliensis* in T cell-specific IL-4Rα-deficient mice despite an impaired T_H2 response.

3.3.7 *Intestine goblet cell hyperplasia in T cell specific IL-4Rα-deficient mice.*

Clearance of *N. brasiliensis* is associated with an increase in IL-4Rα driven intestinal goblet cell hyperplasia and mucus production. WT, iLck^{cre}IL-4R$\alpha^{-/flox}$ and CD4^{cre}IL-4R$\alpha^{-/flox}$ mice all showed an increased mucus production on day 7 and 10. However, mucus production was impaired in IL-4R$\alpha^{-/-}$ mice and supported by previous studies (Barner *et al.* 1998) (Figure 3.3.10). As described for the lung tissue, a quantitative PAS assay for mucus production confirmed histological observations (Figure 3.3.11). No significant differences were detected in WT, iLck^{cre}IL-4R$\alpha^{-/flox}$ and CD4^{cre}IL-4R$\alpha^{-/flox}$ mice but impaired mucus production was observed in IL-4R$\alpha^{-/-}$. These results demonstrate normal mucus production in the intestine of T cell-specific IL-4Rα-deficient mice with no significant difference between the strains. As mucus production was impaired on day 7 in the lung these data suggest a localised mechanism for mucus production.

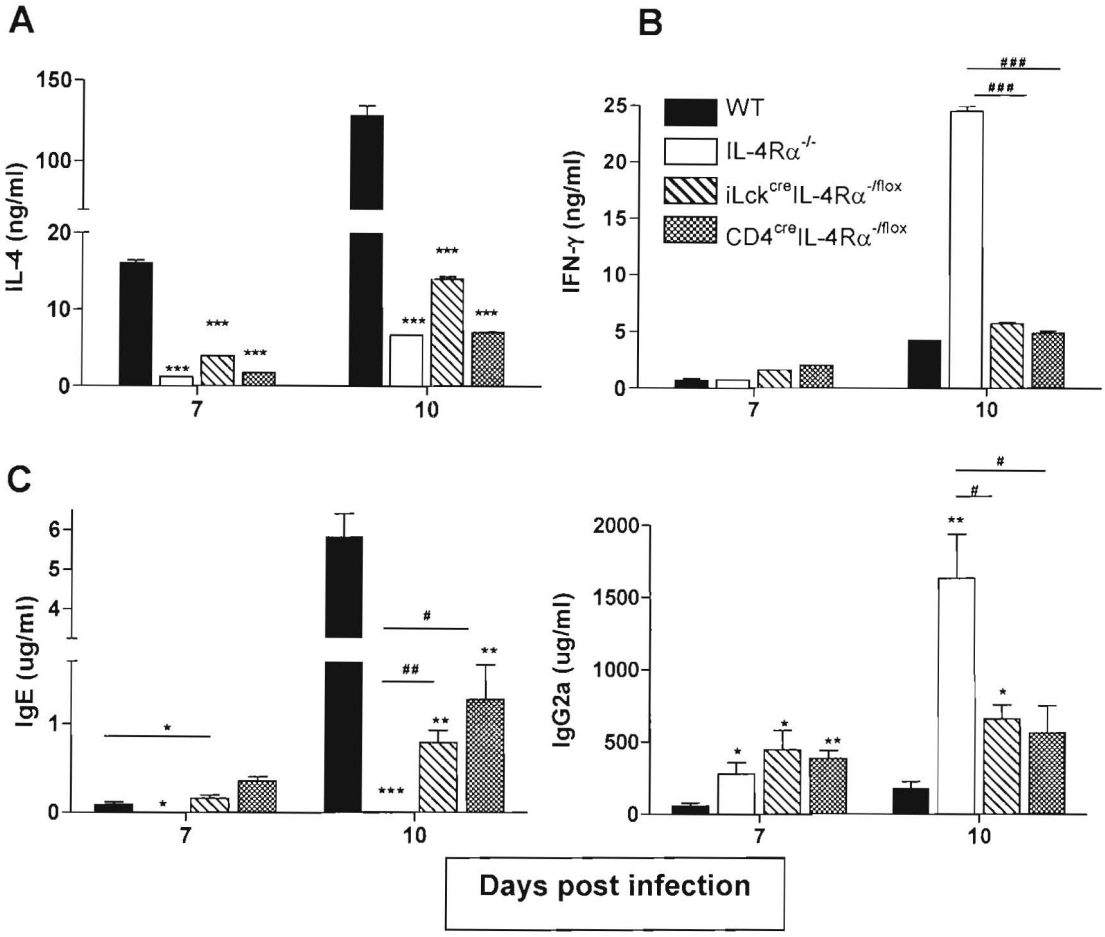

Figure 3.3.9: T cell specific IL-4Rα-deficient mice show an impaired T_H2 response. WT, IL-4Rα$^{-/-}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ BALB/c mice were killed on day 7 and 10 post infection. CD4+ T cells were sorted from pooled mesenteric lymph nodes of 4-5 mice per group. Cells were restimulated with anti-CD3 and supernatants were collected after 72 hrs. IL-4 **(A)** and IFN-γ **(B)** were detected by ELISA. **(C)** IgE and IgG2a were detected in serum by ELISA. For all figures; Mean = ± SD. * = Significance of WT vs all strains and # = IL-4Rα$^{-/-}$ vs T cell-specific IL-4Rα-deficient mice. *, # = $p < 0.05$, **, ## = $p < 0.01$ and *** = $p < 0.001$. Results are from 1 experiment.

