The function of IL-4Rα expression on key immune cells during experimental *Nippostrongylus brasiliensis* infections

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Declaration

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Helen Mearns

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Cover page: Gravid female *Nippostrongylus brasiliensis*. Photograph by J. Claire Hoving.

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Abbreviations

aaMacs	Alternatively activated macrophages
AHR	Airways Hyperresponsiveness
caMacs	Classically activated macrophages
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4Rα	Interleukin-4 Receptor alpha
IL-4Rα$^{-/-}$	Germ line deficient IL-4Rα mice
IL-4Rα$^{-/lox}$	Hemizygous control mice (one floxed and one disrupted IL-4Rα allele)
Lck^{Cre}IL-4Rα$^{-/lox}$	$CD4^+$ T-cell disrupted IL-4Rα expression
LysM^{Cre}IL-4Rα$^{-/lox}$	Macrophage/Neutrophil disrupted IL-4Rα expression
L. major	*Leishmania major*
MLN	Mesenteric
MST	Mediastinal
N. brasiliensis	*Nippostrongylus brasiliensis*
PI	Post Infection
S. mansoni	*Schistosoma mansoni*
STAT	Signal Transducer and Activators of Transcription
Th	Helper T-cells
Tregs	Regulatory T-cells

Abstract

The lifecycle of the parasitic nematode *Nippostrogylus brasiliensis* resembles that of the human hookworms *Necator americanus* and *Ancylostoma duodenale* and as such is a useful murine model for studying hookworm disease. Hookworms are important parasitic nematodes which are estimated to infect approximately 800 million people worldwide. These parasites are especially prominent in developing countries where heavy infestations can result in serious complications including cognitive disorders and malnutrition. Infection with *N. brasiliensis* induces a strong Th2 response which is associated with both host ability to expel parasites from the intestinal tract as well as a prolonged Type 2 inflammatory response in the lungs. IL-4Rα is a key receptor in the signalling pathway responsible for inducing a polarised Th2 response and promoting the alternatively activated macrophage (aaMacs) phenotype. This study utilised transgenic mice deficient in IL-4Rα expression on immune specific cell populations to determine how the spatial expression of IL-4Rα effects *N. brasiliensis* induced pathology. Though mice deficient in $CD4^+$ T-cell IL-4Rα (Lck^{cre}IL-4R$\alpha^{-/lox}$ mice) resolved *N. brasiliensis* infections as control mice (IL-4R$\alpha^{-/lox}$) *N. brasiliensis* associated lung pathology was strikingly reduced. These results demonstrate that successful clearance of *N. brasiliensis* is independent of IL-4 promoted Th2 polarisation while associated pulmonary immuno-pathology is enhanced by this IL-4 promoted Th2 response. We went on to investigate the effect aaMacs have on long term pulmonary inflammation induced by infection with *N. brasiliensis*. Here we used $LysM^{Cre}$IL-4R$\alpha^{-/lox}$ mice, where disrupted IL-4Rα expression on macrophages prevents the generation of aaMacs. Our results indicated that expression of IL-4Rα on macrophages is required for resolution of chronic pulmonary pathology associated with *N. brasiliensis* infections. Together this body of work has demonstrated clear requirements for expression of IL-4Rα on T-cells for initiating lung immuno-pathology and on macrophages for resolving it.

1. Introduction

1.1 The Immune System

An immune response is a complex reaction which occurs when the body is presented with stimuli it recognizes as non-self, initiating a chain of events appropriate to the perceived threat. The complexity of the system is its ability to respond in a variety of ways to a broad range of foreign stimuli (or antigens) in any part of the body with responses which are both general and tailored to their physiological setting. The launch of an immune response requires communication with isolated components of the system including singular cells, cell aggregates or structured organs in order to orchestrate an effective response. Communication and regulation of this system is brought about by messenger molecules which are produced by a variety of cell types. However their production by T helper cells have far reaching effects as these molecules enable T helper cells to act as regulators of the immune system (Roitt, Brostoff et al. 2001).

These messenger molecules are known as cytokines although this is a general term used to include lymphokines, chemokines, interleukins and monokines. Lymphokines are produced by lymphocytes, whilst chemokines have chemotactic properties, interleukins are molecules made by one leukocyte which act upon other leukocytes and monokines are produced by monocytes. Cytokines are small protein ligands which bind specific cell surface receptors, once bound they initiate a signalling cascade and alter gene expression in the cell. The binding of a cytokine to cell can lead to a variety of changes including expansion of cell populations, changes in cell membrane protein expression and the release of effector molecules. These changes are efficiently managed by characteristic cytokine properties including activity at low molecule concentrations, short molecule half-life, signalling redundancy and pleiotropism. Signalling redundancy is the ability of different cytokines to initiate similar functions whilst pleiotropism is the ability of one cytokine to initiate many

functions. There are strictly regulated pathways which certain groups of cytokines activate, eventually determining the physiological phenotype of the immune response. These pathways involve the activation of an assortment of both immune and non-immune cells. The magnitude and variety of cytokines produced determines whether the host's physiological response is protective, ineffective or detrimental. It is therefore important for the host to mount an effective immune response against foreign stimuli by initiating appropriate cytokine production patterns once a stimulus is detected. For this reason cytokine signalling is highly regulated with both production of cytokines and receptor expression rate dependent upon cell types and cell activation states (Roitt I 2001).

1.1.1 Th1/ Th2 dichotomy

In the 1980's *in vitro* studies involving mouse T helper cells demonstrated that two distinct cell populations could be determined after naïve cells received antigenic stimulus. Each cell population secreted a distinct array of cytokines and had specific functions (Mosmann, Cherwinski et al. 1986). This finding led to the classification of T helper subsets into either T helper 1 (Th1) or Th2. Cytokines play a key role in T helper subset proliferation and suppression with mature cells often producing cytokines which suppress the development of other subsets (Tato and O'Shea 2006). The most prominent cell subsets are the Th1 and Th2 although other subsets such as Th17 and regulatory T cells (Tregs) are also formed (Fig. 1) (Glimcher and Murphy 2000).

1.1.1.1 T helper 1

For Th1 polarisation, interleukin-12 (IL-12) signals via STAT-4 and T-bet to generate interferon-γ (IFNγ) the trademark Th1 cytokine. Other cytokines produced by Th1 cells include IL-12, IL-2, Tumour Necrosis Factor (TNF) and Lymphotoxin-α (LTα) (Manetti, Gerosa et al. 1994; Kaplan, Sun et al. 1996; Glimcher and Murphy 2000;

Szabo, Kim et al. 2000; Gadina, Hilton et al. 2001; Szabo, Sullivan et al. 2002). On a cellular level the polarisation of Th1 cells leads to classically activated macrophages and delayed type hypersensitivity reactions. Typically Th1 responses are associated with the resolution of viral, bacterial and protozoan infections (Roitt, Brostoff et al. 2001).

1.1.1.2 T helper 2

Th2 polarisation requires IL-4 binding the IL-4Rα which leads to activation of the GATA-3 and STAT-6 transcription factors. The hallmark Th2 cytokine is IL-4 however IL-13, IL-5, IL-9 and IL-10 are also produced by Th2 cells (Le Gros, Ben-Sasson et al. 1990; Croft and Swain 1995; Shimoda, van Deursen et al. 1996; Takeda, Tanaka et al. 1996; Kubo, Ransom et al. 1997; Zheng and Flavell 1997). Th2 polarisation is associated with alternative activation of macrophages, elevated serum IgE production, activation of mast-cells and tissue infiltration by both basophils and eosinophils. Th2 responses are generally associated as protective against extracellular pathogens such as helminths however they are also associated with allergic type responses such as asthma (Roitt I 2001).

1.1.1.3 Th17 and Tregs

Th17 and Tregs were identified later than Th1 and Th2 subsets. Tregs are both promoted by and differentiated into producers of TGF-β1, utilising the transcriptional regulator FoxP3 for differentiation (Marie, Letterio et al. 2005). Tregs play an important role in regulating and preventing over-activity of the immune system (Powrie and Mason 1990; Groux and Powrie 1999; Sakaguchi, Sakaguchi et al. 2001). Th17 cells are differentiated by IL-6 and TGF-β1 through the transcriptional factor STAT-3, IL-23 promotes this subset expansion, which once differentiated produce IL-17 (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). Th17 cells enhance autoimmune disease and cancer but also play a protective role against infection by extracellular bacteria and parasitic worms (Fallon, Ballantyne et al. 2006; Mangan, Harrington et al. 2006; Owyang, Zaph et al. 2006).

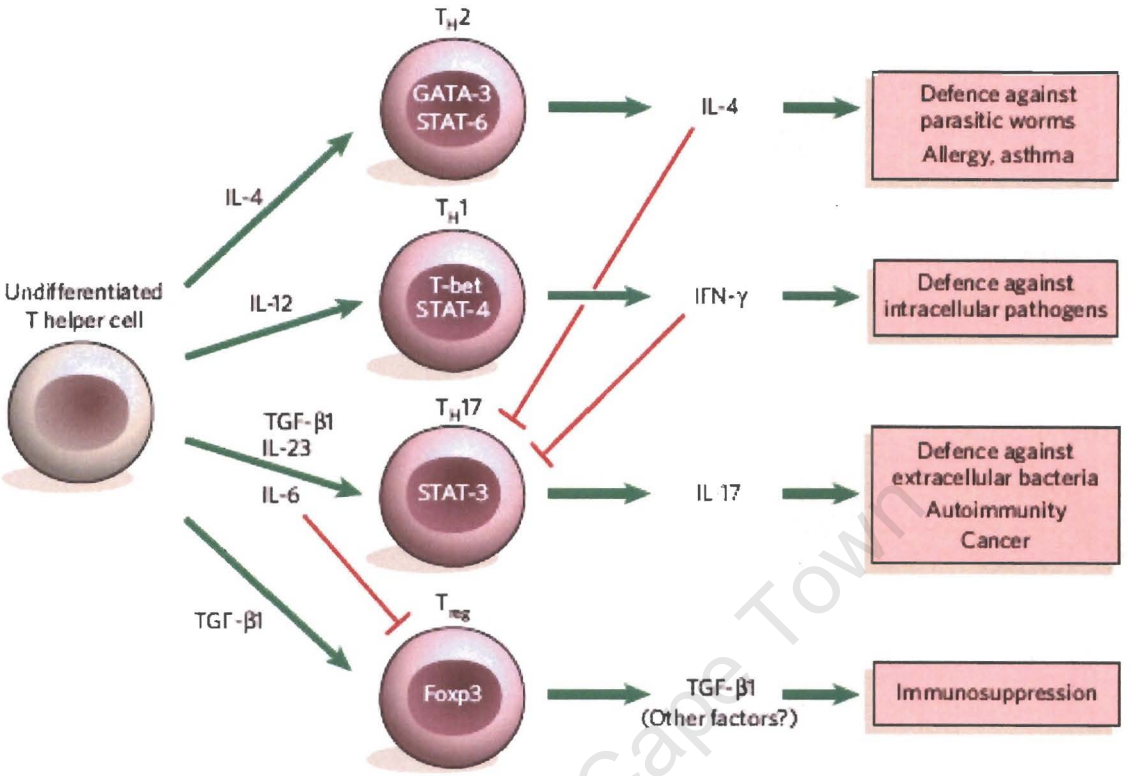

Figure 1: **Cytokines promote the differentiation of naive T helper cells into specific T helper subsets.** Th2 cells are promoted by IL-4 signalling via GATA-3 and STAT-6. Whilst for Th1 cell proliferation IL-12 is the key cytokine acting through T-bet and STAT-4. Th1 cells mediate an immune response to intracellular pathogens while Th2 cells mediate immune responses involving parasitic helminths and allergy. The cytokines produced by Th1 and Th2 cells suppress the development of Th17 cells, whilst TGF-β1 and IL-6 promote the differentiation of this subset. TGF-β1 also promotes the differentiation of Treg cells but if IL-6 is present, this lineage is suppressed. Th17 differentiated cells produce IL-17 and are associated with an inflammatory response whilst Tregs produce TGF-β1 and are involved in immunosuppression (Tato and O'Shea 2006).

1.2 Role of IL-4Rα in cellular responses

The interleukin-4 receptor alpha (IL-4Rα) is a key component of the hetero-dimeric transmembrane receptors through which the cytokines IL-4 and IL-13 signal. IL-4 signalling occurs through type 1 receptors comprised of the common γc and IL-4Rα (Hilton, Zhang et al. 1996; Leonard and Lin 2000), while IL-13 (and IL-4) signal through type 2 receptors comprising an IL-13Rα1 chain along with IL-4Rα (Obiri, Debinski et al. 1995; Miloux, Laurent et al. 1997). Upon ligand binding to the relevant receptor JAK kinase dependent signalling pathways are activated resulting in activation of STAT-6 and GATA-3 transcription factors (Miloux, Laurent et al. 1997). In addition to type 2 receptors IL-13 can also bind to IL-13Rα2, independent of dimerisation with IL-4Rα (Fig. 2) (Caput, Laurent et al. 1996; Zhang, Hilton et al. 1997; Donaldson, Whitters et al. 1998). Previously this receptor was proposed as a decoy receptor for IL-13, recently however a role for signalling via IL-13Rα2 in upregulating TGF-β1 and subsequent fibrosis has been revealed (Fichtner-Feigl, Strober et al. 2006).

Typically, IL-4Rα dependent activation of STAT-6 results in Th2 cell differentiation and IgE class switching (Shimoda, van Deursen et al. 1996). These cellular events mediate physiological changes such as airway hyperresponsiveness, airway mucus hypersecretion and airway inflammation (Akimoto, Numata et al. 1998; Kuperman, Schofield et al. 1998; Miyata, Matsuyama et al. 1999). Though both IL-4 and IL-13 can activate the same transcription factor (STAT-6) and therefore upregulate similar phenotypic responses, certain cellular and physiological changes are driven predominantly or exclusively by one cytokine rather than the other (Hershey 2003). For instance murine Th2 differentiation of naïve $CD4^+$ T cells is promoted by IL-4 not IL-13 as no functional IL-13/ IL-4Rα receptor is expressed on these cell types (Seder, Paul et al. 1992; Zurawski and de Vries 1994).

Mice with a germ line deletion of IL-4Rα have been instrumental in uncovering many *in vivo* functions of this receptor (Mohrs, Holscher et al. 2000). For example, a protective function was illustrated in infection with the gastrointestinal nematode *Nippostrongylus brasiliensis* where it was shown that IL-4Rα signalling is necessary for parasite expulsion (Barner, Mohrs et al. 1998). However IL-4Rα signalling in the protozoan *Leishmania major* infection is beneficial during acute infection but detrimental long term resulting in mice succumbing to the infection (Mohrs, Ledermann et al. 1999). To further characterise receptor signalling at a cellular level, cell specific gene deficient mice were developed using the Cre/ loxP system. Challenging smooth muscle cell specific IL-4Rα deficient mice with *N. brasiliensis* illustrated that IL-4Rα signalling through these cells was necessary for worm expulsion (Horsnell, Cutler et al. 2007). Whilst deletion of the IL-4Rα on $CD4^+$ T-cells rendered usually susceptible BALB/c mice resistant to infection with *L. major* (Radwanska, Cutler et al. 2007). These cell specific gene deficient mice allow for a better understanding of what is occurring *in vivo* at a cellular level.

Of IL-4 and IL-13 it is apparent that the latter is the key cytokine involved in the expulsion of nematodes, however in schistosomiasis IL-13 contributes to damaging pathology, it is also involved in the susceptible phenotype to acute leishmaniasis but plays a protective role in chronic leishmaniasis (Mohrs, Ledermann et al. 1999; Brombacher 2000). In a variety of models which induce pathology similar to that seen in allergic airways diseases, IL-13 has been shown to be the vital cytokine driving this pathology. This pathology includes airways inflammation, mucus hypersecretion, airways hyperactivity, eotaxin production and pulmonary fibrosis (Wills-Karp, Luyimbazi et al. 1998; Zhu, Homer et al. 1999; Mattes, Yang et al. 2001; Shim, Dabbagh et al. 2001; Kuperman, Huang et al. 2002; Singer, Lefort et al. 2002; Webb, Mahalingam et al. 2003). In this study we investigate the role of IL-4Rα on $CD4^+$ T lymphocytes and macrophages in the strong Th2 inflammatory response induced by infection with *N. brasiliensis*.

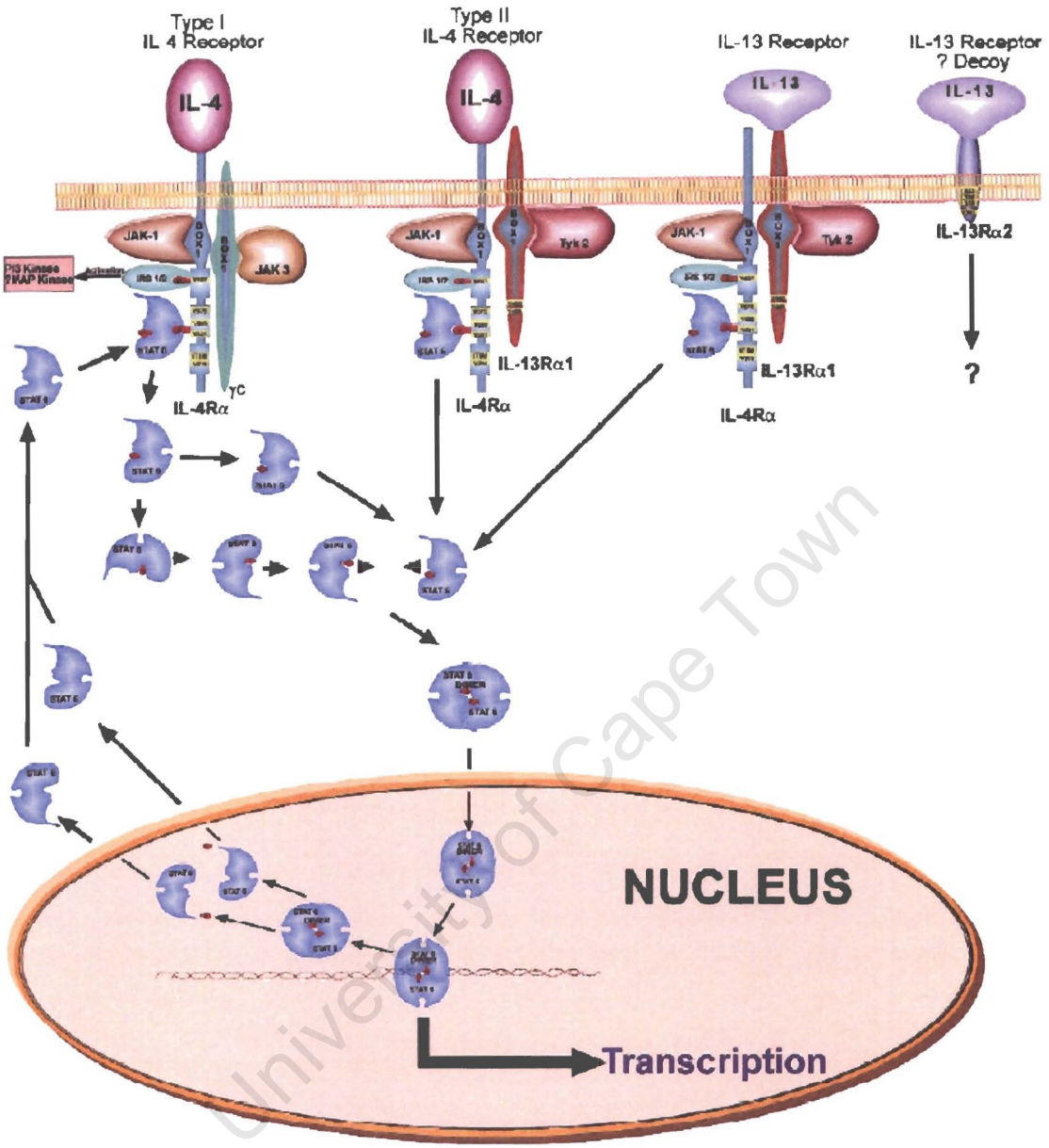

Figure 2: **IL-4 and IL-13 receptor signalling complexes.** IL-4 signals via both the type 1 and type 2 receptor complexes, whilst IL-13 only signals via the type 2 complex. Both type 1 and 2 receptors share a common IL-4Rα chain which dimerises with either the γc chain to form a type 1 receptor or the IL-13Rα1 to form a type 2 receptor. Ligand binding of these complexes results in initiation of the signal cascade involving Jak kinases and activation of STAT-6 transcription factor which upregulates transcription of Th2 associated genes. IL-13Rα2 does not dimerise with IL-4Rα, is only bound by IL-13 and does not initiate activation of STAT-6 (Hershey 2003).

