CD4 T cell responses in lung tissue and their role in Th2 protective immunity

By

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Abstract

The acquisition of protective immunity is a critical feature of the immune system. It is the unique ability of the adaptive immune response to generate and maintain long-lived antigen specific memory cells, which is the key to preventing reinfection and achieving the goal of protective immunity. The importance of secondary lymphoid tissue (such as lymph nodes) as a site of effector CD4 T cell responses and the generation, dissemination and maintenance of memory CD4 T cells is well accepted. However, a key research area needing investigation is the basic biology of the CD4 T cell, particularly the recirculation, distribution and maintenance of CD4 T cells at sites throughout the body.

To address these issues we used *Nippostrongylus brasiliensis* as a model of CD4 mediated protective immunity, combined with G4/IL-4 reporter mice. We show that the lung environment is critical for the priming of CD4 T cells and conferring protective immunity. In contrast to others we find no protective role for the CD4 T cell population of the skin and only a minor role for the population within the gut.

In a separate study we used the drug fingolimod (FTY720) to block the cellular trafficking between lymph node and lung tissue during immune responses. Interestingly, our findings show that protection against *N. brasiliensis* infection is maintained when CD4 T cell recirculation between the lung and lymph node is blocked.

Furthermore, we reveal that peripheral lung residing CD4 T cells are sufficient for conferring protective immunity in the *N. brasiliensis* model, generating support for the model of effector lymphoid tissue. When *N. brasiliensis* experienced CD4 T cells were localised to the lung by intranasal adoptive transfer they were able to confer protection against infection in otherwise naïve animals, as early as 48 hours post infection.

The most striking finding of this work is the discovery that memory CD4 T cells residing in the lung that are sufficient to confer protection against reinfection. Identifying the factors in the lung and lymph node that induce and support this CD4 T cell subset will be an important area of future research given its high relevance to the design of vaccines against parasite infections.

This thesis is dedicated to my grandmother, Audrey Marie Dent, who passed away after a hard fought battle with cancer on December 16th, 2007. She was an avid supporter of the research performed at the Malaghan Institute of Medical Research, and was so excited that I was a part of it. I know that she would be very proud to see this thesis completed.

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List of abbreviations

1°	Primary
2°	Secondary
AAM	Alternately activated macrophage
AICD	Activation induced cell death
Ag	Antigen
APC	Antigen presenting cell
BALT	Bronchial associated lymphoid tissue
BCG	Bacillus Calmette-Guèrin
CD	Cluster of differentiation
CFP	Culture filtrate protein
CFU	Colony forming unit
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CIA	Collagen induced athritis
cIMDM	Complete Iscove's Modified Dulbeccos Medium
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DRG	Dorsal root ganglion
EAE	Experimental autoimmune encephalomyelitis
ELT	Effector lymphoid tissue
FACs	Fluorescently activated cell sorting
FTY720	2-amino-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride
FTY720-P	2-amino-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride phosphate
GALT	Gut associated lymphoid tissue
GFP	Green fluorescent protein

GMCSF	Granulocyte macrophage colony stimulating factor
iBALT	Inducible bronchial associated lymphoid tissue
IBD	Inflammatory bowel disease
i.d.	Intradermal
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iL3	Infective third stage larvae of *N. brasiliensis*
ILF	Isolated lymphoid follicles
IMDM	Iscove's Modified Dulbeccos Medium
iNKT	Invariant natural killer T cell
i.n.	Intranasal
i.p.	Intraperitoneal
iTreg	Inducible regulatory T cell
i.v.	Intraveneous
IVC	Individually ventilated cages
HEV	High endothelial venule
HSV	Herpes simplex virus
L1	First stage larvae of *N. brasiliensis*
L2	Second stage larvae of *N. brasiliensis*
L4	Fourth stage larvae of *N. brasiliensis*
L5	Fifth (adult) stage of *N. brasiliensis*
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
LPS	Lipopolysaccahride
LTα	Lymphotoxin alpha

GI	Gastrointestinal
GVHD	Graft versus host disease
mAb	Monoclonal antibody
MALT	Mucous associated lymphoid tissue(s)
Med	Mediastinal
Mes	Mesenteric
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NALT	Nasal associated lymphoid tissue
Nb	*Nippostrongylus brasiliensis*
NKT	Natural killer T cell
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffered saline
pMHC	peptide MHC complex
PRR	Pattern recognition receptor
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
$S1P_{(R)}$	Sphingosine-1-phosphate (receptor)
SALT	Skin associated lymphoid tissue
s.c.	Subcutaneous
S.E.M.	Standard error of the mean
SPF	Specific pathogen free
SphK	Sphingosine kinase
STAT	Signal transducer and activator of transcription
T_{CM}	Central memory T cell

TCR	T cell receptor complex
Teff	Effector T cell
T_{EM}	Effector memory T cell
TGFβ	Transforming growth factor beta
Th(2)	T helper (2)
TLR	Toll like receptor
T_{MEM}	Memory T cell
Tn	Naïve T cell
Treg	Regulatory T cell
WT	Wild type

Chapter 1:
General Introduction

1.1. Introduction

A unique feature of the adaptive immune response is its ability to mount memory responses when re-exposed to the same pathogen. During an initial encounter with antigen, long-lived memory cells expand and gain functionality, which are capable of rapid response when reinfection occurs. The mechanisms of protective immunity are often broadly defined, but for the purposes of this thesis are defined as protection from reinfection with the invading pathogen (Fig 1.1). Protection may be partial or complete with the gold standard being a 'sterile cure' where pathogens are contained and cleared from the host. The tissues and cells that confer immunity are divided into the innate and adaptive arms of the immune system, which work together to achieve pathogen clearance. A key regulator of immunity is the CD4 T helper cell. CD4 T cells activate and drive the immune response in both innate and adaptive arms through the production of inflammatory cytokines. These secreted messengers have an integral role to play in the orchestration of the immune response, both attracting and interacting with other cells. The recirculation, distribution and maintenance of CD4 T cells at sites throughout the body is of critical importance to defend against pathogenic insult. Lymph nodes provide strong hubs of immune connection where CD4 T cells are able to enter from the blood and sample antigen on antigen presenting cells (APCs) draining via the lymphatics from the surrounding tissues. Antigen experienced CD4 T cells exit from the lymph node to peripheral tissues and as such are able to provide a much faster and effective memory immune response at the site of pathogen exposure. The recirculation and traffic of CD4 T cells between lymph nodes and peripheral tissues is an integral part of the CD4 T cell response.

To study in further detail these aspects of CD4 T cell mediated protective immunity we used an established model of Th2 mediated protective immunity. *Nippostrongylus brasiliensis* is a rodent nematode parasite that induces a strong Th2 immune response with production of cytokines and recruitment of accessory cells. *N. brasiliensis* travels from skin to lung and then gut before being expelled after 10 days in a primary response. Upon reinfection with *N. brasiliensis* there is a reduction of worm burden in the lung and gut, making this a useful model to study the parameters of Th2 mediated protective immunity.

This thesis explores the *N. brasiliensis* model of Th2 mediated protective immunity, using G4/IL-4 reporter mice to clarify and define the key parameters of this protective response with respect to the tissues and IL-4 producing T cells involved. With this goal in mind this

introduction reviews the current literature addressing the development of CD4 mediated protective immunity (in the *N. brasiliensis* model). An understanding of T cell activation, effector function, memory generation and recirculation is required to fully investigate this *N. brasiliensis* model of protective immunity. As CD4 T cells are drivers of other cellular responses a brief overview of adaptive and innate cells and their interactions will be covered also.

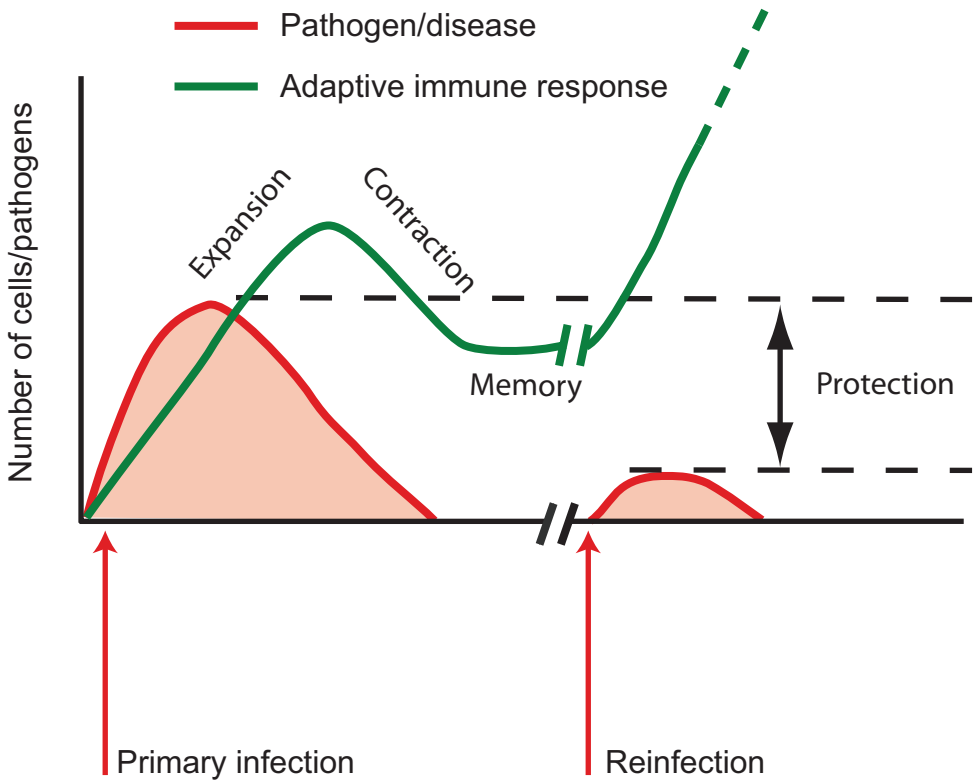

Figure 1.1 The relationship of adaptive cellular response to pathogen infection in protective immunity.

During a primary pathogen infection pathogen numbers increase quickly. As the adaptive immune response is initiated effector cells expand and exert effector function, leading to a decline in pathogen numbers until the infection is controlled. Contraction of the immune cell population occurs as the pathogen numbers decline, however a small number of cells are maintained as memory cells. Upon reinfection, memory cells respond rapidly and the infection is contained, resulting in a reduced pathogen burden and shorter infection that we define as protection.

1.2. Innate immune responses

In evolutionary terms the innate arm of the immune system is the oldest part of the immune system; present in protists, plants and animals it has been conserved for millions of years [1], with the adaptive immune system arising later with the divergence of jawed vertebrates [2]. Innate immunity consists of physical barriers such as skin and mucosal surfaces, physiological barriers like temperature and chemical barriers such as complement. In addition there are cellular responses; phagocytic cells which take up and contain invading pathogens and granulocytes that release chemical mediators to destroy infectious agents.

Although qualitatively different, the innate and adaptive immune responses are intertwined; the innate response is able to work independently and without the involvement of the adaptive response, but is also able to activate the adaptive immune system. Conversely, the adaptive immune response can coordinate and recruit the cells of the innate system.

Upon infection, innate cells directly recognise conserved molecular patterns (PAMPs – Pathogen Associated Molecular Patterns) expressed on microbes through specialised pattern recognition receptors (PRRs) to respond to invading pathogens. There are three types of PRR; phagocytic PRRs like mannose receptor that enable uptake of foreign material; secreted PRRs that can activate complement and PRRs that signal infection such as Toll Like Receptors (TLRs). As well as activation through recognition of PAMPs, innate immune responses can also be activated by the adaptive immune response. The following focuses on the cellular aspect of innate immunity.

1.2.1. Cells of the innate immune system

The cells of the innate immune system are the first cellular line of defence and can act directly, killing pathogens but also function to activate the adaptive immune response.

Granulocytic cells are able to directly kill pathogens via the release of toxic granules, but also carry out other functions. Neutrophils are short-lived phagocytic cells that are recruited to sites of inflammation where they destroy pathogens directly using reactive oxygen and nitrogen species in addition to their toxic granules. Mast cells degranulate in response to ligation of FcεRI surface receptors by IgE releasing histamine, which when released to an innocuous antigen, forms part of an allergic response. Eosinophils (and

basophils) are strongly associated with parasitic infection in addition to asthmatic and allergic responses [3-6].

Phagocytic cells, such as macrophages, 'scavenge' - phagocytosing apoptotic or senescent cells and cell debris via phagocytic PRRs such as the mannose and scavenger receptors. Also by directly engulfing foreign particles and microbes phagocytic cells provide immediate defence against pathogens and also help "clean up" after infection. This is important to control antigen accessibility and the resulting immune response.

1.2.2. Initiation of adaptive responses by innate cells

The initiation of adaptive immunity occurs through presentation of antigen on innate cells for adaptive recognition. T cells, through their T cell receptor (TCR), recognise specific antigenic peptides as a complex with the major histocompatibility complex (MHC) expressed on antigen presenting cells (APC). There are two types of MHC; MHC I that is expressed on all cell types and presents intracellularly derived antigen and MHC II, expressed on professional APCs that presents extracellular antigens. Optimal signalling through the peptide/MHC/TCR complex requires the assistance of co-receptors [7]; cluster of differentiation 4 (CD4) interacts with MHC II molecules, and CD8 interacts with MHC I. Although interaction of the TCR complex and MHC initiates signalling, this signal alone is not enough for naïve T cell activation, and has been shown to render T cells non-functional/anergic. A second signal or costimulation is required for full activation of a functional effector T cell. Costimulatory molecules expressed by T cells include CD28, CD154 and OX40 that interact with CD80/6, CD40 and OX40L respectively on APC. These costimulatory molecules enhance TCR signalling and/or provide additional signals to help expand T cell populations and drive effector function [6].

The presentation of antigen to CD4 T cells to initiate an antigen specific response is restricted to cells expressing MHC II and there are several innate cells that have this capacity. Dendritic cells (DCs), macrophages and monocytes express MHC II, and it has recently been determined that basophils also express MHC II with the ability to initiate CD4 T cell responses [8, 9]. Of these APCs, DCs are well accepted as the most efficient activator and stimulator of CD4 T cell responses, as such the following focuses on DC, but also covers other innate APCs.

1.2.2.1. Dendritic cells as initiators of adaptive immune responses

Dendritic cells bridge innate and adaptive responses, identifying danger from pathogen infection through recognition of PAMPs to become activated themselves and also to activate the specificity of the adaptive immune response through MHC II restricted presentation of antigen and the secretion of soluble mediators [6]. The information received by the DC through pattern recognition allows the DC to influence the corresponding T cell response. In the instance of a microbial infection, DC recognise components of the bacterial cell wall such as lipopolysaccharide (LPS) through pattern recognition, causing secretion of interleukin (IL)-12. The signal of IL-12 in conjunction with T cell/peptide/MHC II (pMHC II) signalling educates the responding cognate T cell to become a Th1 effector cell, the most appropriate lineage to fight such an infection.

One of the properties making DC such efficient activators of T cell immunity is the transport of antigen from peripheral sites of inflammation to the lymph nodes, increasing the likelihood of finding T cells specific for the invading pathogen. In their immature state DC constantly sample the surrounding environment, taking up and presenting antigen on their MHC molecules. In the absence of infection/inflammation these presented antigens can act to tolerise T cells. Upon infection DC receive information through their PRRs and begin to mature; they stop taking up further antigen and present what they already have, they also begin to express a variety of costimulatory molecules and secrete inflammatory mediators [10, 11]. Upon maturation migratory DC travel from the site of inflammation to the draining lymph node via the afferent lymph, carrying their antigenic cargo to the T cells and involving the adaptive immune response [12].

It is important to realise that although some DC are migratory, carrying antigen to the draining lymph nodes, antigen presentation also occurs within the tissues themselves. Memory T cells are reactivated in the periphery by local antigen presentation and effector T cell populations are expanded upon arrival in peripheral tissues. Recent evidence for T cell priming in peripheral tissues DC subsets comes from the work of Wakim *et al* who show that host derived DCs can present antigen to donor T cells residing within the grafted tissue [13].

There are many subsets of DC that have distinct specialised roles. Langerhans cells and dermal DC reside in the skin and carry cutaneous antigen to the lymph nodes [14]. Unique CD8α DC in the lymph nodes specialise in cross presentation of antigen, activating both CD4 and CD8 T cells to fight infection. A recent review of lung DC reveals no less than

five distinct lineages, each functionally specialised [15]. This diversity means that not all functions are shared equally within the DC subsets, some are efficient at tolerisation, others are migratory and yet others are better at antigen uptake.

1.2.2.2. Macrophages and monocytes as initiators of adaptive responses

Macrophages also function as APCs and can present antigen on both MHC I and II. In chronic infections macrophages can be important for the containment and compartmentalisation of microbes through the formation of granulomas, clusters of immune cells including T cells. Macrophages contain infection by taking up microbes but can also directly kill pathogens with antimicrobial products such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). In Th1 mediated protective immunity to Tuberculosis, macrophages act to contain and eventually clear infection through containment within granuloma and direct killing with ROI [16, 17]. Granulomas can also be a feature of Th2 mediated immunity, as in the liver granulomas surrounding the eggs of *Schistosoma* sp [18].

A subset of macrophages, termed alternately activated macrophages, are associated with Th2 immune responses, particularly with parasite infections [19]. These cells differ from classically activated macrophages in that they produce arginase in preference to the iNOS made by classically activated macrophages. Alternately activated macrophages can also be identified by the expression of YM-1 and Fizz-1 (also known as RELMα) [20], and play a role in tissue remodelling. Importantly, nematode elicited macrophages can induce the differentiation of naïve T cells into Th2 cells [21].

Monocytes are circulating mononuclear cells that extravasate to tissues in times of inflammation. Inflammatory monocytes migrate to tissues and differentiate, becoming macrophages or DC's, providing an additional source of APCs at inflamed sites.

1.2.2.3. Basophils as initiators of adaptive responses

Basophils are a relatively rare population of blood circulating granulocytes that are able to bind IgE via FcεRI expressed on the cell surface. Recent studies in humans by Chen et al detail IgD binding by basophils, with a resultant production of cytokines and antimicrobial molecules [22]. A new role for basophils has recently been postulated, suggesting they may act as antigen presenting cells that drive Th2 immunity [8, 9, 23]. Studies by

Yoshimoto *et al*, as well as those by Perrigoue *et al* show that antigen presentation by basophils to CD4 T cells is MHC II dependent [8, 23]. Furthermore, experiments by Sokol *et al* suggest that basophils can replace the role of dendritic cells in the initiation of Th2 immune responses [9]. This is an interesting development in the field of Th2 immunity, but as discussed by Fred Finkelman recently in an Immunology and Cell Biology commentary, "I believe it would be premature to judge the relative importance of basophil vs DC antigen presentation" [24].

1.3. Activation of CD4 T cells and expression of effector function

1.3.1. T cell priming and activation

The adaptive arm of the immune system has evolved in such a way that it is able to mount very specific responses against defined antigens through the expression of a highly diverse repertoire of receptors. The alpha and beta chains of the T cell receptor allow specific recognition of peptide and signal using a series of protein dimers known as CD3, together these molecules make up the T cell receptor complex (TCR). In the thymus, recombination of gene segments for variable (V), diversity (D) and joining (J) regions by RAG enzymes assembles a diverse repertoire of antigen specific αβTCR [25, 26].

Activation of naïve CD4 T cells is contact dependent, requiring TCR-pMHC interaction along with costimulation to ensure sufficient activation [27, 28]. The immunological synapse describes the interface between APC and T cell. Generation of the immunological synapse stabilises the TCR/pMHC interaction allowing for effective activation of the T cell by clustering all the relevant molecules together and allowing sustained signalling [29]. CD4 T cells seem to require longer stimulation by APC than CD8 T cells before differentiating to their effector lineages [30].

When a threshold of stimulatory and costimulatory signals are met and the T cell is activated proliferation occurs – a process called clonal expansion. Clonal expansion describes the generation of many antigen specific T cells from the activation of the initial naïve cell. Early during activation T cells transiently up regulate CD69, which is inversely related to $S1P_1$ expression. The up-regulation of CD69 and corresponding down-regulation of $S1P_1$ retains activated effector cells in the lymph node, enabling more effective activation and prolonged antigen presentation [31]. Activation induces changes in the

expression of cell surface proteins; effector cells down regulate the lymph node homing selectin CD62L and up regulate adhesion molecules such as CD44 in preparation for migration to the site of inflammation in the periphery. Activated cells also gain effector function with production of soluble inflammatory cytokines and in the case of CD8 T cells, production of cytotoxic granules. The effector responses of CD4 T cells are discussed below.

1.3.2. CD4 T cell effector responses

Effector CD4 T cells effectively direct the immune response through the release of soluble cytokine mediators, and expression of CD40L. The initial discovery of the two distinct Th1/Th2 CD4 effector lineages in the early eighties [32, 33] saw the field begin to define the different lineages based on their production of effector cytokines. As technologies have improved the CD4 T cell family has expanded significantly, with Tregs and Th17 cells being revealed as important lineages [34] and increasing evidence suggesting the addition of Tfh and Th9 cells. The following section summarises the development of CD4 T cell lineages and their effector functions upon activation (Fig 1.2). NKT cells are briefly mentioned as they also express CD4 and contribute to T cell effector responses.