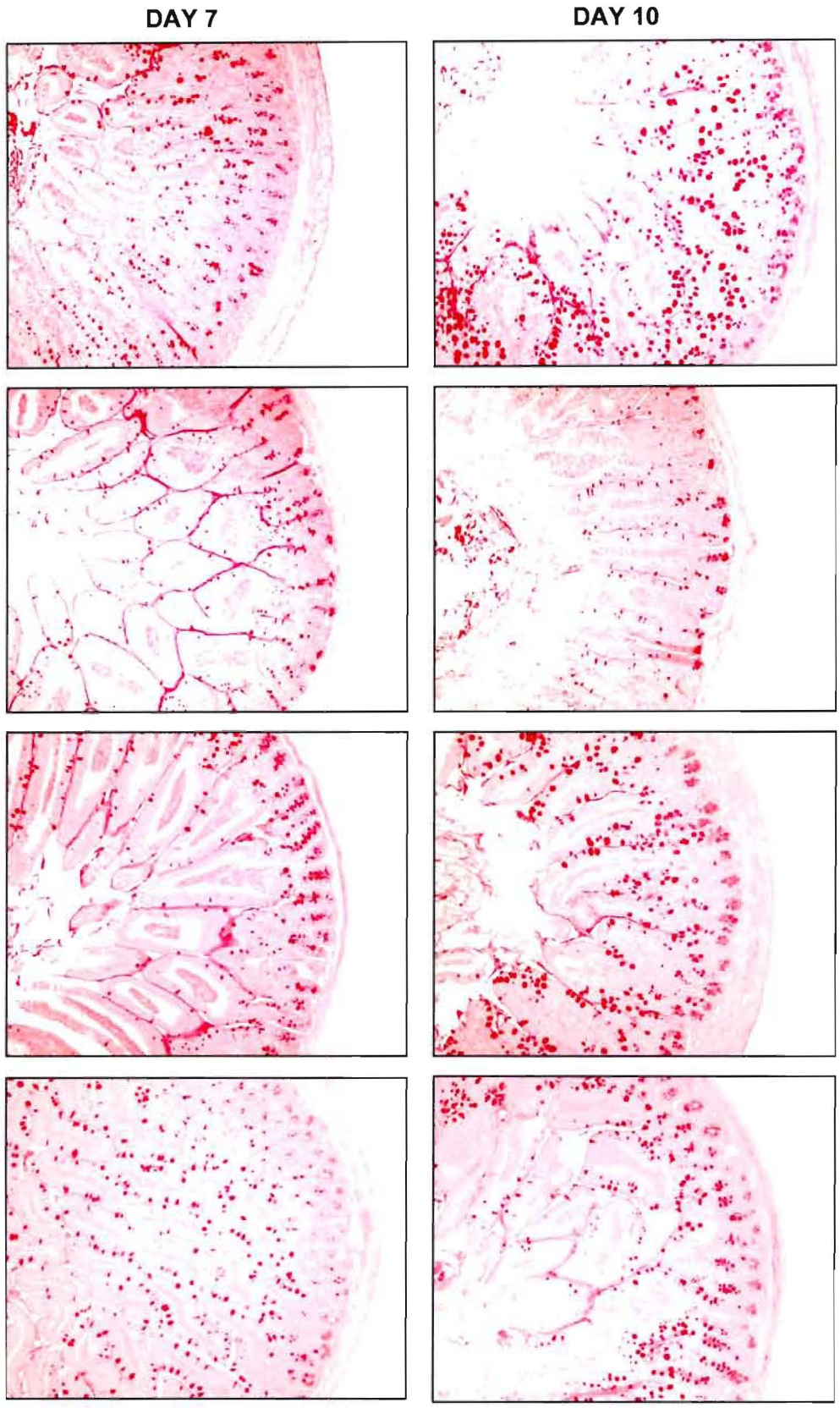

Figure 3.3.10: Unaffected intestine goblet cell hyperplasia in T cell-specific IL-4Rα-deficient mice. Mucus producing goblet cells were visualised (100X magnification) using PAS staining at days 7 and 10 post infection. Representative photographs were taken of individual mice. 3-5 per group

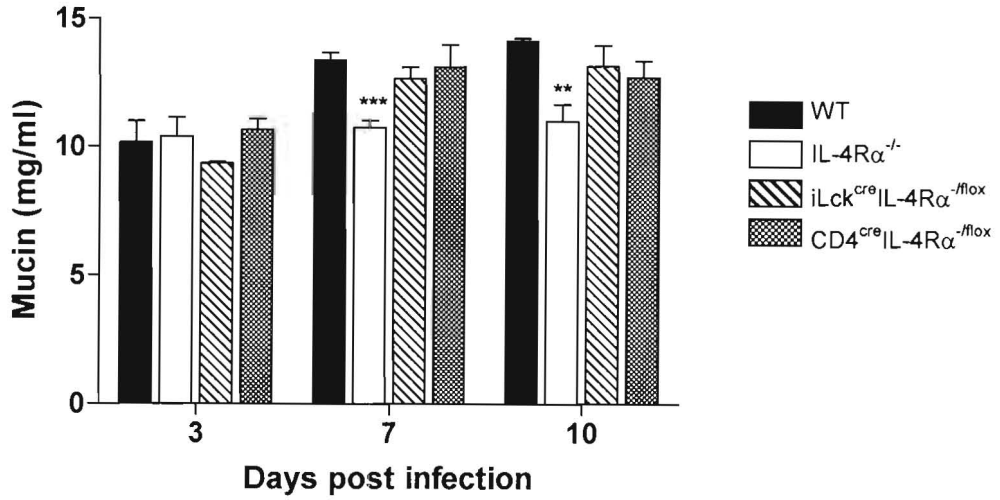

Figure 3.3.11: PAS assay for mucin
Mucus production by *N. brasiliensis* infected goblet cells was detected. Intestine tissue was homogenised and centrifuged, glycoproteins were detected in the supernatant. Values indicate mean ± SEM with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ compared with WT. Data is representative of 1 experiment. Results are from individual mice (4-5 per group).

In summary, a decreased CD3+ infiltration was demonstrated by FACS analysis of lung single cell suspensions in T cell-specific IL-4Rα-deficient mice. These results were supported by a decreased CD3+ infiltration into the lung airways. An impaired cellular infiltration in the lung was due to an impaired T_H2 cytokine secretion by IL-4 responsive CD4+ T cells in the draining lymph node of the lung. Although there was an impaired T_H2 response, T cell polarisation to a T_H1 response did not occur (as with IL-4Rα$^{-/-}$ mice). Furthermore, delayed mucus production in the lung of T cell-specific IL-4Rα-deficient mice was demonstrated. Although, there was a reduced T_H2 response in the lung of all T cell-specific IL-4Rα-deficient mice (with no differences observed between mouse strains), they successfully controlled *N. brasiliensis* infection as WT mice. A reduced T_H2 response was demonstrated in the intestine draining lymph node and confirmed observations in the lung. These data were supported by an impaired T_H2 antibody response in T cell-specific IL-4Rα-deficient mice. Different from the lung observations, T cell-specific IL-4Rα-deficient mice demonstrate normal mucus production in the intestine with no significant difference between the mouse strains. Mucus production was impaired in the intestine of IL-4Rα$^{-/-}$ mice. Our results show that T cell-specific IL-4Rα-deficient mice can successfully clear a *N. brasiliensis* infection as WT mice despite an impaired T_H2 response. This indicates that polarisation to strong IL-4 mediated cellular responses are not crucial for *N. brasiliensis* expulsion and are the cause of increased lung pathology in all T cell subpopulations.

4. Discussion

IL-4 and IL-13 are able to regulate many cell-types and are important in the generation of immunity in human diseases. Both IL-4 and IL-13 share a common signalling pathway through IL-4Rα (Nelms *et al.* 1999). Therefore, the role of both cytokines can be investigated in IL-4Rα deficient mice (Mohrs *et al.* 2000). By using Cre / *Lox*P recombination to delete the IL-4Rα in cell-specific transgenic mice, the role of IL-4 responsive T cells could be investigated. As IL-13 has no functional receptor on T lymphocytes (Mohrs *et al.* 2000), we investigated the beneficial or detrimental role of IL-4 induced T cell responses during *Leishmania major* and *Nippostrongylus brasiliensis* infection models. We described the generation, characterisation and functional analysis of mice lacking the IL-4Rα chain selectively on T lymphocytes. Three T cell-specific IL-4Rα-deficient mouse lines (1 previously characterised and 2 novel strains) using different transgenic promoters to drive cre-recombinase expression were compared in infectious disease models. In the *L. major* infection model, an impaired T_H2 response was protective and resulted in the resolution of disease, with a tendency for increased T_H1 responses. These results suggested that IL-4 / IL-13 responsive non-T cell populations were essential to transform *L. major* infected BALB/c mice to a healer phenotype. An impaired T_H2 response to *N. brasiliensis* infection was protective and allowed for resolution of disease. These results suggested that IL-4 mediated T_H2 cellular responses were not crucial for worm expulsion but instead resulted in increased lung pathology in *N. brasiliensis* infection.