1.3 Helminth Infections and immune responses

Soil transmitted helminth infections and schistosomiasis are the most prevalent parasitic infections in humans with an estimated disease burden at about 2 billion people (WHO and Organization 2006). These diseases are especially prevalent in areas lacking adequate public health systems. Heavy parasitic nematode infections are associated with serious morbidity including anaemia, malnutrition and cognitive disorders (WHO and Organization 2001). Not only do helminths cause serious morbidity they also alter the host's immune response to challenges by other pathogens, environmental antigens and vaccines (Cooper, Chico et al. 2001; Nacher 2001; Su, Segura et al. 2005; Wilson, Taylor et al. 2005). This immune alteration is opportunistically utilised by various diseases including Tuberculosis and HIV/ AIDS (Makgoba, Solomon et al. 2002; Verver, Warren et al. 2004). Relevant in the South African context as these two diseases are especially prevalent in areas where helminths are endemic. For instance in certain areas of the south-western cape up to 90% of the children are infected with worms such as *Ascaris*, *Trichuris* and *Hymenolepis nana* (Adams, Markus et al. 2005). However effects of helminth infections on the host are not always negative, regulatory cytokines produced in response to the worm offer a potential method of treating both Th1 and Th2 driven immune dysfunction and auto-immune diseases (Yazdanbakhsh, Kremsner et al. 2002). This was shown in a study conducted in patients suffering from Crohn's disease, a Th1 driven immune dysfunction. This study utilised infection with helminths and their immunomodulatory effects in an efficacious manner as a means of alternative treatment for this disease (Summers, Elliott et al. 2005). High helminth infection rates and the immune modulatory effects of these parasites illustrate the relevance of studying and understanding the immunological mechanisms which occur during and after an infection.

Infections with helminths result in the host launching an aggressive immune response to the parasite. Infections are controlled by targeting of effector cells including T

cells, eosinophils, macrophages, mast and goblet cells (Finkelman, Shea-Donohue et al. 1997; Daly, Mayrhofer et al. 1999) as well as effector molecules such as fibronectin and complement which are produced by the liver (Shin, Osada et al. 2001). It is also apparent that the worm has evolved strategies to evade aspects of this host response including continuous moulting of their outer cuticle to avoid antibody binding and recognition, rapid movement through the host and the secretion/excretion of molecules which modulate the host's immune system (Maizels and Yazdanbakhsh 2003). These evasion techniques and release of immunomodulatory molecules (Holland, Harcus et al. 2000) could explain the development of a host response which is strong and often damaging not only to the worm but also to the host itself. The host response is driven by the production of Th2 cytokines, characteristically IL-4 and IL-13, driving the polarisation of Th2 cells and type 2 immune responses. In humans the production of these cytokines is correlated with a protective immune response noted as a reduction in worm burden (Turner, Faulkner et al. 2003); data which is backed up in murine models of helminth infections where Th2 cytokines are critical for a protective response which results in effective worm expulsion (Urban, Katona et al. 1991; Urban, Noben-Trauth et al. 1998; Richard, Grencis et al. 2000; Schopf, Hoffmann et al. 2002). However this Th2 response also drives detrimental immune pathology in organs unrelated to the definitive stages of the parasitic infection (Mohrs, Harris et al. 2005), this is a key focus of the project presented here.

1.3.1 *Nippostrongylus brasiliensis*

Infection with *N. brasiliensis* induces a polarised Th2 cytokine response resulting in parasite expulsion as well as immunopathology in the lung (Jankovic, Liu et al. 2001; Gause, Urban et al. 2003; Voehringer, Shinkai et al. 2004). This murine model is analogous to human hookworms such as *Necator americanus* or *Ancylostoma duodenale*, which are important parasitic pathogens infecting around 800 million people worldwide (Bethony, Brooker et al. 2006). The typical hookworm lifecycle consists of infection through the skin of the host by newly hatched L3 larvae, which then migrate via the blood system into the lungs from where they are coughed up and

swallowed resulting in the definitive adult worms becoming established in the small intestine of the host (Bethony, Brooker et al. 2006). *N. brasiliensis* lifecycle is analogous to the human hookworm lifecycle; reaching the lung within hours of infection, establishing the definitive adult stages in the small intestine by about day 5 and by day 9 -11 in immunocompetent mice these parasites are expelled (Fig. 3) (Barner, Mohrs et al. 1998). Expulsion of *N. brasiliensis* relies on signalling through the Th2 pathway which utilises $CD4^+$ T-cells, IL-13, IL-4Rα and STAT-6 (Finkelman, Shea-Donohue et al. 1997; Urban, Noben-Trauth et al. 1998). This Th2 signalling also creates a polarised inflammatory response in the lung which is maintained long after the parasites have been expelled from the gut (Voehringer, Shinkai et al. 2004). The occurrence and persistence of this Th2 pulmonary inflammation is the focus of this study.

1.3.2 *N. brasiliensis* induced immunopathology

N. brasiliensis induced immunopathology was initially described in the lungs of infected rats as lesions not associated with parasite larvae but instead focussed around the vascular system (Salman and Brown 1980). These pulmonary lesions developed after larval lung migration and resultant mechanical damage had healed and continued to persist even after the parasites had been expelled from the intestine; suggesting this pathology is driven by an immunological mechanism rather than a direct response to the migrating parasite (Arizono, Nishida et al. 1996). This inflammatory response has been investigated in both rat and mouse models of *N. brasiliensis* infection and has been shown to be driven by Th2 associated cytokines (Matsuda, Tani et al. 2001; Voehringer, Shinkai et al. 2004). The inflammation was investigated further and shown to persist for up to 4 weeks post infection although little is known about the mechanisms which cause and sustain this immunopathology (Matsuda, Tani et al. 2001).

Although little is known about the mechanisms which cause pulmonary immunopathology part of the cause is likely to be from the parasite itself. As the

parasite passes through the lungs it moults leaving behind antigens which could stimulate an immune response. These antigens have been isolated from various larval stages of *N. brasiliensis* and used in intranasal mouse challenge models (Marsland, Camberis et al. 2005; Trujillo-Vargas, Werner-Klein et al. 2007). Challenges with the secreted/excreted products of *N. brasiliensis* result in Th2 inflammatory responses in the lung (Marsland, Camberis et al. 2005). These secretory products act as adjuvants which enhance Th2 responses in the host (Holland, Harcus et al. 2000). The host immune system has its own set of regulatory and modulatory responses which monitor and suppress overreaction to a stimulus by Th2 cells. This is known as a 'modified Th2-cell response', it was classified when certain individuals were exposed to high levels of antigen and yet did not develop allergic type disease (Platts-Mills, Vaughan et al. 2001). This modified Th2 response is easily identified by the downregulation of specific IgE responses; a factor which has been proposed to be brought about by IL-10 inhibiting B-cell switching to IgE production (Maizels and Yazdanbakhsh 2003). This modulation of the Th2 response is seen in the latter stages of many helminth infections where immune structures such as granulomas or laying down of fibrotic structures are downregulated resulting in less negative pathology to the host (Boros, Pelley et al. 1975; Chiaramonte, Mentink-Kane et al. 2003). On a cellular level Th2 downregulation occurs through the use of cells such as alternatively activated macrophages, altered T helper 2 cells and regulatory T cells. IL-10 and TGFβ have been identified as key regulatory cytokines in the downregulation of host cell-mediated immunity (Sher, Gazzinelli et al. 1992).

In the study presented here we will investigate the role of IL-4Rα expression on $CD4^+$ T-cells and their role in controlling infection with *N. brasiliensis* and the associated lung pathology. We will also investigate the role of IL-4Rα expression on macrophages and their role in controlling the later stages of *N. brasiliensis* associated pulmonary pathology.

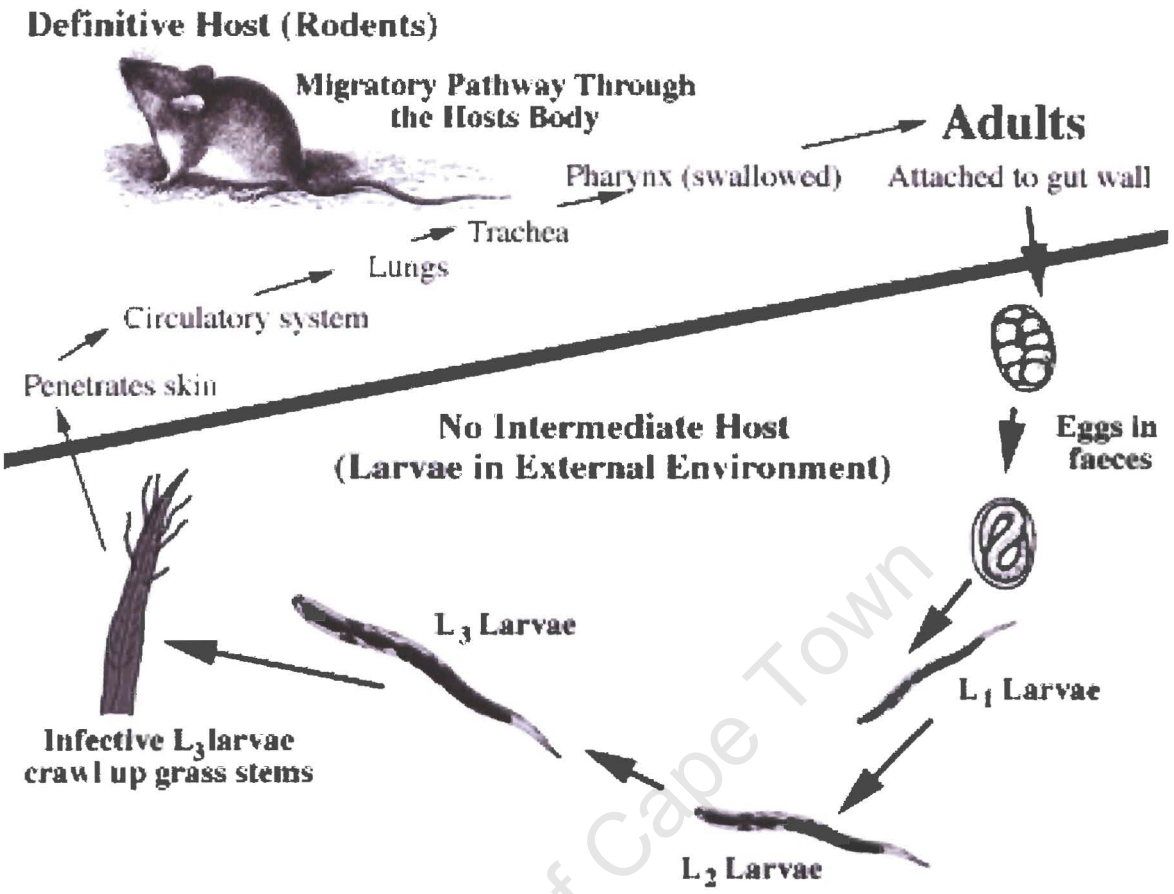

Figure 3: **Lifecycle of *Nippostrongylus brasiliensis*.** L3 infective stage larvae penetrates the skin of the host, circulates via the blood system into the lungs is coughed up and swallowed becoming sexually mature in the small intestine where eggs are released with host faeces (Cambridge 2006).

2 Methods and Materials

2.1 Mice

Male and female 8-12 wk old mice were obtained from the University of Cape Town specific-pathogen-free animal facility. All experiments were approved by the University of Cape Town Animal Ethics Committee. Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice (Radwanska, Cutler et al. 2007) and $LysM^{Cre}$IL-4R$\alpha^{-/lox}$ mice (Herbert, Holscher et al. 2004) were generated as previously described with wild type hemizygous IL-4R$\alpha^{-/lox}$ mice and homozygous IL-4R$\alpha^{-/-}$ mice used as controls (Mohrs, Ledermann et al. 1999).

2.2 Parasite maintenance

N. brasiliensis (kindly provided by Klaus Erb, Wurzburg, Germany) were maintained by routine passage through 5 week old female Wistar rats. 5000 L3 larvae in 0.9% NaCl were subcutaneously injected into the nape of the neck. The faeces were collected at day 6, 7 and 8 post infection (PI), placed in fungisone (Gibco) treated dH_2O and emulsified. The faeces were mixed into a paste with activated charcoal (Sigma, Germany) and placed in the centre of moist Whatman filter paper, allowing eggs to hatch and worms to move to edge of paper. After 7 days the L3 stage larvae were harvested from filter paper and used in infection studies.

2.3 Infection studies

Mice were injected subcutaneously with 750 *N. brasiliensis* L3 larva in 0.9% NaCl. Lck^{Cre}IL-4R$\alpha^{-/lox}$ infected mice were sacrificed at days 7 and 10 PI while $LysM^{Cre}$IL-4R$\alpha^{-/lox}$ infected mice were sacrificed at days 21 and 42 PI by CO_2 asphyxiation.

2.4 Worm burden and egg counts

The number of parasite eggs was determined from days 5 to 15 PI. Mice were separated into individual cages and faeces were collected, weighed and submerged in 0.65% NaCl. 5M NaCl was added to emulsified faeces, centrifuged at 1300rpm/5min and the top meniscus was removed, counted using a McMaster counting chamber (Advanced Equine Product, USA) and normalised relative to weight of faeces. Adult worm burdens were determined from individual mice at day 7 and 10 PI. The small intestine was removed, incised longitudinally and incubated in 0.65% NaCl at 37°C overnight. Saline solution was placed in gridded Petri dishes and total numbers of worms were counted.

2.5 Histology

The Department of Histology, Groote Schuur Hospital prepared and stained all histology sections. Tissue samples were fixed in a neutral buffered formalin solution. Following embedding in paraffin, samples were cut into 5μm sections. Sections were stained with Haematoxylin and Eosin Mayers (H & E), Periodic Acid Schiffs reagent (PAS) or anti-CD3-DAB (Dako). Quantification of intestinal goblet cell hyperplasia was determined in individual mice by counting number of positive goblet cells per 5 villi of the small intestine (Horsnell, Cutler et al. 2007). The Histological Mucus Index (HMI) was determined to indicate the relative amounts of pulmonary goblet cell hyperplasia present in individual mice. All airways were photographed at 100X, placed in Adobe Photoshop and overlaid with a standard grid. The total number of both epithelial cell and mucus positive squares divided by the total number of epithelial cell positive squares determined the HMI (Cohn, Homer et al. 1997). All samples were randomised and counted by a researcher who was blinded to the sample type. Infiltration of inflammatory cells and general pathology was analysed by a registered pathologist, samples were randomised and the pathologist was blinded to sample types.

2.6 Ex Vivo Restimulation of Lymphocytes

$CD4^+$ T-cells were enriched (>98% purity) from pooled mesenteric lymph nodes (MLN) and mediastinal lymph nodes (MST) at days 7 and 10 PI. Single cell suspensions were stained with anti-CD8 (53.6.72), CD11b (M1/70), GR-1 (RB68C5) and B220 (RA36B2). Stained cells were depleted using goat anti-rat IgG coated magnetic beads (Biomag beads, Qiagen, Germany). $CD4^+$ T-cells were restimulated for 72h with anti-CD3 (145-2C11). Supernatants were then collected and stored at -80°C until analysis.

2.7 Fluorescent Activated Cell Sorting (FACS)

Lungs were finely cut and digested in DMEM (Gibco) with 50U/ml collagenase type I (Gibco-Invitrogen) and 13µg/ml DNAse I (Roche, Germany) at 37°C for 90min. Samples were pushed through a 70µm cell strainer, subjected to red blood cell lysis and washed with FACS buffer. $1X10^6$ cells per mouse were blocked with inactivated rat serum and stained on ice with appropriate antibody combinations. T lymphocytes were stained with anti-CD3-FITC (145-2C11), activated T lymphocytes were stained with CD4-Fitc (GK 1.5), CD62-PE (BD-pharmingen) and CD44-APC (BD-pharmingen), macrophages and neutrophils were stained with GR1-Fitc (RB68C5) and CD11c-APC (BD-pharmingen). Macrophages were determined as $CD11c^+GR1^+$ and Neutrophils as $GR1^+$ (de Heer, Hammad et al. 2004). Macrophage and neutrophil populations were confirmed by cytospinning, Rapidiff staining (Clinical Sciences Diagnostics, South Africa) and microscopic identification of FACS Vantage sorted cell populations (Beckton-Dickinson, South Africa) (data not shown). Non-viable cells were excluded from analysis with 7-AAD (Sigma, http://sigmaaldrich.com), acquisition was performed using FACSCalibur and cells were analysed using Cellquest (Beckton-Dickinson, http://www.bd.com).

2.8 Enzyme-linked Immunosorbent assays (ELISA)

Total serum IgE antibody titres and concentrations of IL-4, IL-13 and IFNγ cytokines present in the supernatants were determined by sandwich ELISA. 96 well plates (Nunc-immuno maxisorb, Denmark) were coated overnight (O/N) at 4°C with capturing antibody diluted in PBS (ELISA reagents, Appendix B), after each step the plates were washed 4X with washing buffer. Blocking buffer was added and plates incubated 4°C O/N, standards at appropriate concentrations and sample (in three-fold dilutions) were added and incubated at 4°C O/N. The corresponding biotinylated secondary antibody was added and incubated at 4°C O/N. IL-13 plates were then incubated with strepavidin coupled horseradish peroxidise (HRP) while IgE, IL-4 and IFNγ were incubated with strepavidin coupled alkaline phosphatase (AP) for 1hr at 37°C. Plates were developed with TMB Microwell Peroxidase Substrate System and stopped with 1M H_3PO_3 or p-nitrophenyl phosphate (PNP) and stopped with 1M NaOH respectively. The absorbances were read on a Versamax microplate spectrophotometer (Molecular Devices, Germany), TMB developed plates at 450nm and PNP developed plates between 405 and 492nm.

2.9 Airways Hyperresponsiveness

Conscious unrestrained mice were assessed for changes in enhanced pause (Penh) using a barometric plethysmograph (EMKA Technologies, France). Individual mice were placed in plethysmograph chambers and exposed to aerosolised PBS for 1min followed by 15 min of monitored breathing to obtain a baseline reading. The mice were then challenged with 1mg/ml β-methacholine (MTC) for 1min and monitored for 15min. The Penh readings from the monitored breathing were averaged. The MTC challenge was normalised against the PBS challenge to give an individual average Penh reading per mouse.

2.10 Hydroxyproline Assay

6N HCl was added to pooled weighed lung lobes and hydrolised at 110°C for 18hrs. 8mg/ml Dowex/Norit mixture was added and samples centrifuged at 2000rpm/15min. 1% phenolphthalein in ethanol was added and neutralised with 10N NaOH till colour changed, then back titrated with 3N HCl. Isopropanol, solution A and solution B were then added to standards and samples and incubated at 60°C for 25min. Absorbances were read at 558 and 570nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany).

2.11 Statistics

Values are given as means ± SD and significant differences were determined using the student t test. * indicates $p<0.05$, ** indicates $p<0.01$, ** indicates $p<0.001$ (Graphpad Prism) Three individual experiments were performed for each data set presented unless stated otherwise.

3 Effects of disruption of IL-4Rα expression on $CD4^+$ T-cells in *N. brasiliensis* infection kinetics and associated pathology

3.1 Background

Type 2 immune effector responses are characterised by Interleukin-4 (IL-4) and IL-13 dependent signalling via hetero-dimeric receptors containing an IL-4Rα subunit (Brombacher 2000) and are particularly associated with the resolution of helminth infections (Urban, Katona et al. 1991; Kopf, Le Gros et al. 1993; Svetic, Madden et al. 1993) and the induction of allergy (Holgate 1999). IL-4Rα signalling results in activation of the transcription factor STAT-6 and upregulates GATA-3 to stabilise the Th2 phenotype in the polarised $CD4^+$ T-cell (Nelms, Keegan et al. 1999; Brombacher 2000). This response is characterised by production by B-cells of antibodies such as IgE and IgG1 (or IgG4 in humans) (Vitetta, Ohara et al. 1985; Coffman, Ohara et al. 1986), goblet cell hyperplasia (Madden, Urban et al. 1991) and secretion of Th2 cytokines IL-4, IL-13, IL-5, IL-10 and IL-9 by a number of haematopoietic cells (Mosmann and Coffman 1989).

Though $CD4^+$ T-cells and IL-4Rα are required for optimal differentiation and stability of Th2 responses, the significance of IL-4Rα expression in $CD4^+$ T-cells has yet to be defined. Indeed $CD4^+$ T-cell IL-4Rα independent IL-4 and IL-13 production have been previously described (Noben-Trauth, Shultz et al. 1997; Jankovic, Kullberg et al. 2000; Mohrs, Holscher et al. 2000; Ritz, Cundall et al. 2002; Webb, Mahalingam et al. 2003; Cunningham, Serre et al. 2004). As such $CD4^+$ T-cell IL-4Rα may be associated but not necessarily essential for the production of the Th2 cytokines IL-4 and IL-13 by $CD4^+$ T-cell polarisation to a Th2 phenotype (Mattes, Yang et al. 2001). Studies using mice deficient in $CD4^+$ T-cell IL-4Rα (Lck^{Cre}IL-4$R\alpha^{-/lox}$ mice) have demonstrated important roles for $CD4^+$ T-cell IL-4Rα expression in experimental infection models involving parasites *Leishmania major* (Radwanska, Cutler et al.

2007) and *Schistosoma mansoni* (Leeto, Herbert et al. 2006). Infection of Lck^{Cre}IL-4R$\alpha^{-/lox}$ BALB/c mice with the intra-cellular protozoan parasite *L. major* results in normally susceptible BALB/c mice resolving the infection. Similarly, Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice infected with the trematode *S. mansoni* were protected from parasite induced mortality. Together these data demonstrate that IL-4Rα expression on $CD4^+$ T-cells and the resulting IL-4 promoted Th2 responses are not critical for host survival.