1.3.2.1. Th1

Th1 effector responses are driven by IFNγ and are effective against intracellular bacteria and viruses. The production of IFNγ by Th1 cells plays an important role in resistance to intracellular pathogens such as mycobacteria, with IFNγ activating macrophages to increased metabolic activity able to contain and clear the bacteria. Pro-inflammatory Th1 responses are efficient at clearing pathogens and mounting anti-tumour responses, often involving cell mediated cytolytic activity, but can also cause tissue damage and elicit autoimmunity. IFNγ itself is toxic to cells, including those immune cells that produce it.

The presence of IL-12 during antigen presentation drives the differentiation of a Th1 response. IL-12 signalling through signal transducer and activator of transcription (STAT) 4 can directly stimulate production of IFNγ, inducing an IFNγ positive feedback loop and playing an important role in amplifying Th1 responses. IFNγ stimulates the transcription factor T-bet through STAT 1 mediated signalling. T-bet (known as the master Th1 regulator) goes on to elicit further IFNγ production, amplifying and maintaining the Th1

response [34]. A key function of IFNγ is the inhibition of IL-4 and therefore Th2 immune responses, driving the polarity of the T cell response towards Th1.

1.3.2.2. Th2

Th2 cells mediate effector function through production of IL-4 and also IL-5, IL-10, IL-13 and 25 [34]. Much like it's Th1 counterpart IFNγ, IL-4 inhibits Th1 responses by inhibiting IFNγ production, promoting Th2 polarity. IL-4 also promotes B cell responses and induces isotype switching to IgE. Binding of IgE crosslinks FcεR expressed on the surface of mast cells and basophils, leading to the release of inflammatory mediators such as histamine and also the production of cytokines IL-4 and 13. Eosinophil recruitment is mediated by IL-5 and IL-10 suppresses Th1 proliferation and can also suppress DC function. IL-13 plays an important role in the expulsion of helminth parasites and in the induction of airway hypersensitivity. IL-25 can enhance the production of IL-4, IL-5 and IL-13 by a unique c-kit+ FcεRI- non-lymphocyte population [34]. Innate cells associated with Th2 responses include eosinophils and mast cells, which release toxic granules upon activation.

The events regulating the initiation of Th2 immune responses has been a subject of intense scrutiny, *in vitro* studies showed priming in the presence of IL-4 drives Th2 [35]; however recent work has shown that although IL-4 is necessary for amplification and maintenance of a Th2 immune response (in a similar fashion to that seen for IFNγ and T-bet), induction can occur through IL-4 and STAT 6 independent pathways [36-39]. Th2 induction requires IL-2 to help drive differentiation. IL-2 signals through STAT 5, which has been shown to bind directly to DNase hypersensitivity sites in the *Il4* locus [40]. Strong STAT 5 signalling is required as the loss of STAT 5a causes defects in Th2 differentiation [41, 42]. In the presence of IL-4, GATA 3 is directly activated by STAT 6, however in the absence of IL-4, IL-2 signalling through STAT 5 leads to the production of limited amounts of IL-4 that is then able to signal GATA 3 through STAT 6 dependent pathway to amplify and maintain the response.

1.3.2.3. Th17

The recently discovered lineage of Th17 cells produces IL-17a, IL-17f, IL-21 and IL-22 upon stimulation of effector function. The generation of Th17 cells occurs when naïve T cells are primed in the presence of TGFβ and IL-6 [43-45], activating the transcription

factor RORγt. There are three stages of Th17 differentiation; initiation of differentiation by TGFβ and IL-6, expansion mediated by IL-21 and stabilisation via IL-23 [34]. The IL-21 produced by these cells has a positive feedback, reinforcing the Th17 phenotype [46, 47].

Th17 cells have been found in the autoimmune diseases experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA) and also have a role to play in fungal and bacterial frontline defence [48-50].

1.3.2.4. Th9

Th9 effector cells produce IL-9, which has effects on mast cells and lymphocytes as well as inducing mucin production in epithelial cells [34]. Th9 cells are associated with Th2 immune responses and differentiation of Th9 cells requires the Th2 cytokine IL-4 along with TGFβ [51, 52]. Th9 cells are a relatively poorly characterised T cell subset and as such they are tentatively identified as a new CD4+ lineage [53].

1.3.2.5. Tfh

Follicular helper T cells (Tfh) are a subset of CD4 effector T cells that assist with antibody production and the generation of plasma cells through the production of IL-21. IL-21 is also fundamental in the generation of Tfh [54]. Although Th17 cells also produce IL-21, Tfh are distinct as they differentiate independently of RORγt and TGFβ [53] - requiring the transcription factor Bcl6 [55, 56]. Experiments by Zaretsky *et al* describe how Tfh can differentiate from Th2 cells in a helminth model, highlighting the plasticity of CD4 T effector responses [57].

1.3.2.6. Regulatory T cells

Initially known as suppressor T cells, regulatory T cells (Tregs) are a lineage of CD4 T cells that work to regulate immune responses through suppression of T cell effector function. Activated Tregs regulate the immune response through the production of IL-10 and also in a contact dependent fashion [34].

Improved technologies and the identification of forkhead box transcription factor FoxP3 as the master regulator of Tregs controlling regulatory molecule transcription have facilitated a resurgence in the exploration of regulatory T cells. Studies have revealed there are two

distinct types of CD4+ FoxP3+ Treg, the thymically derived "natural" Tregs and inducible Tregs (iTregs) that arise during infection. Activated CD4 T cells, strongly stimulated by TGFβ in the absence of proinflammatory cytokines, develop into Foxp3+ iTregs that are antigen specific.

The cytokines produced by effector T cells in addition to driving each of the lineages also have an important suppressive role to inhibit other lineages to maintain a polarised immune response.

1.3.2.7. NKT cells

NKT cells are a unique CD4 T cell subset that can rapidly produce large amounts of cytokine, making either IFNγ or IL-4 depending on the stimuli. Interestingly NKT cells have been found to contribute to the amplification of Th2 responses in a parasite model [58].

Although not a traditional CD4 effector cell, NKT cells that express αβTCR can also express CD4 and therefore can be included in the CD4 T cell family. NKT cells are unique in that they recognise lipid and glycolipid antigens when they are presented on the CD1d complex, unlike traditional CD4 T cells that recognise antigen presented in the context of MHC II. Diversity of the TCR is limited in NKT cells.

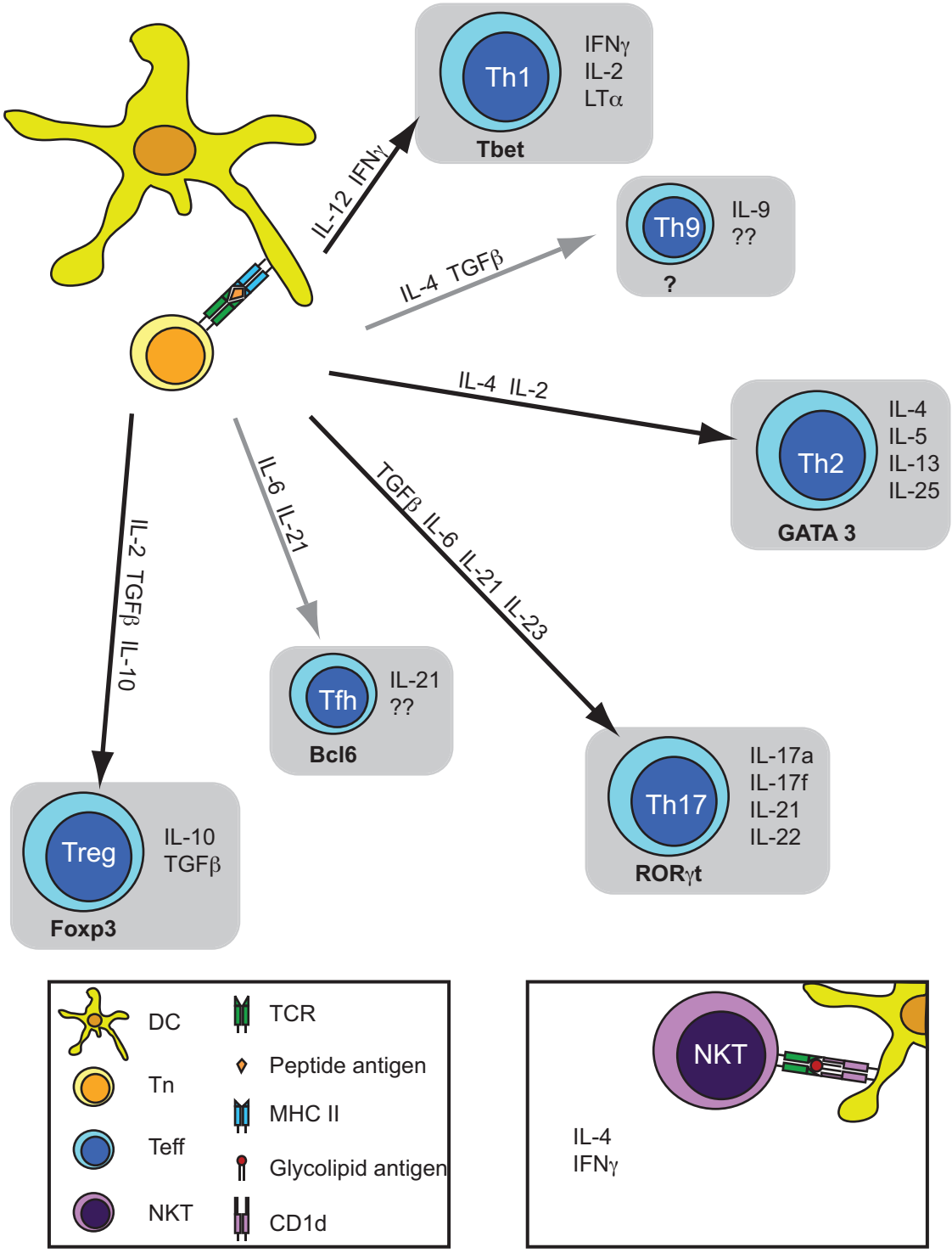

Figure 1.2 CD4 T cell effector lineages.

Naïve CD4 T cells (Tn) are activated by antigen presented on MHC II by APCs, in the presence of coreceptors and costimulatory molecules to become effector T cells (Teff). The black arrows indicate the four commonly accepted CD4 T cell lineages, indicated by grey arrows are two newly suggested CD4 T cell subsets. **Th1 cells:** IL-12 production by DC drive a Th1 response. Effector functions of Th1 cells are controlled by the transcription factor Tbet which drives production of IFNγ. Th1 cells also produce IL-2 and LTα. **Th2 cells:** IL-4 and IL-2 help to drive a Th2 response, although Th2 immune responses can be initiated independently of IL-4[39]. Transcription factor GATA 3 initiates the production of effector cytokines including IL-4, IL-5, IL-13 and IL-25. **Th17 cells:** Generation of Th17 cells occurs in the presence of TGFβ and IL-6. IL-21 mediates cellular expansion and IL-23 is necessary to stabilise the response. Transcription factor RORγt drives the production of IL-17a, IL-17f, IL-21 and IL-22. **Treg cells:** T regs can be derived from the thymus (natural Tregs) or induced upon infection (iTregs) and express the transcription factor Foxp3. Strong TGFβ stimulation on the absence of proinflammatory cytokines can induce antigen specific iTregs. Tregs can act to suppress through production of IL-10 and also produce TGFβ. **Th9 cells:** Associated with Th2 responses, little is known about the Th9 cell. Th9 cells are thought to arise in the presence of IL-4 and TGFβ, and produce IL-9. **Tfh cells:** A recently characterised CD4 cell type Tfh cells can differentiate independently of other CD4 lineages in the presence of IL-6 and IL-21. Tfh differentiation requires the transcription factor Bcl6, and differentiated Tfh assist with antibody production through the release of IL-21. **Inset: NKT cells** also express CD4, but differ from conventional CD4 T cells in that they recognise glycolipid antigen presented on CD1d molecules. NKT cells can produce large quantities of both IFNγ and IL-4.

1.4. Memory

1.4.1. Generation of memory T cells

The generation of cellular memory is a unique feature of the adaptive immune system enabling protective immunity. Both memory T and B cells have important roles to play in protective responses, however this thesis focuses on T cell mediated responses. Memory T cells are long-lived and able to respond rapidly to reinfection. Current dogma accepts two lineages of memory T cell, effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) [59]. T_{CM} preferentially reside in the secondary lymphoid organs and are able to proliferate rapidly upon re-exposure to antigen generating effector cells that can then migrate to the site of inflammation. They are characterised as CCR7+ and CD62L+. T_{EM} reside in the periphery and are characterised by CCR7- and CD62L-. These cells are able to rapidly regain effector function and proliferate upon re-exposure to antigen [60].

The generation of memory T cells is controversial and many models have been put forward. The models are diverse; incorporating the linear model that describes the development of memory T cell from effector precursors and also the intriguing divergent model that shows unequal cell division of an activated naïve precursor gives rise to both effector and memory daughter cells. Other models suggest memory T cells arise at various times during infection and their potency depends on the conditions at their generation. The truth behind the generation of memory T cells is most likely to involve several of the current models (summarised in Fig 1.3) – perhaps dependent on the infectious organism protection is being developed against. Current models of memory T cell lineage generation are well reviewed in Kaech *et al* [30].

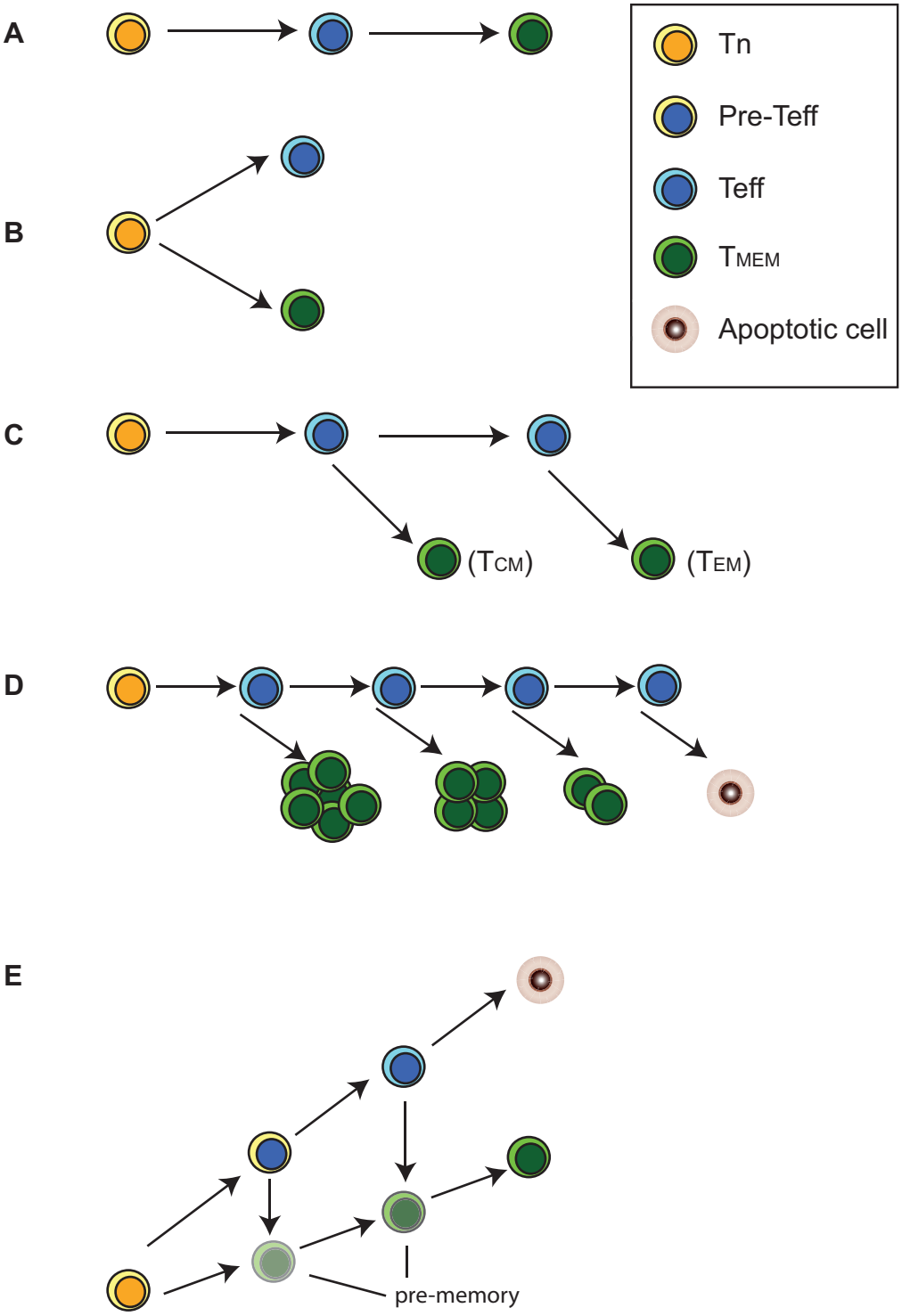

Figure 1.3 Models for the generation of T cell memory.

A. Linear model of memory generation. The linear model of memory T cell generation proposes that naïve T cells (Tn) are activated and differentiated during primary infection to become T effector cells (Teff) before the population contracts and a small subset of cells develop into T_{MEM}. Studies by Jacob and Baltimore and Hu *et al* support to this model [61, 62]. **B.** Divergent model of memory generation. The divergent model of memory T cell generation proposes that naïve T cells give rise to distinct precursors of Teff or T_{MEM} lineage in a branched fashion. Experiments by Chang *et al* support the divergent model of memory generation [63]. **C.** Early and late activation model of memory generation. The early and late model of memory generation advocates that memory subsets (T_{EM}, T_{CM}) are differentially generated depending on whether they arise from early or late Teff. **D.** Decreasing potential model of memory generation. The decreasing potential model of memory generation is based on the idea that T cell effector functions steadily decrease with persisting antigen exposure and memory T cells can be generated at varying times giving rise to functional and non-functional memory cells. This model proposes that the key to memory development is the level and duration of antigen exposure, which dictates whether a cell goes on to become a functional T_{MEM} cell or dies. **E.** Intersecting pathway model of memory generation. The intersecting pathway model incorporates the ideas of antigen exposure, proliferative response and effector potential in the development of memory cells. This model suggests that pre- memory cells (lighter colour) can arise at different times during activation and effector response when T cells have different effector potential and variance in the number of rounds of proliferation they have undergone. Development of memory cells is speculated to occur upon withdrawal of antigen making it antigen independent in contrast to the antigen driven development of effector cells.

1.4.2. Maintenance of memory T cells

An important issue relevant to T cell memory is how both the pool of memory T cells and the diversity of the TCR repertoire within that pool is maintained. The number of memory T cells appears to be stable and the memory T cell pool is under strict homeostatic control to remain at equilibrium. After cell expansion from an immune response a contraction of the memory pool is required, if T cells are lost (as in some viral infections) there is expansion to compensate. Homeostatic control of memory T cells is controlled independently to that of naïve cells, for example, depleting all naïve cells will not cause an increased number of memory cells, although they will represent a higher proportion of total T cells [64].

The pool of memory T cells appears to be maintained by competition for survival factors between each of the antigen specific memory cells. Neither cognate antigen or MHC molecules seem to be required for the long term maintenance of CD4 or CD8 memory cells [65, 66]. Homeostasis of CD8 memory T cells is maintained in part by IL-15; mice that are deficient in IL-15 show a significant reduction in the number of CD8 memory cells. IL-7 also plays a role in maintenance of CD8 memory T cells. Homeostasis of CD4 T cells is not entirely clear. CD4 T cells are unaffected by IL-15 deficiency, however depletion of IL-7 leads to a loss of both naïve and memory T cells. Studies of IL-7 requirements in memory homeostasis are complicated by the fact that an excess of IL-7 will lead to expansion of T cells [67]. Further support for a role for IL-7 in CD4 memory T cell survival comes from the experiments of Garcia *et al* where recovery of adoptively transferred memory cells in IL-7 deficient RAG -/- recipients was impaired [68].

Diversity of the TCR repertoire can itself influence the diversity of the memory TCR repertoire. Low diversity of the memory TCR repertoire can influence the expansion of naïve T cells with novel TCR to achieve a memory compartment with a more diverse repertoire. This adds a further level of complexity to the maintenance of memory T cells. Min *et al* show that monoclonal memory T cells fail to suppress proliferation of polyclonal naïve T cells, whereas polyclonal memory CD4 T cells are capable of suppressing proliferation of naïve T cells [69]. The size of the memory compartment remains constant, so limited diversity in the TCR repertoire would lead to a larger proportion of memory cells specific for a given antigen, and higher diversity to fewer clones of the same specificity.