4.1 Characterisation of T-cell specific IL-4Rα-deficient mice

Two novel T cell-specific IL-4Rα-deficient mouse strains ($iLck^{cre}$IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$) were characterised and compared with a previously characterised (Lck^{cre}IL-$4R\alpha^{-/flox}$) mouse strain (Radwanska *et al.* 2006, submitted). As previously described, T cell-specific IL-4Rα-deficient mouse strains were generated using the Cre / *lox*P recombination system in BALB/c ES cells (Radwanska *et al.* 2006. submitted). Furthermore, the efficiency of T cell-specific IL-4Rα deletion was increased by mating hemizygous IL-$4R\alpha^{-/flox}$ mice with transgenic Lck^{cre}, $iLck^{cre}$ and $CD4^{cre}$ mice. PCR genotyping confirmed transgene-bearing, hemizygous Lck^{cre}IL-$4R\alpha^{-/flox}$, $iLck^{cre}$IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$ mouse strains (Figure 3.1.1). Efficient T cell specific IL-4Rα deletion was confirmed by flow cytometry analysis. A loss of IL-

4Rα expression on T cell subpopulations was observed, with variation between Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains. In Lck^{cre}IL-4Rα$^{-/flox}$ mice the IL-4Rα was completely disrupted in CD3+CD4+ T cells and partially deleted in CD8+ T cells, while NK T and γδ T cells maintained receptor expression. In comparison, the IL-4Rα was completely disrupted from all T cell subpopulations (CD4, CD8, NK T and γδ T cells) in iLck^{cre}IL-4Rα$^{-/flox}$ mice. The expression of IL-4Rα on γδ T cells in CD4^{cre}IL-4Rα$^{-/flox}$ mice was the only difference between these two novel mouse strains. The normal expression of IL-4Rα on NK and B cells confirmed the cellular specificity of disruption in all three T cell specific IL-4Rα-deficient mice (Figure 3.1.2). Furthermore, normal expression of the receptor was observed on macrophages and dendritic cells in Lck^{cre}IL-4Rα$^{-/flox}$ mice, shown by Radwanska, *et al.* 2006 (submitted). Due to an enhanced transcription of the Lck gene (by insertion of a Kozak consensus sequence in Cre) and a nuclear localisation signal in Cre, targeting the IL-4Rα on T cells was more effective in the iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains (Dr. Chris Wilson personal communication). FACS analysis of IL-4Rα expression was confirmed at a genomic level for CD4+ and CD8+ T cells on all three T cell specific IL-4Rα-deficient mouse strains. Intron-exon 8 allelic expression was similar between Lck^{cre}IL-4Rα$^{-/flox}$ and IL-4Rα$^{-/flox}$, but reduced in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice on CD19+ cells. As amplification of the β-actin housekeeping gene revealed similar DNA amplification between mouse stains (data not shown), the difference could be due to the sensitive nature of Real Time PCR (Figure 3.1.4).

The effective impairment of IL-4Rα-mediated functions on T cells was demonstrated by an impaired IL-4-induced CD4+ T cell proliferation in all T cell-specific IL-4Rα-deficient mice. This proliferation was slightly increased compared with IL-4Rα$^{-/-}$ CD4+ cells and could be due to slight contaminating CD4+ population as bead sorts were 95-98% pure. When stimulated with IL-2 all mouse strains demonstrated a dose-dependent proliferative response. Compared with previous experiments (Mohrs *et al.* 1999; Radwanska *et al.* 2006, submitted) the variability of IL-2 stimulated proliferation was due to assay inconsistency and not mouse strain differences. Impaired *in vitro* T_H2 differentiation of CD4+ T cells mediated by IL-4 supported the observations of impaired IL-4Rα functions. T_H1 polarisation was unimpaired in all mouse strains; interestingly the level of IFN-γ production was lower in all knockout strains. Again, compared with previous experiments (Herbert *et al.* 2004; Radwanska *et al.* 2006, submitted) the variability could be due to assay inconsistency and not mouse strain

differences. Normal IL-4 responsiveness on non-T cell populations was demonstrated by IgE production in response to Ova-alum sensitisation (Figure 3.1.5). In conclusion, IL-4Rα was shown to be specifically and functionally inactivated on CD4+ T cells of all three T cell-specific IL-4Rα-deficient mouse strains.

4.2 IL-4/IL-13 responsive non-T cell populations are essential to transform *Leishmania major* infected BALB/c mice to a healer phenotype

To define the role of IL-4 responsive T cell subpopulations in *L. major* infection, three T cell-specific IL-4Rα-deficient mouse strains (Lck^{cre}IL-$4R\alpha^{-/flox}$, $iLck^{cre}$IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$) were used in a comparative infection study with WT and IL-$4R\alpha^{-/-}$ mice. In *L. major* infection it is accepted that, healer strains (C57/BL6) develop dominant T_H1 responses with high IFN-γ and low IL-4 production. In contrast, nonhealer strains (BALB/c) develop dominant T_H2 responses with high IL-4 and low IFN-γ production (Heinzel *et al.* 1989). Furthermore, our results confirmed a previous study that IL-$4R\alpha^{-/-}$ mice were able to control *L. major* infection in the early phase of disease but developed progressive disease compared with C57/BL6 mice in the late phase (Mohrs et al. 1999) (Figure 3.2.1). Previous studies in our lab have shown Lck^{cre}IL-$4R\alpha^{-/flox}$ mice to develop a healing phenotype comparable with C57/BL6 mice (Radwanska *et al.* 2006, submitted). These results were observed when comparing this strain to two T cell-specific IL-4Rα-deficient mice ($iLck^{cre}$IL-$4R\alpha^{-/flox}$ and $CD4^{cre}$IL-$4R\alpha^{-/flox}$). No difference in footpad swelling was determined between the three T cell-specific IL-4Rα-deficient mice. Therefore, all T cell-specific IL-4Rα-deficient mice control *L. major* disease progression as C57/BL6 controls.

Another indicator of disease progression and control of infection was parasite burden in the footpad and popliteal lymph node. As early as 3 weeks post infection, all mouse strains had significantly lower parasite numbers in the footpad compared with the WT mouse suggesting control in the early phase of disease (Figure 3.2.2). By week 6, the T cell-specific IL-4Rα-deficient mice retained parasite numbers comparable with C57/BL6 mice, while the parasite numbers for IL-$4R\alpha^{-/-}$ mice gradually increased. After killing WT mice by week 12 (due to uncontrolled footpad swelling and parasite burden), IL-$4R\alpha^{-/-}$ mice maintained parasite burden and all the other strains had controlled the infection. Parasite numbers in the draining popliteal lymph node (PLN) of T cell-specific IL-4Rα-deficient mice were significantly reduced compared with IL-$4R\alpha^{-/-}$ mice (from week 6). Interestingly, the parasite numbers

remained significantly higher than C57/BL6. These results were previously shown in $Lck^{cre}IL\text{-}4R\alpha^{-/flox}$ mice (Radwanska *et al.* 2006, submitted). Therefore, control of *L. major* at the site of infection in T cell-specific IL-4Rα-deficient mice was comparable to C57/BL6 controls and demonstrated as early as 3 weeks post infection.

Cytokines produced by CD4+ T cells in response to *L. major* infection can directly influence the outcome of disease (Sacks and Noben-Trauth 2002; Radwanska *et al.* 2006, submitted). Unsorted draining LN cells stimulated with SLA demonstrated a significant increased IFN-γ secretion in all T cell-specific IL-4Rα-deficient strains. The difference in IFN-γ production between the three strains had no effect on the outcome of disease. CD4+ T cells sorted from draining LN maintained a trend towards an increased IFN-γ secretion, suggesting that these cells are IFN-γ producers in response to *L. major* infection. IFN-γ was lower in $iLck^{cre}IL\text{-}4R\alpha^{-/flox}$ and $CD4^{cre}IL\text{-}4R\alpha^{-/flox}$ mice compared with $Lck^{cre}IL\text{-}4R\alpha^{-/flox}$. However, it has been previously demonstrated in $IL\text{-}4R\alpha^{-/-}$ mice infected with a different strain of *L. major* (IR173), that residual levels of IFN-γ were critical for the control of infection (Noben-Trauth *et al.* 1999). The absence or reduction in T_H2 cytokines (IL-4 and IL-13) resulted in the ratio of IFN-γ to IL-4 and IL-13 favouring a T_H1 response. Therefore, even low levels of IFN-γ became sufficient to control parasite growth in the infected footpad. Although CD8+ (Muller *et al.* 1991) or γδ T (Ferrick *et al.* 1995) cells have been shown to produce IFN-γ, sorted CD4+ T cells maintained the trend of increased IFN-γ,.