In the study presented here we examined the response to infection with the intestinal parasitic nematode *N. brasiliensis* in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice. Our results show that the Lck^{cre}IL-4R$\alpha^{-/lox}$ mice resolve *N. brasiliensis* infections as wild-type mice (IL-4R$\alpha^{-/lox}$). However, Lck^{cre}IL-4R$\alpha^{-/lox}$ mice do show decreased extra-intestinal (lung) pathology associated with *N. brasiliensis*, demonstrating that IL-4 responsive T-cells are not responsible for worm expulsion but do play a role in *N. brasiliensis* driven lung pathology. These results demonstrate that successful clearance of *N. brasiliensis* is independent of IL-4 promoted Th2 polarisation while associated pulmonary immuno-pathology is enhanced by this IL-4 promoted Th2 response.

3.2 Results

3.2.1 Expulsion of adult *N. brasiliensis* from the intestine is not dependent on IL-4Rα responsive $CD4^+$ T-cells

To investigate a possible role for IL-4Rα expression on $CD4^+$ lymphocytes in resolving infections with *N. brasiliensis* IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice were infected with 750 L3 *N. brasiliensis* larva. Worm burdens and egg production were measured at days 7 and 10 PI (Fig. 4A & B). At day 7 PI all mouse groups demonstrated comparable worm burdens. As previously shown (Horsnell, Cutler et al. 2007), at day 10 PI IL-4Rα$^{-/-}$ mice had significant intestinal adult worm burdens. In contrast IL-4Rα$^{-/lox}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice cleared the infection at this time point. Additionally worm fecundity, as demonstrated by faecal egg counts, was also comparable between IL-4Rα$^{-/lox}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice with no eggs present in either group after day 10 PI in contrast to IL-4Rα$^{-/-}$ mice. Together, these results clearly show that expulsion of *N. brasiliensis* from in Lck^{Cre}IL-4Rα$^{-/lox}$ mice is equivalent to that seen in IL-4Rα$^{-/lox}$ mice.

A key host response for expulsion of adult *N. brasiliensis* from the intestine is increased IL-4Rα dependent goblet cell hyperplasia and mucus production. Intestinal mucus production, as demonstrated by PAS staining, was also comparable between IL-4Rα$^{-/lox}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice (Fig. 4C). Peak mucus production was seen at day 7 PI in IL-4Rα$^{-/lox}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice, with IL-4Rα$^{-/-}$ mice having significantly ($p<0.05$) lower intestinal mucus production. At day 10 PI all mice had equivalent levels of intestinal mucus production.

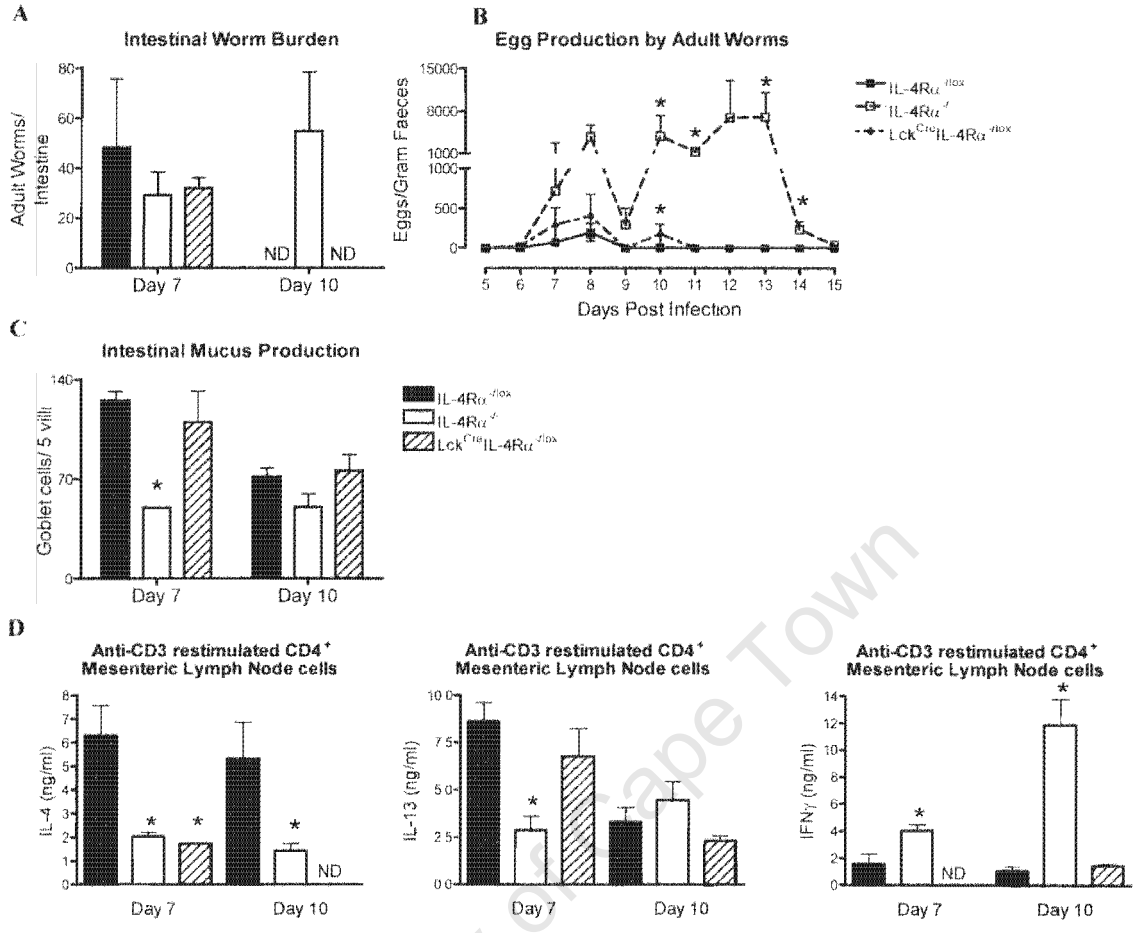

Figure 4: ***N. brasiliensis* infection and intestinal immune responses.** A) Mice were infected with 750 L3 *N. brasiliensis*, at days 7 and 10 PI worm burden of the small intestine was assessed to determine expulsion kinetics. B) Faeces from days 5 to 15 PI were collected and egg production by *N. brasiliensis* was calculated using the modified McMaster technique. C) Intestinal mucus production was assessed by counting the total number of PAS positive goblet cells per 5 villi on histological sections of the small intestine at days 7 and 10 PI. D) Supernatant cytokine levels of $CD4^+$ T cells from the mesenteric lymph nodes were anti-CD3 stimulated for 72 hours and detected via ELISA. (Significant differences from IL-4Rα$^{-/lox}$ mice *$p<0.05$, n=4, data representative of 3 individual experiments)

3.2.2 IL-4Rα independent Th2 and type 2 responses

Worm expulsion and goblet cell hyperplasia are associated with the host generating a $CD4^+$ lymphocyte-induced Th2 cytokine response. Cytokine levels from restimulated mesenteric lymph node derived $CD4^+$ cells demonstrated the expected Th2 polarisation in IL-4Rα$^{-/lox}$ mice, with higher levels of both IL-4 and IL-13, at day 7 PI when compared to IL-4Rα$^{-/-}$ mice (Fig. 4D). $CD4^+$ T-cells in IL-4Rα$^{-/-}$ mice shifted to their typical Th1 polarisation with elevated IFNγ, when compared to IL-4Rα$^{-/lox}$ mice. Lck^{Cre}IL-4Rα$^{-/lox}$ mice showed, as expected, a significant impairment of IL-4 production when compared to IL-4Rα$^{-/lox}$ mice. However, IL-13 levels were equivalent between both IL-4Rα$^{-/lox}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice. Together these data demonstrated sufficient IL-4 but not IL-13 production is dependent on $CD4^+$ T-cell IL-4Rα mediated responses as demonstrated by the Lck^{Cre}IL-4Rα$^{-/lox}$ mice.

Analysis of pulmonary cytokine production in response to *N. brasiliensis* infection in $CD4^+$ lymphocytes isolated from mediastinal lymph nodes (MST) demonstrated a delayed onset in anti-CD3 induced IL-4 cytokine production (Fig. 5A). MST Th2 secreted cytokine levels of IL-4 and IL-13 in IL-4Rα$^{-/-}$ and Lck^{Cre}IL-4Rα$^{-/lox}$ mice were significantly lower than in IL-4Rα$^{-/lox}$ mice at day 7 PI. As expected IL-4Rα$^{-/-}$ mice displayed significantly higher levels of IFNγ, than IL-4Rα$^{-/lox}$ mice, suggesting a shift towards Th1 polarisation. At day 10 PI IL-4 and IL-13 levels secreted from MST $CD4^+$ lymphocytes in Lck^{Cre}IL-4Rα$^{-/lox}$ mice were comparable to levels in IL-4Rα$^{-/lox}$ mice. Systemic IgE levels were also unaffected in Lck^{Cre}IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/lox}$ mice (Fig. 5B). Together these results suggest that $CD4^+$ IL-4Rα independent IL-4 and IL-13 production in *N. brasiliensis* infections leads to type 2 B-cell responses.

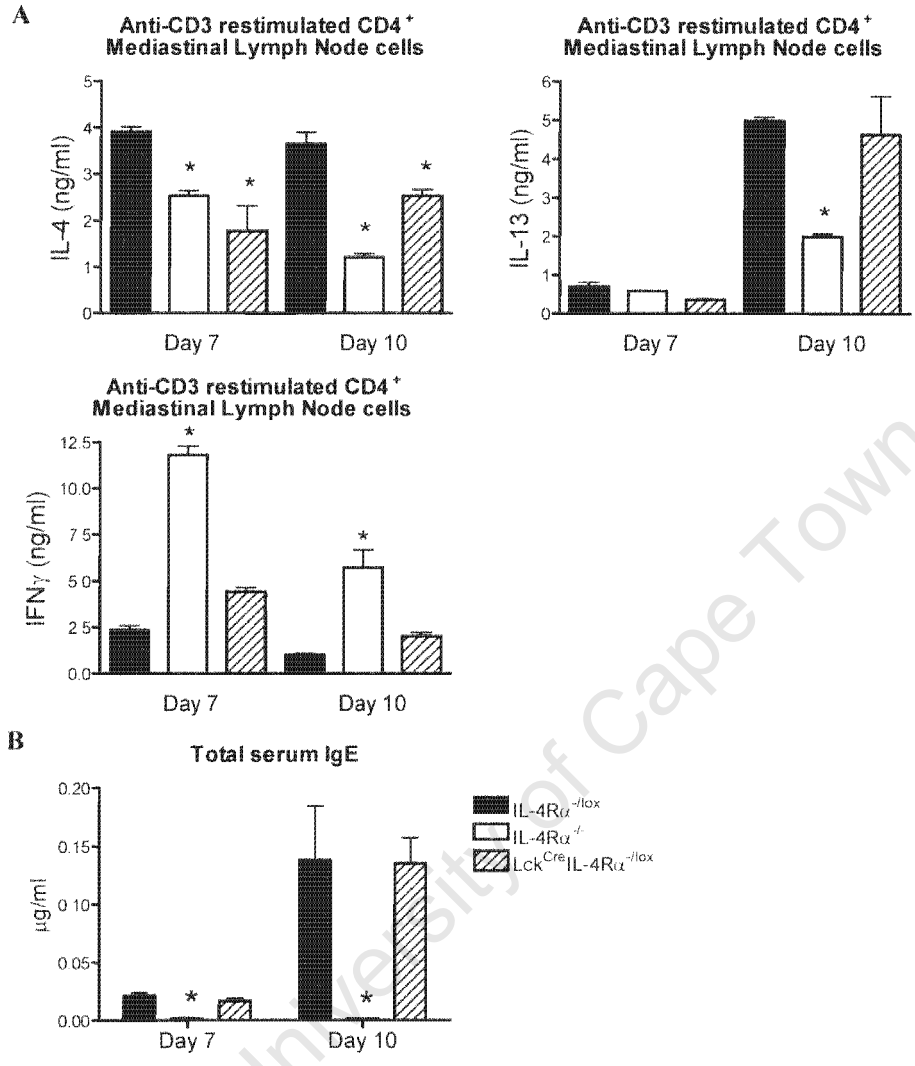

Figure 5: **Cytokine levels in the draining lung lymph node of LckCreIL-4Rα-/lox mice show a reduced Th2 response.** A) Supernatant cytokine levels of $CD4^+$ sorted mediastinal lymph nodes were anti-CD3 stimulated for 72 hours and detected via ELISA. B) Antibody production in the serum was assessed by ELISA at days 7 and 10 PI. (Significant differences from IL-4Rα$^{-/lox}$ mice *$p<0.05$, n=4, data representative of 3 individual experiments)

3.2.3 Depressed *N. brasiliensis* induced pulmonary immuno-pathology in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice

Airway mucus production was demonstrated by PAS staining (Fig. 6A). Day 7 PI results were similar between IL-4R$\alpha^{-/lox}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice. In IL-4R$\alpha^{-/lox}$ mice airway mucus production increased further at day 10 PI, this was not seen in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice which had significantly less mucus at this time point. As expected IL-4R$\alpha^{-/-}$ mice had significantly reduced airway mucus production at days 7 and 10 PI.

In order to investigate the role for IL-4Rα responsive T-cells in this abrogated pulmonary pathology we examined the distribution of $CD3^+$ T-cells in the lung. We found that T-cells in IL-4R$\alpha^{-/lox}$ mice formed localised foci associated with vascular systems and airways whilst in the IL-4R$\alpha^{-/-}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice this localisation was less apparent with $CD3^+$ T-cells being dispersed throughout the tissue (Fig. 6C). Moreover, FACS analysis of T-cell recruitment to the lung showed significantly lower numbers of $CD3^+$ T-cells in the lungs of both IL-4R$\alpha^{-/-}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice at days 7 and 10 PI when compared to IL-4R$\alpha^{-/lox}$ mice (Fig. 6B). Antigen-induced airways hyperresponsiveness (AHR) has been shown to be dependent upon both $CD4^+$ T-cells (Gavett, Chen et al. 1994) and STAT-6 signalling (Kuperman, Schofield et al. 1998). For this reason we investigated the role of IL-4Rα responsive T-cells in AHR to acetylcholine stimulation, although no differences were noted between IL-4R$\alpha^{-/lox}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice at day 7 or day 10 PI. IL-4R$\alpha^{-/-}$ mice showed a significantly reduced AHR in comparison IL-4R$\alpha^{-/lox}$ mice at day 7 PI however by day 10 PI no significant differences were observed (Fig. 6D). These results indicate that IL-4 promotion of $CD4^+$ T-cells via the IL-4Rα has no effect on AHR during acute stage *N. brasiliensis* lung inflammation.

The lower mucus production observed in the lung of *N. brasiliensis* infected Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice may be related to decreased numbers and disrupted distribution of T-

cells in the lung. Though $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mouse $CD4^+$ T-cells maintain IL-13 production levels similar to that of $IL\text{-}4R\alpha^{-/lox}$ mice $CD4^+$ T-cells, these cells are dispersed throughout the lung which would account for ineffective stimulation of goblet cell mucus hypersecretion. Together the data suggests that IL-4Rα responsive $CD4^+$ T-cells are required for adaptive immune responses associated with pulmonary immuno-pathology in experimental *N. brasiliensis* mouse infections.

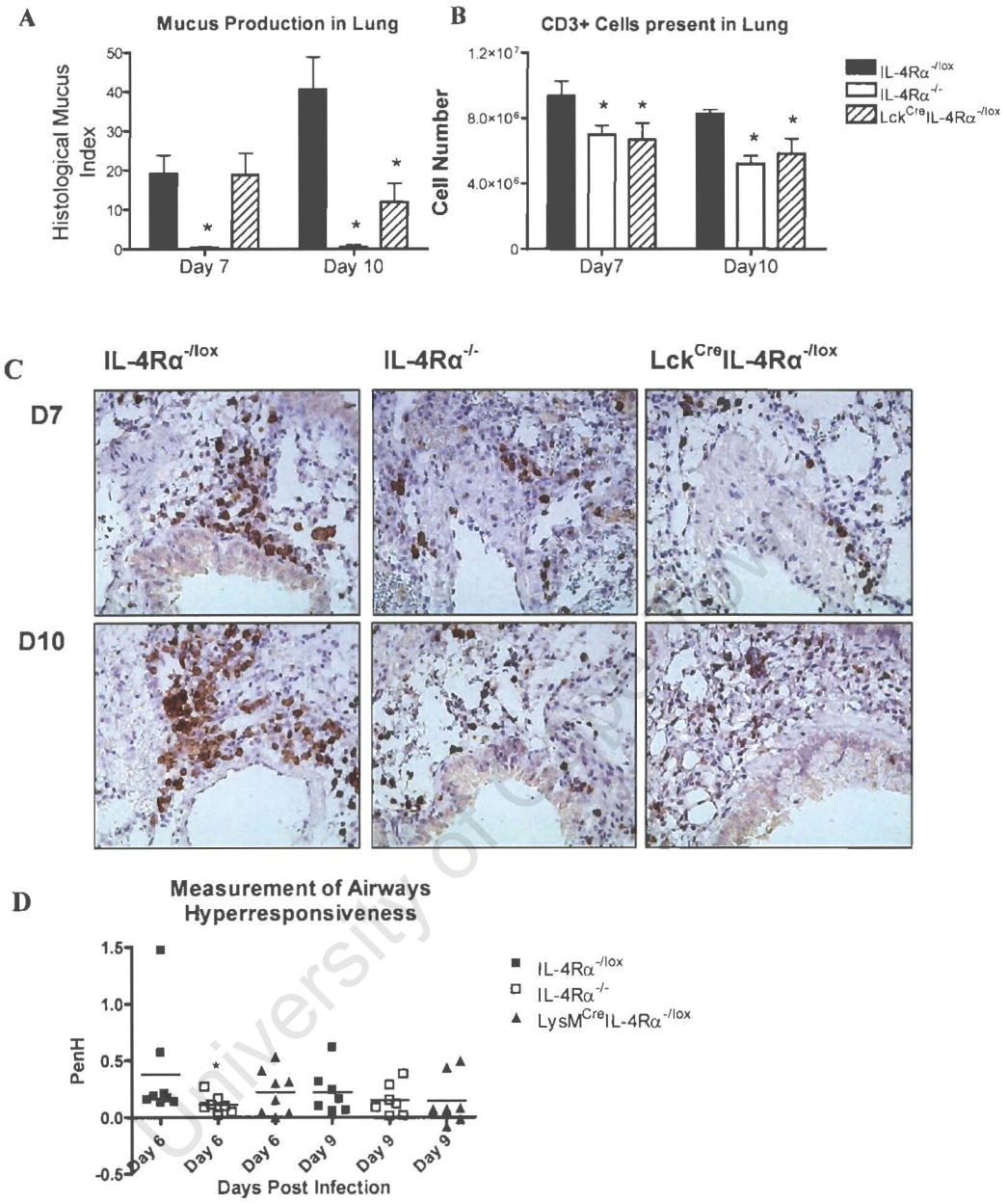

Figure 6: **Depressed *N. brasiliensis* induced pulmonary immuno-pathology in Lck^{Cre}IL-4Rα$^{-/lox}$ mice.** A) The histological mucus index was determined using PAS stained lung sections in order to compare mucus production by airway goblet cells in the various mouse groups B) Single cell suspensions of whole lung were analysed by FACS for numbers of CD3+ cells present in the various mouse types. (Significant differences from IL-4Rα$^{-/lox}$ mice *p<0.05, n=4, data representative of 3 individual experiments) C) Lung tissue removed at days 7 and 10 PI formalin fixed and stained with anti-CD3 DAB highlighting the formation of lymphocyte foci around the airways and vascular systems in IL-4Rα$^{-/lox}$ mice whilst Lck^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice lymphocytes were dispersed throughout the tissue at both time points. (Data is representative of T-cell distribution at multiple airways throughout the lung, 400X magnification, n=4, data representative of 3 individual experiments) D) The mouse groups were challenged with acetylcholine and the enhanced pause (PenH) was measured as an assessment of airways hyperresponsiveness. (Significant differences from IL-4Rα$^{-/lox}$ mice *p<0.05, n=8, data representative of 1 experiment)

3.3 Discussion

Using Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice, we clearly demonstrate that IL-4Rα responsive $CD4^+$ T-cells are not required for resolving *N. brasiliensis* infections. Furthermore, cytokine production from restimulated mesenteric lymph node (MLN) derived $CD4^+$ cells confirms expulsion to be independent of IL-4 secretion (Barner, Mohrs et al. 1998), which we also found to be significantly reduced. Somewhat surprisingly, IL-13, the key cytokine in worm clearance (McKenzie, Bancroft et al. 1998), showed equivalent levels in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice to those of IL-4R$\alpha^{-/lox}$ mice demonstrating IL-13 production by $CD4^+$ T-cells independent of signalling via IL-4Rα on these cells. IL-4Rα independent IL-13 production by $CD4^+$ T-cells has been demonstrated previously in ovalbumin induced airway hyper-activity (Webb, Mahalingam et al. 2003) and our data further extends this observation to *N. brasiliensis* infection with differential regulation of IL-4 and IL-13. The absence of a measurable effect on worm expulsion in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice is in agreement with studies which demonstrate that although $CD4^+$ cells (Horsnell, Cutler et al. 2007) and IL-4Rα (Urban, Noben-Trauth et al. 1998) are required for expulsion of *N. brasiliensis*, signalling through the STAT-6 pathway in $CD4^+$ cells is not required (Voehringer, Reese et al. 2006). Expulsion is driven by smooth muscle cell contraction (Horsnell, Cutler et al. 2007) and goblet cell hyperplasia (McKenzie, Bancroft et al. 1998). Both effector functions are primarily induced by IL-13 signalling through the IL-4Rα (Zhao, McDermott et al. 2003). Mechanistically, effective worm expulsion independent of IL-4Rα responsive $CD4^+$ T-cells was highlighted by IL-4R$\alpha^{-/lox}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice having equivalent levels of intestinal mucus and goblet cell hyperplasia. The comparable levels of IL-13 are probably sufficient to drive the similar levels of intestinal mucus production seen in the IL-4R$\alpha^{-/lox}$ and Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice.