1.5. Immune system structure and function

The anatomy and structural organisation of the immune system allows it to be efficient at pathogen surveillance and response to infection. The requirements of immunity are not only to respond appropriately to contain and resolve new infections but also to remember foreign pathogens and protect against reinfection with them. Development of lymphocytes from T cell precursors to long-lived memory cells happens within the defined tissues of the immune system. T cell precursors enter the primary lymphoid tissues to develop into antigen specific naïve T cells. The secondary and tertiary lymphoid organs are where induction of immune responses and the development of memory occur as well as activation and effector function [70-72]. That these events occur in secondary lymphoid tissues is clear however the role of tertiary tissues is less defined.

1.5.1. Primary Lymphoid Organs

The primary lymphoid organs are where lymphocytes mature from B- and T-precursors into naïve B and T cells. The thymus has a key role in T cell development and B cells develop in the bone marrow, the remainder of this section focuses on the development of αβ T cells in the thymus.

T cell precursors migrate from the bone marrow to the thymus where they undergo TCR generation in the subcapsular area. Once TCRs are successfully assembled thymocytes begin to express both CD4 and CD8 coreceptors [73, 74] before undergoing the processes of positive and negative selection. Positive selection ensures the TCR is capable of interacting with antigen presented on MHC and also drives lineage development of different T cell subsets based on the interaction of MHC with coreceptors; either MHC I/CD8 or MHC II/CD4 [75].

The now single positive cells (expressing either CD4 or CD8) are subject to the process of negative selection, which causes T cells with high affinity for self-peptides to apoptose in order to eliminate self-reactive cells, which could otherwise lead to autoimmune disease [76]. T cells meeting these criteria emerge into the circulation as single positive non-self reactive naïve T cells [77]. These stringent processes allow for the generation of a population of naïve T cells with a diverse repertoire of antigen specificities against non-self antigens.

1.5.2. Secondary lymphoid tissues

The traditional view of the events involved in the initiation of an immune response is that they are confined to highly organised and structured lymphoid tissues. Secondary lymphoid organs are organised, encapsulated compartmentalised tissues, which are arranged as nodes through which lymphatic fluid draining all peripheral tissues must pass through before entering the bloodstream via the thoracic duct (Fig 1.4). This structural arrangement ensures that the lymph node is the place of first contact between pathogenic antigens and immune cells. Secondary lymphoid tissues are dispersed around the body and include lymph nodes, spleen and Peyers patches as well as some mucosal associated lymphoid tissues. This section will focus on lymph nodes as the major secondary lymphoid organs, however similar structure and processes exist for other secondary lymphoid organs also.

The characteristic anatomical structure and organisation of secondary lymphoid organs creates a tightly regulated yet productive environment where many cell types are drawn together to facilitate the efficient creation of an effector response to invading pathogens. Frequency of naïve cells specific for any one given antigen is estimated at two to 200 cells per mouse [78]. Secondary lymphoid organs are sites where naïve T cells are concentrated, enhancing opportunities to meet antigen presenting cells (APCs) loaded with specific antigen, enabling maturation into effector and memory T cells.

Lymph nodes are organised structurally so that the expression of key chemokines and adhesion molecules directs the arriving cells to migrate into specific areas within the node. Entry into the lymph node is restricted to those T cells that express CD62L (L-selectin) [79], LFA-1 and CCR7 [80], as these molecules mediate rolling, adhesion and diapedesis from the high endothelial venules (HEVs) into the lymph node. The CCR7 ligands CCL21 (expressed on stromal cells) and CCL19 (expressed by macrophages, DCs and some non-hemopoietic cells) are present within T cell areas and direct T cell migration and maintain the organisation of the T cell areas of the secondary lymphoid organs. The expression of CCL21 and CCL19 also facilitate the migration of CCR7 expressing mature DCs to interact with and present antigen to the T cells.

The fate of a naïve T cell within the lymph node is dependant on activation, however effector, memory and naïve T cells all exit through the efferent lymphatics. The sphingophospholipid sphingosine-1-phosphate (S1P) has recently been shown to regulate lymphocyte migration into efferent lymph [81, 82]. A gradient of S1P attracts T cell

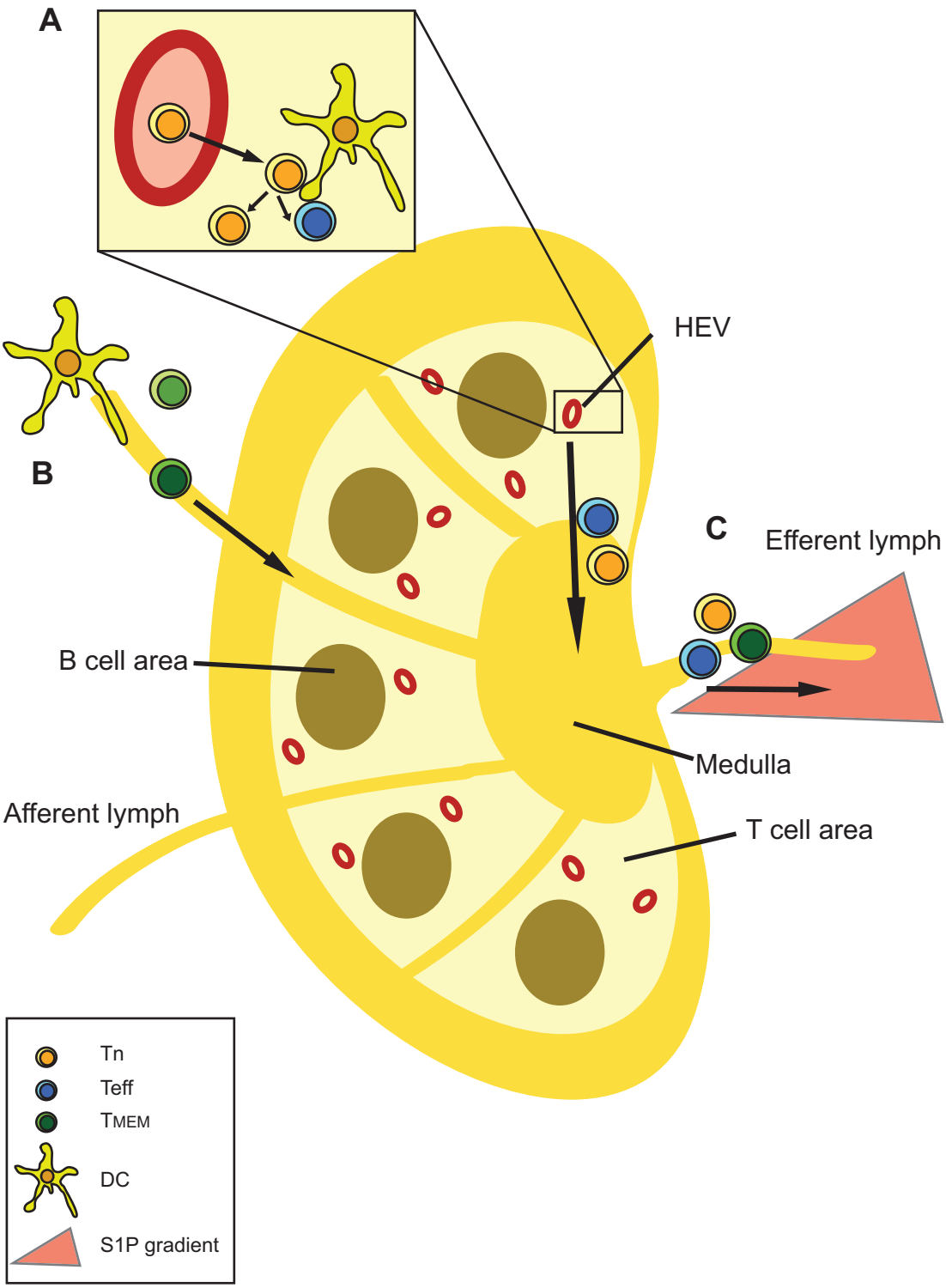

Figure 1.4 Secondary lymphoid anatomical structure and lymphocyte trafficking.

A. Naïve T cells (Tn) enter lymph nodes in a CD62L dependent manner through high endothelial venules (HEV). If Tn meet an APC bearing their cognate antigen they form an immune synapse, and if stimulatory and costimulatory signals are met they become activated effector T cells (Teff) before leaving the lymph node. If Tn do not detect their cognate antigen they exit the lymph node. **B.** APCs and T_{MEM} drain from the tissues through the afferent lymphatics into the lymph node. **C.** T cells egress the lymph node into the efferent lymphatics in response to a chemotactic gradient of sphingosine-1-phosphate.

migration out of the node and into the efferent lymphatics [82], where they return to the blood via the thoracic duct.

1.5.3. Tertiary (peripheral) lymphoid tissues

Tertiary lymphoid tissues are concentrated accumulations of lymphoid cells in non-lymphoid tissues sometimes referred to as peripheral lymphoid tissues. The tissues of mucosal associated lymphoid tissue (MALT) can fall within this category and will be discussed later. Defining secondary vs. tertiary lymphoid tissues can be problematic and controversial, as there is a distinct plasticity to these tissues. A major difference between secondary and tertiary lymphoid tissues is the fact that tertiary lymphoid tissue can arise in almost any organ and seems to be inducible upon infection. A tentative descriptor is that tertiary lymphoid tissues arise when necessary to deal with localised insult, and in some cases can be resolved by treating the initial inflammation instigator (for example with antibiotics).

Tertiary lymphoid tissue can be found in any tissue and provides an important immediate defence, as it is located at the point of pathogen entry in the periphery. The structure of tertiary lymphoid tissue is loosely similar to that of secondary lymphoid tissue, with B and T cell areas (although ratios of T:B cells differ), naïve lymphocytes, DC's and lymphoid chemokines like CCL21. HEV like vessels have been observed in tertiary lymphoid structures and lymphatic vessels have also been detected although whether acting as efferent or afferent is unknown [6].

T cell entry to peripheral tissues is restricted to the T cells recognising the correct "addressins" expressed on endothelial cells of HEVs, similarly to secondary lymphoid tissues. Although homing is still incompletely understood, combinations of selectin and ligand expression by T cells and endothelial cells as well as other myeloid cells determines the homing potential of T cells to peripheral tissues. Studies of T cells in the bronchus associated lymphoid tissue (BALT) have shown a requirement for $\alpha 4\beta 1$integrin as well as L-selectin (CD62L) for T cell entry, with strong staining of VCAM-1 and PNAd on HEVs entering the lung [83]. Skin entry is restricted to T cells that are capable of binding ELAM-1 [84] (expressed in cutaneous venules during chronic inflammation), and $\alpha 4\beta 7$ integrin allows access to the gut tissues [85]. The expression of both selectin and ligand can change in response to the state of inflammation the tissue is undergoing [86] (with the corresponding T cell requirements) and also depend on the activation state of the T cell

itself. Activated effector and memory T cells express different integrin and selectin molecules and therefore respond to different ligands than naïve cells and as such have different tissue accessibility.

Recent evidence suggests that priming of T cell responses is possible in tertiary lymphoid tissue. Naïve T cell proliferation has been demonstrated in islets of NOD mice after the removal of pancreatic lymph node [87]. Bertolino *et al* demonstrate that adoptively transferred transgenic auto reactive CD8 T cells are able to infiltrate the liver of recipient mice, expressing activation markers within two hours of transfer. In addition within 24 hours these cells have divided up to four times and started to recirculate in the blood, where CD8 T cells that were retained in the lymph nodes took between two-three days to recirculate, although showing similar activation and division to the liver based lymphocytes [88]. Feuerer *et al* show the ability of T cells to home to and be primed in the bone marrow [89]. A recent paper by Gelman *et al* discovers that alloreactive T cells are primed within lung tissue independently of secondary lymphoid organs, leading to transplant rejection [90]. Experiments by Wakim *et al* support the proliferation of T cells in peripheral tissues, demonstrating the antigen specific proliferation of HSV specific graft residing T cells after transplantation of previously infected Dorsal Root Ganglion (DRG) into naïve hosts [13]. Research into skin immunity is increasingly finding an important role for tissue residing T cells, independent of those recruited from the circulation [14]. A recent paper by Greter *et al* [91] describes how cell mediated immunity can be initiated in mice lacking secondary lymphoid tissues, with the generation of productive CD4 and CD8 systemic responses and the ability of liver tissue to support T cell priming. Although T cell responses were found to be able to be generated independently of secondary lymphoid tissues, B cell responses were not. Collectively these papers add weight to the idea that T cell priming and initiation of an immune response can occur outside of secondary lymphoid organs.

1.5.3.1. Mucosal Associated Lymphoid Tissue (MALT)

Mucosal Associated Lymphoid Tissues (MALT) are considered to be secondary lymphoid tissues for the most part. MALT structures such as Peyers patches in the Gut Associated Lymphoid Tissue (GALT) and Nasal Associated Lymphoid Tissue (NALT) are well established, encapsulated and structured, similar to secondary lymphoid tissues. However, the categorisation of Bronchial Associated Lymphoid Tissue (BALT) is unclear.

The presence of BALT in mice is diffuse and does not share the same regularity of structure as other MALT such as Peyers patches or NALT [92]. Often BALT does not appear until mice are older, in the case of Balb/c and C57BL/6 mice more than one year, but can be more easily found in NOD mice, especially those older than seven months [83]. Only 43% of four month old mice kept in special pathogen free (SPF) conditions exhibit BALT [93] – suggesting that it is an inducible tissue arising after infection (iBALT) rather than a pre-exisiting tissue such as the Peyers patches present in the Gut Associated Lymphoid Tissue (GALT). Isolated Lymphoid Follicles (ILFs) in the GALT have also been considered as tertiary lymphoid tissue as they are inducible and shaped by microbial stimuli [94]. Splenectomised Lta -/- mice lack secondary lymphoid organs, however these mice can develop iBALT and are capable of mounting effective immune responses [70]. The inducibility of BALT after infection along with its ability to arise in a compensatory fashion for a lack of secondary lymphoid organs in splenectomised Lta -/- mice and lack of consistent structure and location cause me to consider it as a tertiary lymphoid structure/tissue.

1.6. Recirculation of immune cells between lymphoid tissues

The recirculation of immune cells between the different lymphoid tissues allows cells to travel to sites of specialised immune activity. The recirculation of adaptive immune cells between lymphoid tissues is distinct from the migration of innate cells to sites of inflammation. Migration of innate cells such as neutrophils to inflammatory sites happens in a unidirectional fashion. The innate cells are attracted to signals of inflammation, which they follow to the inflamed tissue, exert effector function and perish.

In contrast to innate cells, after development in the primary lymphoid tissues T cells travel to the secondary and tertiary lymphoid tissues where APC are screened for their specific antigen. APCs travel between tertiary and secondary lymphoid tissues presenting antigen to large numbers of naïve T cells. Recirculation and mixing of these immune cells throughout the different tissues and organs of the body is required for the immune system to efficiently detect and respond to infection. We have chosen to focus on T cells as they orchestrate the immune response.

1.6.1. T cell recirculation

In the early 1990's, work by Mackay *et al* investigated the recirculation patterns of naïve and memory T cells in sheep[95]. T cells were divided into naïve or memory phenotype based on the expression of adhesion molecules CD2, CD11a, CD58, CD44 and importantly CD45R; memory T cells exhibited increased CD2, CD11a, CD58 and CD44 expression, but lacked CD45R, where naïve T cells exhibited low adhesion molecules and were CD45R+. T cells from the blood, afferent and efferent lymph were collected and analysed for their activation phenotype, and also for their proliferative capacity. Memory-like T cells were found to accumulate in the afferent lymph, where most cells in the efferent lymph (~90%) exhibited a naïve phenotype. Blood T cells showed a somewhat mixed population. The memory-like T cells in the afferent lymph did not express CD45R, expressed increased adhesion molecules and were shown to proliferate in response to recall antigen. This data led to the conclusion that naïve and memory T cells trafficked differentially; naïve T cells circulating through the blood and HEV to the lymph node, with memory T cells patrolling tissues and returning to the lymph node via the afferent lymphatics [95]. Further work showed that subsets of memory T cells show tissue selective migration patterns which likely develop after antigen encounter in a particular environment, such as the skin (reviewed in [96]) or gut [97], with differential expression of adhesion molecules between the differentially homing cell subsets. A review of lymphocyte recirculation and homing by Butcher *et al* reinforces this view, discussing the ability of memory and effector T cells to recirculate through extra-lymphoid sites, while naïve T cells remain confined to recirculation through secondary lymphoid tissues [79].

Addition of antigen into the immune system understandably alters T cell migration patterns. An increase in T cells migrating through inflamed lymph nodes and a drop in cells travelling through the efferent lymph was observed in experiments by Mackay *et al* [98]. This increased lymph node traffic was largely due to an increase in the number of memory-type T cells recirculating through the node [98]. Experiments by Weninger *et al* followed the recirculation of adoptively transferred *in vitro* generated CD8 effector and memory T cells in the steady state and in inflammatory conditions. They found that memory but not effector cells homed to secondary lymph node and Peyers patches and that this lymph node homing required CCR7 and CD62L for entrance through HEV. Both effector and memory T cells were able to respond to inflammatory chemokines and migrate to sites of inflammation [99]. This data highlights the different migration

capacities of T cells depending on their activation status. Interestingly further experiments by Weninger *et al* suggest that naïve T cells can migrate to peripheral tissues under inflammatory conditions, due to interactions with CCL21 expressed on inflamed endothelium [100], this is particularly interesting in light of the findings of Mackay *et al* [98] where naïve T cells were observed trafficking in the afferent lymph of antigen challenged animals. T cell recirculation between primary, secondary and tertiary lymphoid tissues is described in Fig 1.5.

A recent paper by Cose *et al* demonstrates that a population of naïve T cells can be found in a large number of non-lymphoid tissues under steady state conditions including lung, liver, skin and brain [101]. These cells have been classified as naïve, effector or memory based on the expression of CD11a and CCR7 and further characterised as to CD62L and CD44 expression. The T cells were assessed as to their function and effector cytokine production was observed only in those cells that were $CD11a^{hi}$, and not in the $CD11a^{int}$ $CCR7^{hi}$ naïve subset. To assess the migration of naive cells through peripheral tissues the drug FTY720 was used. FTY720 blocks egress of naïve cells from the lymph node and causes lymphopenia in the blood, it was reasoned that if the $CD11a^{int}$ $CCR7^{hi}$ population seen in peripheral tissues was naïve then the drug would selectively deplete it. The cell populations of the lung, liver and lamina propria were assessed and both CD4 and CD8 percentages were greatly reduced; importantly it was the naïve subset of cells that disappeared following treatment [101]. This preferential depletion of naïve cells from peripheral tissues has also been observed in other independent studies [102]. Further testing of this hypothesis was carried out by generating bone marrow transgenic chimeras; TCR x RAG-/- chimeras were created generating cells which are TCR specific and lacking RAG - ensuring they remain naïve unless stimulated with their specific antigen. These chimeras revealed that although naïve cells predominantly remain within lymphoid tissues, transgenic T cells could be found in non-lymphoid tissues including the brain and testes. Treatment with pertussis toxin did not prevent naïve cells entering peripheral tissues, suggesting that entry is not mediated by chemokine receptors. The implications of this work are that naïve T cells enter non-lymphoid organs as part of a normal migratory pathway [101].

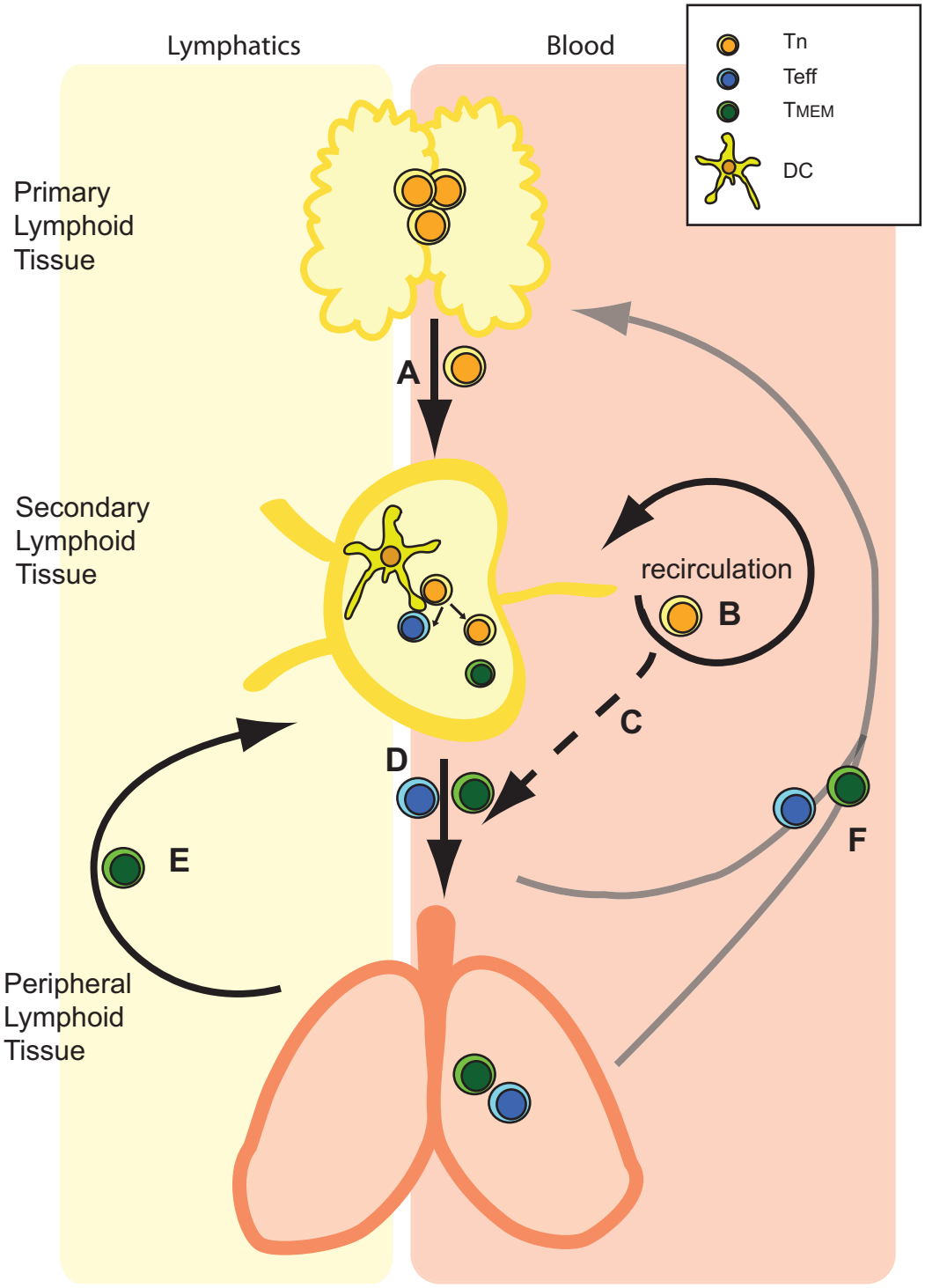

Figure 1.5 T cell recirculation between primary, secondary and peripheral lymphoid tissues.