In contrast, early IL-4 secretion was significantly decreased in all three T cell-specific IL-4Rα-deficient strains (comparable with C57/BL6) in comparison to WT and $IL\text{-}4R\alpha^{-/-}$ mice. The reduction in IL-4 secretion was maintained 6 weeks post infection and corresponded with reduced IL-13 secretion at week 12. Although, significantly less IL-4 was produced in $iLck^{cre}IL\text{-}4R\alpha^{-/flox}$ and $CD4^{cre}IL\text{-}4R\alpha^{-/flox}$ mice compared with $Lck^{cre}IL\text{-}4R\alpha^{-/flox}$ this had no effect on the control of infection. Together these results demonstrate an impaired T_H2 response. Interestingly, reduced levels of IL-4 detected at week 6 post infection in $IL\text{-}4R\alpha^{-/-}$ mice were not supported by previous data where unimpaired IL-4 production was detected (Mohrs *et al.* 2000). However, CD4+ T cells were restimulated with SLA in the absence of APCs. Our results obtained using SLA pulsed CD4+T cells in the presence of APCs resembled those in which cells were restimulated with anti-CD3. Furthermore, decreased IL-4 message was demonstrated in CD4+ T cells compared with BALB/c mice 56 days post infection (Noben-Trauth *et al.* 1997). As IL-13 has been described as a susceptibility factor in *L. major* infection (Matthews *et al.* 2000) the impaired IL-13 secretion in all T cell-specific IL-4Rα-

deficient mice (comparable with C57/BL6) could be expected. Furthermore, the significantly increased levels of IL-13 shown in IL-4Rα$^{-/-}$ mice (compared with C57/BL6 controls) could be associated with a type 2 response but could not have influenced susceptibility of these mice as all cells lack IL-4 / IL-13 responsiveness. As described in the literature, C57/BL6 controls demonstrated a polarised T_H1 response (with increased IFN-γ and impaired IL-4 and IL-13), while WT (IL-4Rα$^{-/flox}$) controls demonstrated a polarised T_H2 response (with increased IL-4 and impaired IFN-γ). T cell-specific IL-4Rα-deficient mice were similar to C57/BL6 controls with a trend towards T_H1 polarisation and an impaired T_H2 response associated with resistance to infection.

While much work has focused on IL-4 and IL-13, a role for IL-10 and Regulatory T (Tregs) cells in susceptibility have been reported (Noben-Trauth *et al.* 1997; Kane and Mosser 2001). Although a trend towards decreased IL-10 production at week 6 was demonstrated in SLA stimulated CD4+ T cells from all T cell-specific IL-4Rα-deficient mice compared with IL-4Rα$^{-/-}$, no difference was detected when compared with WT controls (also demonstrated at week 3 post infection -data not shown) (Figure 3.2.4). These results were supported by a recent study with Lck^{cre}IL-4Rα$^{-/flox}$ mice in our lab (Radwanska, *et al.* 2006, submitted). However intricate studies in the same paper, suggested that extensive IL-10 production by Tregs in WT and IL-4Rα$^{-/-}$ mice were possibly responsible for their inability to control parasite growth. Anti-CD3-stimulated CD4+ T cells from C57/BL6 and Lck^{cre}IL-4Rα$^{-/flox}$ mice demonstrated significantly less IL-10 compared with susceptible WT and IL-4Rα$^{-/-}$ mouse strains (Radwanska *et al.* 2006, submitted). Analysis of the PLN response in chronically infected IL-4Rα$^{-/-}$ mice revealed an increased number of antigen-specific IL-10 secreting CD4+CD25+ T cells displaying an activated T cell / natural Treg phenotype. Furthermore, 5-fold more CD4+FoxP3+ and CD4+CTLA-4+ Treg cells were seen in IL-4Rα$^{-/-}$ mice compared with Lck^{cre}IL-4Rα$^{-/flox}$ suggesting that Treg cells were responsible for the IL-10 production in susceptible strains. Direct involvement of IL-10 in suppressing the *L. major* antigen-specific DTH was demonstrated by neutralisation of IL-10 signalling, which allowed susceptible, non-DTH responder BALB/c and IL-4Rα$^{-/-}$ mice to mount a significant response to SLA (Radwanska *et al.* 2006, submitted). Therefore, IL-10 secretion was associated with high parasite burden in the infected footpads of WT and IL-4Rα$^{-/-}$ mice.

Delayed-type hypersensitivity (DTH) is associated with resistance to *L. major* (De Rossell *et al.* 1987) and is driven by IL-12 induced IFN-γ producing T_H1 cells (Mattner

et al. 1996). Non-healing lesions were associated with either negative DTH (BALB/c mice) or DTH that peaked at 24 hrs but had subsided by 48hrs. Our results resembled the above model with resistant C57/BL6 controls developing DTH within 24hrs (as early as 3 weeks post infection) which was maintained at 48hrs and subsided by 72 hrs (Figure 3.2.5). In contrast, WT controls demonstrated a slight response at 24hrs, which had completely subsided at 48hrs. Even though IL-4Rα$^{-/-}$ mice controlled acute infection with *L. major* (demonstrated by a delay in footpad swelling) they were unable to develop a DTH response. Interestingly, all T cell-specific IL-4Rα-deficient mice developed DTH by 24hrs which remained significantly higher than WT and IL-4Rα$^{-/-}$ at 48hrs. A protective DTH was maintained in C57/BL6 and all T cell-specific IL-4Rα-deficient mice at week 6 post infection. These results support the observation that all T cell-specific IL-4Rα-deficient mice are resistant to *L. major* infection. Although IL-4 and IL-10 have been demonstrated to inhibit DTH in susceptible strains (Powrie *et al.* 1993) a more extensive study would be required to determine the role of IL-10 in the three T cell-specific IL-4Rα-deficient mice, as described by Radwanska *et al.* 2006, submitted.

Besides its activity as a T cell growth factor, IL-4 stimulates B cells to produce IgG1 and IgE. As previously described (Heinzel *et al.* 1989), WT controls developed a dominant T_H2 antibody response with increased production of IgG1 and IgE at weeks 6 and 8 (Figure 3.2.6). In contrast, IL-4Rα$^{-/-}$ mice were unable to produce T_H2 antibodies (due to the lack of IL-4Rα on B cells). Furthermore, a reduced level of IgG1 and IgE provides further evidence of an impaired T_H2 polarisation in T cell-specific IL-4Rα-deficient mice. Interestingly, IgG antibodies have been demonstrated to contribute to disease progression in *L. major* infected BALB/c mice and induce IL-10 production from macrophages (Miles *et al.* 2005). BALB/c mice with a targeted deletion in the Ig heavy chain locus were more resistant to infection with footpad swelling similar to that of IL-4Rα$^{-/-}$ mice. Therefore, the inability of IL-4Rα$^{-/-}$ mice to produce T_H2 antibodies may contribute to their resistance in the early phase of *L. major* infection. However, this mechanism may be mediated by T_H1 antibodies, in which case it would not affect IL-4Rα$^{-/-}$ mice. C57/BL6 controls demonstrated very low antibody levels for all isotypes and could be due to the type of immune reaction. The impaired production of T_H2 antibodies in T cell-specific IL-4Rα-deficient mice support previous data that T_H2 responses to *L. major* are reduced in these mice.