Interestingly, in this study *N. brasiliensis* induced pulmonary immuno-pathology was strikingly affected in Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice. Here we found a significantly lower airway mucus response in the lung which was associated with disrupted T-cell

recruitment and localisation to the airway in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice. These results clearly demonstrate the $CD4^+$ T-cell IL-4Rα expression is playing a significant role in T-cell targeting and recruitment to the airways in N. brasiliensis induced pulmonary pathology. The decreased airway mucus production and T-cell recruitment to the lung in $Lck^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice is indicative of disrupted T-cell targeting being an important component for driving T-cell mediated airway mucus production. This would be in agreement with previous work which demonstrated recruitment of Th2 $CD4^+$ T-cells into the lungs of *N. brasiliensis* infected mice being dependent upon their expressing STAT-6 (Mohrs, Shinkai et al. 2001; Voehringer, Shinkai et al. 2004). Moreover ovalbumin induced Th2 $CD4^+$ T-cell recruitment to the lung requires T-cell IL-4 responsiveness (Cohn, Homer et al. 1997). However, ovalbumin induced airway mucus itself is not wholly dependent on IL-4 (Cohn, Tepper et al. 1998) but does require IL-13 (Whittaker, Niu et al. 2002) and IL-4Rα (Cohn, Homer et al. 1999). Indeed, IL-13/ IL-4Rα interaction directly with airway epithelial cells are sufficient for inducing airway mucus production (Whittaker, Niu et al. 2002). Together these studies indicate a common requirement for STAT-6/ IL-4Rα and IL-13 (and to a lesser extent IL-4) in recruitment of T-cells to the lung and induction of airway mucus production. Our data supports and expands on these findings by demonstrating that $CD4^+$ T-cell IL-4Rα is a requirement for effective T-cell recruitment to the lung and localisation to the airway. We propose that diminished T-cell recruitment and airway localisation is responsible for the decreased levels of airway mucus production reported in this study.

In conclusion, this study has demonstrated, for the first time that expression of IL-4Rα on $CD4^+$ T-cells is not required for the resolution of the definitive intestinal stage of the infection. However, IL-4Rα expressing $CD4^+$ T-cells play a significant role in driving *N. brasiliensis* induced pulmonary immuno-pathology through orchestrating T-cell recruitment to the lung and localisation to the airway.

4 Effects of disruption of IL-4Rα expression on macrophages in *N. brasiliensis* infection

4.1 Background

Infection with *N. brasiliensis* induces a chronic polarised Th2 inflammatory response in the lungs (Sher and Coffman 1992; Allen and Maizels 1996; MacDonald, Araujo et al. 2002; Voehringer, Shinkai et al. 2004). The cellular infiltrate that modulates this response is composed primarily of macrophages, which owing to the Th2 cytokine milieu present in the tissue are of the alternatively activated phenotype (Reece, Siracusa et al. 2006), as opposed to the classical phenotype. Classically activated macrophages (caMacs) are typically activated by Th1 cytokines (IFNγ) resulting in macrophages which secrete pro-inflammatory cytokines, release toxic radicals and phagocytose bacterial and protozoan pathogens (Dalton, Pitts-Meek et al. 1993; Bach, Aguet et al. 1997). Alternatively activated macrophages (aaMacs) are activated by the Th2 cytokines (IL-4 and IL-13), resulting in macrophages which release anti-inflammatory cytokines and act as immunosuppressor cells in both an antigen specific and non-specific manner (Fig. 7) (Goerdt and Orfanos 1999; Nelms, Keegan et al. 1999; Gordon 2003).

Alternatively activated macrophages can be identified by the expression of particular molecular markers. These markers may include the mannose receptor, chitinase-like lectin (Ym1), arginase 1 (Arg1) and resistin-like secreted protein (Fizz1) (Welch, Escoubet-Lozach et al. 2002; Gordon 2003; Misson, van den Brule et al. 2004; Raes, Brys et al. 2005). Although the precise roles of these markers in the cell biology of aaMacs are relatively undefined, a growing body of evidence suggests their involvement in proposed aaMac tissue repair functions. For example Ym1 and Fizz1 are both associated with extracellular matrix remodelling – indicative of a role in tissue remodelling (Chang, Hung et al. 2001; Liu, Dhanasekaran et al. 2004). Whilst

aaMacs are also associated with angiogenesis, the removal of tissue debris and fibroblast recruitment - all processes necessary for wound repair (Gordon 2003).

Proliferation of aaMacs is associated with damaged tissue, particularly during the healing phase of both acute and chronic inflammatory diseases (Goerdt, Bhardwaj et al. 1993; Szekanecz, Haines et al. 1994; Djemadji-Oudjiel, Goerdt et al. 1996), as well as in tissue which undergoes wound healing following damage such as that caused by surgical procedures (Nair, Gallagher et al. 2005). The resolution of inflammation by aaMacs is associated with induction of pro-inflammatory lymphocyte apoptosis by IL-4/IL-13/IFNγ responsive aaMacs as well as lymphocyte suppression (Bronte, Serafini et al. 2003). These findings indicate a role for aaMacs in effectively clearing inflammation from tissues; a vital process which if done ineffectively could lead to a sustained diseased state and permanent damage to the organ.

In this study we investigate the effect alternatively activated macrophages have on the long term pulmonary inflammation induced by infection with *N. brasiliensis*. Here we used $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice, which have disrupted IL-4Rα expression on macrophages and as such are unable to generate aaMacs (Herbert, Holscher et al. 2004). Previous studies in our laboratory have confirmed this phenotype in the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mouse and investigated the role of aaMacs in *N. brasiliensis* expulsion (Herbert, Holscher et al. 2004). Here no effect on expulsion was seen, illustrating that aaMacs are not required for resolving *N. brasiliensis* infection, however the role of aaMacs in the associated lung inflammatory responses was not investigated. The potential role aaMacs play in clearing inflammation poses an interesting question as to whether these cells play a role in this disease model, the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mouse grants the opportunity to look into this. Our results indicate a pivotal role for aaMacs in clearance of chronic inflammatory lesions associated with *N. brasiliensis* infection, as well as an immuno-suppressive function in dampening an over-reactive Th2 cytokine and antibody response.

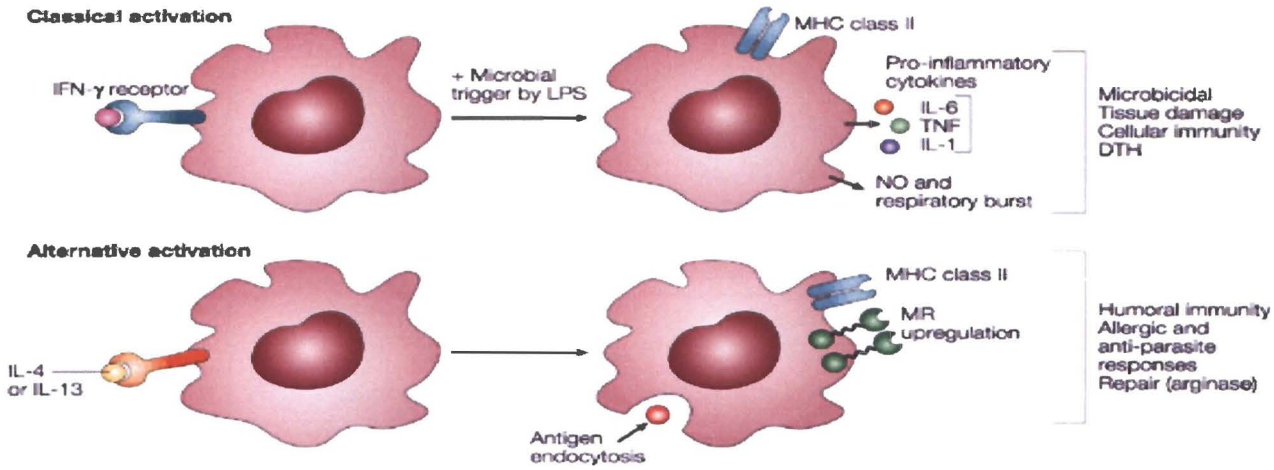

Figure 7: **Alternative and classical activation states of macrophages.** Classically activated macrophages are stimulated by IFNγ to produce pro-inflammatory cytokines and free oxygen radicals. Alternatively activated macrophages are induced by signalling through the IL-4Rα complexes by IL-4 or IL-13 which results in upregulation of mannose receptor and arginase 1 and is associated with wound repair and tissue remodelling (Gordon 2003).

4.2 Results

4.2.1 *N. brasiliensis* induced pulmonary pathology is prolonged in LysM^{Cre}IL-4Rα$^{-/lox}$ mice

N. brasiliensis larval migration through the host lung causes mechanical damage which heals rapidly however chronic immunopathology, characterised by perivascular inflammatory lesions, persist (Salman and Brown 1980; Arizono, Nishida et al. 1996). This inflammation consists of Th2 cells of which macrophages compose a major proportion (Voehringer, Shinkai et al. 2004; Reece, Siracusa et al. 2006). To investigate whether aaMacs play a role in the resolution of these inflammatory lesions, LysM^{Cre}IL-4Rα$^{-/lox}$ mice and control mice (IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$) were infected with *N. brasiliensis* and the resulting cellular infiltrates were assessed. Lung pathology was analysed histologically at days 21 and 42 PI. At both days 21 and 42 PI an emphysematous-like phenotype was clearly apparent in all the mouse groups. The emphysematous phenotype was characterised as dilation of the distal airways and alveolar sacs of the infected lungs. At day 21 PI in all the lungs of the mouse groups, inflammatory cells were focused predominantly around the vascular and bronchial systems but were also present in the alveolar walls (Fig. 8A). Of interest were differences in cellular composition of these lesions. In LysM^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice the inflammation consisted predominantly of lymphocytes, plasma cells, neutrophils and eosinophils (Figures 8Ai & iii, lower panel). However IL-4Rα$^{-/-}$ mouse lesions were composed predominantly of lymphocyte and plasma cells with fewer neutrophils and eosinophils than in the other mouse types (Figure 8Aii, lower panel).

At 42 days PI variability in the inflammatory response between LysM^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice was clearly apparent (Figure 8B). Here significantly reduced inflammation, when compared to 21 days PI, was noted in IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. This inflammation was predominantly interstitial and consisted primarily of

lymphocytes (Figure 8Bi & ii, lower panel). However, the inflammatory response in LysM^{Cre}IL-4Rα$^{-/lox}$ mice still resembled that of day 21 PI, namely continued perivascular and peribronchial lesions composed of lymphocytes, plasma cells, neutrophils and eosinophils (Figure 8Biii, lower panel). The prolonged presence of inflammatory lesions in LysM^{Cre}IL-4Rα$^{-/lox}$ mice illustrates a delayed ability to resolve pulmonary inflammation.

To quantify these cellular infiltrates, single cell suspensions of lungs were FACS analysed for macrophages, neutrophils and activated CD4^{+} T-cells populations. At day 21 PI there was no significant difference between the numbers of macrophages in LysM^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. The numbers of macrophages in IL-4Rα$^{-/-}$ mice were significantly less than that of IL-4Rα$^{-/lox}$ mice. However at day 42 PI there were no significant differences between the macrophage numbers in the various mouse groups (Fig. 9A). Neutrophils numbers peaked at day 21 in all mouse groups significantly dropping off by day 42 PI. The IL-4Rα$^{-/-}$ mice showed a trend towards a higher neutrophil infiltrates at both time points when compared to IL-4Rα$^{-/lox}$ mice although this difference was not significant (Fig. 9A). At day 21 PI activated T cells (identified as CD4^{+}CD62^{low}CD44^{high}) in the lungs of LysM^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice were similar in population size whilst the IL-4Rα$^{-/-}$ mice had significantly lower cell numbers. The numbers of activated T cells decreased significantlyfrom day 21 to day 42 PI in the IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice, which was not seen in the LysM^{Cre}IL-4Rα$^{-/lox}$ mice. The maintenance of activated T-cell numbers in the LysM^{Cre}IL-4Rα$^{-/lox}$ mice could explain the prolonged inflammatory lesions seen in these animals (Fig. 9A) (Activated T cells were investigated in one experiment only).

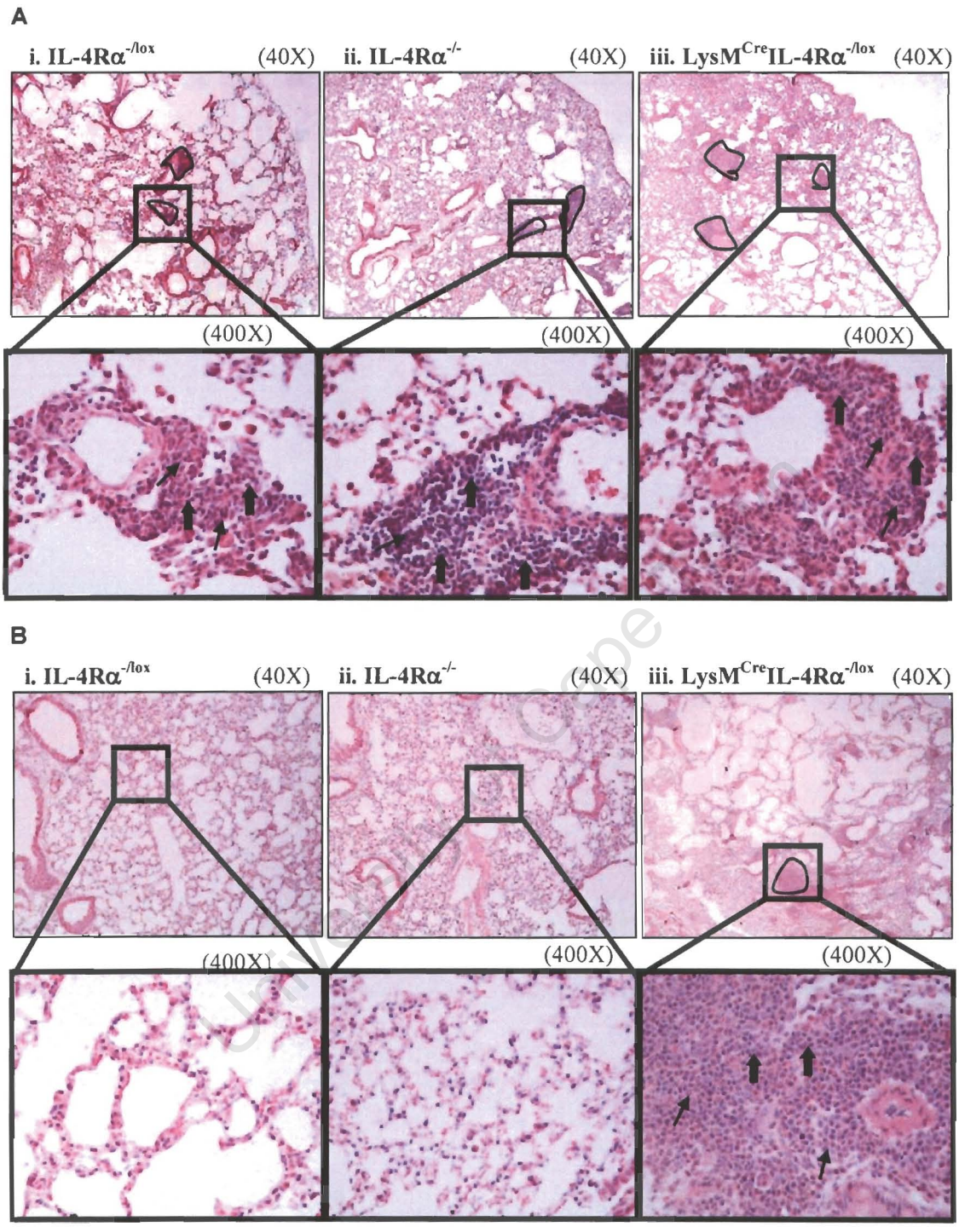

Figure 8: **Persistent inflammatory lesions in LysM^{Cre}IL-4Rα$^{-/lox}$ mice at the later time point.** Lungs were removed at 21 and 42 days PI, stained with H&E and analysed. A: Day 21 PI; LysM^{Cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice showed inflammatory response in the interstitial tissue along with perivascular and peribronchial lesions. B: Day 42 PI; LysM^{Cre}IL-4Rα$^{-/lox}$ mice showed persistent perivascular and peribronchial lesions whilst the IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice only had inflammation in the interstitial tissue (Analysed by double blinded pathologist; Data representative of 3 individual experiments n=6) Key: Upper panel - solid lines indicate outline of lesion, lower panel - block arrow indicates lymphocytes; thin arrow indicates granulocytes).

aaMacs are associated with increased arginase 1 expression, an enzyme which drives the process of fibrosis. Fibrosis is an extracellular matrix remodelling system involved in the deposition of collagen fibres in tissues. The major component of these collagen fibres is a crystalline amino acid called hydroxyproline. Fibrosis has previously been described in association with chronic lung pathologies. We found no differences in the levels of hydroxyproline at day 21 or day 42 PI between the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice or the control groups (Two individual experiments were performed) (Fig. 9B). AHR is associated with Th2 inflammation and allergic type lung diseases. AHR was measured by challenging the mice with acetylcholine and measuring enhanced pause. There were no significant differences in AHR measured between the mouse groups (One experiment performed with 6 individual mice per group) (Fig. 9C).

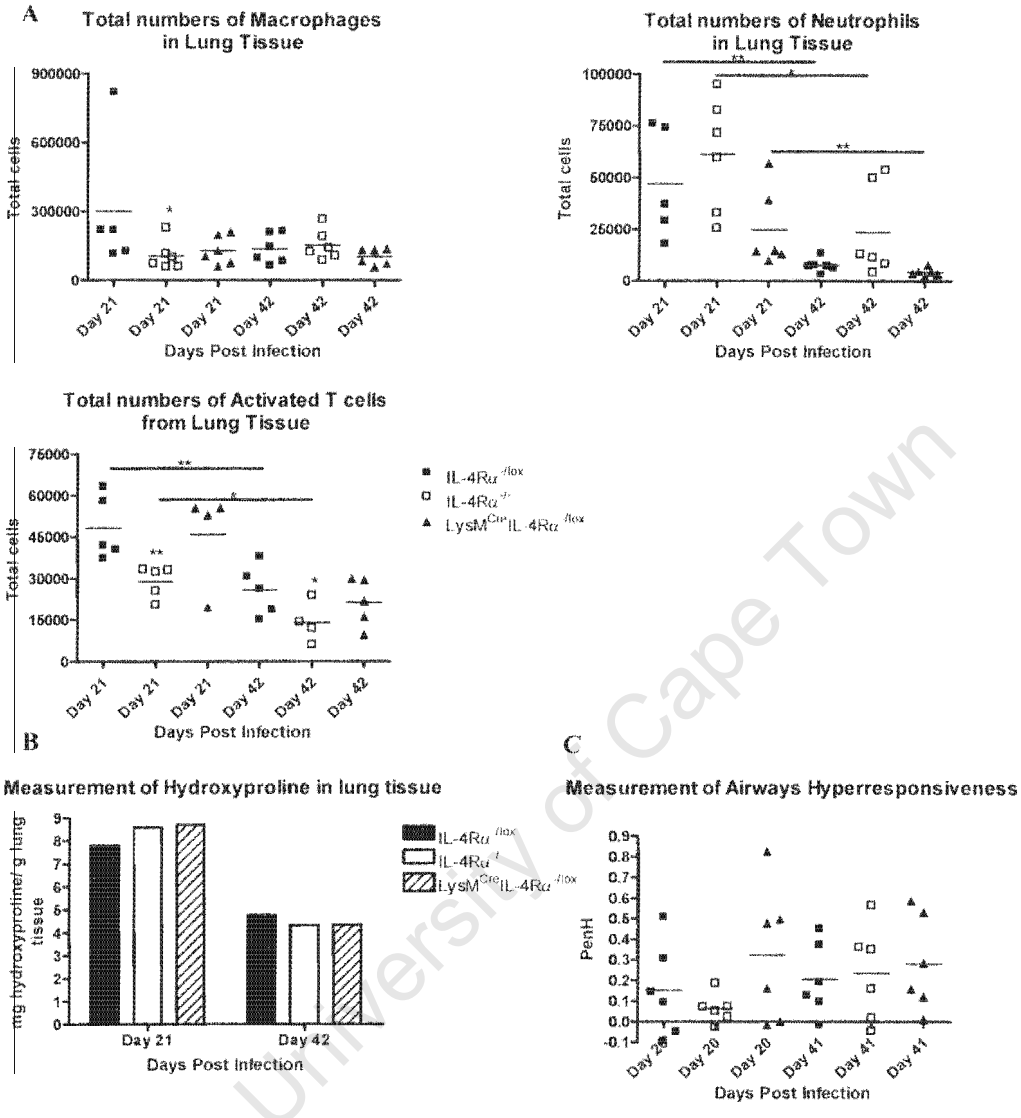

Figure 9: **Quantification of inflammatory cell infiltrates, collagen deposition and airways hyperresponsiveness.** A: Single cell suspensions of lung cells were quantified for macrophages, neutrophils and activated T cells by FACS analysis (All figures are representative of 3 individual experiments. * $p<0.05$, n=6) B: Lungs were assayed for hydroxyproline levels as a measurement of fibrosis (Data representative 2 individual experiments, n=6 pooled samples) C: The mouse groups were challenged with acetylcholine and the enhanced pause (PenH) was measured as an assessment of airways hyperresponsiveness. (Data representative of a single experiment, n=6)

4.2.2 Enhanced type 2 and CD4$^+$ MST IL-4 responses in LysM^{Cre}IL-4Rα$^{-/lox}$ mouse

IL-4, the hallmark cytokine of a Th2 response, drives class switching of B-cells to IgE production (Mandler, Finkelman et al. 1993). Increased IL-4 secretion by CD4$^+$ T-cells along with high levels of non-specific IgE is associated with *N. brasiliensis* infection (Barner, Mohrs et al. 1998; Urban, Noben-Trauth et al. 1998). In this study we compared serum IgE antibody production in LysM^{Cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. As expected IgE production in IL-4Rα$^{-/-}$ mice, which fail to generate high IL-4 levels (Barner, Mohrs et al. 1998), remained significantly lower at days 21 and 42 PI (Fig. 10A). However IgE titres in LysM^{Cre}IL-4Rα$^{-/lox}$ mice were significantly higher than IL-4Rα$^{-/-}$ levels at both time points (Fig. 10A). Such significantly higher IgE production in LysM^{Cre}IL-4Rα$^{-/lox}$ mice, suggests a role for IL-4/IL-13 promoted macrophages in reducing total production of IL-4, possibly through negative regulation of lymphocyte populations, resulting in lower levels of IgE in the IL-4Rα$^{-/lox}$ mice.