A. Single positive naïve T cells egress the thymus and enter circulation. Naïve T cells enter lymph nodes from the blood in a CD62L dependant manner. **B.** Naïve T cells egress lymph nodes through the efferent lymphatics and rejoin the circulation through the thoracic duct. **C.** Recent evidence suggests that naïve cells can migrate through peripheral tissues [101]. **D.** Effector (Teff) and memory T cells (T_{MEM}) exit the lymph nodes through the efferent lymphatics and traffic to peripheral sites of inflammation or infection. **E.** T_{MEM} can return to the lymph nodes from the tissues through the afferent lymphatics. **F.** Recent studies suggest that mature memory like T cells migrate back to the thymus to mediate positive selection [103]. Grey arrows indicate controversial recent publications.

Recirculation of antigen bearing DCs is important in the generation of efficient T cell effector responses. Not all DCs are migratory but under inflammatory conditions, mature antigen loaded DC's drain to the lymph node via the afferent lymph. This migration ensures the efficient activation of the immune response in the draining lymph node, although some soluble antigens may drain to the lymph node independently. Other APCs and DC subsets remain in the periphery stimulating the immune response there.

1.7. Current paradigms of protective immunity

Recently a proposition was put forward that the development of a memory population in the tertiary lymphoid tissue is an important goal of the immune response to drive protection against reinfection [104]. This Effector Lymphoid Tissue (ELT) paradigm suggests that the local tissue response plays a critical role in protective immunity by mounting a swift effector response at the site of infection.

In the original opinion paper, it was postulated that ELT could form T_{EM} from Teff cells that had migrated from the lymph node during primary infection. Upon reinfection the T_{EM} could be reactivated rapidly becoming Teff, lymph node derived T_{CM} maintain the supply of Teff to the inflamed tissue. Experiments by David Woodlands group have led to the development of a three phase model for the immune response to control viral reinfection in the lungs [105]. They propose that the recall response to reinfection is mediated by memory T cells (T_{MEM}) residing in the lung airways (phase 1), non-proliferating T_{MEM} that are recruited to the lungs (phase 2) and proliferating T_{MEM} that are recruited to the lung airways (phase 3). Roberts *et al* maintain that the contribution of T_{EM} and T_{CM} changes over time; early after infection Tem dominate the memory pool, however at later times after infection T_{CM} dominate [106]. Using a sendai virus model of infection Ely *et al* show that the CD8 Tem population in the lung is maintained by continual recruitment, indicating a requirement for lymph node derived T cells [107].

Recent work by Wakim *et al* [13] demonstrates in a Herpes simplex viral (HSV) model that locally resident T cells can be presented with antigen in the periphery to initiate and maintain protective immunity. Previously infected dorsal root ganglia (DRG) were grafted to the kidney capsule of recipient mice that were then reinfected with HSV. It was shown that the HSV specific memory CD8 T cells transferred within the graft were able to proliferate after being stimulated by recipient derived DCs, in a CD4 dependent manner.

Further work by this same group demonstrates that when memory T cells are recruited from the circulation to the site of reinfection they are able to proliferate locally rather than being continuously recruited [108]. A recent publication by Gebhardt *et al* describes a unique skin resident protective memory T cell population [109]. Collectively these experiments strengthen the theory that development of localised ELT in peripheral tissues is essential in pathogen containment and protection after reinfection.

1.8. Separating lymph node and peripheral responses: S1P and FTY720

1.8.1. S1P

The naturally occurring small lysosphingophosolipid molecule sphingosine-1-phosphate (S1P) has been found to be involved in a number of cellular processes. The phosphorylation of sphingosine *in vivo* by sphingosine kinases (SphK 1 and 2) [110] generates biologically active S1P. S1P is produced in the plasma by erythrocytes [111] by platelets [112] and is found naturally in the plasma at 0.1-1μM [113] and is produced in the lymph by a distinct radiation resistant cell type [111]. S1P signals through a number of G-protein coupled receptors $S1P_{1-5}$ (first noticed on endothelial cells and named Endothelial differentiating gene - Edg - receptors), with each receptor coupled to a different type of G protein to give a distinct signal transduction (well reviewed in [114]).

S1P receptors are differentially expressed on the cells of the immune system and are associated with a variety of outcomes including chemotaxis, polarity of the immune response and cell proliferation [115]. CD4 T cells predominantly express $S1P_1$ and to a lesser extent $S1P_4$. CD8 T cells also express these receptors and have additionally been shown to express $S1P_5$ mRNA [116, 117].

1.8.2. FTY720

The natural immune suppressive properties of myriocin, an immunosuppressant derived from the fungus *Ischaria sinclairii* led to the development of a less toxic yet more suppressive synthetic analog, FTY720 (2-amino-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride) [118]. Interestingly it was soon discovered that FTY720 had the ability to bind to S1P receptors [82, 117]. Phosphorylation of FTY720 by sphingosine kinases *in vivo* gives the active FTY720-P form; a potent direct agonist of four of five S1P receptors ($S1P_2$ unaffected by FTY720 treatment) [110]. Low nanomolar concentrations of the drug

have been demonstrated to inhibit signalling through receptors 1, 2 and 5 but not 3 and 4 by rapidly inducing prolonged internalisation of the $S1P_R$ [117]. Affected receptors remain internalised up to several days post treatment [117]. FTY720 does not stimulate the activation of the receptor; it simply causes recycling without directly competing with ligand [117]. FTY720 has varying affinity to each of the S1P receptors and receptor ligation by FTY720 can have different outcomes.

1.8.3. Effects on lymphocyte recirculation

S1P has a critical involvement in lymphocyte recirculation, with several hypothesised mechanisms. It has been postulated that the lymphopenic effects of S1P are due to increased chemokine dependent homing to lymphoid organs [119]. Another proposed mechanism behind reduced lymphocyte recirculation was S1P strengthening of endothelial tight junctions (reviewed in Rosen *et al* [120]) however experiments in mice lacking secondary lymphoid organs showed that T cells remained sequestered and reduced in the circulation, showing that a physical endothelial barrier to lymph node egress was unlikely [121].

It has been convincingly shown that S1P is chemotactic to CD4 T cells through $S1P_1$ [122, 123]. A chemotactic gradient of S1P controls lymphocyte egress from the lymph nodes [81, 82], inducing lymphopenia by preferentially depleting naïve T cells from circulation [102]. High S1P levels in the blood facilitate binding to $S1P_1$, which is then internalised. The lymph node environment has very low levels of S1P present, so after entry into the lymph node S1P receptors are re-expressed over time. This re-expression of $S1P_1$ allows the cells to become responsive to moderate levels of S1P emanating from the efferent lymph, causing migration out of the lymph node and into the lymph along a chemotactic gradient [82]. This is shown diagrammatically in Fig 1.6. The chemotactic gradient of S1P is established and maintained by the S1P degrading enzyme S1P-lyase that ensures low S1P levels in lymphoid tissues, with S1P-lyase deficiency also preventing lymphocytes egress (through S1P gradient destruction) [81]. Oral FTY720 treatment has been shown to induce the sequestration of lymphocytes from peripheral blood and spleen, causing homing to lymph nodes [119, 124]. The internalisation of $S1P_1$ caused by FTY720 treatment leads to a blockade of lymphocyte egress by preventing the T cells following the S1P chemotactic gradient into the efferent lymphatics [125].

Activation of T cells has been shown to suppress S1P receptor expression. *In vitro* studies show that activation of T cells with αCD3/αCD28 significantly suppresses S1P receptor expression [116]. TCR dependent activation of T cells results in a decrease in $S1P_1$ expression making the cells less responsive to signal through this receptor [122]. Furthermore it has been shown that $S1P_1$ expression is inversely related to the expression of C-type lectin CD69 (associated with early T cell activation), with surface expression of CD69 suppressing S1P expression [31]. It is thought that this may relate to an extended stay in the lymph node for activated cells allowing them to interact longer in the lymph node environment with Ag bearing APCs – enabling the efficient generation of effector T cells.

The egress of mature T cells from the thymus is also mediated by S1P, specifically CD4 T cells, Tregs and iNKT cells (reviewed in Drennan *et al* [126]). $S1P_R$ agonist administration has been shown to down modulate CD69 expression in thymocytes and also to inhibit thymocyte egress [127]. $S1P_1$ in particular has been shown to play a role in the thymic egress of T cells, as egress of T cells was prevented in a T cell specific $S1P_1$ knock out mouse [128] and also by FTY720 induced retention of $S1P_1$ [125]. Allende *et al* show that $S1P_1$ expression is necessary for dissemination of NKT cells into peripheral tissues, although not essential for their development within the thymus. Furthermore FTY720 treatment did not seem to alter the pre-existing distribution of NKT cells within peripheral tissues [129]. However varying results have been observed with iNKT and Treg cells – "it may be that the relationship between T cell export from the thymus, peripheral recirculation and $S1P_1$ may differ depending on the T cell subset being investigated" [126].

The low S1P concentrations in the tissues allow cells to recirculate through tissues and then to the lymph node. Recent findings by Ledgerwood *et al* speculate that agonism of $S1P_1$ causes lymphocyte retention within the peripheral tissues, due to an inability for lymphocytes to traffic via the afferent lymphatics [130]. The experiments show FTY720 treated lymphocytes are able to initially adhere to, but not cross the basement membrane of the lymphatic endothelium into the afferent lymphatics [130]. A paper by Hofmann *et al* follows the lymphopenia induced by FTY720; finding that while naïve T cells do pass through peripheral tissues during their recirculation, FTY720 treatment preferentially depletes these naïve cells from the periphery [102] and sequesters them in the peripheral lymph nodes [119], with effector/memory phenotype cells being less affected by the drug. It can also be noted that this depletion of effector/memory type cells varied depending on

the organ or tissue being analysed, as lung tissue seemed to be depleted of memory/effector cells more readily than liver [102]. FTY720 treatment was shown to enhance T cell accumulation in Peyers patches due to increased integrin mediated arrest in HEVs. This effect was not shown in $S1P_1$ -/- cells, showing it to be an FTY720 effect independent of $S1P_1$ [131]. An intriguing study performed by Sugito *et al* looks at the effects of FTY720 treatment on mice lacking secondary lymphoid organs. Using splenectomised aly/aly mice, which lack lymph nodes and Peyers patches, it was found that FTY720 treatment still induced lymphopenia in the peripheral blood; prolonging graft survival in this model by preventing infiltration of CD4 lymphocytes into grafts. However the fate of these sequestered lymphocytes is unclear [121].

The accumulating data shows a key role for S1P in regulating the recirculation of lymphocytes between primary, secondary and tertiary lymphoid tissues. This regulation can be exploited experimentally as administration of FTY720 effectively ceases S1P mediated migration of lymphocytes between the lymphoid tissues.

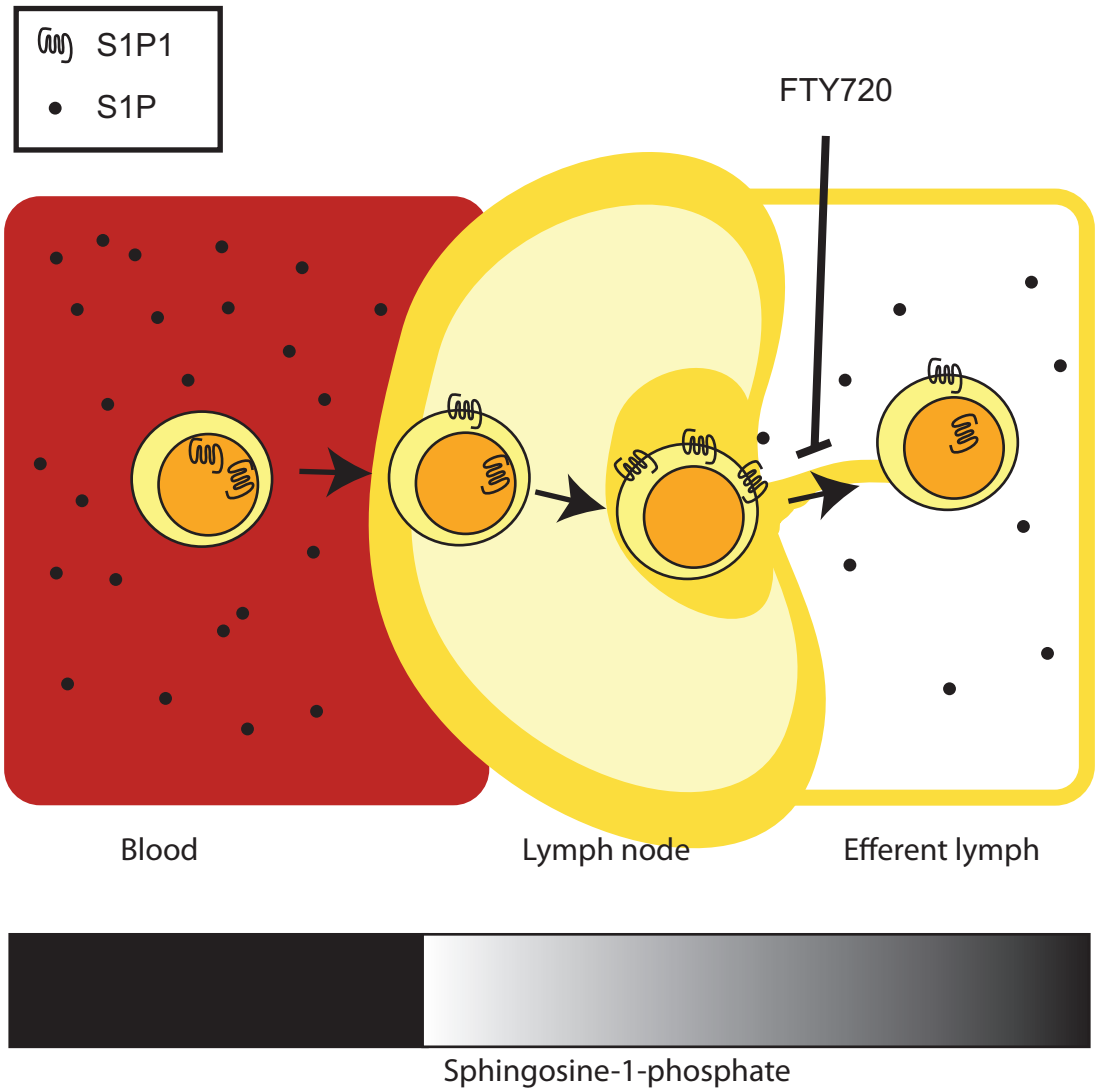

Figure 1.6 S1P controls lymphocyte egress from lymph nodes.

S1P concentrations are high in the blood, leading to $S1P_1$ receptor internalisation. Low S1P in the lymph nodes allows $S1P_1$ receptor re-expression, making the lymphocytes responsive to a S1P chemotactic gradient trafficking out through the efferent lymphatics. FTY720 binds to $S1P_1$ and prevents lymphocyte egress by maintaining receptor internalisation, thus making lymphocytes unresponsive to the S1P chemotactic gradient.

1.8.4. Immunological effects

A review of the immunological effects of signalling through S1P receptors reveals a variety of effects. Inhibition of proliferation has been observed in *in vitro* studies using S1P. T cells stimulated to proliferate with αCD3/αCD28, PMA and ionomycin or allogeneic DC's were inhibited by the addition of S1P [115]. Additionally S1P signalling through $S1P_4$ has been shown to mediate suppression of T cell proliferation and inhibition of effector cytokine production using a combination of $S1P_R$ deficient and transfected cell lines and *ex vivo* splenocytes [132].

FTY720 treatment leads to a reduced expansion of antigen specific CD4 T cells in the draining lymph node, however both *in vitro* and *in vivo* proliferation of antigen specific T cells is unimpaired, as shown using a local antigen-challenge mouse model by Xie *et al* [133]. They suggest that this reduced expansion is due to a low precursor frequency of naïve Ag specific T cells being present in the draining lymph node as a result of the FTY720 mediated reduction in recirculating naïve T cells. The observation that both naïve and activated cells were reduced equally in the peripheral blood, implies that both T cell subsets were unable to egress from the lymph node. Hofmann *et al* show preferential sequestering of naïve T cells from peripheral tissues, this is most likely because the naïve cells are recirculating, whereas peripheral memory cells are tissue residing and not recirculating through lymph nodes for FTY720 to affect them [102]. Lymph node based expansion of CD8 T cells and induction of cytotoxic activity remained unaffected by FTY720 treatment in an LCMV model (as shown *ex vivo*), although footpad inflammation is reduced due to the sequestering effects of FTY720 preventing T cells entering and inflaming the peripheral site [134].

Long term FTY720 treatment (up to six months at ~0.5mg) causes a decline in peripheral T cell numbers over time and a consequent increase in homeostatic proliferation is observed. This in turn changes the composition of naïve/memory T cell pools to smaller populations that have a higher proportion of memory phenotype cells. Memory phenotype T cells are over-represented in FTY720 enhanced homeostatic proliferation. The increased proportion of memory cells correlated with increased functional activity as measured by numbers of IFNγ producing cells after stimulation with phorbol ester and ionomycin; FTY720 treated animals had two-three fold more pre-existing memory cells capable of producing IFNγ than matched untreated mice [135].

Optimal suppressive function of CD4+ CD25+ Tregs requires S1P. Tregs express $S1P_1$ and $S1P_4$ similarly to other CD4 T cell subsets. Using *in vitro* assays Wang *et al* show that Tregs could better inhibit the proliferation and IL-2 production of αCD3/αCD28 stimulated CD4+ CD25- T cells, when exposed to S1P at concentrations consistent with that found in normal blood and lymph. Using FTY720 to down regulate $S1P_1$ also decreased the suppressive activity of these cells, implicating an important role for $S1P_1$ in their suppressive function. Neutralisation of the IL-10 generated by these Treg cells abolished the previously observed suppression. Regulatory T cells are differentially sequestered by FTY720, with CD4+ CD25+ Tregs seen in increased numbers in the blood and spleen of FTY720 treated animals, in contrast to CD4 and CD8 T cells, which are depleted in blood and spleen and accumulate in the lymph nodes [136]. In a Th1 mediated model of colitis FTY720 treatment improved disease by enhancing Treg function. Increased IL-10, TGF-β and FoxP3 expression was measured in FTY720 treated animals and the therapeutic effects of treatment were abrogated by administration of anti-IL-10 or anti-CTLA-4 [137].

S1P dependent migration of APCs can be impaired by FTY720 treatment. FTY720 has been shown to inhibit migration of CFSE labelled skin DC's to the draining lymph node. Czeloth *et al* [138] found that mature, but not immature DC migrated in response to S1P – correlating with an upregulation of $S1P_1$ and $S1P_3$, which was abrogated by FTY720 treatment. Subsequently mature murine dendritic cells have been shown to migrate in a $S1P_3$ dependent manner using *in vitro* DC's prepared from $S1P_3$ deficient mice [139]. This group also demonstrated that pre treatment with FTY720-P significantly inhibited the migration of these DC's. In an *in vivo* model of OVA induced experimental asthma, Idzko *et al* found a direct intratracheal administration of FTY720 into the lung inhibited the trafficking of antigen bearing DC's to the mediastinal lymph node. This method of treatment also rendered the DC's less effective at activating T cells due to a reduced ability to form stable interactions for immunological synapse [140]. In *in vitro* human monocyte derived DC populations, both immature and mature DC's show reduced chemotaxis in the presence of FTY720 and FTY720-P, as well as reduced expression of $S1P_1$ and $S1P_4$ [141]. Work by Martino *et al* looked at the role of S1P during the differentiation of human monocytes into dendritic cells. Including S1P in cultures with GMCSF and IL-4 generated DC's that did not express CD1a, but acquired some aspects of mature DC in the absence of maturation stimuli [142]. The above data clearly show that FTY720 treatment can affect

APC, however more studies will be required to reveal the effects of FTY720 on different APC subsets.