A comparison between the three T cell-specific IL-4Rα-deficient mice (Lck^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$) demonstrated that all three mouse strains

were able to control disease progression and parasite numbers as C57/BL6 controls. Subtle differences were apparent between the strains such as; higher IFN-γ secretion by CD4+ cells in Lck^{cre}IL-4R$\alpha^{-/flox}$ mice and reduced parasite number and IL-10 secretion in iLck^{cre}IL-4R$\alpha^{-/flox}$. However, these differences were not significant and not supported by subsequent experiments. As IL-4Rα deletion is limited to CD4+ T cells in Lck^{cre}IL-4R$\alpha^{-/flox}$ mice, it can be concluded that; IL-4 responsive CD4+ T cells are responsible for susceptibility of BALB/c mice to *L. major* with no role for IL-4 responsive γδ or NK T cells.

In summary, all T cell-specific IL-4Rα-deficient mice control *L. major* disease progression and parasite burden as C57/BL6 controls. A trend towards T_H1 polarisation with an impaired T_H2 response was associated with resistance to infection. T_H1 polarisation was demonstrated by increased IFN-γ production by CD4+ draining LN cells and the development of a protective DTH. An impaired T_H2 response was determined by reduced (but not abrogated) levels of IL-4, IL-13, IgG1 and IgE. *L. major* initiates early IL-4 production (Belkaid *et al.* 2000) which is successfully redirected by IL-12-dependant mechanisms to a T_H1 response in resistant mice. In susceptible mice IL-4 production is maintained and drives a T_H2 response which leads to disease progression (Reiner *et al.* 1994). The inability of T cell-specific IL-4Rα-deficient mice to respond to this early IL-4 may prevent them from developing a polarised T_H2 response. Therefore, macrophages can respond to IL-12 / IFN-γ and produce iNOS (and not IL-4 / IL-13 derived Arginase). This is demonstrated by the increased iNOS production in Lck^{cre}IL-4R$\alpha^{-/flox}$ mice (Radwanska *et al.* 2006, submitted). Furthermore, the inability of IL-4R$\alpha^{-/-}$ mice to resist infection suggests that abrogation of IL-4Rα from all cell types is not appropriate to confer immunity. Rather, abrogating IL-4Rα responsiveness on T cell subpopulations and retaining IL-4 / IL-13 mediated function on non-CD4+ T cells confers immunity comparable with the C57/BL6 strain.

As described by Radwanska *et al.* 2006 (submitted), WT CD4+ T cells respond to IL-4 driving a T_H2 response with IL-10 production. IL-10 enhances proliferation and function of regulatory T cells, promoting acute disease progression in *L. major* infected mice. With T cell-specific IL-4Rα-deficient mouse strains, non-CD4 T cells retain IL-4 / IL-13 responsiveness and IL-12 is produced. Il-12 may protect activated T cells from regulation and promote protective immune responses against *L. major* infection. Due to the lack of T_H2 polarisation, the small T_H1 response observed in all T

cell-specific IL-4Rα-deficient mouse strains may control *L. major* infection at the site of infection.

4.3 IL-4 responsive T cells are not crucial for worm expulsion and increase lung pathology in *Nippostrongylus brasiliensis* infection

To define the role of IL-4 responsive T cell subpopulations in *N. brasiliensis* infection, two novel T cell-specific IL-4Rα-deficient (iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$), WT and IL-4Rα$^{-/-}$ mouse strains were used in a comparative infection study with Lck^{cre}IL-4Rα$^{-/flox}$ mice. In *N. brasiliensis* infection it is accepted that, BALB/c mice resist infection and develop polarised T_H2 responses with high IL-4 / IL-13 and low IFN-γ production (Urban *et al.* 1991; Kopf *et al.* 1993; Svetic *et al.* 1993). In contrast, IL-4Rα$^{-/-}$ mice develop a polarised T_H1 response with high IFN-γ and low IL-4 / IL-13 production in the absence of IL-4Rα signalling and develop chronic infection (Barner *et al.* 1998; Herbert *et al.* 2004).

The life cycle of *N. brasiliensis,* requires an obligate migration pathway through the lung. Although the intestine stage of infection is well characterised, early immune responses in the lung have not been fully investigated. We determined the lung gross pathology at day 1, 3 and 7 post infection. Although the lungs were severely damaged at day 1 and 3, no differences were observed in damage between the mouse strains. These observations suggest that although IL-4Rα$^{-/-}$ mice fail to control the intestinal stage of infection, the worms migrate through the lung as in WT mice and T cell responsiveness to IL-4 is not necessary (Figure 3.3.1). At the cellular level, decreased infiltration of CD3+ T cells in IL-4Rα$^{-/-}$ and T cell-specific IL-4Rα-deficient mice suggest that T cells require IL-4 responsiveness to be recruited to the lung by day 7 post infection. CD4^{cre}IL-4Rα$^{-/flox}$ mice had a higher number of CD3+ T cells compared to iLck^{cre}IL-4Rα$^{-/flox}$ and Lck^{cre}IL-4Rα$^{-/flox}$. The only difference between the two novel T cell-specific IL-4Rα-deficient strains is IL-4 responsive γδ T cells in CD4^{cre}IL-4Rα$^{-/flox}$ mice. However if γδ T cells were responsible for CD3+ lung infiltration, comparable results would be expected in Lck^{cre}IL-4Rα$^{-/flox}$ mice. Increased granulocyte infiltration was observed in IL-4Rα$^{-/-}$ mice only, therefore granulocyte infiltration may be regulated by an IL-4 / IL-13 dependent mechanism (Figure 3.3.3).

Broncho-alveolar lavage (BAL) is a useful method to determine the infiltration of airway immune cells. *N. brasiliensis* induces extravasation of effector cells from the blood vessels into the alveolar lining (Maizels and Yazdanbakhsh 2003). The observations seen in recruitment of cells to the lungs of *N. brasiliensis* infected mice

were also demonstrated in the lung airways and supported by previous studies of *N. brasiliensis* infected WT mice (Coyle *et al.* 1998). Very few eosinophils or basophils were observed in the BAL of all mouse strains. These observations were surprising and unexplainable as it is well established that eosinophils are recruited in response to helminth infections (Coyle *et al.* 1998; Yamaguchi *et al.* 1988; Coffman *et al.* 1989). The decreased number of CD3+ cells in the lungs and airways of T cell-specific IL-4Rα-deficient mice comparable with IL-4Rα$^{-/-}$, could be due to the inability of T cells to respond to IL-4 and would be associated with an impaired type 2 response.

IL-4 has been implicated in driving a polarised T_H2 response against *N. brasiliensis,* demonstrated by a diminished type 2 response in IL-4$^{-/-}$, IL-4Rα$^{-/-}$ and STAT-6$^{-/-}$ mice (Kopf *et al.* 1993; Takeda *et al.* 1996; Noben-Trauth *et al.* 1997). We asked whether T_H2 responses in T cell-specific IL-4Rα-deficient mice were impaired. The cytokine secretion profile of CD4+ T cells from the draining lymph node of the lung was determined. As described in the literature, IL-4 production was induced in WT mice and impaired in IL-4Rα$^{-/-}$ (Figure 3.3.5). The impaired IL-4 production in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains suggested that IL-4 secretion was dependent on IL-4Rα expression on T cells. However, the diminished but measurable levels of IL-4 secretion by CD4+ T cells in all the knockout mouse strains implied that cells were able to produce IL-4 independently of IL-4Rα (Noben-Trauth *et al.* 1997).