Type 2 lesions are characteristic of the pulmonary immuno-pathology associated with infection with *N. brasiliensis* (Matsuda, Tani et al. 2001). In this study we investigated Th2 cytokine production by CD4$^+$ T-cells isolated from lung draining lymph nodes (MST lymph node). As expected IL-4Rα$^{-/-}$ mice had characteristically low levels of both IL-4 and IL-13 in comparison to IL-4Rα$^{-/lox}$ mice (Fig 10B). LysM^{Cre}IL-4Rα$^{-/lox}$ mice showed similar levels of IL-13 to IL-4Rα$^{-/lox}$ mice but significantly higher IL-4 production at the later time point (Fig 10B). The differences between LysM^{Cre}IL-4Rα$^{-/lox}$ mice CD4$^+$ T-cell IL-4 production and that of IL-4Rα$^{-/lox}$ mice CD4$^+$ T-cells, illustrates a possible role of IL-4/IL-13 promoted macrophages in modulating T-cell responses.

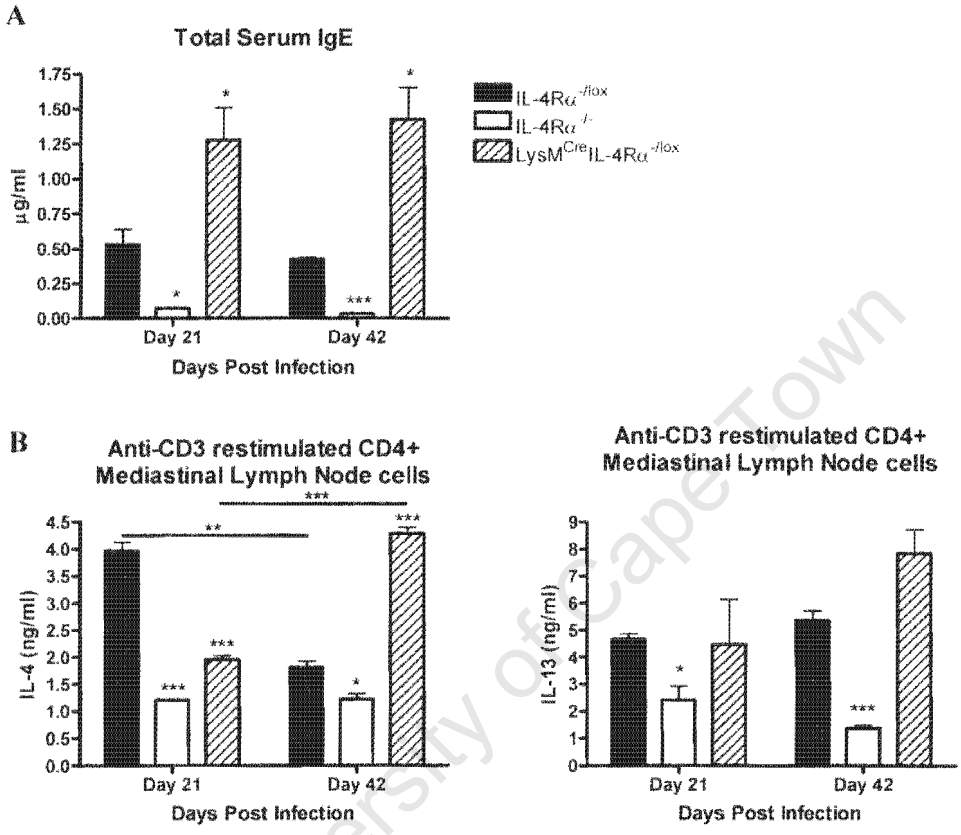

Figure 10: **$CD4^+$ MST IL-4 cytokine levels and IgE serum antibody levels were enhanced in the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mouse.** A: Total serum IgE was measured using ELISA B: $CD4^+$ T-cells from mediastinal lymph nodes were anti-CD3 stimulated and IL-4 and IL-13 supernatant cytokine levels were measured using ELISA (All figures are representative of 3 individual experiments. * $p<0.05$, $n=6$).

4.3 Discussion

An understanding of lung immuno-pathology following infection with *N. brasiliensis* has yet to be fully defined, however advances have been made in understanding immune cell function in the lung during innate and acute phases of the infection. Innate lung responses occur until 4 days PI where the upregulation of chitinase family and arginase 1 genes - hallmarks of aaMacs - are noted (Reece, Siracusa et al. 2006). Prolonged and selective expression of these genes, which is dependent upon T cell activation indicates a role for these genes in the development of associated changes in lung physiology (Nair, Gallagher et al. 2005; Reece, Siracusa et al. 2006). In the acute lung inflammatory phase, inflammation peaks at 9 days PI with recruitment of IL-4 producing inflammatory cells dependent upon signalling via STAT-6 on resident bone-marrow derived cells – possibly alveolar macrophages (Voehringer, Shinkai et al. 2004). Following the inflammatory peak of the acute stage, inflammation persists in the lung. This chronic inflammation is poorly understood, in the rat model of *N. brasiliensis* infection predominantly lymphocytic Type 2 granulomatous lesions have been shown to persist for up to 4 weeks PI (Matsuda, Tani et al. 2001). The Th2 cytokine environment and possible role of macrophages in the initiation of this chronic inflammation led us to investigate any possible role played by these cells in changes in lung physiology. Examination of lung histopathology and humoral immune responses at days 21 and 42 PI in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice illustrated an important role for aaMacs in the resolution of chronic inflammatory lesions and the suppression of hyperactive Th2 responses.

We found pulmonary lesions to be present in all mouse groups at 21 days PI, however by 42 days PI $IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice resolved inflammatory lesions yet these lesions persisted in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice. The key phenotype of $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice is their inability to generate aaMac dependent responses (Herbert, Holscher et al. 2004). In a different helminth model, the impaired ability to generate aaMacs was associated with prolonged inflammation (Herbert, Holscher et al. 2004) indicative of important roles played by aaMacs in the regulation of inflammatory responses. The

inflammatory lesions at day 21 PI were predominantly lymphocytic and similar to those previously described in rats (Matsuda, Tani et al. 2001). Although all the mouse groups had predominantly lymphocytic lesions, the total cellular compositions between the groups differed. The composition of lesion cellular infiltrates in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ mice were similar at day 21 PI, consisting primarily of lymphocytes but also a notable infiltrate of granulocytes. In $IL\text{-}4R\alpha^{-/-}$ mice the lesions were predominantly lymphocytic with markedly lower numbers of granulocytes. Furthermore all mouse groups at day 21 PI had high levels of activated T-cells in the lung, though these levels were significantly lower in $IL\text{-}4R\alpha^{-/-}$ mice when compared to $IL\text{-}4R\alpha^{-/lox}$ mice. We propose this decreased recruitment of activated T-cells to be a function of the inappropriate Th1 polarisation of $IL\text{-}4R\alpha^{-/-}$ mice, this may also explain the differences we describe in the lesions of these mice.

The similarity in lung immuno-pathology between $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/lox}$ mice did not persist to 42 days PI. Inflammatory lesions were no longer present in either $IL\text{-}4R\alpha^{-/lox}$ or $IL\text{-}4R\alpha^{-/-}$ mice but persisted in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice. The numbers of macrophages and granulocytes present in the lungs had no effect on the presence of inflammatory lesions, indicating that the resolution of inflammatory lesions was independent of effector cell numbers. It is likely that the resolution of these lesions was dependent rather upon immune activation status of these cells and the resultant effector mechanisms brought about by activated effector cells. *Brugia malayi*, another nematode infection model illustrated that there was no correlation between the numbers of effector cells and their immune activation status, illustrating that although effector cells are present in the tissue these cells are not necessarily activated (Loke, Gallagher et al. 2007). The proposal that activated effector cells play an important role in suppressing lung immuno-pathology is supported by our demonstration of maintained numbers of activated T-cells in the lungs of $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice; in $IL\text{-}4R\alpha^{-/lox}$ mice such numbers decline significantly. T lymphocytes are thought to maintain inflammatory lung responses in certain diseases such as chronic obstructive pulmonary disease (Barnes and Cosio 2004) and asthma (Corrigan and Kay 1992; Wills-Karp 1999), as well as playing a vital role in the formation of

granulomatous lesions in infection with a different helminth, *Schistosoma mansoni* (Mathew and Boros 1986; Angyalosi, Pancre et al. 1998). We propose that the disruption of signalling via the IL-4Rα on macrophages in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice and the subsequent lack of activation via this receptor results in lower numbers of aaMacs and hence an inability to decrease the number of activated T-cells. aaMacs have been shown to both alter T-cell activation and cause T-cell apoptosis (Loke, MacDonald et al. 2000; Bronte, Serafini et al. 2003).

aaMacs prevent the proliferation of T lymphocytes either through the depletion of L-Arginine which results in changes in the microenvironment and alterations on T-cell surfaces (Bronte, Serafini et al. 2003) or in a cell-to-cell contact manner (Loke, MacDonald et al. 2000). Another form of lymphocyte suppression by IL-4/IL-13/IFNγ stimulated macrophages is the production of peroxynitrite, a potent nitrating and oxidising molecule, which induces apoptosis in T lymphocytes (Bronte, Serafini et al. 2003). We propose that the lack of suppression of T lymphocyte activity and T lymphocyte proliferation by aaMacs is probably responsible for the immunopathology, cytokine and antibody responses seen in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice.

Th2 cytokines induce arginase 1 in macrophages, this enzyme converts L-Arginine into proline (a precursor of collagen) and subsequent tissue fibrosis (Hesse, Modolell et al. 2001). In our model the lack of functional aaMacs in $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice had no effect on the amount of fibrosis present in the tissue, this phenotypic response was also noted in liver fibrotic responses to *Schistosoma mansoni* infected $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice (Herbert, Holscher et al. 2004). AHR in an antigen-induced model is dependent upon signalling through STAT-6 (Kuperman, Schofield et al. 1998), however induction of AHR in $IL\text{-}4R\alpha^{-/-}$ mice has also been shown (Webb, Mahalingam et al. 2003). We investigated if any effect would occur if IL-4Rα and therefore STAT-6 signalling was abrogated in macrophages. However increases in AHR, in comparison to naïve animals (data not shown), was uniformly detected in all the mouse groups at these late time points. This is possibly due to sufficient endogenous levels of IL-13 acting directly on epithelial cells (Kuperman, Huang et al.

2002) or due to the emphysematous phenotype which is noted in the lungs of all mouse groups. Emphysema is characterised by the dilation of the distal airways, irreversible lung damage and limited airflow (Pauwels, Buist et al. 2001), changes which would likely to cause serious airflow limitations and changes in enhanced pause.

In conclusion, this study has demonstrated that the expression of IL-4Rα on macrophages is required for the long term resolution of pulmonary pathology associated with *N. brasiliensis* infections. Furthermore our data is indicative of the likely aaMac component to this response being dependent on their proposed inhibition of T-cell proliferation as opposed to their wound healing phenotype.

5 Conclusion

Helper T-cells and macrophages are important producers of cytokines in an immune response. These cytokines initiate, sustain and mediate both the molecular and cellular responses which ultimately lead to the physiological changes associated with pathogenic/antigenic stimuli. In this study we focussed on the cytokines IL-4 and IL-13, the classical Th2 cytokines. Both IL-4 and IL-13 share a common chain in their signalling receptors - the IL-4Rα chain (Nelms, Keegan et al. 1999). Through the use of IL-4Rα$^{-/-}$ mice it has been illustrated that signalling via the IL-4Rα is critical for the production of a polarised Th2 response (Barner, Mohrs et al. 1998). Th2 immune responses are generally regarded as protective responses generated by the host against parasitic helminths. The requirement for IL-4Rα dependent polarisation to Th2 in resolving parasitic helminth infections has also been well documented in a number studies using a variety of helminth models. For example *S. mansoni* infected IL-4Rα$^{-/-}$ mice succumb to infection (Herbert, Holscher et al. 2004), whilst *Trichinella spiralis* infected IL-4Rα$^{-/-}$ mice show a significant delay in parasite expulsion (Scales, Ierna et al. 2007) whilst IL-4Rα$^{-/-}$ mice infected with *N. brasiliensis* were unable to expel the parasites (Barner, Mohrs et al. 1998). In the study presented here we further define the role of IL-4Rα in a parasitic nematode infection through the use of cell-specific IL-4Rα deficient mice. Specifically we have investigated the role of signalling via IL-4Rα on CD4^{+} T-cells and macrophages in *N. brasiliensis* expulsion kinetics and associated pulmonary pathology.

Using mice deficient in CD4^{+} T-cell IL-4Rα expression we have demonstrated that IL-4 promoted Th2 cells are not critical for expulsion of *N. brasiliensis*. However, they are associated with an important role in mediating pulmonary pathology. Here the disrupted signalling in CD4^{+} T-cells and resultant dampening of the Th2 response strikingly reduced this pulmonary pathology. This indicates that although IL-4Rα signalling and CD4^{+} T-cells are critical for *N. brasiliensis* expulsion, IL-4 promotion and expansion of the Th2 cell population is not necessary for expulsion kinetics but

instead leads to a large activated T-cell population, a population which we propose to be the major contributor in promoting acute lung immuno-pathology. This is well demonstrated in our work; illustrated by reduced T-cell recruitment to the lung and localisation to the airways which results in a reduction in pathological airway mucus production. This is in agreement with previous work demonstrating the importance of Th2 cells for the generation of airway mucus responses (Cohn, Homer et al. 1997; Mathew, MacLean et al. 2001). Hence in this acute inflammatory response, T-cells which are not activated in an IL-4Rα dependent manner are not recruited to the airways and therefore less immuno-pathology is noted.

In the chronic inflammatory response demonstrated by our studies using the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mice the persistence of activated T-cells was correlated with perpetuated inflammatory lesions. This persistence of activated T-cells appeared to be due to the lack of IL-4/IL-13 promoted macrophages/ aaMacs. We propose that these aaMacs act in an immunosuppressive manner on these cells. aaMacs have been documented to regulate T-cells through inducing their apoptosis and by altering their activation status (Loke, MacDonald et al. 2000; Bronte, Serafini et al. 2003). These macrophages have also been shown to reduce inflammation in a chronic inflammatory renal disease model (Wang, Wang et al. 2007). Such effects result in lower numbers of activated T-cells as we demonstrate in the $IL\text{-}4R\alpha^{-/lox}$ mouse in comparison to the $LysM^{Cre}IL\text{-}4R\alpha^{-/lox}$ mouse, which lacks aaMacs. Furthermore this data appears to further demonstrate a role for aaMacs in T-cell regulation as opposed to wound healing.

Taken together these two studies clearly demonstrate activated $CD4^+$ T-cells being vital in the initiation and maintenance of the pulmonary pathology which is associated with infection with *N. brasiliensis*. Furthermore, these data are in agreement with previous studies using ovalbumin induced asthma where the recruitment of T-cells to the airway has been demonstrated to play a major role in the resulting asthmatic pathology (Webb, Mahalingam et al. 2003). This study demonstrates the requirement

for the expression of IL-4Rα on T-cells for initiating the pathology and on macrophages for resolving it.

6 Future Work

Although the Th2 response generated by signalling through the IL-4Rα on $CD4^+$ T-cells appears to be an over-reaction, this proliferation and differentiation of Th2 cells may be necessary to generate an effective memory response against the parasites. This could be investigated by challenging Lck^{Cre}IL-4R$\alpha^{-/lox}$ mice with a secondary infection of *N. brasiliensis* and comparing the efficiency of these mice in stopping the secondary infection in comparison to IL-4R$\alpha^{-/lox}$ mice.

The macrophage phenotype in the $LysM^{Cre}$IL-4R$\alpha^{-/lox}$ mice studies would be interesting to further investigate. This could be done by cell-sorting the population, performing RNA extraction and analysing gene expression, to investigate whether these macrophages have cell-surface receptors for IL-4/IL-13/IFNγ. Furthermore immunohistochemical analysis could be carried out to determine the spatial orientation of these macrophages. Macrophages and lymphocytes could be cell-sorted and co-cultured in order to determine whether the macrophages from the different mouse groups show different abilities to suppress lymphocytes. This would confirm our hypothesis that aaMacs are the cell type responsible for decreasing the T-lymphocyte population in the lung.

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8 Appendices

Appendix A: Solutions

Blocking Buffer
20g Milk powder (spar instant)
Make up to 1L with 1XPBS

Chloramine T
7g chloramine T
Make up to 100ml with dH_2O

Citrate-Acetate buffer
57g NaAcetate
37.5g trisodium citrate
5.5g citric acid
385ml isopropanol
Make up to 1L with dH_2O

Dilution Buffer
10g BSA (Roche)
0.2 g NaN_3 (Merck)
Make up to 1L with 1XPBS

Dowex/Norit mixture
40g Dowex (AG1-X8-200-400 mesh)
20g Norit (Decolourising carbon)
Wash 2-3X in Buchner funnel with 6N HCl
Wash 2X in 95% Ethanol
Dry under hood

Erlich's reagent
25g p-dimethylaminobenzaldehyde (Sigma)
37.5ml 60% perchloric acid

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

Hydroxyproline Solution A
Mix
1 part chloramine T
4 parts citrate acetate buffer

Hydroxyproline Solution B
Mix
3 parts Erlich's reagent
23 parts isopropanol

PBS (10X)
80g NaCl
2g KCl
14.4g K_2PO_4
2.4g KH_2PO_4
Make up to 1L with dH_2O

Red Cell Lysis Buffer
5mM EDTA
150mM NaCl
10% glycerol
25mM Tris-Cl pH 5.7
0.1% SDS
1% Triton-X 100
0.5% Non idet P-40
0.5% Deoxycholate
5mM PMSF

Washing Buffer
20g KCl
20g KH_2PO_4
144g $Na_2HPO_4.H_2O$
800g NaCl
50ml Tween 2 (Sigma)
100ml 10% NaN_3
Make up to 5L with dH_2O

Appendix B: ELISA Reagents

	Capture	Detection	Standard
IL-4	Rat anti-mouse (Pharmingen International) BVD4-1D11	Rat anti-mouse (Pharmingen International) BVD6-24G2	Recombinant (Pepro Tech EC LTD, London)
IL-13	Anti-mouse (R&D Systems, Germany) 38213.11	Rat anti-mouse (R&D Systems, Germany)	Recombinant (BD Biosciences)
IFNγ	Rat anti-mouse (Pharmingen International) R4-6A2	Rat anti-mouse (BD Biosciences) XMG1.2	Recombinant (BD Biosciences)
IgE	Anti-mouse (Pharmingen, USA) 84.1C	Rat anti-mouse (Southern Biotechnology Associates, USA) 23G3	Recombinant (Pharmigen)

Appendix C: Published paper

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Delayed Goblet Cell Hyperplasia, Acetylcholine Receptor Expression, and Worm Expulsion in SMC-Specific IL-4Rα–Deficient Mice

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Interleukin 4 receptor α (IL-4Rα) is essential for effective clearance of gastrointestinal nematode infections. Smooth muscle cells are considered to play a role in the type 2 immune response–driven expulsion of gastrointestinal nematodes. Previous studies have shown in vitro that signal transducer and activator of transcription 6 signaling in response to parasitic nematode infection significantly increases smooth muscle cell contractility. Inhibition of the IL-4Rα pathway inhibits this response. How this response manifests itself in vivo is unknown. In this study, smooth muscle cell IL-4Rα–deficient mice (SM-MHC^{Cre}IL-4Rα$^{-/lox}$) were generated and characterized to uncover any role for IL-4/IL-13 in this non–immune cell type in response to *Nippostrongylus brasiliensis* infection. IL-4Rα was absent from α-actin–positive smooth musde cells, while other cell types showed normal IL-4Rα expression, thus demonstrating efficient cell-type–specific deletion of the IL-4Rα gene. *N. brasiliensis*–infected SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice showed delayed ability to resolve infection with significantly prolonged fecal egg recovery and delayed worm expulsion. The delayed expulsion was related to a delayed intestinal goblet cell hyperplasia, reduced T helper 2 cytokine production in the mesenteric lymph node, and reduced M3 muscarinic receptor expression during infection. Together, these results demonstrate that in vivo IL-4Rα–responsive smooth muscle cells are beneficial for *N. brasiliensis* expulsion by coordinating T helper 2 cytokine responses, goblet hyperplasia, and acetylcholine responsiveness, which drive smooth muscle cell contractions.