1.8.5. FTY720 in disease models

FTY720 is an excellent candidate drug for a variety of diseases due to its immunosuppressive properties; as such FTY720 has been administered in many disease models with varied success. Kurashima *et al* have abrogated allergic diarrhoea in a food allergy model by preventing trafficking of pathogenic CD4 T cells and mast cells through FTY720 treatment [143]. Eosinophil recruitment and IgE response remained unaffected by the treatment. Using a diabetes model mediated by CD8+ T cells Pinschewer *et al* showed that although CD8 memory induction remained unaffected, the lymphocyte sequestration induced by FTY720 treatment prevented autoimmune destruction of pancreatic islets and corresponding development of diabetes in the majority of animals [134]. Diabetes in NOD mice has also been prevented by FTY720 administration [144]

FTY720 has been evaluated for use in avoiding graft rejection (GVHD) and was found to prevent lymphocyte infiltration into grafts, thereby delaying rejection (reviewed in [145-147]). Experiments by Taylor *et al* [148] suggest that the delay of GVHD caused by FTY720 treatment is due to a number of mechanisms including reducing donor effector cells through lymph node trapping and apoptosis; reduction in splenic DC's thereby reducing effector expansion in the spleen and that the observed delay is additive to the effect of donor Treg cells.

FTY720 treatment reduced severity of disease in a Th2 mediated oxalone colitis model. The therapeutic effects of FTY720 treatment correlated with a reduction of Th2 cytokines IL-4, 5 and 13, but also an inhibition of expression of GATA3 and T1/ST2; which are associated with induction and secretion of Th2 cytokines respectively [149]. FTY720 treatment prevented the development of colitis in SCID mice after transfer of CD4+ CD44 high CD62L - effector memory T cells suggesting that FTY720 treatment may potentially treat memory cell mediated autoimmune conditions [150].

The work of Sawicka *et al* investigates the effects of FTY720 on Th1 and Th2 mediated airway inflammation, delivering *in vitro* generated OVA specific Th1 or Th2 differentiated T cells and oral FTY720 treatment. Both Th1 and Th2 inflammatory infiltrates were reduced after OVA sensitisation with FTY720 treatment and bronchial hyper responsiveness was also reduced in this model [151]. Interestingly a recent paper

investigates the effect sphingosine kinase plays in OVA induced allergic asthma. Using a knockdown of SphK1 they show a reduction in IL-4, 5, eotaxin and IgE [152].

The treatment of wistar rats with FTY720 completely prevented the development of EAE (experimental autoimmune encephalomyelitis) [145] leading to the subsequent testing of FTY720 as a treatment for multiple sclerosis (MS) in humans. FTY720 (known also as Fingolimod) has shown great promise as a treatment for MS with success in phase II clinical trials (proof of concept studies) [153, 154] and is currently in phase III clinical trials.

1.9. Nippostrongylus brasiliensis

N. brasiliensis is a rodent model of hookworm infection. *N. brasiliensis* is an excellent parasitic model to study Th2 immune responses as it generates a very rapid and robust Th2 immune response with production of cytokines, antibodies and recruitment of accessory cells. The generation of this strong Th2 response is CD4 dependent and can occur independently of B cells, eosinophils and CD8 T cells [155]. Additionally *N. brasiliensis* provides a good model to study protective immunity as mice are protected against reinfection.

1.9.1. Parasite lifecycle

The lifecycle of *N. brasiliensis* occurs mostly within the host tissues. Like many parasites, *N. brasiliensis* migrates extensively throughout the host during its life cycle, inducing immune responses at various peripheral sites (Fig 1.7). Infective 3rd stage larvae (iL3) invade the host by burrowing through the skin before migrating through the vasculature until they burst through into the lung parenchyma, where they mature into 4th stage larvae (L4). These L4 are swept up into the oesophagus via the mucocilliary ladder prior to being ingested and establishing a population in the gastrointestinal tract. Here they mature to 5th stage adult worms (L5), mate and lay eggs before being expelled by an immunocompetent host in a “self cure” reaction by day 10 post-infection. The eggs go on to hatch in the infected faeces into 1st stage larvae (L1) before maturing into 2nd stage larvae (L2), and then maturing further into the iL3 parasite.

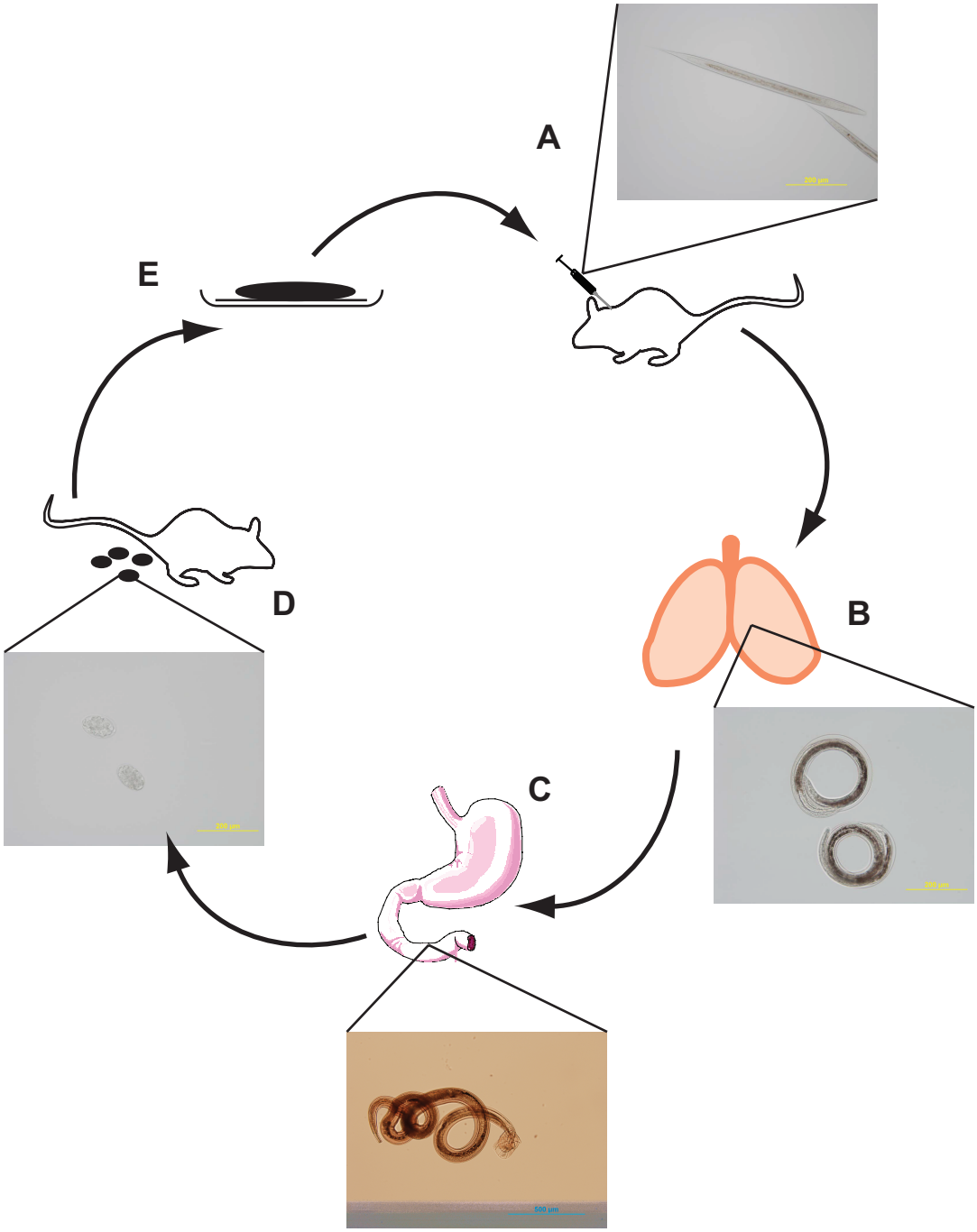

Figure 1.7 Lifecycle and experimental model of *N. brasiliensis.*

The infectious third stage larvae (iL3) of *N. brasiliensis* infects the host through the skin **(A)**. Experimental infection is performed via intradermal or subcutaneous injection in order to reduce variation in number of infecting parasites. From the skin site of infection the worms migrate to the lung tissue **(B)**, arriving from 16 hours post infection. Here the migrating worms mature into fourth stage larvae (L4). Worms can be found in the lung tissue up to day three post infection, after which worms travel up the trachea before being swallowed and establishing a population in the intestine **(C)** which peaks at day six post infection. In the intestine the L4 mature to the adult stage (L5), mate and lay eggs prior to expulsion from the host by day ten post infection (primary infection). The eggs are passed in the faeces **(D)** and when they hatch the resulting L1, and later L2 stage larvae develop within the faecal matter. We recreate this experimentally by making faecal "patties" [156] that are incubated in a moist environment at 26°C. Within one week of incubation, iL3 migrate away from the faecal culture and are ready to infect a new host.

Previous studies with *N. brasiliensis* have explored the mechanisms and requirements of gut expulsion in great detail. Experiments by Finkelman, Urban and colleagues have defined roles for CD4 T cells with IL-4, IL-13 and STAT 6 playing major roles in worm expulsion [157-159]. Studies by Dent and colleagues focus more on the immune response at the skin. Using an air pouch model of *N. brasiliensis* infection in combination with IL-5 transgenic mice and eosinophil deleted mice they propose that boosting eosinophil numbers can confer resistance to reinfection most particularly at the skin site and lung [3, 160, 161]. Shin *et al* also support a protective role for eosinophils in immune responses against *N. brasiliensis* [162].

1.9.2. Immune evasion by parasites

Parasites have evolved with their hosts for millions of years to achieve a fine balance of immune response and infectious load. Too high a burden of parasites will cause host mortality, whilst too much of an immune response will kill the parasites, therefore it is in the best interest of the parasite to maintain a low level chronic infection without inducing too strong an immune response. To this end many parasites have developed immunoevasion strategies, from rapid tissue migration to the manipulation of host immune regulatory mechanisms in an effort to maximise survival.

The rapid tissue migration of parasites has been postulated as a method of escaping the host immune response [163]. *N. brasiliensis* passes rapidly from skin site of infection to the lung before further migration into the gastrointestinal (GI) tract. From studies with this model we know that the during primary infection the Th2 immune response induced by *N. brasiliensis* is maximal well after the worm has left the affected tissue (G. LeGros, M. Camberis, M. Prout, M. Harvie unpublished observation).

Regulatory T cells have been reported as a feature of many parasitic responses [164-168]. The role of Tregs in parasite infection may be to protect the parasite from the host immune response, but also to protect the host tissue against inflammatory damage [163]. Parasite induced Tregs have been shown to suppress unrelated immune responses using bystander suppression [169, 170].

The development of alternately activated macrophages (AAM) in *N. brasiliensis* infection has been documented [19, 171, 172]. They appear to function in a regulatory role and it has been suggested that AAMs are active in parasitic infection in three different ways; firstly suppressing the inflammatory response to the parasite, secondly releasing molecules that

directly affect the parasite and lastly repairing tissue damage caused by the parasite infection [165]. Physical changes to the lung after *N. brasiliensis* are marked [171, 173]. The delicate tissues of the lung are damaged during worm migration and the postulated role of AAMs in wound repair and tissue remodelling would be advantageous to the host.

1.10. Aims

While the development of cellular memory is critical to establish protective immunity against many pathogens, the relative importance of peripheral tissues in this process is unclear. The *N. brasiliensis* model is a useful tool to study the relevance of peripheral immune responses in protective immunity, as during parasite migration immune responses are initiated at multiple peripheral tissue sites within the host. Use of G4/IL-4 reporter mice provides a sensitive measure of the *in vivo* immune response with minimal *ex vivo* manipulation. Drug treatment with FTY720 provides a tool to separate the immune responses of secondary and tertiary lymphoid tissues *in vivo*, to determine their roles in protective immune responses. Separation of the immune responses of secondary and tertiary lymphoid tissues using FTY720 in a Th1 mediated model will further investigate the roles of these different lymphoid tissues in protective immunity. The use of G4/IL-4 reporter mice and FTY720 treatment in the *N. brasiliensis* model provides us with the framework to address the following aims;

- To determine which tissues are key for priming protective CD4 T cell responses against *N. brasiliensis*.
- To compare the relative contribution of CD4 T cell responses of the lung and lymph node using FTY720 to block T cell recirculation.
- To characterise the key CD4 T cell subtypes of the protective response against *N. brasiliensis*.
- To compare these findings in a Th1 mediated model of protective immunity

Chapter 2:
Materials and Methods

2.1. Materials

2.1.1. Labware

Product	Supplier/Distributor
Acrodisc® 25mm syringe filter with 0.2μm Supor® membrane	PALL lifeSciences, Cornwall U.K
Axygen Microtubes 1.7 mL Falcon® Polystyrene	Axygen Scientific Inc., Union City, CA, USA
BD 10, 5, 2 mL Syringes & BD 1 mL tuberculin syringes and BD Ultra-fine™ 29 gauge needle & syringe	BD BioSciences, Bedford, MA, USA
Cheesecloth/muslin fabric	Spotlight
Eppendorf Combitips plus	Eppendorf AG, Hamburg, Germany
Falcon® Polystyrene round bottom tubes: 5mL	BD BioSciences, Bedford, MA, USA
Falcon® Polystyrene tissue culture plates: 6 well plates, Multiwell plates & Microtest™ U-bottom 96 well plates, V-bottom	BD BioSciences, Bedford, MA, USA
Falcon® Polystyrene conical tubes: Blue max 50 mL and Blue Max Jr 15 mL	BD BioSciences, Bedford, MA, USA
Falcon® Polystyrene serological pipettes	BD BioSciences, Bedford, MA, USA
Nylon cell strainers (70μm and 40μm)	BD BioSciences, Bedford, MA, USA
Nylon gauze (70μm)	NZ Filter specialists Ltd., Onehunga, Auckland, NZ
PrecisionGlide™ Needles (25, 23, etc gauge)	BD BioSciences, Bedford, MA, USA
TitreTubes® Micro Tubes	BioRad, Hercules, CA, USA
Transfer pipettes (1 mL)	Samco Scientific, Mexico
Ultra-Fine™ needle Insulin syringes (29 gauge)	BD BioSciences, Bedford, MA, USA

2.1.2. Reagents and buffers

2.1.2.1. Cell culture and flow cytometry reagents

Alsever's Solution

Dextrose (BDH Laboratory Supplies, Poole, England), NaCl (BHD Laboratory Supplies, Poole, England) and sodium citrate.2 H_2O (BHD Laboratory Supplies, Poole, England) were added to 1 L distilled (MilliQ) H_2O to give final concentrations of 20.5 mg/mL dextrose, 4.2 mg/mL NaCl and 8.0 mg/mL sodium citrate.2 H_2O. The pH was adjusted to 6.1 with 1 M citric acid (BDH Laboratory Supplies, Poole, England). The solution was filter-sterilised and stored at room temperature.

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)

CFSE was purchased from Molecular Probes (Eugene, OR, USA) and was stored at -20°C, as a 10 mM solution in DMSO (Sigma, St. Louis, MO, USA).

Collagenase I

Collagenase I was purchased from Invitrogen (Auckland, NZ). The lyophilised powder was stored at 4°C and made up fresh in IMDM to a concentration of 2.4 mg/mL before use.

DNase I

DNase I was purchased from Roche (Mannheim, Germany). The lyophilised powder was dissolved to a concentration of 10 mg/mL in PBS and stored at -20°C.

Enzymatic digestion solution

Collagenase I and DNAse I were added to cIMDM, to give a final concentration of 2.4 mg/mL collagenase I and 120μg/mL DNAse I. The solution was prepared fresh as required.

Fluorescence Activated Cell Sorting (FACS) Buffer

NaN_3 (Sigma, St. Louis, MO, USA) and FBS were added to 1 L PBS, to give final concentrations of 0.01 % NaN_3 and 2 % FBS. The buffer was stored at 4°C.

FACS sort buffer

FBS and DNAse I were added to PBS to give final concentrations of 5% FBS and 0.002% DNAse. The buffer was stored at 4°C.

Foetal Bovine Serum

Mycoplasma and virus screened and performance tested FBS, with 27 EU/mL endotoxin, was purchased from GIBCO (Invitrogen, Auckland, NZ), and stored at -20°C.

Iscove's Modified Dulbecco's Medium (IMDM) IMDM supplemented with GlutaMAX™, 25 mM HEPES buffer and 3.024 mg/L $NaHCO_3$ was purchased from GIBCO (Invitrogen, Auckland, NZ).

Complete Iscove's Modified Dulbecco's Medium (cIMDM)

cIMDM was made by adding Penicillin-Streptomycin (GIBCO, Invitrogen, Auckland, NZ), 2 ME and FBS to 0.5 L IMDM to give final concentrations of 100 U/mL Penicillin, 100 μg/mL Streptomycin, 55 μM 2 ME (GIBCO, Invitrogen, Auckland, NZ) and 5 % FBS. cIMDM was stored for up to 1 month at 4°C.

Magnetic Cell Sorting (MACS) Buffer

EDTA, BSA and DNAse I were added to 1 L PBS to give final concentrations of 2 mM EDTA, 0.5 % BSA and 0.012% DNAse I. The buffer was filter-sterilised and stored at 4°C.

2 Mercaptoethanol (2 ME)

2 ME was purchased from Sigma (St. Louis, MO, USA) as a 55 mM (1000 x) solution in PBS and stored at 4°C.

Phosphate Buffered Saline (PBS)

One 47.8 g bottle of Dulbecco's PBS without $CaCl_2$ and $MgCl_2$ (GIBCO, Invitrogen, Auckland, NZ) was dissolved in 0.5 L distilled (MilliQ) H_2O to make a 10 x solution or in 5 L d H_2O to make a 1 x solution. pH was adjusted to 7.4. The solutions were filter sterilised and stored at 4°C.

Red Blood Cell Lysis Buffer

Purchased from Sigma (St. Louis, MO, USA) as a 8.3g/L NH_3Cl solution in 0.001M Tris-HCl, pH 7.5.

2.1.2.2. Antibodies and flow cytometry reagents

Antibodies specific for murine markers

Specificity	Clone	Source
FcγRII/III (CD32/CD16)	2.4G2	Purified from a B cell hybridoma line
B220	RA3-6B2	Purchased from BD Pharmingen™
CD3	2C11	eBioscience
CD4	GK1.5 RM4-5	Purchased from Invitrogen
CD8	56-6.7	Purchased from BD Pharmingen™
CD44	IM7	Purchased from BD Pharmingen™
CD45	30-F11	Purchased from BD Pharmingen™
CD45RB	16A	Purchased from BD Pharmingen™
CD62L	Mel 14	Purchased from BD Pharmingen™
CD127	A7R34	eBioscience
TCRβ	H57-597	Purchased from BD Pharmingen™

Intracellular and Isotype Control Antibodies

IFNγ	XMG1.2	Purchased from BD Pharmingen™
Rat IgG1 - APC		Purchased from BD Pharmingen™

Flurophores

Specificity	Source
SA-Qdot 565	Purchased from Molecular Probes
SA-PE	Purchased from BD Pharmingen™
DAPI	Purchased from Invitrogen

2.2. Methods

2.2.1. Mice

2.2.1.1. Maintenance and Ethical approvals

All mice used in these experiments were bred and maintained in the specific pathogen free (SPF) Biomedical Research Unit (BRU), Malaghan Institute of Medical Research, Wellington, New Zealand, with the exception of inbred athymic nude mice. Nude mice were imported from the Animal Resource Centre (Canning Vale, Australia), quarantined according to MAF regulation for 30 days and maintained in the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. Where indicated mice were housed in individually ventilated cages (IVC) maintained at 60-100 air changes per hour.

Age and sex matched mice were used in all experiments, from the age of six weeks. All experimental procedures described in this study were approved by the Victoria University Animal Ethics Committee (AEC #2006R20, #2006R19, #2009R14M, 2009R8M) and carried out in accordance with the guidelines of the Victoria University of Wellington, New Zealand, and the standard operating procedures (SOP) of the BRU, Malaghan Institute of Medical Research, Wellington, New Zealand.

2.2.1.2. Mouse strains

B6Aao (MHC II-/-) mice were from breeding pairs obtained from Biological Research Laboratories Ltd. Wolferstrasse 4, Switzerland [174]. These mice are maintained in IVC units.

Balb/c mice were from breeding pairs obtained from the Jackson Laboratory (Bar Harbour, ME, USA).

Balb/c STAT 6 -/- mice were from breeding pairs originally derived from mice generated by Kaplan et al [175].

C57BL6 mice were from breeding pairs obtained from the Jackson Laboratory (Bar Harbour, ME, USA).