IL-13 has been described as responsible for worm expulsion in *N brasiliensis* infected mice (McKenzie *et al.* 1998). A preliminary study in our lab using iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice demonstrated a tendency towards impaired production of IL-13 by lung draining LN CD4+ T cells (data not shown). These results correspond with the impaired T_H2 response described above. Although IFN-γ secretion in IL-4Rα$^{-/-}$ mice was higher than iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains, the IFN-γ was not increased compared with WT (previously demonstrated, Noben-Trauth *et al.* 1997). The lower levels of IFN-γ in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice indicated that no default T_H1 responses were stimulated due to an impaired T_H2 response. Therefore, decreased CD3+ infiltration into the lungs of iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice was associated with an impaired T_H2 response.

Although *N. brasiliensis* eventually emigrates from the lung, a T_H2 immune response characteristic of allergic airway inflammation remains for an additional 14 days, possibly due to the deposition of allergens (Marsland *et al.* 2005). However, the systemic dissemination of immune responses derived from the intestinal stage of

infection should be considered. This was demonstrated in a study with *Heligmosomoides polygyrus,* a strictly enteric nematode, when CD4+/GFP+ T_H2 cells were found to accumulate in the liver and lung airways (Mohrs *et al.* 2005). On day 3 post infection all mouse strains demonstrated severe haemorrhage and destruction of the alveolar wall. As previously described WT controls developed goblet cell hyperplasia and mucus production from day 7 and maintained at day 10, while IL-4Rα$^{-/-}$ mice demonstrated similar mucus production as naïve mice (supported by quantitative PAS assay). This was probably due to the lack of IL-4 / IL-13 responsive airway epithelial cells. The delayed mucus production in all T cell-specific IL-4Rα-deficient mice could be associated with the observed impaired T_H2 response (Figure 3.3.6) with airway epithelial cells maintaining the ability to respond to IL-4 / IL-13. Therefore, allergic inflammation was associated with mucus production and T_H2 cellular infiltration. Together these results demonstrate a delayed allergic response to *N. brasiliensis* in all T cell-specific IL-4Rα-deficient mice associated with reduced T_H2 cytokine secretion and CD3+ infiltration.

Despite a reduced type 2 response, T cell-specific IL-4Rα-deficient mouse strains were able to control *N. brasiliensis* infection as WT, demonstrated by worm and egg clearance by day 14 post infection (Figure 3.3.8). Moreover, the impaired T_H2 response demonstrated in the lungs was comparable to the draining LN of the intestine in all IL-4Rα-deficient strains with impaired IL-4. IL-4 production was reduced but not abrogated and can be explained by the ability of non-T cells such as; basophils (Min *et al.* 2004), mast cells (Plaut *et al.* 1989) and eosinophils (Sabin *et al.* 1996) to produce IL-4. As seen in the lung of iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice, low levels of IFN-γ secretion were maintained suggesting IL-4Rα indepenent IL-4 production. In contrast to the lung, CD4+ cells in the intestine draining LN of IL-4Rα$^{-/-}$ mice produced significantly higher levels of IFN-γ compared with all mouse strains. Low IgE (T_H2) and IgG2a (T_H1) antibody levels in the sera of iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mice, confirmed an impaired type 2 and a low T_H1 response. Maintaining a low T_H1 response despite an impaired T_H2 response could be explained by non-T cell IL-4 producing cells or a small percentage of T cells expressing the IL-4Rα. As detectable levels of IgE were demonstrated, it can be suggested that the low levels of IL-4 produced were sufficient to stimulate antibody production by B cells. Furthermore, an IL-4 independent pathway for IgE production has been observed in IL-4$^{-/-}$ mice (Morawetz *et al.* 1996). Previous studies demonstrated IFN-γ secretion to be equivalent between WT and IL-4Rα$^{-/-}$ mice. As

IFN-γ induced IgG2a antibodies a T_H1 polarisation was described by increased IgG2a (Noben-Trauth *et al.* 1997; Barner *et al.* 1998).

Clearance of *N. brasiliensis* is associated with an increase in IL-4Rα dependent intestinal goblet cell hyperplasia. As demonstrated in the lung, IL-4Rα$^{-/-}$ mice were unable to produce mucus (induced by IL-13) and developed chronic infection (Figure 3.3.10). In contrast, iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ showed mucus production comparable with WT controls (supported by PAS assay). Due to unavailable IL-13 results for CD4+ intestine draining LN cells in T cell-specific IL-4Rα-deficient mice, two conclusions could be drawn (i) Low levels of IL-13 present are sufficient for goblet cell hyperplasia and mucus production or (ii) Compensatory mechanisms produce normal levels of IL-13 responsible for worm expulsion. Therefore, we demonstrate the successful clearance of *N. brasiliensis* in iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ mouse strains despite an impaired T_H2 response.

A comparison between iLck^{cre}IL-4Rα$^{-/flox}$ and CD4^{cre}IL-4Rα$^{-/flox}$ demonstrated that both strains were able to control *N. brasiliensis* infection as WT controls (comparable with Lck^{cre}IL-4Rα$^{-/flox}$ mice). Furthermore, both strains demonstrated impaired type 2 responses and reduced lung pathology. When comparing subtle differences, CD4^{cre}IL-4Rα$^{-/flox}$ mice had a higher number of CD3+ cells infiltrating the lung and airways (Figure 3.3.4) but iLck^{cre}IL-4Rα$^{-/flox}$ demonstrated an accelerated worm expulsion (Figure 3.3.8) (not supported by egg production data). As most of the results shown are representative of only one experiment, no conclusions can be made without repeat experiments. An interesting repeated observation (data not shown), was an increased number of γδ T cells (γδ TCR+/CD3+/β-chain-) in draining lymph nodes of the intestine in iLck^{cre}IL-4Rα$^{-/flox}$, but not Lck^{cre}IL-4Rα$^{-/flox}$ or CD4^{cre}IL-4Rα$^{-/flox}$ mice. This observation was specific to *N. brasiliensis* and not seen in *L. major* infection studies. As shown in the characterisation of IL-4Rα surface expression on T cell subpopulations, iLck^{cre}IL-4Rα$^{-/flox}$ mice were the only T cell-specific IL-4Rα-deficient mice with complete abrogation on γδ T cells. These results suggest an IL-4 regulatory mechanism for the recruitment or proliferation of these cells. Therefore, the differences demonstrated between the three T cell-specific IL-4Rα-deficient mice had no affect on worm clearance or the cytokine milieu.

In summary, a decreased cellular infiltration in the lungs and airways due to the inability of T cells to respond to IL-4 was associated with an impaired type 2 response in all three T cell-specific IL-4Rα-deficient mice. Furthermore, these mice were able to clear *N. brasiliensis* despite an impaired T_H2 response and lung pathology was delayed. A reduced T_H2 response was confirmed by a reduction in IgG1 and IgE antibodies and the differences in cellular specificity of deletion between the three T cell-specific IL-4Rα-deficient mice had no affect on worm clearance. Therefore, CD4+ T cell T_H2 polarisation was not necessary for worm expulsion and was responsible for lung pathology. By disrupting IL-4 / IL-13 responsiveness on T cells a polarised T_H2 response was impaired. However, non-T cells retain the ability to respond to IL-4 / IL-13 and may also be responsible for the low levels of T_H2 cytokines detected. The low levels T_H2 cytokines may be sufficient to confer immunity to *N. brasiliensis* infected mice with reduced lung pathology. Global IL-4Rα$^{-/-}$ mice are unable to respond to IL-4 / IL-13 and the low levels of T_H2 cytokines detected would be insufficient to confer immunity.