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Introduction

The interleukin (IL)-13/IL-4 receptor α (IL-4Rα)/signal transducer and activator of transcription 6 (STAT-6) signaling pathway is essential in the control of a number of infectious diseases as well as being a key factor in the induction of allergic responses. Signaling through this pathway can either confer protective immunity or mediate tissue damage depending on the antigenic stimuli and the cell-specific response [1]. Previously, our laboratory provided the first description of the effect of a cell-specific deletion of IL-4Rα from macrophages and neutrophils on the host's ability to respond to two parasitic infections [2]. It was demonstrated that such a deletion failed to affect resolution of infection by the nematode *Nippostrongylus brasiliensis*, while mice demonstrated an increased susceptibility to infection by the trematode *Schistosoma mansoni*. The work presented here describes the effect of a smooth muscle–specific disruption of IL-4Rα expression on the immune response to *N. brasiliensis*.

Murine infection with *N. brasiliensis* induces a strong protective host T helper 2 (T_H2) response for which IL-13 production and signaling through IL-4Rα are essential for successful clearance of infection [3,4]. Infective third-stage *N. brasiliensis* larva penetrate the skin and migrate via the blood system, to the lungs. Larva emerge from blood vessels and enter the airways, from which they are coughed up and swallowed. Upon reaching the intestine, larva develop into egg-producing adult worms that attach to the small intestine epithelium. BALB/c mice clear *N. brasiliensis* infection after approximately 9 d [5].

Although essential for expulsion of *N. brasiliensis* from the intestine, the precise role of IL-4Rα in coordinating the immune and physiological response remains unclear [6]. IL-13/IL-4Rα/STAT-6 signaling is required for the host to produce an effective goblet cell hyperplasia [7]. Disruption of this response impairs the host ability to resolve an *N. brasiliensis* infection. Additionally, acetylcholine-driven contractions of longitudinal smooth muscle in the intestine are

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Abbreviations: FACS, fluorescence-activated cell sorting analysis; GMF, geometric mean fluorescence; IL-4R, interleukin 4 receptor; MLN, mesenteric lymph node; PI, postinfection; SM-MHC, smooth muscle myosin heavy chain; STAT-6, signal transducer and activator of transcription 6; T helper 2, T_H2

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Author Summary

Intestinal parasitic worm infections are a major public health concern, with more than 1 billion people infected worldwide. Symptoms associated with these infections are similar to that of other intestinal illnesses, including irritable bowel syndrome. It is likely that the immune response required to expel the worm can also, when activated inappropriately, cause the symptoms of irritable bowel syndrome. This makes understanding parasitic worm infections important in their own right and also as a model for other intestinal illnesses. In previous studies, we demonstrated the crucial importance of interleukin 4 receptor α (IL-4Rα) responsiveness for worm expulsion in global IL-4Rα–deficient mice. In this study, we specifically addressed the role of IL-4Rα responsiveness in a novel smooth muscle cell–specific IL-4Rα–deficient mouse model. These mice showed decreased ability to control the worm infection, with delayed expulsion and reduced protective immune responses. These data provide compelling evidence for smooth muscle cell IL-4Rα being an important coordinator of both the immune and physiological responses to intestinal worm infections. A proposed model is suggested with IL-4Rα responsiveness on smooth muscle cells coordinating T helper 2 cytokine responses, goblet hyperplasia, and acetylcholine-driven smooth muscle contractions for optimal worm expulsion.

also implicated in playing a role in worm expulsion [8]. A number of in vitro studies have shown that intestinal segments and intestinal smooth muscle cells previously exposed to infection by murine nematode models have increased contractile ability. This contractile ability of intestinal segments and/or smooth muscle cells is abrogated in STAT-6$^{-/-}$ mice. Therefore, the IL-13/IL-4Rα/STAT-6 pathway is necessary for elevated smooth muscle cell contractility required to aid worm expulsion [6,9,10]. Additionally, IL-13/IL-4Rα/STAT-6–dependent smooth muscle cell signaling can induce responses in surrounding tissues [11], as well as inducing smooth muscle cell release of chemokines, such as thymus- and activation-regulated chemokine [12], in order to coordinate early host responses to pathogens. From these studies, it is apparent that both goblet cell and smooth muscle cell responses to nematode infections are coordinated by the host immune response to infection and that this coordination is essential for optimal disease resolution [13].

To date, no studies have been able to demonstrate in vivo the effect of a cell-specific inhibition of the IL-13/IL-4Rα/STAT-6 pathway in smooth muscle cells. Using smooth muscle myosin heavy chain (SM-MHC)^{Cre}IL-4Rα^{-flox} mice, we demonstrate that disrupted IL-4Rα expression in smooth muscle cells influences host immunity to an intestinal nematode infection. The absence of smooth muscle IL-4Rα delays worm expulsion and goblet cell hyperplasia. Furthermore, induction of T_H2 cytokines is delayed and/or reduced, as is intestinal expression of the M3 acetylcholine receptor, in response to infection with *N. brasiliensis*.

Results

Transgenic mice, expressing Cre recombinase under the control of the smooth muscle cell–specific myosin heavy chain promoter (SM-MHC^{Cre}), were backcrossed to the BALB/c genetic background for nine generations and then intercrossed with IL-4Rα$^{-/-}$ and "floxed" IL-4Rα$^{lox/lox}$ BALB/c mice to establish smooth muscle cell–specific IL-4Rα–deficient BALB/c mice (SM-MHC^{Cre}IL-4Rα^{-flox}), with one deleted and one floxed IL-4Rα allele (SM-MHC^{Cre}IL-4Rα^{-flox}) to increase the efficiency of Cre-mediated site-specific recombination. Mutant mouse strains were identified by PCR genotyping (Figure 1A), and cell specificity of disrupted IL-4Rα expression was confirmed by fluorescence-activated cell sorting analysis (FACS).

IL-4Rα expression was analyzed on α-actin–positive cells derived from aortic cells (Figure 1B). Surface expression of IL-4Rα on α-actin–positive cells was equivalent in SM-MHC^{Cre}IL-4Rα^{-flox} (geometric mean fluorescence [GMF]: 11.02) and global IL-4Rα$^{-/-}$ (GMF: 11.2) mice (Figure 1B). Low levels of expression were present in IL-4Rα^{-flox} mice (GMF: 18.37). IL-4Rα expression on α-actin–positive smooth muscle cells isolated from small intestine and lung was too low to detect using FACS analysis (unpublished data). However, Cre mRNA was highly expressed in tracheal and intestinal tissue in the SM-MHC^{Cre}IL-4Rα^{-flox} mice. As expected, IL-4Rα^{-flox} mice demonstrated no Cre expression. In agreement with the smooth muscle specificity of the deletion, IL-4Rα mRNA expression was substantially depressed in both tracheal and intestinal tissue in SM-MHC^{Cre}IL-4Rα^{-flox} mice compared to IL-4Rα^{-flox} mice (Figure 1C). Importantly, IL-4Rα expression was maintained on CD3$^+$ T cells, CD19$^+$ B cells (Figure 1D), and macrophages (unpublished data) in smooth muscle cell–specific IL-4Rα knockout mice and equivalent to levels expressed on transgenic Cre-negative IL-4Rα^{-flox} control littermates. Functional analysis confirmed IL-4Rα responsiveness in these cell types (unpublished data). Together, these results provide convincing support for the specificity of smooth muscle cell disruption of IL-4Rα in SM-MHC^{Cre}IL-4Rα^{-flox} mice, in agreement with previously published data on the characterization of SM-MHC^{Cre} transgenic mice [14].

To investigate a possible role of IL-4/IL-13–stimulated smooth muscle cells in nematode infections, comparative infection studies with the gastrointestinal nematode *N. brasiliensis* were performed. Worm fecundity in the host was followed by determination of egg production in a time kinetic (Figure 2A). As previously demonstrated [2], control IL-4Rα^{-flox} mice behaved as BALB/c mice with peak fecal egg production found at day 7 and subsequently declining thereafter due to a functional host protective immune response [1,3]. Both the IL-4Rα$^{-/-}$ and SM-MHC^{Cre}IL-4Rα^{-flox} mice demonstrated prolonged egg production, with SM-MHC^{Cre}IL-4Rα^{-flox} mice having eggs present in their feces until day 12 postinfection (PI). As expected, IL-4Rα$^{-/-}$ mice demonstrated a chronic infection with eggs present in feces at day 14 PI. Determining the number of worms in the intestine at various time points following infection with *N. brasiliensis* resulted in comparable worm burdens between IL-4Rα^{-flox}, IL-4Rα$^{-/-}$, and SM-MHC^{Cre}IL-4Rα^{-flox} mice at days 4 and 7 PI. However, at day 10 PI, IL-4Rα^{-flox} control mice, but not SM-MHC^{Cre}IL-4Rα^{-flox} or IL-4Rα$^{-/-}$ mice, had cleared the worm (Figure 2B), explaining the extended worm fecundity. SM-MHC^{Cre}IL-4Rα^{-flox} mice, but not IL-4Rα$^{-/-}$ mice, showed complete worm expulsion at day 14 PI (Figure 2A). Examination of total serum IgE antibody (Figure 2C) levels showed that SM-MHC^{Cre}IL-4Rα^{-flox} mice responded like the IL-4Rα^{-flox} mice. Together, these results demonstrate increased susceptibility to *N. brasiliensis* in smooth muscle cell-

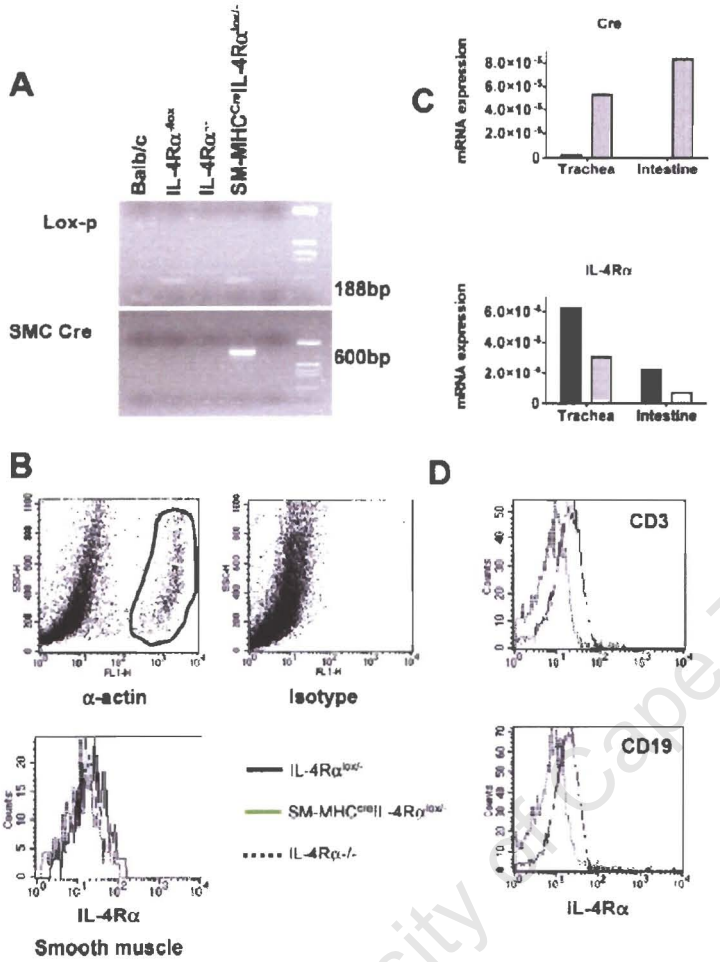

Figure 1. IL-4Rα Expression Is Impaired on Smooth Muscle Cells in SM-MHC^{Cre}IL-4Rα ^{lox} Mice

(A) Genomic integrity of the SM-MHC^{Cre}IL-4Rα ^{lox} hemizygous mice was established by PCR.

(B) Smooth muscle cells were identified by intracellular α-actin (i) staining versus isotype control (ii). IL-4Rα surface expression was analyzed on gated α-actin–positive cells (iii).

(C) cDNA levels in trachea and small intestine of IL-4Rα ^{lox} (black bars) and SM-MHC^{Cre}IL-4Rα ^{lox} (hatched bars). Data are derived from pooled tissue samples from three mice and are representative of two experiments.

(D) IL-4Rα expression on lymphocyte subpopulations of T cells and B cells is unaffected in SM-MHC^{Cre}IL-4Rα ^{lox} mice.

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specific IL-4Rα–deficient mice with increased parasite burden and delayed worm expulsion.

T_H2 cytokines drive protective mechanisms following *N. brasiliensis* infection [4]. Therefore, cytokine production by anti-CD3–stimulated CD4$^+$ T cells purified from mesenteric lymph nodes (MLNs) was analyzed at days 4, 7, and 10 PI. A reduction ($p < 0.05$) of T_H2 cytokine responses was observed from CD4$^+$ T cells of SM-MHC^{Cre}IL-4Rα^{-flox} mice at all time points compared to IL-4Rα^{-flox} control mice, including IL-4, IL-5, IL-9, and IL-13 (Figure 3). Impairment was comparable to mesenteric CD4$^+$ T cell from IL-4Rα$^{-/-}$ mice at day 7 PI. Whereas global IL-4Rα$^{-/-}$ mice shifted to a polarized T_H1 cytokine response, indicated by the production of interferon γ, this was not observed in infected SM-MHC^{Cre}IL-4Rα^{-flox} mice, which had similar interferon γ levels as IL-4Rα^{-flox} control mice. In order to ascertain any compensatory cytokine production in the intestine, we examined IL-13 levels from small intestine tissue at days 4, 7, and 10 PI (Figure 4). At days 4 and 7 PI, IL-13 levels were significantly elevated in IL-4Rα^{-flox} mice compared to IL-4Rα$^{-/-}$ and SM-MHC^{Cre}IL-4Rα^{-flox} mice ($p < 0.05$). By day 10 PI, intestinal IL-13 levels were reduced in IL-4Rα^{-flox} mice but still

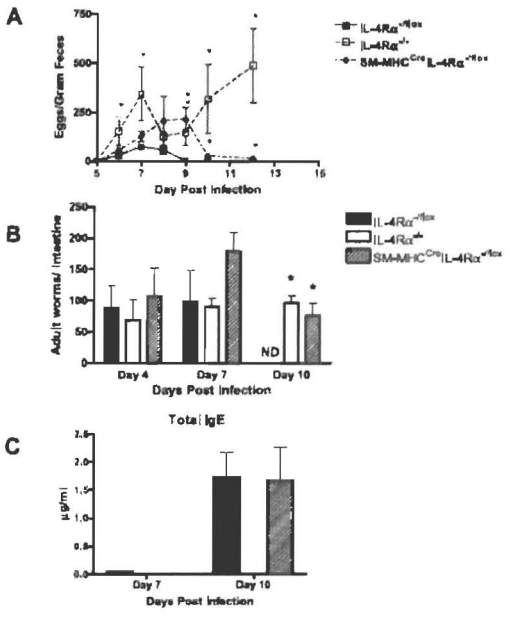

Figure 2. SM-MHC^{Cre}IL-4Rα ^{flox} Mice Have a Delayed Adult Worm Expulsion from the Intestine

(A) *N. brasiliensis* egg production in infected mice was assessed daily from day 5 PI using the modified McMaster technique.
(B) Worm burden was established on days 4, 7, and 10 PIn by counting worms in intestines removed from infected mice.
(C) Serum IgE antibody responses in IL-4Rα ^{flox} and SM-MHC^{Cre}IL-4Rα ^{flox} mice are equivalent. Serum from infected mice was taken on days 7 and 10 PI and analyzed for antibody production by ELISA as described in Materials and Methods. *Significant differences from IL-4Rα ^{flox} mice ($p < 0.05$); data are representative of four separate experiments.
doi:10.1371/journal.ppat.0030001.g002

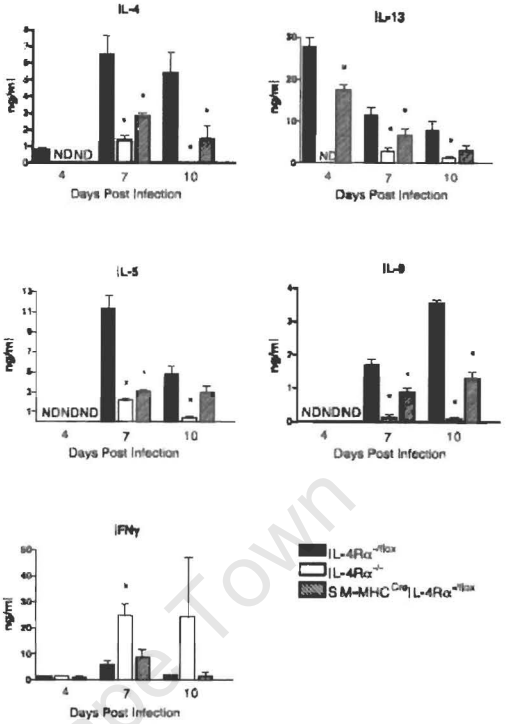

Figure 3. CD4$^+$ Lymphocytes Are Essential for *N. brasiliensis* Clearance

MLNs were removed from infected mice on days 4, 7, and 10 PI. CD4$^+$ cells were isolated and stimulated with CD3 for 72 h. Supernatants were then analyzed for cytokine production by ELISA as described in Materials and Methods. *Significant difference ($p < 0.05$) from IL-4Rα ^{flox} mice. Data are representative of four repeated experiments.
doi:10.1371/journal.ppat.0030001.g003

significantly higher than those in IL-4Rα$^{-/-}$ mice ($p < 0.05$). SM-MHC^{Cre}IL-4Rα^{-flox} mice, however, also showed significantly higher levels of IL-13 than did IL-4Rα$^{-/-}$ mice at day 10 PI ($p < 0.05$) in accordance with the delayed worm expulsion.

Reduced T_H2 responses in the MLNs had no influence on systemic type 2 antibody responses, as there were similar total serum IgG_1 (unpublished data) and IgE (Figure 2) concentrations in infected SM-MHC^{Cre}IL-4Rα^{-flox} and IL-4Rα^{-flox} mice. Effective clearance of *N. brasiliensis* is associated with a CD4-driven T_H2 cytokine response with IL-13 playing an essential role [1]. In order to confirm a requirement for CD4$^+$ T cells in conferring protection, we carried out a CD4$^+$ antibody-driven depletion of these cells. Depletion was confirmed using FACS analysis (unpublished data). As expected [15], IL-4Rα^{-flox}–treated mice were unable to clear infection, and CD4$^+$ T cells were also essential for clearance in SM-MHC^{Cre}IL-4Rα^{-flox} mice, as depletion resulted in increased adult worm burdens in SM-MHC^{Cre}IL-4Rα^{-flox} mice (Figure 5).

T_H2 cytokine–driven expulsion of *N. brasiliensis* infections is associated with a concomitant increase in IL-4Rα–dependent intestinal goblet cell hyperplasia and mucus production, a process impaired in IL-4Rα$^{-/-}$ mice [3]. Interestingly, impairment of goblet cell hyperplasia was observed in SM-MHC^{Cre}IL-4Rα^{-flox} mice. At day 7 PI, where SM-MHC^{Cre}IL-4Rα^{-flox} mice showed comparable worm burdens and egg production as Cre-negative IL-4Rα^{-flox} control mice (Figure 2), qualitative analysis of intestine histology sections, stained with periodic–acid Schiff reagent to visualize goblet cell mucus production, indicated abrogated mucus production in global IL-4Rα$^{-/-}$ mice and a transient reduction of goblet cell hyperplasia in SM-MHC^{Cre}IL-4Rα^{-flox} mice, compared to IL-4Rα^{-flox} control mice (Figure 5). The mucus production was delayed in SM-MHC^{Cre}IL-4Rα^{-flox} mice as by day 10 PI in goblet cell hyperplasia was comparable to levels observed in IL-4Rα^{-flox} control mice at day 7 PI (Figure 6).