CD1 -/- mice (on a C57BL6 background) were obtained from C.R. Wang (the Department of Pathology, University of Chicago, IL, USA). Generated as described in Chen et al [176].

eGFP mice [177] (on a C57BL6 background) were from breeding pairs obtained from the Hercus Taieri Research Unit, Dunedin, NZ.

Foxp3^{gfp} mice were from breeding pairs obtained from and were used with permission of A. Rudensky [178].

G4/IL-4 mice were from breeding pairs obtained from W. E. Paul at the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda MD 20892-1892, USA [179].

Nu/Nu homozygous mice on a Balb/c background (BALB/c-Foxn1nu/ARC) were purchased from the Animal Resources Centre (Canning Vale, Western Australia).

2.2.1.3. Drug treatments

FTY720 treatment Lyophilised FTY720 (Novartis Biopharma) was dissolved into sterile milli Q water and frozen in 10mg/mL aliquots at -80°. FTY720 was further diluted for use in *in vivo* experiments and stored at required concentration at -20°C. FTY720 was administered by daily gavage at a dose of ~0.5mg/kg unless otherwise stated.

Anti-helmintic treatment pyrantel embonate was administered where specified by oral gavage daily, at a concentration of 1mg/mouse.

Antibiotic treatment To clear remaining bacilli after BCG vaccination, mice received 1mg/mL of isoniazid (Sigma-Alderich, St Louis MO) in their drinking water, daily for 4 weeks. Following treatment, CFU of BCG were undetectable in lung or spleen homogenates from vaccinated mice.

Anaesthetic Administration Mice were injected i.p. with a ketamine/xylazine mix at a final concentration of 8.6mg/mL ketamine and 0.26mg/mL xylazine. Anaesthetic was administered at 10μL/g for restraint purposes, or at 1mL/mouse for lethal overdose.

2.2.2. Nippostrongylus brasiliensis model

N. brasiliensis is maintained by passage through Lewis rats [156].

2.2.2.1. Parasite preparations

Preparation of infective L3 Infective L3 were harvested from faecal cultures and washed (worms suspended in sterile PBS, briefly pulsed in the centrifuge to "pellet", PBS discarded, then worms resuspended in sterile PBS) three times before being counted and suspended in PBS at the correct concentration for infection.

Preparation of dead infective L3 Infective L3 were harvested from faecal cultures and washed five times in sterile PBS (as above). Larvae were made up to a concentration of ~600 iL3 per 30μl in PBS and put through three freeze-thaw cycles. Larvae were then checked for viability.

Recovery of L4 lung dwelling larvae The lungs of *N. brasiliensis* infected mice (or rats) were collected at day two post infection, diced, placed on cheesecloth and suspended in PBS at 37°C for at least 2 hours. Viable worms were migrated out and accumulated at the bottom of the container. These recovered worms were then washed at least three times (as above) in sterile PBS before being used for infection.

Recovery of L5 adult worms The gut tissues of *N. brasiliensis* infected mice (or rats) were collected at day 5-6 post infection, diced, placed on cheesecloth and suspended in PBS at 37°C for at least 2 hours. Viable worms migrated out and accumulated at the bottom of the container. These recovered worms were then washed at least three times in sterile PBS (as above) before being used for infection.

2.2.2.2. Parasite infections

Primary infections, unless otherwise stated, primary infections consist of ~600 infective L3 administered subcutaneously in the scruff of the neck.

Challenge infections mice were reinfected at 30 days post primary infection (unless otherwise stated) with ~600 infective L3 administered subcutaneously in the scruff of the neck (unless otherwise stated).

For intradermal infections, mice were anaesthetized using xylazine and ketamine (Phoenix, New Zealand) and 30µl sterile PBS containing 600 dead or live infective L3 were injected into the ear pinnae using a B-D Ultra-fine™ 29 gauge needle & syringe (Becton-Dickinson, NSW, Australia).

For subcutaneous infections, mice were physically restrained and injected with 200µL sterile PBS containing ~600 infective L3 subcutaneously into the scruff of the neck using a 1mL syringe and 25 gauge needle (PrecisionGlide).

For intranasal infections, mice were anaesthetized using xylazine and ketamine (Phoenix, New Zealand) and 50µL sterile PBS containing either L4 worms was instilled into the nostrils using a glass pipette.

Helminth segregated priming Mice were infected with 600 live infective L3 on day 0 and re-infected with live iL3 on day 30 to investigate protection. L4 priming was achieved by intranasal administration of ~200 freshly harvested L4 stage. L5 priming was achieved by gavage of ~300 freshly harvested L5 adult worms. Anti helminthic pyrantel embonate was administered where specified by oral gavage daily, 1mg/mouse.

2.2.2.3. Parasite migration assays

***In vitro* Skin Penetration Assay**. Abdomen skin was shaved and 2cm^2 was harvested from naïve or previously infected mice. Skin was placed on 15ml tubes containing PBS. 200 L3 were added to each tube and incubated overnight at 37°C. Larvae penetrating the skin accumulated at the bottom of the tube. Method adapted from Brown *et al* 1999 [180].

Tissue migration assay. Tissue (lung or gut) was diced, placed on cheesecloth and suspended in PBS at 37°C for at least 2 hours. For ears, dorsal and ventral sheets were separated placed on cheesecloth and suspended in PBS at 37°C. Viable worms migrated out and accumulated at the bottom of the container, before being counted on gridded plate.

CFSE labelling of parasites. Infective L3 stage were washed several times in PBS then incubated at room temperature for 8 minutes in 2.5mM carboxyl fluorescein succinimidyl ester (CFSE). Worms were then washed three times in PBS before infection into host.

Imaging of larvae in ear tissue. CFSE labelled L3 were injected intra-dermally into the ear pinna. Ears were excised and dorsal and ventral sheets separated. Images were captured using Olympus BX51 microscope, Olympus DP70 camera and AnalySIS LS software.

2.2.3. Bacillus Calmette-Guèrin (BCG) model

2.2.3.1. Infections

BCG subcutaneous vaccination *M. bovis* BCG Pasteur strain 1173P2 was briefly sonicated and diluted in phosphate buffered saline solution (PBS). Mice were vaccinated s.c. on the left flank with 100µL of PBS containing 1 $x10^6$ colony forming units (CFU) of BCG.

BCG intranasal infection Mice were anaesthetized with ketamine and xylazine i.p. prior to an intranasal challenge with 1 $x10^4$ CFU of BCG in 50µL of PBS.

2.2.3.2. Determination of bacterial load

Tissue samples were mechanically homogenized in 3mL of PBS with 0.5% Tween 80 and plated in tenfold serial dilutions on selective Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (BD, Franklin Lakes NJ). Plates were incubated at 37°C for 2-3 weeks before counting colonies.

2.2.3.3. T cell restimulation

To evaluate IFNγ production by $CD4^+$ T cells, $CD45^+$ cells from individual lungs were cultured overnight at a concentration of 2 $x10^6$ cells/mL in supplemented IMDM with 10µg/mL *M. bovis* culture filtrate protein (CFP) (AgResearch, Hopkirk Building, Palmerston North, New Zealand) and 2µg/mL anti-CD28 mAb (clone 37.51). Monensin was added to the cultures 2 hours prior to harvest to enable intracellular staining.

2.2.4. Preparation of lymphocyte suspensions from tissue

Blood was collected (either from the tail vein or from the vena cava of euthanised animals) into 1.5mL microtubes (eppendorf). A known volume was pipetted into 500µL of Alsievers solution and stored on ice. Collected blood was centrifuged at 1200 rpm for 8

min and the resulting soft pellet was incubated with red blood cell lysis buffer (Sigma, St. Louis, MO, USA) at 37°C, before being washed and resuspended in IMDM.

Lung tissue was perfused with 10mL sterile PBS (with the exception of those mice receiving BCG infection) before being stored in IMDM on ice. Lungs were finely minced and enzymatically digested in 5mL of enzymatic digestion solution for 1 hour at 37°C. Lung cells were resuspended using a 1mL pipette to break up any clumps before being passed through a 70μM cell strainer, washed in IMDM and then counted. The resulting single cell suspension was incubated with CD45+ magnetic microbeads (Miltenyi Biotec, Germany) as per manufacturers instructions. After incubation with MACs beads, cells were washed in MACs buffer and passed through a 40μM cell strainer before being separated on an AutoMACs separator (Miltenyi Biotec, Germany) using POSSEL programme for positive selection of CD45+ cells. Collected CD45+ cells were then counted using trypan blue exclusion to exclude dead cells.

Lymph Nodes were harvested into IMDM before being pressed through a 70μM cell strainer with a sterile 3mL syringe plunger for a single cell suspension. Cells were then washed in IMDM and counted using trypan blue exclusion to exclude dead cells.

Alternatively, after harvest into IMDM the lymph nodes were enzymatically digested. Lymph nodes were placed into 1mL of enzymatic digestion buffer and the capsule was physically disrupted with a fine gauge needle before being incubated for 1 hour at 37°C. After digestion cells were resuspended using a 1mL pipette to break up any clumps before being passed through a 70μM cell strainer, washed in IMDM and then counted using trypan blue exclusion to exclude dead cells.

Spleens were harvested into IMDM before being pressed through a 70μM cell strainer with a sterile 3mL syringe plunger for a single cell suspension. Red blood cells were lysed using Red Blood Cell Lysis buffer (Sigma) at 5mL/spleen for 2 min at 37°C. Cells were then washed in IMDM and counted using trypan blue exclusion to exclude dead cells.

2.2.5. Adoptive transfer of T cells

Preparation and adoptive transfer of immune CD4 lymph node T cells into the lungs of mice. Donor mice were infected with ~600 infective L3. After at least 28 days rest mediastinal lymph nodes were harvested and enzymatically digested for one hour at 37°C.

Cells were washed and then enriched for CD4+ cells using MACsbeads (as per manufacturers instructions) and an AutoMACs separator (Miltenyi Biotec, Germany). Recipient mice were anaesthetised using Xylazine and Ketamine (Phoenix, New Zealand). CD4 enriched cells were adoptively transferred intranasally to recipients in a volume of 50μL PBS.

CFSE labelling of cells Cells were washed in PBS before suspension at a concentration of $2x10^7$/ml. Immediatey prior to use CFSE was diluted in PBS to give a final concentration of 2.5μM before being added in an equivalent volume to the cells, to a new tube and vortexed to mix well. Cells were then incubated for 8min at room temp, and mixed occasionally. To quench staining an equal volume of FBS was added before four washes in cIMDM, transferring to a new tube with every wash.

2.2.6. Flow Cytometry

2.2.6.1. Detection of surface expressed molecules

To evaluate surface expression of molecules cells were resuspended in FACS buffer and Fc receptors blocked before staining with primary monoclonal antibodies. Cells were washed and if required a secondary antibody was applied. Once stained cells were suspended at a maximum number of 1 x 10^7 and collected on a four colour flow cytometer. If cells were to be collected on the LSR II multicolour flow cytometer (Beckton-Dickinson, Mountain View, CA, USA) addition of DAPI immediately prior to collection allowed exclusion of dead cells.

2.2.6.2. Detection of intracellularly expressed molecules

To evaluate IFNγ production by CD4 T cells, cells were restimulated as described above. Monensin was added to the cultures 2 hours prior to harvest. Cells were washed and blocked for FcγRI/II using anti-CD32/16 mAb and then labelled with anti-CD4-PerCP before being fixed and permeabilized for intracellular staining with anti-IFNγ-APC or isotype control APC -labelled antibodies (BD Pharmingen™). At least 100000 events were collected from the lymphocyte gate for each sample.

2.2.6.3. Acquisition and analysis

Live cells were resuspended in 200 μL of FACS buffer, for acquisition. Live cells were identified based on Forward Scatter (FSC) and Side Scatter (SSC) properties. When using the LSR II for collection, cells were diluted with a solution of DAPI at a final concentration 50ng/mL immediately prior to collection. Unlabelled samples and single labelled controls for each fluorochrome were included in all cases for calibration of voltages and compensation. In some cases isotype control antibodies were used to control for background fluorescence caused by non-specific antibody binding. Data was acquired using a FACSort, FACScalibur (both four colour capacity) or LSR II flow cytometer (Beckton-Dickinson, Mountain View, CA, USA) and analysed using FlowJo software (Tree Star, San Carlos, CA, USA).

2.2.6.4. FACS sorting of cells

Cells were sorted by the staff of the flow cytometric suite on a FACSVantage cell sorter, using DIVA software (Beckton-Dickinson, Mountain View, CA, USA).

2.2.7. Statistics

Statistical calculations were performed using the Graphpad Prism® Version 4 statistical package (Graphpad Software Inc., San Diego, CA, USA).

Student's t test analysis was used when comparing between two samples

One-way ANOVA was used to analyse the variance of multiple groups

Tukey post test was used to compare variances between groups

Dunnets post test was used to compare variances to a selected control

The assumptions of a one-way ANOVA are

- Normal distribution of sample
- Independent samples
- Equal variances

If these assumptions are not met then a more stringent non-parametric Kruskal Wallis or Mann-Whitney test was used.

Two-way ANOVA was used to analyse the variance of multiple groups in relation to a second X variable, usually time (days). When samples were repeatedly taken from the same animal a repeated measures analysis of variance was used, with time as the repeated factor and group as the between subjects factor.

Bonferroni post test was used to compare variances between groups

The assumptions of a two way ANOVA are

- Normal distribution of sample
- Independent samples
- Equal variances

If these assumptions are not met then a more stringent non-parametric Kruskal-Wallis or Mann-Whitney test was used.

Specific statistical tests used are detailed in figure legends.

Chapter 3:
The lung is a key site for protective immunity in a *Nippostrongylus brasiliensis* model of infection

3.1. Introduction

This thesis investigates the recirculation of protective CD4 T cells in a parasitic model of protective immunity. In the *N. brasiliensis* model immune responses are initiated at multiple peripheral sites. The chapter herein examines the critical peripheral tissues required for the priming of CD4 T cells to confer immune protection.

3.1.1. Nippostrongylus brasiliensis

During migration through the host *N. brasiliensis* induces immune responses at three distinct peripheral sites; skin, lung and gut. Exposure to an infection with *N. brasiliensis* confers immunity to the host upon reinfection with the parasite, resulting in a reduced worm burden in mucosal sites. As such *N. brasiliensis* can be used as a model of protective immunity and the following experiments describe the levels of protection seen in the various tissues and the corresponding immune response.

The skin is the initial site of infection where iL3 invade the skin barrier and enter the host. iL3 are the only stage capable of penetrating through otherwise intact skin. Experiments have been performed in IL-5 transgenic and deficient animals using air pouch models to study the immunity against parasites at the skin site. From data collected using an air pouch model, Dent and colleagues have proposed a role for eosinophils in parasite killing at the infection site using knock in IL-5 transgenic mice [3].

iL3 burst through into the lung parenchyma as early as 16 hours post-infection. The lung migration is traumatic for the host tissue as the migrating worms cause damage to the delicate lung tissue [171], damage that can easily be seen upon dissection as red petechia on the lung surface. It is here in the lung that the iL3 mature into L4 stage parasites. There are few reported experiments investigating lung immune responses in *N. brasiliensis* infection, particularly examining protective immunity. However, work investigating vaccination strategies in other parasite infections have highlighted the importance of this organ. A recent publication by Knott *et al* [160] proposes that damage during the lung phase, or pre-lung phase is responsible for reduced worm burdens in FVB/N mice, a strain found to be resistant to *N. brasiliensis* infection. Experiments by Reece *et al* investigate the impact of *N. brasiliensis* infection on the lung environment at mRNA transcript level and find that *N. brasiliensis* infection induces the development of alternately activated macrophages in a STAT 6 dependent manner [173]. This finding builds on the earlier work

of Marsland *et al* who also investigated lung pathology and saw the induction of alternately activated macrophages after *N. brasiliensis* infection [171]. Both of these groups focused on the physical pathological changes in the lung and related the parasite induced alternately activated macrophages to the ongoing repair of tissue damage.

Extensive research has focused on investigating immune responses at the gut site of infection in the *N. brasiliensis* model. Dependent on CD4 T cells and STAT 6, gut expulsion is mediated by the production of IL-4 and IL-13 by immune cells that acts on goblet cells to increase mucous secretion and also causes contraction of smooth muscle leading to the expulsion of the worms[158]. IL-5 and IL-9 are also thought to play a role in worm expulsion[181].

3.1.2. Peripheral tissue sites of N. brasiliensis *migration*

The initial site of *N. brasiliensis* infection is the skin. The skin is of course a site of much host interaction with foreign pathogens. The skin offers a physical barrier to protect the host, but this is easily penetrated by the iL3 during *N. brasiliensis* infection. Similarly to the lung and gut mucosal sites, the skin is well vascularised and immune cells traffic in a directed manner to the skin. Many immune cells are present within the skin, leading to the theoretical concept of skin associated lymphoid tissue (SALT) [182], similar to the previously introduced MALT tissues (chapter 1). Specialised subsets of APC reside within the skin tissues, including Langerhans cells and dermal DC. These APC are activated upon infection and can present antigen locally to induce proliferation of skin residing T cells [13]. Recently it has been proposed that skin resident T cells rather than recruited T cells, have a major role in skin immune homeostasis and pathology, from work with human psoriasis [183]. The skin is also a site of allergic Th2 mediated responses including dermatitis, psoriasis and eczema.

The lung tissue is also traversed by *N. brasiliensis* larvae as they pass through the host. Similarly to the skin the lung is a site that is constantly exposed to inhaled foreign pathogens. The lung has a significant population of immune cells present, including APC such as lung resident alveolar macrophages and alveolar DC, and also T cells. Recently it has been proposed there are as many as five distinct subsets of DC present within the lung [15]. The formation of BALT has been documented in many species including mouse [71, 92, 93]. In the absence of secondary lymphoid organs, protective immune responses have been shown to occur within the lung tissues [70].

The final host tissues of the *N. brasiliensis* migration are the gut tissues. The gut is also an important immune site that is frequently exposed to ingested foreign material. The gut contains MALT in the form of Peyers patches, isolated lymphoid follicles and cryptopatches [92], all containing specialised APC and gut resident T cells. Similarly to the immune response at the skin and lung, the immune response at the gut site needs to be carefully regulated to avoid allergic responses such as colitis, food allergies or inflammatory bowel disease (IBD).

All of these peripheral tissues traversed by *N. brasiliensis* during infection are highly immune responsive with resident APC and T cells ready to respond to infection. These peripheral tissues are primary sites of pathogen exposure, which all contain their own lymphoid tissue. Any of these peripheral sites of *N. brasiliensis* migration could be considered as potential candidates in conferring protective immunity against reinfection with this parasite.

3.1.3. Aims

The following experiments were designed to investigate *N. brasiliensis* infection as a model for protective immunity. In particular these experiments aim to reveal the tissue location where priming of protective immunity against reinfection by *N. brasiliensis* occurs by:

- Creating robust and unique experimental methods for tissue specific infection and analysis
- Identify the role of different tissues in priming protective immune responses using tissue specific infection

3.2. Results

3.2.1. N. brasiliensis as a model of Th2 protective immunity

3.2.1.1. Mice are protected from secondary infection in both the lung and gut

To establish the levels of protection against reinfection in the *N. brasiliensis* model, G4/IL-4 mice were infected with 600 iL3, rested for 30 days and then reinfected with 600 iL3 worms. Over the course of infection lung and gut tissues were harvested and viable worms migrated out using the viable worm recovery assay (as described in materials and methods,

chapter 2) and enumerated (Fig 3.1A and B). To assess the level of protection in reinfected mice, tissue worm burdens were compared to those of primary infected mice (Fig 3.1 A and B).

During primary infection worms could be recovered from the lung from as early as day one, with peak numbers in this tissue being observed at day two post-infection (Fig 3.1A). As worm numbers decrease in the lung tissue at day three post-infection, they can be detected in the gut tissue, with numbers peaking at day six post-infection (Fig 3.1B). When mice are reinfected with *N. brasiliensis* the number of viable worms recovered from both lung and gut tissues are dramatically reduced, with a 75% reduction in lung worm burden and 90% reduction in gut worm burden (Fig 3.1 A and B).

The results from these experiments detail the kinetics of worm migration through host tissues during primary infection and reinfection. A dramatic reduction in worm burden of both lung and gut tissue is observed upon reinfection with *N. brasiliensis*, facilitating a model that can be used to study protective immunity.

3.2.1.2. Protection from *N. brasiliensis* correlates with induction of a Th2 immune response

In order to further characterise the *N. brasiliensis* model of protective immunity the development of Th2 immune responses were followed using G4/IL-4 reporter mice. Mice were infected and challenged with *N. brasiliensis* as described above. During the course of infection lymph nodes draining the lung and gut (mediastinal and mesenteric lymph nodes respectively) were harvested and assessed for the development of IL-4 producing CD4 T cells, using a GFP reporter gene (as described in materials and methods, chapter 2).

Development of a Th2 immune response during primary infection follows worm infection. By day three post-infection worms have migrated through the lungs, yet the number of GFP+ cells present in the mediastinal lymph node peaks at day nine post infection (Fig 3.1C). A similar trend is seen in the mesenteric lymph node where GFP+ CD4 T cell numbers peak at day nine (Fig 3.1D), yet the peak of worm burden is day six post-infection, with the majority of worms expelled from the gut by day nine to ten post-infection (Fig 3.1B).