4.4 Future Studies

To completely characterise the deletion of IL-4Rα on all T cell subpopulations, proliferation of CD8+, γδ and NK T cells in response to IL-4 should be determined. PCR amplification on the genomic DNA of purified γδ and NK T cells of IL-4Rα would confirm FACS analysis data. Furthermore, the level of IL-4Rα expression can be quantified by determining the amount of mRNA produced by T cell subpopulations.

No apparent differences were evident between the three T cell-specific IL-4Rα-deficient strains in the outcome of *L. major* and *N. brasiliensis* infections. However it has been shown in a study by Rosat *et al.* 1993 that the expansion of gamma delta T cells in BALB/c mice is dependent upon type 2 CD4+ T cells, with the lack of IL-4 reducing the expansion by 40 %. The precise role of IL-4 on innate T cell responses in infection and allergy remains unclear and by implementing these mouse strains in models such as contact hypersensitivity, of which γδ T cells are thought to play an important role, the function of this cytokine can be further explored. As γδ T cells are predominantly found in the epithelial tissue a disease model utilising this infection / sensitisation route would be more significant. Contact hypersensitivity is an autoimmune skin disease mediated by inflammation that occurs when a sensitising chemical comes in contact with the skin surface. The chemical interacts with proteins of the body, altering them to appear foreign to the immune system. The mechanism of most autoimmune skin diseases is still elusive; however, infiltration by significant number of γδ T cells has been shown. It is important to further explore these interactions.

4.5 Conclusion

L. major infection represents a highly characterised model of T_H1 immunity with IFN-γ activating infected macrophages for intracellular killing, with complex mechanisms responsible for acquired immunity. *N. brasiliensis* infection represents a highly characterised model of T_H2 immunity critically dependent on CD4+ T cells, IL-4 / IL-13 IL-4Rα and STAT-6 and demonstrates an immune response to a complex array of diverse parasite antigens. T cell-specific IL-4 receptor-alpha deficient mice ($CD4^{cre}$IL-$4R\alpha^{-/flox}$, Lck^{cre}IL-$4R\alpha^{-/flox}$ and $iLck^{cre}$IL-$4R\alpha^{-/flox}$) were generated to study the importance of IL-4 responsive T cells in *Leishmania major* and *Nippostrongylus brasiliensis* infection models.

The cellular specificity of IL-4Rα deletion was confirmed by quantitative PCR and FACS analysis and IL-4Rα expression compared on T cell subpopulations (CD4+, CD8+, γδ and NK T cells) between the three strains. This work has defined three distinct T cell-specific IL-4Rα–deficient mouse models; (i) where IL-4Rα is deleted from all T cell subpopulations, (ii) deleted from all T cell subpopulations other than γδ T cells and (iii) only deleted from CD4+ T cell subpopulation. Although no differences were determined in the outcome of *L. major* and *N. brasiliensis* infections, these novel research tools will enable investigation into of the effects of IL-4 responsive γδ and CD8+ T cells in infection and hypersensitivity.

All T cell specific IL-4Rα-deficient mouse strains became resistant to *L. major* infection dependent on an impaired T_H2 response, while total abrogation of IL-4 / IL-13 responsiveness was associated with chronic disease progression. T_H1 polarisation was demonstrated by increased IFN-γ production by CD4+ draining LN cells and the development of a protective DTH. An impaired T_H2 response was determined by reduced (but not abrogated) levels of IL-4, IL-13, IgG1 and IgE. Abrogating IL-4Rα responsiveness on T cell subpopulations and retaining IL-4 / IL-13 mediated function on non-CD4+ T cells conferred immunity comparable with the C57/BL6 strain.

Furthermore, IL-4 mediated T_H2 cellular responses were not crucial for worm expulsion but were responsible for increased lung pathology in *N. brasiliensis* infection. Impaired T_H2 polarisation was demonstrated by reduced IL-4 and IgE, fewer cells infiltrating the lungs and a decrease in airway goblet cell hyperplasia. Other studies in our lab have shown that the deletion of IL-4Rα specifically from T cells prevents OVA-induced anaphylaxis despite the maintenance of a T_H2 response (Nieuwenhuizen *et al.* 2006, submitted). Also, unlike global IL-$4R\alpha^{-/-}$ mice, Lck^{cre}IL-

$4R\alpha^{-/flox}$ survive acute schistosomiasis; where T_H2 responses are essential for host survival (Herbet, *et al.* 2006, submitted).

T cell responses to IL-4 are responsible for susceptibility to *L. major* infection and are the cause of hypersensitivity to *N. brasiliensis*. By targeting IL-4Rα signalling specific to T cells, a better understanding of the immune mechanisms involved in diseases will assist in developing effective cytokine therapy to achieve immunity.

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6. Appendices

Appendix A: SOLUTIONS

Anaesthetic

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

Blocking Buffer

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

Make up to 1 L with 1X PBS

Carbonate Buffer

20 ml	10X PBS
8g	BSA (Merck)

Make up to 1 L in ddH_2O

Concavalin A

Dilute

5mg	Stock into
5 ml	1X PBS to get a concentration of 1 mg/ml

Decolourised Schiff's reagent

100mg	Sodium Metabisulphite
6ml	Schiff's reagent

Digestion Buffer

50mM	Tris
10mM	EDTA
100mM	NaCl
0.5%	SDS

Dilution Buffer

10g	BSA (1 %) (Roche)
0.2g	NaN_3 (0.02 %) (Merck)

Make up to 1L with 1X PBS

FACS Buffer

0.1%	BSA (Roche)
0.05%	NaN_3 (Merck)

Made up in 1X PBS

Iseove's Modified Dulbeco's Medium (IMDM)

1 tube	IMDM (Gibco)
750 ml	ddH_2O
81.7 ml	$NaHCO_3$ (37g / L)
2 ml	Penicillin/streptomycin (500X)

Adjust the pH to 7.2 – 7.4

Make up to 1 L with ddH_2O and filter sterilize

OPD (o-pheylenediamine dihydrochloride)

16 mM	o-pheylenediamine dihydrochloride
0.01 %	H_2O_2 in Tris-triton buffer

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 ddH_2O

Periodic acid

10ml	7% Acetic acid
20µl	Periodic acid

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

Salt Solution (0.65 %)

13g	NaCl

Make up to 2 L with ddH_2O and filter sterilize

Schiff's Reagent

10g	Pararosaniline Chloride (dissolved in boiling)
1ltr	dd H_2O
200ml	HCl (1M)
30g	Activated charcoal (filtered and repeat)

Schneiders medium

1 X	Scheider's media (Sigma)
0.4g	Sodium Bicarbonate
0.795g	$CaCl_2.2H_2O$

Make up to 1 L with dd H_2O

SOB Medium

2%	Tryptone
0.5%	Yeast extract
10mM	NaCl
2.5mM	KCl
10mM	$MgCl^{2+}$

SOC Medium

Sob	
2M	Glucose (1/100)
1M	Mg^{2+} (1/100)