In addition to goblet cell hyperplasia, another proposed mechanism of expulsion of *N. brasiliensis* from the host is an increased contractile ability of smooth muscle cells [6]. Induction of such contractility is primarily mediated through an acetylcholine-driven cholinergic response mediated by the M3 muscarinic receptor [6,16,17]. We examined mRNA expression levels of the M3 receptor in the intestine of mice at days 4, 7, and 10 PI (Figure 7). At day 4 PI, no significant difference was noted between groups, although a trend for

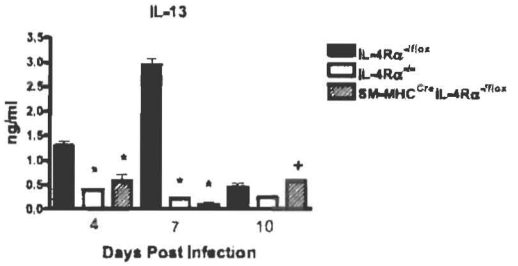

Figure 4. Intestinal IL-13 Levels Are Disrupted in SM-MHC^{Cre}IL-4Rα ^{flox} Mice

Intestinal supernatants were analyzed for IL-13 cytokine production by ELISA as described in Materials and Methods. *Significant difference ($p < 0.05$) from IL-4Rα ^{flox} mice; +significant difference from day 10 PI IL-4Rα mice. Data are representative of three repeated experiments.
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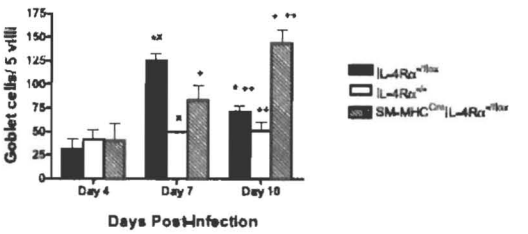

Figure 6. Intestinal Goblet Cell Hyperplasia Is Delayed in SM-MHC^{Cre}IL-4Rα ^{flox} Mice following Infection with *N. brasiliensis*

Mucus-producing goblet cells were visualized using periodic–acid Schiff reagent staining at days 4, 7, and 10 PI. The number of hyperplasic goblet cells per five villi was calculated. Values indicate mean ± SD, with *, x, +, and ++ indicating significant differences between groups ($p < 0.05$). *Significant decrease in hyperplasic goblet cells in IL-4Rα ^{flox} mice at day 10 PI compared to IL-4Rα ^{flox} mice at day 7 PI. x, Significantly less hyperplasic goblet cells in IL-4Rα $^{-/-}$ mice than in IL-4Rα ^{flox} mice at day 7 PI. +, SM-MHC^{Cre}IL-4Rα ^{flox} mice had significantly more hyperplasic goblet cells at day 10 PI than did SM-MHC^{Cre}IL-4Rα ^{flox} mice at day 7 PI. ++, SM-MHC^{Cre}IL-4Rα ^{flox} mice had significantly more hyperplasic goblet cells than did IL-4Rα ^{flox} mice at day 10 PI. Data are representative of four separate experiments.
doi:10.1371/journal.ppat.0030001.g006

higher expression in IL-4Rα^{-flox} mice was noted. We found that at peak infection (day 7 PI), IL-4Rα^{-flox} mice had significantly higher ($p < 0.05$) expression levels of M3 than both IL-4Rα$^{-/-}$ and SM-MHC^{Cre}IL-4Rα^{-flox} mice. By day 10 PI, IL-4Rα$^{-/-}$ mice still showed a significantly lower level of M3 mRNA expression compared to IL-4Rα^{-flox} mice. However, SM-MHC^{Cre}IL-4Rα^{-flox} mice showed increased M3 expression compared to that on day 7 PI. This important result is the first report of IL-4Rα expression having an effect on the expression of acetylcholine receptors in vivo.

Together, these results show smooth muscle IL-4Rα plays an important role in the regulation of both draining lymph and intestinal cytokine production, goblet cell hyperplasia, and acetylcholine responsiveness. Disruption of these responses in the SM-MHC^{Cre}IL-4Rα^{-flox} mice results in delayed expulsion of the parasites.

Discussion

This work provides the first description of the generation, characterization, and functional analysis of a smooth muscle cell–specific IL-4Rα–deficient mouse model. Disruption of IL-4Rα expression in smooth muscle cells was applied to a disease model where smooth muscle cells are proposed to play an important role in the resolution of infection, namely, a gastrointestinal nematode infection [6].

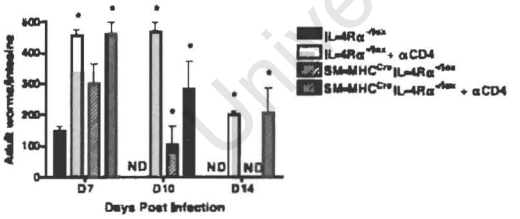

Figure 5. T_H2 Cytokine Responses Are Impaired in SM-MHC^{Cre}IL-4Rα ^{flox} Mice

Mice were injected IP with depleting anti-CD4 antibody 3 d before infection and subsequently every 4 d. Intestinal worm counts were examined at days 7, 10, and 14 PI in IL-4Rα ^{flox} mice and SM-MHC^{Cre}IL-4Rα ^{flox} mice. *Significant differences compared to IL-4Rα ^{flox} mice ($p < 0.05$); data are representative of a single experiment.
doi:10.1371/journal.ppat.0030001.g005

Clearance of nematode pathogens from the intestine is considered to require a number of physiological and immunological responses by the host. Increased intestinal contractions [6], increased mucus production [18], and elevated levels of T_H2-associated antibodies and cytokines [3] are all mechanisms induced by nematode infection. Wild-type mice infected with *N. brasiliensis* cleared the infection at day 9 PI, while SM-MHC^{Cre}IL-4Rα^{-flox} mice had an impaired ability to clear the nematode until day 12 PI. We demonstrated this impairment to be associated with a delay in goblet cell hyperplasia and the subsequent influx of mucus into the lumen of the host intestine. These physiological disruptions were related to an inability of the host to amplify appropriate cytokine production both locally and by CD4$^+$ T cells from the draining MLNs.

A number of authors have demonstrated nematode-induced amplification of intestinal smooth muscle contractions to be dependent on IL-13/IL-4Rα/STAT-6 signaling. Isolated strips of smooth muscle from the small intestine of *N. brasiliensis*–infected STAT6$^{-/-}$ mice have a significantly decreased tensile potential in vitro [6]. Depressed contractile ability was also observed in other nematode models in the absence of STAT-6 [6,9]. The significance of these nematode-induced contractions in the resolution of infection remains unclear. Recent work has demonstrated that the serotonin receptor 5-HT_{2a} is a potent inducer of IL-13– and *N. brasiliensis*–dependent intestinal contractions. However, specific inhibition of 5-HT_{2a} failed to affect the ability of the host to resolve infection [19]. We demonstrate a striking reduction in the expression of the acetylcholine M3 receptor in SM-MHC^{Cre}IL-4Rα^{-flox} and IL-4Rα$^{-/-}$ mice following *N. brasiliensis* infection. The M3 expression data we present here

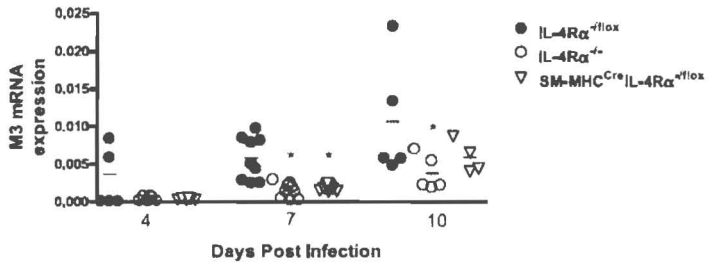

Figure 7. Intestinal Expression of M3 Receptor Is Inhibited by Disrupted Smooth Muscle Cell IL-4Rα Expression

mRNA was extracted from intestines of *N. brasiliensis* infected mice at days 4, 7, and 10 PI. Synthesized cDNA was probed with primers to M3. Increases are normalized against α-actin. Data are representative of two to five experiments per time point. $n \geq 4$ mice per group. $^{*}p < 0.05$.

doi:10.1371/journal.ppat.0030001.g007

are similar to those of 5-HT$_{2a}$ in response to *N. brasiliensis* infection. However, the potential role of M3 in mediating expulsion of intestinal parasites is more compelling. M3$^{-/-}$ mice are incapable of eliciting smooth muscle contractions [16]; this is not the case in 5-HT$_{2a}^{-/-}$ mice [20]. Previous studies have demonstrated IL-13– and STAT-6–dependent increases in acetylcholine-induced smooth muscle contractions in tissue from *N. brasiliensis*–infected mice [6]. M3 is the principal acetylcholine receptor in smooth muscle and drives 75% of the contractile response in the small intestine [16]. As such, our demonstration of significant inhibition of M3 expression in IL-4Rα–deficient mice is compelling in vivo evidence of IL-4Rα–muscarinic receptor interactions contributing to proposed muscle hypercontractility-aided nematode expulsion [8].

In addition to contractile responses, host epithelial responses constitute a second major physiological response to the parasite. This response varies according to parasite; in the case of the intraepithelial nematode *Trichuris muris*, expulsion is driven by epithelial cell turnover [21]. The principal aspect of this response to the luminal dwelling *N. brasiliensis* is induction of goblet cell–driven mucus production. Goblet cell–derived mucus is essential for clearance of *N. brasiliensis* infection [22,23]. Secreted mucus directly affects viability of the worms through inhibition of parasite motility [24,25] and ability to feed [26]. Pathogen-induced mucus production is strongly influenced by the host immune response. A deficiency in T$_H$2 polarization severely impairs the ability of goblet cells to secrete mucus and expel *N. brasiliensis* [18]. Mucus production is also modulated by the enteric nervous system via innervation of mucosal mast cells [27] and goblet cells [28]. Innervation of epithelial mucus-producing cells is also important for the host mucosal response to *N. brasiliensis* infection [29,30]. Previous studies have established the importance of this epithelial response, the most significant cells for effective expulsion being the mucus-producing goblet cells. This body of work combined with the data we present suggests that smooth muscle cells may represent an intermediate zone of signal transduction between the epithelium and MLNs. Disruption of the ability of the smooth muscle cells to respond to IL-4Rα ultimately results in a delayed mucosal response and depressed MLN cytokine production.

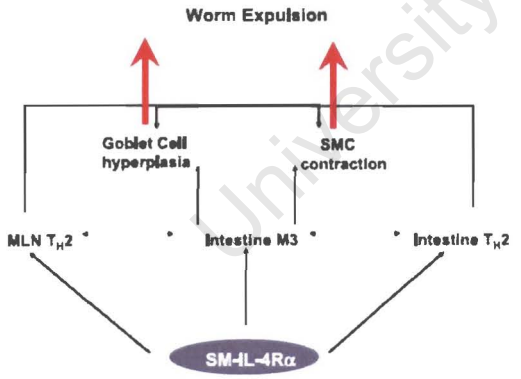

Figure 8. Role of Smooth Muscle IL-4Rα in *N. brasiliensis* Infection

Solid arrows represent demonstrated effects of smooth muscle IL-4Rα on the host response to *N. brasiliensis* infection. Dotted arrows indicate other potential and/or likely interactions.

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Prolonged *N. brasiliensis* infection, due to a deficiency in smooth muscle cell expression of IL-4Rα, may therefore be a result of the host's inability to mount an effective mucus response. Delayed mucus responses to *N. brasiliensis* infection are associated with an impaired T$_H$2 response [18]. The delayed expulsion we report here is then explained by the reduced MLN CD4^{+} T$_H$2 response (Figure 3), delaying mucus production (Figure 6) through inhibition of smooth muscle responsiveness to neurotransmitters (Figure 7) and cytokines. The depressed T$_H$2 response we suggest to be a result of smooth muscle cells being unable to react effectively to the key smooth muscle contraction amplifying cytokine IL-13 and the neurotransmitter acetylcholine [6] sufficiently to stimulate rapid cytokine production in the MLNs. Parasite clearance would then be more reliant on local effector lymphoid tissue responses [31]. The resulting recovery in response to infection and its eventual clearance in the SM-MHC^{Cre}IL-4Rα^{-flox} mouse may then be explained by local responses in the intestine providing a sufficient, albeit delayed and reduced compensatory response which induces the eventual disease-resolving response (Figure 4).

In conclusion, we have demonstrated in vivo a significant role for smooth muscle cell IL-4Rα in the optimal resolution of a gastrointestinal nematode infection. Deletion of smooth

muscle IL-4Rα significantly disrupts the host ability to resolve infection with *N. brasiliensis*. We demonstrate severe disruption of both known and proposed mediators of expulsion. Depressed M3 receptor expression, delayed goblet cell hyperplasia, disruption of $CD4^+$ MLNs, and intestinal cytokine production provide compelling evidence for an important role in the induction of both physiological effector mechanisms and immunological mediators of expulsion. Together, these data are suggestive of smooth muscle IL-4Rα being an important inducer of T_H2 cytokine signaling from the lymph node and tissue and goblet cell hyperplasia and having a striking effect on the key smooth muscle contraction–inducing M3 muscarinic receptor (Figure 8).

Materials and Methods

Generation and genotyping of conditional IL-4Rα–deficient mice. SM-MHC^{Cre} mice were a kind gift from Gary K. Owens, Charlottesville, Virginia, United States [14,32]. SM-MHC^{Cre} mice were backcrossed to Balb/c for nine generations and then intercrossed with IL-4Rα^{lox} mice ($n = 30$). These mice were then mated with IL-4Rα^{lox} mice ($n = 2$) to generate SM-MHC^{Cre}IL-4Rα^{lox} mice. Transgene negative littermates (IL-4Rα^{lox}) were used as controls in all experiments. Mice were housed under specific pathogen-free barrier conditions in the University of Cape Town animal facility. All work was approved by the University of Cape Town animal ethics board.

Genotyping. Specific PCR primer pairs were for the IL-4Rα, 5′-GTACAGCGCACATTGTTTTT-3′ and 5′-CTCGGCGCACTGACCCATCT-3′; deletion, 5′-GGCTGCCCTGGAATAACC-3′ and 5′-CCTTTGAGAACTGCGGGCT-3′; LoxP, 5′-CCGTTCCTGGCCCTGAATTT-3′ and 5′-GTTTCCTCGTACCGCTGATT-3′; and Cre, 5′-ATGCCCAAGAAGAAGAGGAAGGT-3′ and 5′-GAAATCAGTGCGTTCGAACGCTAGA-3′. PCR conditions were as follows: 94 °C for 1 min, 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min for 40 cycles on an MJ thermocycler (Biozym Diagnostik, http://www.biozym.com).

Analysis of IL-4Rα expression by FACS. A single cell suspension of smooth muscle cells was prepared as previously described [33] along with lymph node cells. For the intracellular stain, cells in single cell suspension were incubated with 1% normal rat serum and stained with rat anti-mouse IL-4Rα–PE (mIL4RM-1; BD Biosciences, http://www.bdbiosciences.com). Stained cells were then washed, fixed in 2% paraformaldehyde, permeabolized with saponin, preblocked with 2% NRS and Fc block (2.4G2), and stained with anti–α-actin FITC (Abcam, http://www.abcam.com) or isotype control IgG2a-FITC (BD Biosciences). For lymphocyte staining, anti–CD3-FITC, anti–CD19-PE, and anti–IL-4Rα biotin in combination with SA-APC were used to identify lymphocyte subsets (BD PharMingen, http://www.bdbiosciences.com). Nonviable cells were stained with 7-AAD and excluded from analysis (Sigma, http://www.sigmaaldrich.com). Acquisition was performed using FACSCalibur and cells analyzed using Cellquest (Becton Dickinson, http://www.bd.com).

Infection studies. Mice were injected subcutaneously with 750 *N. brasiliensis* L3 larva (kindly provided by Klaus Erb, Wurzburg, Germany). Analysis of parasite eggs in feces was carried out using the modified McMaster technique [34]. Adult worm burdens were determined as previously described [3].

$CD4^+$ T-cell depletion. $CD4^+$ T cells were depleted from mice by intraperitoneal injection of 0.5 mg of anti-$CD4^+$ monoclonal antibody (clone GK1.5) 3 d prior to infection. Mice received booster injections every 3 d to maintain depletion. Effective depletion was confirmed by FACS analysis.

Ex vivo restimulation of lymphocytes. $CD4^+$ T cells were purified from pooled MLNs at days 7 and 10 PI. Enrichment was carried with a negative selection. Briefly, cells in single cell suspension were stained with anti-CD8, CD11b, GR-1, B220, and CD16/32. Stained cells were depleted using goat anti-rat IgG–coated magnetic beads (Biomag Beads; Qiagen, http://www.qiagen.com). Cell purity was approximately greater than 98%. $CD4^+$ T cells were restimulated for 72 h with anti-CD3 (clone 145–2C11; 20 μg/ml). Supernatants were then collected and stored at −80 °C until analysis.

ELISA analysis. Cytokines in supernatants and serum antibody isotype levels from infected animals were determined as previously described [35].

Histology. Tissue samples were fixed in a neutral buffered formalin solution. Following embedding in paraffin, samples were cut into 5-μm sections. Sections were stained with hematoxylin and eosin or periodic–acid Schiff reagent. The number of positively stained cells per five villi were counted by light microscopy. All samples were randomized and counted in a blinded fashion.

RT-PCR. RNA was extracted from the intestine of infected mice with the use of Tri-reagent (Sigma), and cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, http://www.promega.com). M3 cDNA was amplified using the following primers: 5′-CGG AAA AGG ATG TCG-3′ and 5′-GGC ACT CGC TTG TGA A-3′. Data were normalized using the α-actin housekeeping gene.

Statistics. Values are given as mean ± SD, and significant differences were determined using the Mann-Whitney *U* test.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the genes and gene products discussed in this paper are IL-13 (16163), IL-4Rα (16190), STAT-6 (20852), M3 (12671), smooth muscle myosin heavy chain (17880), and α-actin (11475).

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Author contributions. WGCH and FB conceived and designed the experiments. WGCH, AJC, CJH, HM, EM, and BA performed the experiments. WGCH, AJC, and CJH analyzed the data. FDF, GKO, and DF contributed reagents/materials/analysis tools. WGCH and AJC wrote the paper.

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Appendix D: Paper submitted for publication (Am J Path)

IL-4 promoted T-helper responses are not required for *Nippostrongylus brasiliensis* expulsion but enhance pulmonary immuno-pathology.

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Short Title: CD4 IL-4Rα and *N. brasiliensis* infection

Abstract

Signalling through the IL-4Rα and STAT-6 by IL-13 is essential for protection against intestinal parasitic nematodes. $CD4^+$ T-cells are also required for clearance of these infections with IL-4 promoted T-helper responses being associated with the protective response.

To establish the role of $CD4^+$ T-cell IL-4Rα expression in resolving a nematode infection, we infected mice with impaired IL-4 promoted T_H2 cell responses using the recently established $CD4^+$T-cell specific IL-4RαKO, with *N. brasiliensis*. Worm burdens, egg counts and intestinal mucus production were comparable in both control and $CD4^+$T-cell IL-4RαKO mice, but impaired in IL-4RαKO mice. Interestingly IL-4 but not IL-13 was strikingly reduced in the draining lymph nodes of infected $CD4^+$T-cell specific IL-4RαKO suggesting the production of IL-4 but not IL-13 is dependent on IL-4Rα responsiveness in $CD4^+$ T-cells. Extra-intestinal pathology in the lungs of $CD4^+$T-cell specific IL-4RαKO mice showed impaired airway mucus production along with lower numbers of T-cells recruited into the lung.

Together these data demonstrate that though IL-4 responsive $CD4^+$ T-cells are not required for worm expulsion polarised T_H2 responses play a significant role in *N. brasiliensis* driven extra-intestinal pathology.

Introduction

Type 2 immune effector responses are characterised by Interleukin-4 (IL-4) and IL-13 dependent signalling through hetero-dimeric receptors containing an IL-4Rα subunit [1] and are particularly associated with the resolution of helminth infections [2-4] and the induction of allergy [5]. IL-4Rα signalling results in activation of the transcription factor STAT-6 and upregulates GATA-3 to stabilise the T_H2 phenotype in the polarised $CD4^+$ T-cell [1,6]. IL-4Rα mediated T_H2 differentiation is specific to IL-4 owing to T-cells lacking a functional IL-13 receptor [7]. This response is characterised by production by B-cells of antibodies such as IgE and IgG1 (or IgG4 in humans) [8,9], goblet cell hyperplasia [10] and secretion of T_H2 cytokines IL-4, IL-13, IL-5, IL-10 and IL-9 by a number of haematopoietic cells [11].