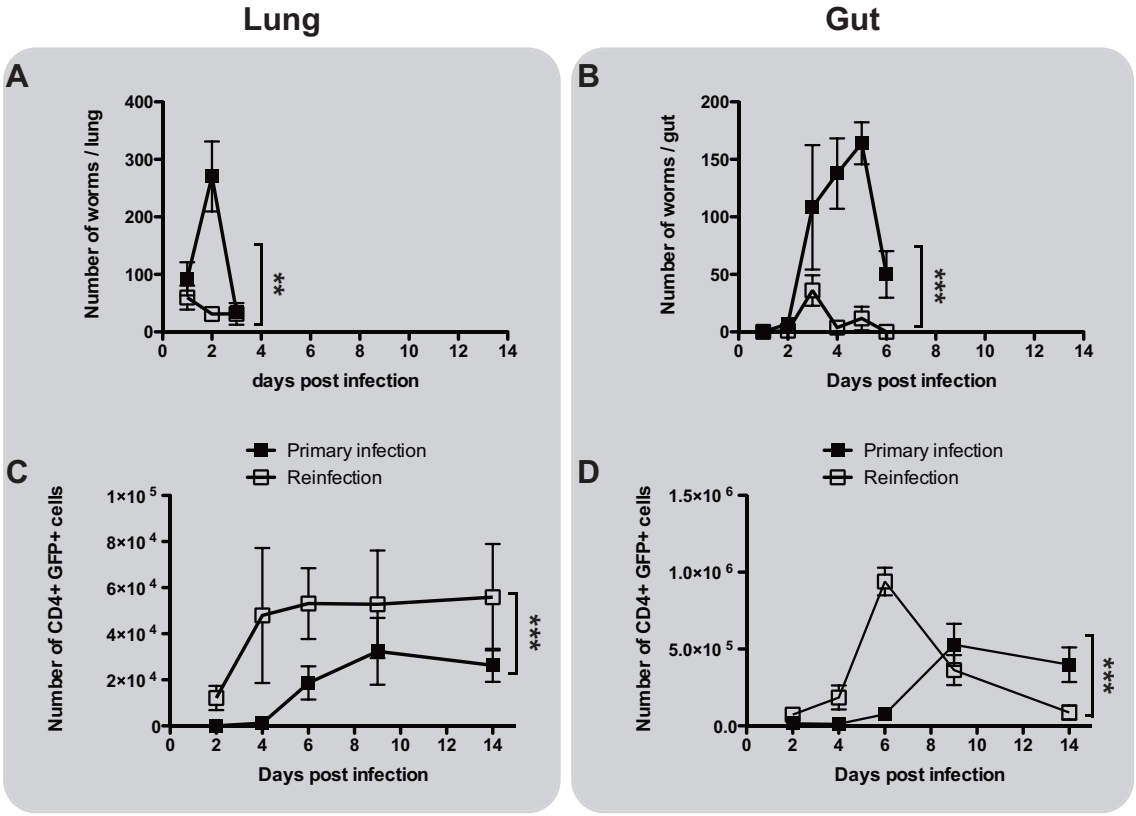

Figure 3.1 *N. brasiliensis* infection as a model of Th2 mediated protective immunity.

G4/IL-4 mice (n=4/group/time point) were infected with *N. brasiliensis* (closed squares) and 30 days post-infection mice were reinfected with *N. brasiliensis* (open squares). **A.** Parasite burden in the lung during primary *N. brasiliensis* infection and reinfection. At the indicated time points lung tissues were harvested and viable worms migrated out and enumerated. Values represent mean number of worms recovered ± S.E.M. **B.** Parasite burden in the gut during primary *N. brasiliensis* infection and reinfection. At the indicated time points gut tissues were harvested and viable worms migrated out and enumerated. Values represent mean number of worms recovered ± S.E.M. **C.** GFP+ CD4 T cell responses during primary *N. brasiliensis* infection and reinfection. At the indicated time points mediastinal lymph nodes from *N. brasiliensis* infected animals (n=4/group/time point) were harvested and surface stained for CD3 and CD4 expression. Values represent mean number of cells ± S.E.M. **D.** GFP+ CD4 T cell responses during primary *N. brasiliensis* infection and reinfection. At the indicated time points mesenteric lymph nodes were harvested and surface stained for CD3 and CD4 expression. Values represent mean number of cells ± S.E.M. Statistics (**A-D**) represent differences between primary infection and secondary infection for each data set. Statistics were calculated using two-way ANOVA with Bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Data from **A-D** are representative of at least three independent experiments.

During a secondary infection, however, the Th2 immune response is initiated faster and is of greater magnitude than that seen in the primary response in both the mediastinal and mesenteric lymph nodes (Fig 3.1C and D). This stronger, faster immune response correlates with the reduced worm burden seen during reinfection (Fig 3.1A and B).

This data suggests that during reinfection with *N. brasiliensis* there is a rapid Th2 immune response initiated in the mediastinal and mesenteric lymph nodes that is of increased magnitude and that also coincides with a reduced worm burden in both the lung and gut tissues.

3.2.2. Protection does not occur at the skin site of infection

In the life cycle of *N. brasiliensis* the larvae travel through many tissues of the host, beginning with penetration through the skin. Published work has suggested that immunity against reinfection occurs at the skin site of infection [3] and the following experiments were undertaken to determine if the skin plays an important role in protection from reinfection with *N. brasiliensis*.

An *in vitro* assay was developed to assess the ability of *N. brasiliensis* iL3 to penetrate naïve or previously primed (immune) skin. Skin was harvested from naïve animals or from previously primed animals (from the site of priming) and placed securely over PBS filled tubes. Tubes were incubated in a 37°C water bath and ~200 iL3 were placed onto the skin and allowed to migrate through (Fig 3.2). Worms that had successfully migrated were collected from the bottom of the tube and enumerated.

When the number of worms migrating through naïve skin was compared to the number of worms migrated through immune skin, there was no difference in the ability of worms to traverse immune skin. This suggests that immunity against reinfection is not due to an inability of the worms to penetrate through the skin of previously primed immune mice. However the *in vitro* nature of this assay does not allow for the migration of immune cells towards the site of worm penetration, which has been shown by Daly et al [3]to occur over a two hour time period.

To investigate protection at the skin site in a more physiologically relevant way, an *in vivo* assay was developed where iL3 were injected intradermally into the ear pinnae of naïve or previously primed mice and their migration from the site of injection was evaluated. At various time points post-infection, ears were removed and worms migrated out using the

viable worm recovery assay and enumerated. An initial study used CFSE labelled worms to enable visualisation under the fluorescent microscope (Fig 3.3A), which allowed us to test the efficiency of the viable worm recovery assay.

Migration of iL3 away from the injection site in the ear was rapid, with more than 50% of larvae leaving the ear within 30 min (Fig 3.3B). There was no difference in migration between naïve or previously primed animals implying that worms are able to migrate away from the site of infection in both naïve or immune mice and are not trapped by scar tissue or immune responses as has been previously suggested [3].

These *in vivo* experiments support our *in vitro* findings that the skin is not the site where protective immunity against *N. brasiliensis* occurs. The observation that equivalent numbers of worms are able to be recovered from the lungs of primary or reinfected mice at day one post infection (Fig 3.1 A) also supports this finding.

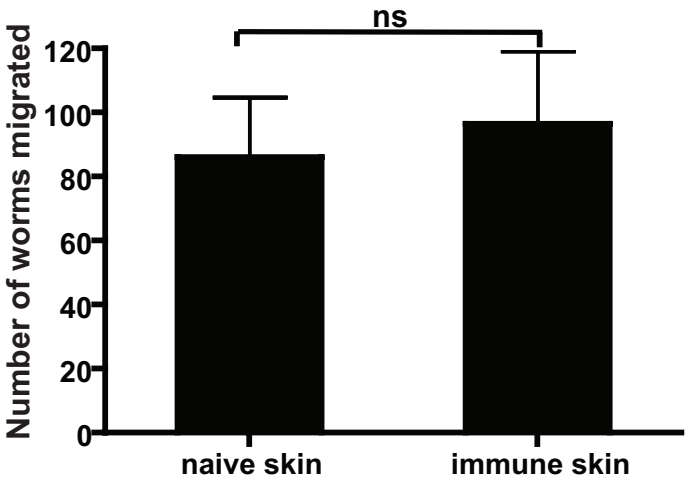

Figure 3.2 *N. brasiliensis* penetrate the skin of both naïve and immune animals.

Efficiency of iL3 skin migration *in vitro*. Skin harvested from naïve or previously infected mice (n=4/group) was suspended over PBS. iL3 *N. brasiliensis* were placed on the skin samples and numbers of iL3 *N. brasiliensis* able to migrate through naïve or immune skin were enumerated. Values represent mean number of larvae migrated ± S.E.M. Statistics were calculated using Student's t test.

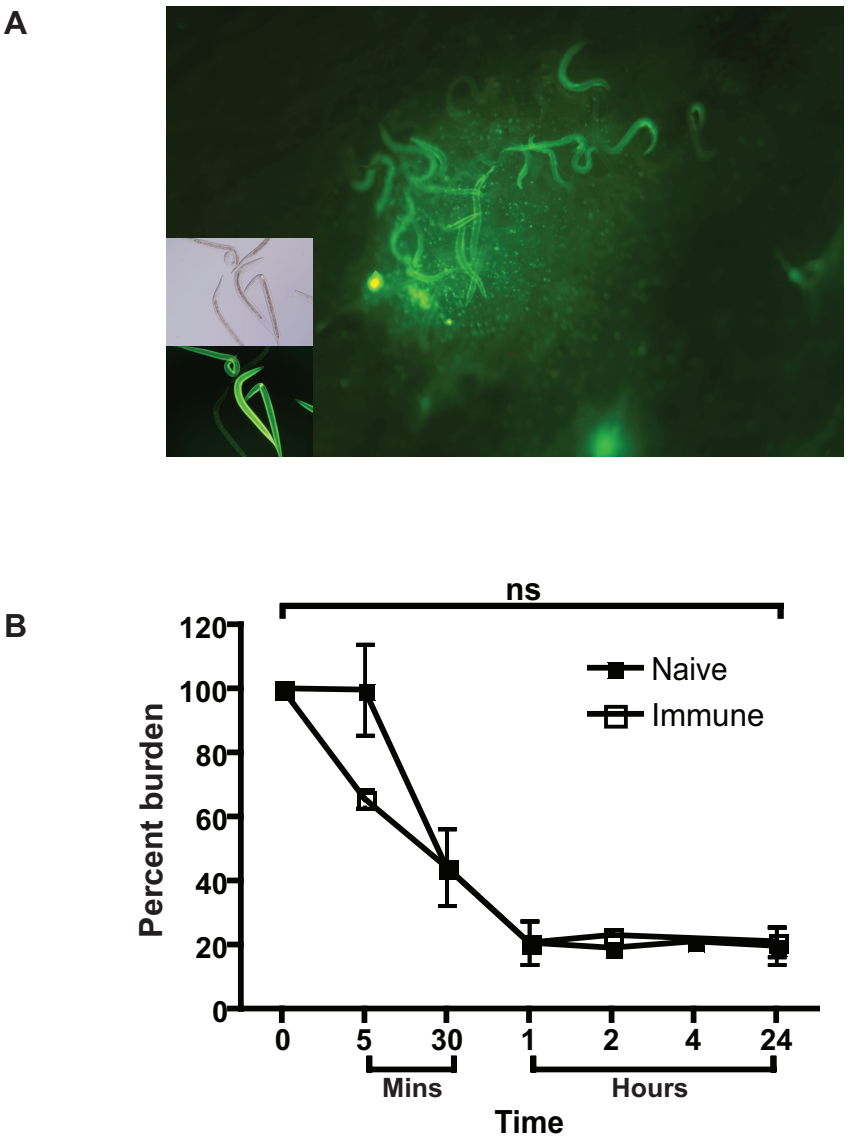

Figure 3.3 *N. brasiliensis* migrate from the infection site in immune animals.

A. Representative photomicrograph of CFSE labelled iL3 *N. brasiliensis* injected intradermally into ear at five minutes post-infection. **Inset**, DIC vs fluorescence. Pictured worms are 0.5mm in length. **B**. Number of larvae migrating from skin site of infection. G4/IL-4 mice (n=3/time point) were infected intradermally into the ear pinna with *N. brasiliensis (open squares)* or left uninfected *(closed squares)*, and at day 30 post-infection were reinfected with *N. brasiliensis*. At indicated time-points, viable larvae were migrated out from excised ears & enumerated. Values expressed as percent burden ± S.E.M. and are representative of three independent experiments. Statistics were calculated using two-way ANOVA with Bonferroni post test and data was found to be not significantly different.

3.2.3. Priming in the lung and not the gut can confer protection against N. brasiliensis *reinfection*

Having established that the skin is not a critical site conferring protective immunity against *N. brasiliensis* we next sought to investigate the relative importance of the mucosal sites in generating protective immunity. *N. brasiliensis* passes through both the lung and gut tissues during migration, so it was important to develop a method of separating the migration through these sites to assess where priming of protective immunity is occurring. The following experiments describe the development of a partial infection model where the lung and gut phases of the *N. brasiliensis* life cycle are isolated. This model was then used for segregated priming to ascertain where protective immunity is initiated.

3.2.3.1. Tissue specific priming – development of the partial infection model

With the knowledge that prior infection significantly reduced the number of tissue migrating *N. brasiliensis* iL3/L4 that gain access to the lung, it was important to determine what immune priming mechanisms mediated this effect. Therefore, we exploited the secondary infection model using distinct phases of the *N. brasiliensis* life cycle (illustrated in Fig. 3.4) and examined the relative contribution of immune priming within the skin, the lung or the gut towards protective immunity against reinfection. To do this, *N. brasiliensis* of different stages were harvested; infective iL3 worms were harvested as usual, L4 worms were harvested from the lungs of infected mice and adult L5 worms were harvested from the gut tissues. Tissue specific priming was then achieved as follows: (Group A) skin and lung priming was achieved by infection with iL3 and infection of the gastrointestinal tract prevented by anti-helmintic treatment from days two to nine of infection; (Group B) Lung only priming was achieved by intranasal administration of L4 and infection of the GI tract prevented by anti-helmintic treatment from days two to nine of infection; (Group C) Gut priming was achieved by gavage of adult L5; (Group D) skin, lung and gut priming was achieved by conventional infection with iL3. Once primary infections had cleared and immune responses dissipated (days 25 - 30 post-infection – see appendix Fig A.1), all groups were reinfected with iL3 and a group of naïve mice (Group E) was included for comparison of worm burden, cellular response and protective immunity.

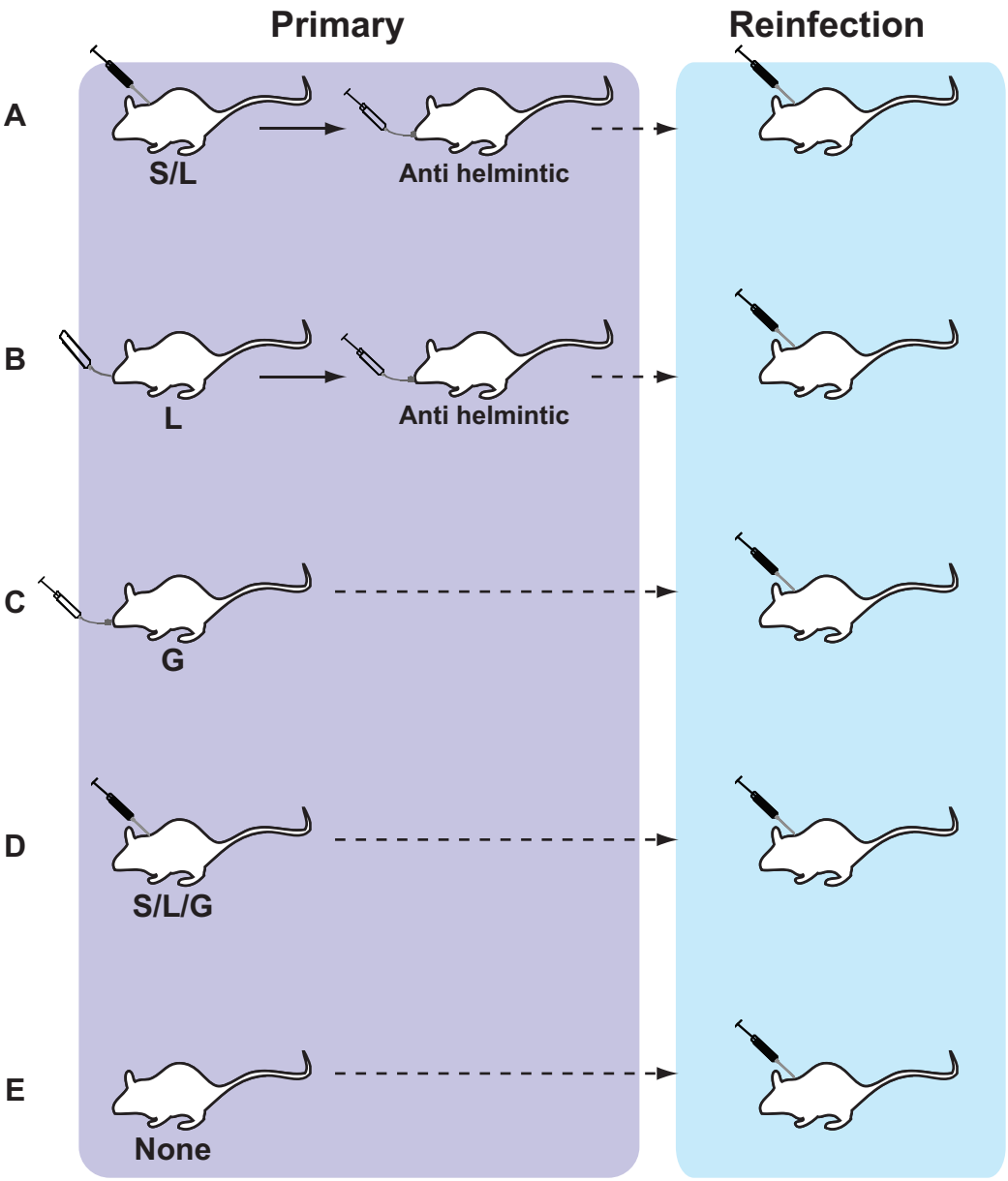

Figure 3.4 Experimental design to create partial infections restricted to individual peripheral tissues.

A. Skin and lung priming (S/L). Mice were infected with ~600 iL3 *N. brasiliensis* subcutaneously and gavaged daily from days two to nine with anti-helmintic to prevent establishment of gut population. Mice were re-infected with 600 iL3 *N. brasiliensis* at day 30 post infection. **B.** Lung priming (L). Mice were intranasally administered ~ 200 L4 *N. brasiliensis* and gavaged daily from days two to nine with anti-helmintic to prevent establishment of gut population. Mice were re-infected with 600 iL3 *N. brasiliensis* day 30 post infection. **C.** Gut priming (G). Mice were gavaged with ~300 L5 *N. brasiliensis* worms. Mice were challenged with 600 iL3 *N. brasiliensis* day 30 post infection. **D.** Skin, lung and gut priming (S/L/G). Mice were infected with ~600 iL3 *N. brasiliensis* subcutaneously. Mice were re-infected with ~600 iL3 *N. brasiliensis* day 30 post infection. **E.** No priming (Primary control) Mice were infected with ~600 iL3 *N. brasiliensis* subcutaneously at the same time as other groups were re-infected to allow for measurement of protection. The reduced numbers of worms used for priming groups B and C were due to difficulty recovering enough worms for infection, as these stages of worm had to be recovered from passage through mice rather than from faecal cultures.