Substrate Buffer

0.2g	NaN_3 (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

Tris-triton buffer

100 mM	Tris
0.01 %	Triton 100x pH 8

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: FACS ANTIBODIES

Antibody	Format	Dilution	Type	Company	Cat #
IL-4Rα	Biotin	1:400	Rat IgG2a	BD-pharmingen	552508
IL-4Rα	PE	1:400	Rat IgG2a	BD-pharmingen	552509
IL-4	PE	1:50	Rat IgG2b	BD-pharmingen	554389
CD4	PE	1:640	Rat IgG2b	BD-pharmingen	557308
CD4	FITC	1:160	Rat IgG2b	Home-grown	
CD3e	PE	1:80	Hamster IgG1	BD-pharmingen	553064
CD3e	FITC	1:640	Hamster IgG	Home-grown	
CD3e	Biotin	1:80	Hamster IgG1	BD-pharmingen	553060
CD8a	PE	1:80	Rat IgG2a	BD-pharmingen	553033
CD19	PE	1:400	Rat IgG2a	BD-pharmingen	553783
β-chain	Biotin	1:400	Hamster IgG2	BD-pharmingen	553169
β-chain	FITC	1:80	Mouse IgG2a	Home-grown	
γδ-chain	Biotin	1:200	Hamster IgG2	BD-pharmingen	553176
B220	PE	1:320	Rat IgG2a	BD-pharmingen	553090
DX-5	PE	1:50	Rat IgM	BD-pharmingen	553858
DX-5	Biotin	1:80	Rat IgM	BD-pharmingen	553856
F4/80	PE	1:80	Rat IgG2b	Caltag	RM2904
CD11b	PE	1:640	Rat IgG2b	BD-pharmingen	553311
CD11c	FITC	1:80	Hamster IgG1	BD-pharmingen	553801
CD11c	Biotin	1:200	Hamster IgG1	BD-pharmingen	553800
GR-1	FITC	1:80	Rat IgG2b	BD-pharmingen	553125
Thy1.2	FITC	1:160	Rat IgG2a	BD-pharmingen	553004
MHC II	Biotin	1:200	Mouse IgG3	BD-pharmingen	553609
Isotype	PE	1:200	Hamster IgG	BD-pharmingen	554711
Isotype	PE	1:80	Rat IgG2b	BD-pharmingen	556925
Isotype	FITC	1:80	Rat IgG2b	BD-pharmingen	553988
SA-APC	APC	1:400	N/A	BD-pharmingen	554067
SA-PercP	PercP	1:2	N/A	BD-pharmingen	340130
FcγRII/III	purified	1 %	Rat IgG2b	BD-pharmingen	553141
FcγRII/III	purified	1:400	Rat IgG2bK	Home-grown	
CD8 (Ly2)	purified	1:200	Rat IgG2a	Home-grown	
CD11b (MAC1)	purified	1:800	Rat IgG2b	Home-grown	
GR-1	purified	1:100	Rat IgG2b	Home-grown	
B220	purified	1:200	Rat IgG2b	Home-grown	

Appendix C: Cytokine / Antibody ELISA

	Capture	Detection	Standard	Sensitivity
IL-4	**1:500**	**1:1000**	**250ng/ml**	**2pg/ml**
Type	Rat anti-mouse	Biotinylated Rat anti-mouse	Recombinant	
Company & Clone	Pharmingen International BVD4-1D11	Pharmingen International BVD6-24G2	PeproTech EC LTD London	
IL-5	**1:500**	**1:1000**	**250ng/ml**	**38pg/ml**
Type	Rat anti-mouse	Biotinylated Rat anti-mouse	Recombinant	
Company & Clone	Pharmingen International TRFK5	Pharmingen International TRFK4	BD Biosciences	
IL-13	**1:500**	**1:500**	**100ng/ml**	**46pg/ml**
Type	Anti-mouse	Biotinylated Anti-mouse	Recombinant	
Company & Clone	R&D Systems, Germany 38213.11	R&D Systems, Germany	BD Biosciences	
INF-γ	**1:500**	**1:1000**	**100ng/ml**	**46pg/ml**
Type	Rat anti-mouse	Biotinylated Rat anti-mouse	Recombinant	
Company & Clone	Pharmingen International R4-6A2	BD Biosciences XMG1.2	BD Biosciences	

	Capture	Detection	Standard
IgE	**1:1000**	**1:1000**	
Type	Anti-mouse	Rat Anti-Mouse, AP conjugate	Recombinant
Company & Clone	Pharmingen (USA 84.1C	Southern Biotechnology Associates. Birmingham, (USA) 23G3	Pharmingen (USA)
IgG1	**1:1000**	**1:1000**	
Type	Goat Anti-mouse	Goat Anti-Mouse, AP conjugate	Recombinant
Company & Clone	Southern Biotechnology Associates. Birmingham (USA) 15H6	Southern Biotechnology Associates. Birmingham (USA) 15H6	Southern Biotechnology Associates. Birmingham (USA)
IgG2b	**1:1000**	**1:1000**	
Type	Goat Anti-mouse	Goat Anti-Mouse, AP conjugate	Recombinant
Company & Clone	Southern Biotechnology Associates. Birmingham (USA) A-1	Southern Biotechnology Associates. Birmingham (USA) A-1	Southern Biotechnology Associates. Birmingham (USA)

Appendix D: Supplement to Material and Methods

1. Splenocyte and Lymphocyte isolation

Pooled spleens or lymph nodes were teased through a 70 µm cell strainer (Falcon, NJ USA) and the single cell suspension was collected by centrifugation at 1200 rpm for 5 min at 4 °C in a 15 ml Falcon tube. The pelleted cells were resuspended in 1 ml Red Cell Lysis Buffer (see appendix A) and the centrifugation step was repeated. The cells were resuspended in 2 ml IMDM / 10 % FCS and stained with Trypan Blue (Sigma-Aldrich, Irwine, UK) to count viable cells in a haemocytometer.

2. Bicinchoninic Acid Protein Estimation (BCA)

The BCA protein assay (Pierce, Rockford, USA) was used to determine the protein concentration of all samples. The principle of this method is the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The rate of BCA colour formation (purple) is dependent on the incubation temperature, the types of protein present in the sample and the amounts of reactive amino acids in the proteins. The protein standard was prepared by diluting Bovine Serum Albumin (BSA) in the same diluent as the samples (diluted 10 fold down). The BCA working solution was added and after incubation at 37 °C for 30 min the absorbance was measured at 540 nm using a microplate spectrophotometer (Molecular devices, California, USA).

Appendix C: Histology Techniques

Preparation of sections

The tissues were dehydrated in an automated processor and embedded in wax as indicated below:

70 % alcohol	30 min
96 % alcohol (2X)	45 min
100 % alcohol (4X)	45 min
xylol (2X)	60 min
wax (55 °C to 60 °C)(2X)	45 min with vacuum

The tissues were sectioned at 2 µm with a microtome, floated onto glass slides and fixed by incubation at 37 °C overnight. The wax was removed from the sections by incubation at 60 °C for 2 – 18 hours and they were rehydrated for staining as follows:

Xylol	30 min
Xylol (2X)	1 min
100 % alcohol	1 min
96 % alcohol	1 min
70 % alcohol	1 min
water	1 min

Periodic Acid Schiffs (PAS)

(stain for carbohydrates – glycogen)
The sections were brought to water (as described above) and oxidized in 1 % aqueous periodic acid for 5 – 10 min. They were washed in running H_2O for 5 min and treated with Schiff reagent for 15 min. They were then washed for 10 min in running H_2O and counterstained with Mayers Haematoxylin for 5 min. The sections were 'blued' in Scotts H_2O, dehydrated and the cover slip was mounted.

Alcian Blue

The sections were brought to water and rinsed for 1 min in 3 % acetic acid. They were stained in alcian blue for 30 min and rinsed in running H_2O. They were then counterstained with Mayers Haematoxylin for 1 min and 'blued' in Scott's H_2O. They were placed in eosin for 20-30 sec and rinsed in H_2O. The sections were then dehydrated and the cover slip was mounted.