Though $CD4^+$ T-cells and IL-4Rα are required for optimal differentiation and stability of T_H2 responses, the significance of IL-4Rα expression in $CD4^+$ T-cells has yet to be defined. Indeed $CD4^+$ T-cell IL-4Rα independent IL-4 and IL-13 production have been previously described [12-17]. As such $CD4^+$ T-cell IL-4Rα may be associated but not necessarily essential for the production of the T_H2 cytokines IL-4 and IL-13 by $CD4^+$ T-cell polarisation to a T_H2 phenotype [18]. Studies using mice deficient in $CD4^+$ T-cell IL-4Rα ($CD4^+$T-cell IL-4Rα KO) have demonstrated important roles for $CD4^+$ T-cell IL-4Rα expression in experimental infection models involving parasites *Leishmania major* [19] and *Schistosoma mansoni* [20]. Infection of $CD4^+$T-cell IL-4Rα KO BALB/c mice with the intra-cellular protozoan parasite *L. major* renders non-healer BALB/c mice to a healer phenotype. Unexpectedly, $CD4^+$T-cell IL-4Rα KO mice infected with the trematode *S. mansoni* were protected from parasite induced

mortality. Together these data demonstrate that IL-4Rα expression on $CD4^+$ T-cells and the resulting IL-4 promoted T_H2 responses is not critical for host survival.

In the study presented here we examined the response to infection with the intestinal parasitic nematode *N. brasiliensis* in $CD4^+$T-cell IL-4RαKO mice. Our results show that the $CD4^+$T-cell IL-4RαKO mice resolve *N. brasiliensis* infections as wild-type mice. However, $CD4^+$T-cell IL-4RαKO mice do show decreased extra-intestinal (lung) pathology associated with *N. brasiliensis*, demonstrating that IL-4 responsive T-cells are not responsible for worm expulsion but do play a role in *N. brasiliensis* driven lung pathology. These results demonstrate that successful clearance of *N. brasiliensis* is independent of IL-4 promoted T_H2 polarisation while the associated pulmonary immuno-pathology is enhanced by this IL-4 promoted T_H2 response.

Materials and Methods

Mice. 8-12 wk old mice were obtained from the University of Cape Town specific-pathogen-free animal facility. All experiments were approved by University of Cape Town Animal Ethics Committee. $CD4^+$T-cell IL-4RαKO mice were generated as previously described [19] with hemizygous IL-$4R\alpha^{-/lox}$ mice (control mice) and homozygous IL-$4R\alpha^{-/-}$ mice (IL-4Rα KO mice) used as controls.

Infection studies. Mice were injected subcutaneously with 750 *N. brasiliensis* L3 larva (kindly provided by Klaus Erb, Wurzburg, Germany). Analysis of parasite eggs in faeces was carried out using the Modified McMaster technique [21]. Adult worm burdens were determined as previously described [22].

Histology. Tissue samples were fixed in a neutral buffered formalin solution. Following embedding in paraffin, samples were cut into 5µm sections. Sections were stained with Periodic Acid Schiffs reagent (PAS) where quantification of intestinal and pulmonary goblet cell hyperplasia was carried out as previously described [23,24]. All samples were randomised and counted by a blinded observer. For CD3 staining; sections were left 56°C overnight, rehydrated through zylols, alcohol to water. Blocked with 3% H_2O_2 in methanol, antigen retrieval in citrate buffer in the pressure cooker, block with 5% Goat serum, incubate with CD3 (Dako, A0452), secondary anti Rabbit Envision (Dako, K4003) and visualise with DAB substrate (Dako, K3466). Counterstain with Mayer's Haematoxylin.

***Ex Vivo* Restimulation of Lymphocytes.** $CD4^+$ T-cells were enriched (>94% purity) from pooled mesenteric lymph nodes (MLN), mediastinal lymph nodes (MST)

and spleens at days 7 and 10 PI. Single cell suspensions were stained with anti-CD8 (53.6.72), anti-CD11b (M1/70), anti-GR-1 (RB68C5) and anti-B220 (RA36B2). Stained cells were depleted using goat anti-rat IgG coated magnetic beads (Biomag beads, Qiagen, Germany). $CD4^+$ T-cells were restimulated for 72h with anti-CD3 (145-2C11). Supernatants were then collected and stored at -80°C until analysis.

Fluorescent activated cell sorting (FACS). Lungs were finely cut and digested in DMEM (Gibco) with 50U/ml collagenase type I (Gibco-Invitogen) and 13µg/ml DNAse I (Roche, Germany) at 37°C for 90min. Samples were pushed through a 70µm cell strainer, subjected to red blood cell lysis and washed with FACS buffer (PBS with 0.1% sodium azide and 1% bovine serum albumin (BSA; Roche)). T lymphocytes were stained with anti-CD3-FITC (145-2C11), non-viable cells were excluded from analysis with 7-AAD (Sigma, http://sigmaaldrich.com), acquisition was performed using FACSCalibur and cells were analysed using Cellquest (Beckton-Dickinson, http://www.bd.com).

ELISA Analysis. Cytokines in supernatants and serum antibody isotype levels from infected animals were determined as previously described [25].

Statistics. Values are given as means ± SD and significant differences were determined using the Mann-Whitney U test.

Results

Expulsion of adult *N. brasiliensis* from the intestine is not dependent on IL-4Rα responsive CD4$^+$ T-cells

To investigate a possible role for IL-4Rα expression on CD4$^+$ lymphocytes in resolving infections with *N. brasiliensis* control, IL-4RαKO and CD4$^+$T-cell IL-4RαKO mice were infected with 750 L3 *N. brasiliensis* larva. Worm burdens and egg production were measured at days 7 and 10 PI (Figures 1A & B). At day 7 PI all mouse groups demonstrated comparable worm burdens. As previously shown [23], at day 10 PI IL-4RαKO mice had significant intestinal adult worm burdens. In contrast control and CD4$^+$T-cell IL-4RαKO mice cleared the infection at this time point. Additionally worm fecundity, as demonstrated by faecal egg counts, was also comparable between control and CD4$^+$T-cell IL-4RαKO mice with no eggs present in either group after day 10 PI in contrast to IL-4RαKO mice. Together, these results clearly showed that expulsion of *N. brasiliensis* from in CD4$^+$ T-cell IL-4Rα KO mice is equivalent to that seen in control mice.

A key host response for expulsion of adult *N. brasiliensis* from the intestine is increased IL-4Rα dependent goblet cell hyperplasia and mucus production. Intestinal mucus production, as demonstrated by PAS staining, was also comparable between control and CD4$^+$T-cell IL-4RαKO mice (Figure1C). Peak mucus production was seen at day 7 PI in control and CD4$^+$T-cell IL-4RαKO mice, with IL-4RαKO mice having significantly ($p<0.05$) lower intestinal mucus production. At day 10 PI all mice had equivalent levels of intestinal mucus production.

IL-4 and IL-13 have a differential dependency on IL-4Rα responsive CD4$^+$ T-cells.

Worm expulsion and goblet cell hyperplasia are associated with the host generating a CD4$^+$ lymphocyte-induced T_H2 cytokine response. Cytokine levels from restimulated mesenteric lymph node derived CD4$^+$ cells demonstrated the expected T_H2 polarisation in control mice, with higher levels of both IL-4 and IL-13, at day 7 post infection when compared to IL-4RαKO mice (Figure 1D). CD4$^+$ T-cells in IL-4RαKO mice shifted to their typical T_H1 polarisation with elevated IFNγ, when compared to control mice. CD4$^+$T-cell IL-4RαKO mice showed, as expected, a significant impairment of IL-4 production when compared to control mice. However, IL-13 levels where equivalent between both control and CD4$^+$T-cell IL-4RαKO mice. Together these data demonstrated sufficient IL-4 but not IL-13 production is dependent on IL-4Rα mediated responses.

Analysis of pulmonary cytokine production in response to *N. brasiliensis* infection in CD4$^+$ lymphocytes isolated from mediastinal lymph nodes (MST) demonstrated a delayed onset in anti-CD3 induced T_H2 cytokine production (Figure 2). MST T_H2 secreted cytokine levels of IL-4 and IL-13 in IL-4RαKO and CD4$^+$T-cell IL-4RαKO mice were significantly lower than in control mice at day 7 PI. As expected IL-4RαKO mice displayed significantly higher levels of IFNγ, than control mice, suggesting a shift towards T_H1 polarisation. At day 10 PI IL-4 and IL-13 levels secreted from MST CD4$^+$ lymphocytes in CD4$^+$T-cell IL-4RαKO mice were comparable to levels in control mice. Systemic IgE levels were also unaffected in CD4$^+$T-cell IL-4RαKO mice when compared to control mice (Figure 2). Together

these results suggest that $CD4^+$ IL-4Rα independent IL-4 and IL-13 production in *N. brasiliensis* infections leads to type 2 B-cell responses.

Reduced pulmonary immuno-pathology in CD4+T-cell IL-4RαKO mice

Airway mucus production demonstrated by PAS staining (Figure 3A) was similar between control and $CD4^+$T-cell IL-4RαKO mice at day 7 PI. In control mice airway mucus production increased further at day 10 PI, this was not seen in $CD4^+$T-cell IL-4RαKO mice which had significantly less mucus at this time point. As expected IL-4RαKO mice had significantly reduced airway mucus production at days 7 and 10 PI.

In order to investigate the role for IL-4Rα responsive T-cells in this abrogated pulmonary pathology we examined the distribution of $CD3^+$ T-cells in the lung. We found that T-cells in control mice formed localised foci associated with vascular systems and airways whilst in the IL-4RαKO and $CD4^+$T-cell IL-4RαKO mice this localisation was less apparent with $CD3^+$ T-cells being dispersed throughout the tissue (Figure 4C-H). Moreover, FACS analysis of T-cell recruitment to the lung showed significantly lower numbers of $CD3^+$ T-cells in the lungs of both IL-4RαKO and $CD4^+$T-cell IL-4RαKO mice at days 7 and 10 PI when compared to control mice (Figure 4B).

The lower mucus production observed in the lung of *N. brasiliensis* infected $CD4^+$T-cell IL-4RαKO mice may be related to decreased numbers and disrupted distribution of T-cells in the lung. Though $CD4^+$T-cell IL-4RαKO mouse $CD4^+$ T-cells maintain IL-13 production levels similar to that of control mice $CD4^+$ T-cells, these cells are dispersed throughout the lung which would account for ineffective stimulation of

goblet cell mucus hypersecretion. Together the data suggests that IL-4Rα responsive $CD4^+$ T-cells are required for adaptive immune responses associated with pulmonary immuno-pathology in experimental *N. brasiliensis* mouse infections.

Discussion

Using $CD4^+$ T-cell IL-4RαKO mice, we clearly demonstrate that IL-4Rα responsive $CD4^+$ T-cells are not required for resolving *N. brasiliensis* infections. Furthermore, cytokine production from restimulated mesenteric lymph node (MLN) derived $CD4^+$ cells confirms expulsion to be independent of MLN $CD4^+$ T-cell IL-4 secretion [22], which we also found to be significantly reduced. Somewhat surprisingly, IL-13, the key cytokine for worm clearance [26], showed equivalent levels in $CD4^+$ T-cell IL-4RαKO mice to those of control mice demonstrating IL-13 production by $CD4^+$ T-cells independent of signalling via IL-4Rα on these cells. IL-4Rα independent IL-13 production in $CD4^+$ T-cells has been demonstrated previously in ovalbumin induced airway hyper-reactivity [17] and our data further extends this observation to *N. brasiliensis* infection with differential regulation of IL-4 and IL-13. The absence of a measurable effect on worm expulsion in $CD4^+$T-cell IL-4RαKO mice is in agreement with studies which demonstrate that although $CD4^+$ cells [23] and IL-4Rα [27] are required for expulsion of *N. brasiliensis*, signalling through the STAT-6 pathway in $CD4^+$ cells is not required [28]. Expulsion is driven by smooth muscle cell contraction [23] and goblet cell hyperplasia [26]. Both effector functions are primarily induced by IL-13 signalling through IL-4Rα [29]. Mechanistically, effective worm expulsion independent of IL-4Rα responsive $CD4^+$ T-cells was highlighted by control and $CD4^+$T-cell IL-4RαKO mice having equivalent levels of intestinal mucus and goblet cell hyperplasia, which would be explained by $CD4^+$ T-cell IL-4RαKO mice having comparable levels of IL-13 to control animals which would be sufficient to drive the intestinal mucus production.

Interestingly, in this study *N. brasiliensis* induced pulmonary immuno-pathology was strikingly affected in $CD4^+$ T-cell IL-4RαKO mice. Here we found a significantly lower airway mucus response in the lung which was associated with disrupted T-cell recruitment and localisation to the airway in $CD4^+$ T-cell IL-4RαKO mice. These results clearly demonstrate $CD4^+$ T-cell IL-4Rα expression to be playing a significant role in T-cell targeting and recruitment to airways in *N. brasiliensis* induced pulmonary pathology. This decreased airway mucus production and T-cell recruitment to the lung in $CD4^+$ T-cell IL-4RαKO mice is then indicative of disrupted T-cell targeting being an important component for driving T-cell mediated airway mucus production. This would be in agreement with previous work which demonstrated recruitment of T_H2 $CD4^+$ T-cells into the lungs of *N. brasiliensis* infected mice being dependent upon their expressing STAT-6 [30,31]. Moreover, ovalbumin induced T_H2 $CD4^+$ T-cell recruitment to the lung requires T-cell IL-4 responsivness [24]. However, ovalbumin induced airway mucus itself is not wholly dependent on IL-4 [32] but does require IL-13 [33] and IL-4Rα [34]. Indeed, IL-13/ IL-4Rα interactions directly with airway epithelial cells are sufficient for inducing airway mucus production [33]. Together these studies indicate a common requirement for STAT-6/ IL-4Rα and IL-13 (and to a lesser extent IL-4) in recruitment of T-cells to the lung and induction of airway mucus production. Our data supports and expands on these findings by demonstrating that $CD4^+$ T-cell IL-4Rα is a requirement for effective T-cell recruitment to the lung and localisation to the airway. We propose that diminished T-cell recruitment and airway localisation is responsible for the decreased levels of airway mucus production reported in this study.

In conclusion, this study has demonstrated, for the first time that expression of IL-4Rα on $CD4^+$ T-cells is not required for the resolution of the definitive intestinal stage of the infection. However, IL-4Rα expressing $CD4^+$ T-cells play a significant role in driving *N. brasiliensis* induced pulmonary immuno-pathology through orchestrating T-cell recruitment to the lung and localisation to the airway.

Figure Legends

FIGURE 1

N. brasiliensis infection and intestinal immune responses.

$CD4^+$T-cell IL-4RαKO mice (hatched bars/ line); control mice (solid bars/ line); IL-4RαKO mice (empty bars/ line).

A) Mice were infected with 750 L3 *N. brasiliensis*, at days 7 and 10 PI worm burden of the small intestine was assessed to determine expulsion kinetics.

B) Faeces from days 5 to day 15 PI were collected and egg production by *N. brasiliensis* was calculated using the modified McMaster technique.

C) Intestinal mucus production was assessed by counting the total number of PAS positive goblet cells per 5 villi on histological sections of the small intestine at days 7 and 10 PI.

D) Supernatant cytokine levels of $CD4^+$ sorted mesenteric lymph nodes were anti-CD3 restimulated for 72hours and detected via ELISA.

*Significant differences from control mice ($p<0.05$), 4 mice per group, data representative of 3 separate experiments.

FIGURE 2

Cytokines levels in the draining lung lymph node of $CD4^+$T-cell IL-4RαKO mice show a reduced T_H2 response.

$CD4^+$T-cell IL-4RαKO mice (hatched bars); control mice (solid bars); IL-4RαKO mice (empty bars).

A) Supernatant cytokine levels of $CD4^+$ sorted mediastinal lymph nodes were anti-CD3 restimulated for 72hours and detected via ELISA.

B) Antibody production in the serum was assessed by ELISA at days 7 and 10 PI.

*Significant differences from IL-4Rα$^{-/lox}$ mice ($p<0.05$), 4 mice per group, data representative of 3 individual experiments.

FIGURE 3

Cytokines levels in the draining lung lymph node of CD4$^+$T-cell IL-4RαKO mice show a reduced T_H2 response.

CD4$^+$T-cell IL-4RαKO mice (hatched bars); controls mice (solid bars); IL-4RαKO mice (empty bars).

A) The histological mucus index was determined using PAS stained lung sections in order to compare mucus production by airway goblet cells in the various mouse types.

B) Single cell suspensions of whole lung were analysed by FACS for numbers of CD3$^+$ cells present in the various mouse types.

C) Lung tissue removed at days 7 and 10 PI fixed in formalin and stained with anti-CD3-DAB highlighting the formation of lymphocyte foci around airways and vascular systems in the control mice whilst in the CD4$^+$T-cell IL-4RαKO and IL-4RαKO mice lymphocytes were dispersed throughout the tissue. (C, F) control mice day 7 and 10 PI respectively (D, G) IL-4RαKO mice day 7 and 10 PI respectively (E, H) CD4$^+$T-cell IL-4RαKO mice day 7 and 10 PI respectively

*Significant differences from control mice ($p<0.05$), 4 mice per group, data representative of 3 individual experiments.

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Figure 1

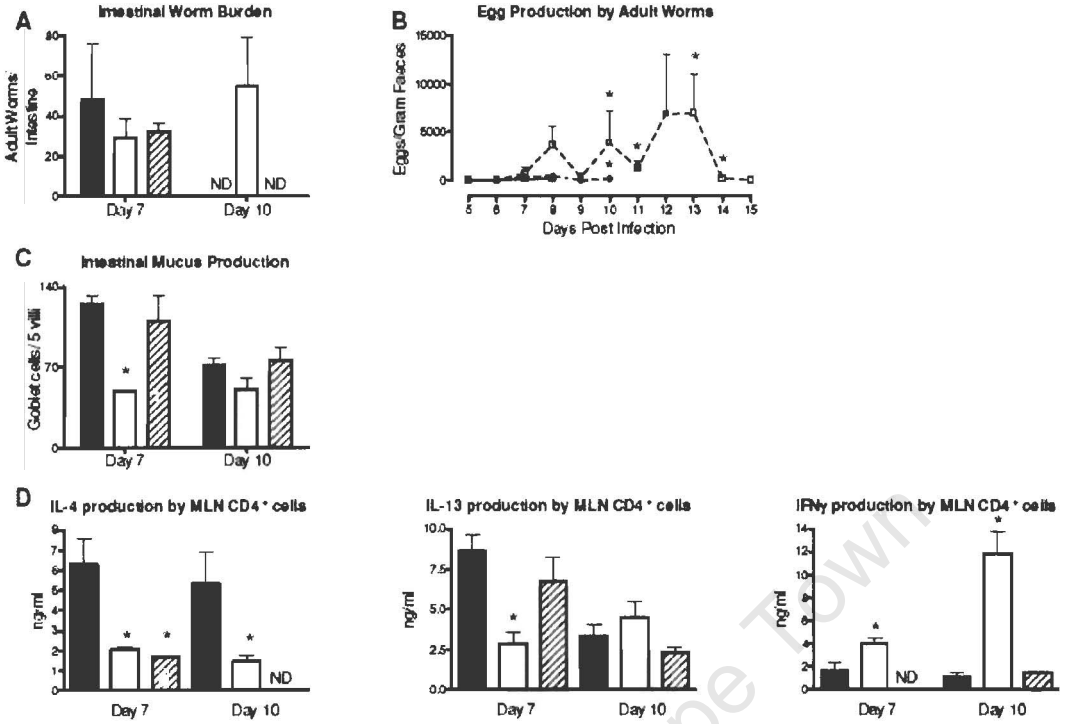

Figure 2

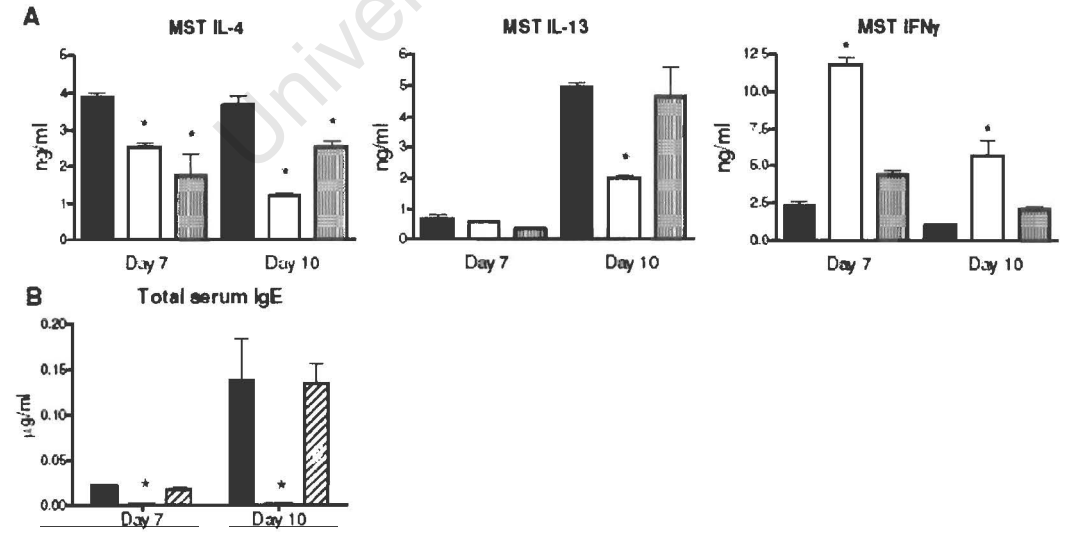

Figure 3