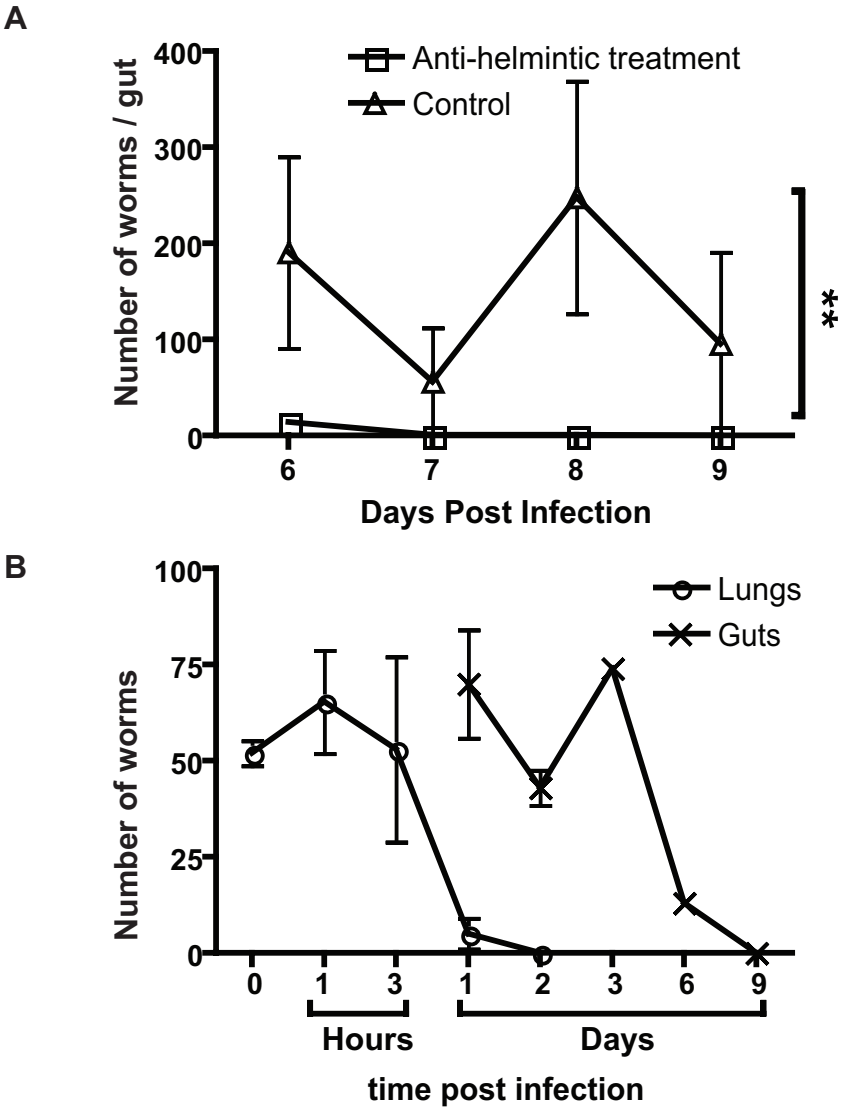

Figure 3.5 Validation of tissue specific priming.

A. Treatment with anti-helmintic prevents establishment of a gut residing worm population. G4/IL-4 mice (n=3/time point) were infected with ~600 iL3 *N. brasiliensis*, one group received anti-helmintic treatment from day 2 post infection (squares) and the other did not (triangles). At the indicated time points guts were excised, worms migrated out and enumerated. Values represent mean number of larvae present ± S.E.M. Data is representative of two independent experiments. Animals treated with anti-helmintic had significantly (**) less worms recovered than untreated animals. Statistics were calculated using two-way ANOVA with Bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. **B**. Worm burden in the lung (circles) & guts (crosses) following i.n. administration of lung larvae. G4/IL-4 mice (n=3/time point) were intranasally infected with 150 freshly harvested lung larvae and on indicated time points, lungs and guts were excised, worms migrated out and enumerated. Values represent mean number of larvae present ± S.E.M.

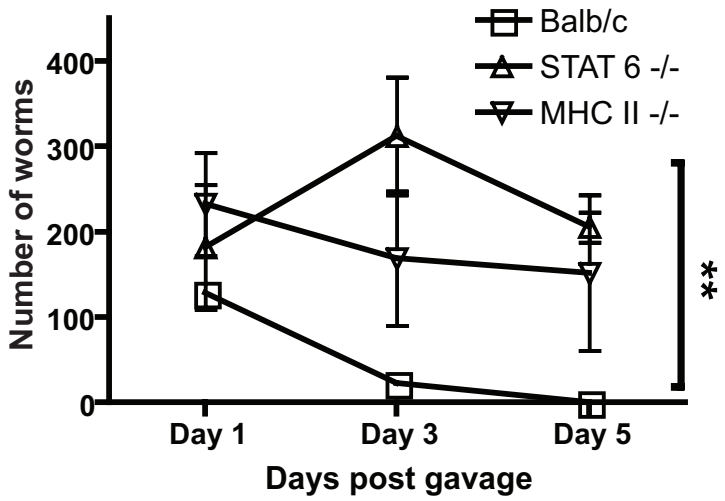

Figure 3.6 Gavaged L5 worms are not cleared from immune deficient animals.

Balb/c (squares), STAT 6 -/- (triangles) and MHC II -/- (inverted triangles) mice (n=2/time point) were gavaged with ~300 freshly isolated and washed L5 *N. brasiliensis* worms. At the indicated time points guts were excised, worms migrated out and enumerated. Values represent mean number of worms present ± S.E.M. Data is representative of two independent experiments. The difference in number of worms recovered between Balb/c and STAT 6 -/- or MHC II -/- strains is significant (**), in that the worms are cleared from the Balb/c strain over time, but not the immune deficient mice. Statistics were calculated using two-way ANOVA with Bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

It is important to note that anti-helmintic treatment restricted infection, preventing the establishment of a worm population within the gut (Fig 3.5A). L4 introduced intranasally to non-drug treated mice were able to complete their life cycle to the L5 stage and to egg production, suggesting that they were not affected by the extraction procedure (Fig 3.5B). Also, adult L5 gavaged into normal mice were cleared with normal kinetics (expulsion day 6-10) while those gavaged into MHC II -/- or STAT 6 -/- mice failed to be cleared, indicating that the L5 worms were not affected by the extraction and gavage procedure (Fig 3.6).

3.2.3.2. The effects of tissue specific priming on protective immunity against *N. brasiliensis* reinfection

Mice whose primary infection was confined to the skin and lung (S/L), or to the lung alone (L) significantly reduced the number of migrating larvae in their lungs when compared with primary infected naive mice (Fig 3.7A). In contrast, mice whose primary infection with *N. brasiliensis* was restricted to gut infection (G) failed to significantly reduce their lung worm burden when reinfected. Analysis of the lymph nodes draining the infected sites revealed that the presence of more than 10^4 CD4 GFP+ cells per mediastinal lymph node (Fig 3.7B) in groups that were primed in the lung alone (L), skin and lung (S/L), or skin, lung and gut (S/L/G). This correlated with a significant reduction in worm burden in the lung (Fig 3.7A). By comparison significantly lower numbers of CD4 GFP+ cells were found in the mediastinal lymph nodes of gut-primed and naive mice (G and Primary respectively, Fig 3.7B).

In contrast to the distinct effect of different priming regimens on the lung worm burden of reinfected mice, all mice that had received either skin, lung or gut only primary infection significantly reduced the numbers of gut dwelling adult L5 at day six following reinfection (Fig 3.7 C). This protective effect was associated with high numbers of CD4 GFP+ T cells in the mesenteric lymph node (Fig 3.7 D) of all reinfected mice. Interestingly, it would appear that lung only priming is sufficient to protect mice from reinfection with the adult L5, as mice primed in the lung and reinfected with gut (L5) worms are able to maintain protection (Fig 3.8).

The observation that protection in the lung was only achieved in mice primed with worms that migrated through the lungs implies that lung priming is critical for the establishment of protective immunity against *N. brasiliensis*. In contrast, immune protection in the gut

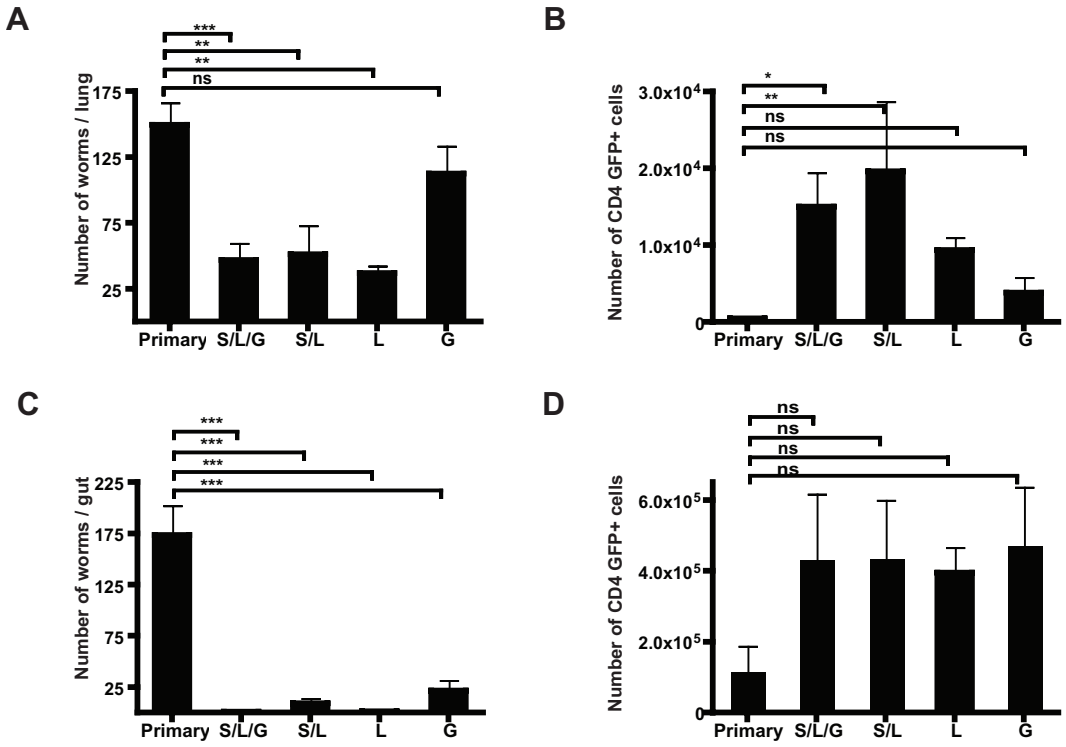

Figure 3.7 Protection against reinfection and corresponding immune response in tissue specific primed animals.

G4/IL-4 mice (n=4/group/time point) were infected via skin & lung (S/L), lung only (L), gut only (G), complete infection (S/L/G), or remained uninfected (Primary) as described in Fig 3.4. At day 30 post infection, mice were reinfected with ~600 *N. brasiliensis* iL3 **A**. Worm burden in the lung following priming with distinct phases of *N. brasiliensis* life cycle. At day two post reinfection lungs were excised, worms migrated out and enumerated. Values represent mean number of larvae present in the lung ± S.E.M. **B**. GFP+ CD4 T cell responses in mediastinal lymph node during distinct phases of *N. brasiliensis* life cycle. Mediastinal lymph nodes were harvested on day two post reinfection and surface stained for CD3 and CD4. Values represent mean cell numbers ± S.E.M. **C**. Worm burden in the gut following priming with distinct phases of *N. brasiliensis* life cycle. At day six post reinfection guts were excised, worms migrated out and enumerated. Values represent mean number of larvae present in the gut ± S.E.M. **D**. GFP+ CD4 T cell responses in mesenteric lymph node during distinct phases of *N. brasiliensis* life cycle. Mesenteric lymph nodes were harvested at day six post reinfection and surface stained for CD3 and CD4. Values represent mean cell numbers ± S.E.M. Data are representative of three independent experiments. Statistics were calculated using one-way ANOVA with Dunnets post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

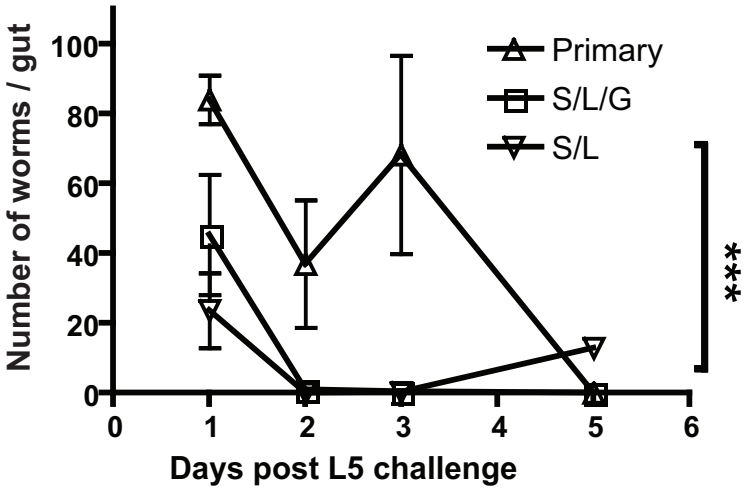

Figure 3.8 Priming in the lung can protect from reinfection in the gut tissues.

G4/IL-4 mice (n=3/group/time point – last time point n = 1/group) were infected in the skin and lung, or the skin/lung and gut as described in Fig 3.4. At 30 days post infection mice were reinfected with ~300 gavaged L5 *N. brasiliensis* worms, also at this time a naïve group received ~300 gavaged L5 *N. brasiliensis* worms to measure any protection conferred by previous infection. At the indicated time points mice were sacrificed and gut tissues harvested. Worms were migrated out and enumerated. Values represent mean number of worms present in the gut ± S.E.M. Statistics were calculated using two-way ANOVA with Bonferroni post test and the difference in worm burden between Primary infected mice and those receiving a secondary infection (S/L/G and S/L) was significant (***) * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

could be achieved when the primary infection was restricted to either the lung or gut (Fig 3.7C).

3.2.3.3. Priming with dead *N. brasiliensis* does not confer protection from reinfection

Following on from the characterisation of live *N. brasiliensis* infection a question was raised whether dead *N. brasiliensis* could act as an antigen depot and prime a protective immune response, conferring protection against reinfection with live parasites. To address this question mice were injected intradermally in the ear pinnae with ~600 dead iL3 worms, rested for 30 days and then reinfected with ~600 live iL3. The number of worms present in the lung tissue was then assessed using the viable worm recovery assay (as described in Chapter 2).

As seen previously, mice primed with a live infection prior to reinfection were protected exhibiting a reduced worm burden within the lung, however animals that had been primed with dead iL3 were unprotected(Fig 3.9 A).

A similar experiment was carried out with the L4 stage lung dwelling larva. Mice were primed intranasally (i.n.) with ~200 dead L4, rested for 30 days and then reinfected subcutaneously with ~600 live iL3. Mice that had been primed with dead L4 were not protected from reinfection when compared to mice that had received a live primary infection, animals primed with dead L4 exhibited a worm burden similar to that of mice which had a primary infection (Fig 3.9 B).

Taken together these data show that a live infection is required to generate a protective response against *N. brasiliensis* as priming with dead iL3 or L4 does not confer protection against reinfection.

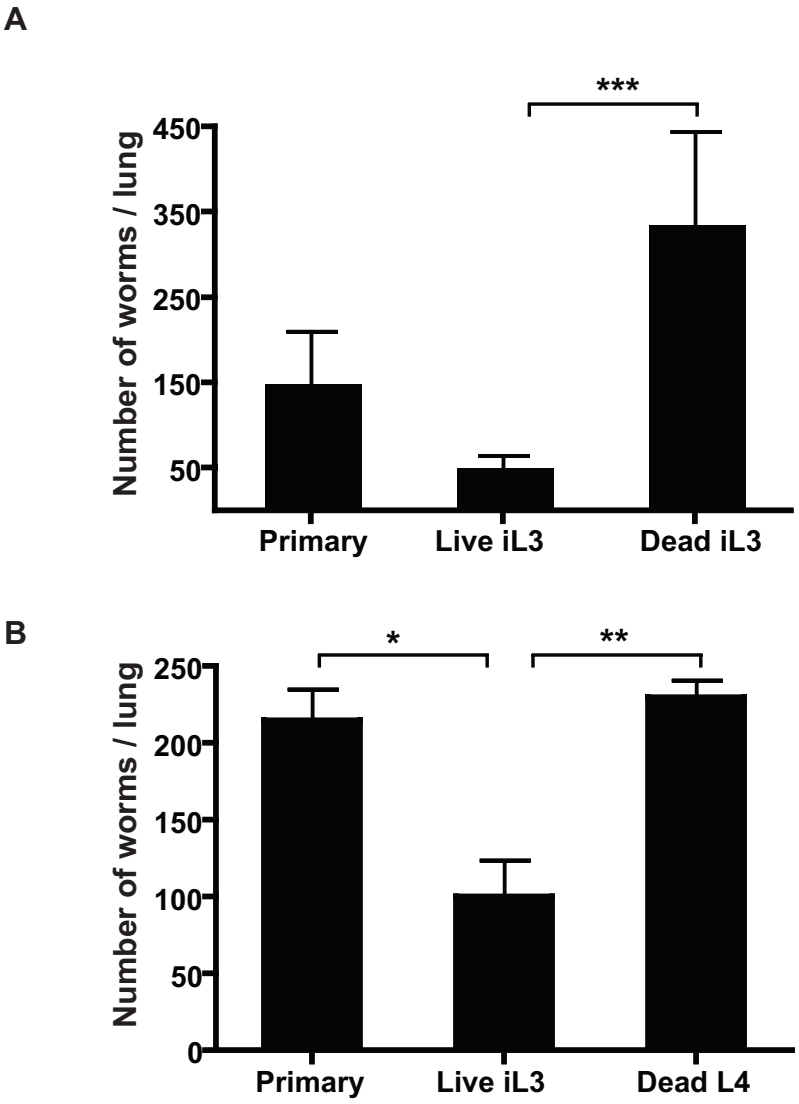

Figure 3.9 Infection with dead worms does not confer protection against reinfection with *N. brasiliensis*.

A. Worm burden in the lung following intradermal priming with dead iL3 *N. brasiliensis* worms. G4/IL-4 mice (n=3) were primed intradermally in the ear with ~600 live or dead iL3 *N. brasiliensis* worms. At 30 days post-infection, mice were reinfected with ~600 iL3 *N. brasiliensis* worms. At day two post-reinfection, lungs were excised worms migrated out and enumerated. Values represent mean number of worms present in the lung ± S.E.M. **B.** Worm burden in the lung following intranasal infection with dead lung larvae. G4/IL-4 mice (n=3) were primed with ~200 dead L4 or ~600 iL3 *N. brasiliensis* worms. At 30 days post infection, mice were infected with ~600 iL3 *N. brasiliensis* worms. At day two post-reinfection, lungs were excised worms migrated out and enumerated. Values represent mean number of worms present in the lung ± S.E.M. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

3.3. Discussion

The primary aims of the work described in this chapter were to create a robust and unique assay to measure priming of immune responses in specific tissues and use the assay to assess the role of different tissues in priming for protection against reinfection with *N. brasiliensis*. We have developed a unique assay restricting primary *N. brasiliensis* infection to specific tissue sites during infection. We have used this assay to pinpoint the critical peripheral site in conferring protective immunity against *N. brasiliensis* infection.

The data in this chapter clearly demonstrate that the lung has the potential to be a very important site for conferring protection against reinfection with *N. brasiliensis*. We show that not only is the lung an important site for reducing the numbers of migrating larvae but that it is an important site for priming the protective CD4 Th2 immune response that occurs in the lung.

Strikingly, priming in the lung tissue was sufficient to confer gut protective immunity to adult stages of the worm. This result indicates that there is a qualitative difference between priming in the lung tissue and priming in the gut tissue. Although priming in the gut tissue is sufficient to generate Th2 immune responses, protection is not as successful as the protection observed when mice are primed in the lung. The fact that only lung priming is able to achieve this level of protection indicates that the lung immune environment has a unique capacity to generate protective responses that can protect even at a distant site. Our conclusions are supported by other findings that suggest the lung is a critical tissue for protection from reinfection [160]. Work with schistosome parasites has revealed that there exists an important need for pulmonary T lymphocytes in establishing protection using an attenuated cercarial vaccination [184, 185]. Studies involving *Strongyloides* species have previously indicated that there is a role for parasites in modulating lung function and conferring protection from reinfection [186]. Investigation into immunisation against *Ascaris* migration has found intranasal administration to be most effective in protecting against parasite infection [187].

Although we found that the skin is not a site of protection or priming for protection in our studies, as is commonly thought, this does not mean that it may not be relevant for both priming and protection in the natural environment. Although we found limiting the primary immune response to the skin (through the injection of dead worms) did not confer

protection against reinfection in the lung, it could be imagined that under special circumstances of high infection rates or vaccination boosting that lung immunity could be generated [3, 161, 188, 189].

Priming at the gut site was sufficient to confer local immunity upon reinfection, however it did not confer protection against the lung stage parasite. The mechanisms of gut expulsion have been a focus of much research in the *N. brasiliensis* field, with roles for CD4 T cells, IL-4, IL-13 and STAT 6 being confirmed [157-159]. The finding that the gut is not a critical site for protection offers new avenues for the study of protective immunity against *N. brasiliensis* infection.

3.4. Conclusions

The aims of this chapter were to develop an assay to isolate tissue priming in the *N. brasiliensis* model and to use this model to assess the role of different tissues in priming protective immune responses. The development of a model assay using segregated priming infection has enabled us to dissect out the relative contributions of priming at the peripheral sites of migration to protective immunity.

We have shown the lung to be an important site for immune priming to confer protective immunity. We find not only that worm numbers are reduced within lung tissues during reinfection, but also that it is an important site for priming the immune response to respond to reinfection both locally and at distant sites. We have provided strong evidence that the skin is not a candidate for the site of protective immunity using *in vitro* and *in vivo* methods. Priming at the gut site, while able to confer local immunity to reinfection, does not appear to be as critical as the lung, which can protect both locally and at a distant site.

Chapter 4:
Contribution of the lymph node vs lung CD4 T cell response in protection against *N. brasiliensis*

4.1. Introduction

The studies outlined in chapter three reveal that primary infection in the lung tissue is important in the generation of protective immunity against *N. brasiliensis*. Mice primed solely at the lung site were protected from reinfection both locally and at the distant gut site. In this chapter we sought to build on this finding by exploring whether the cellular responses underpinning this protective immunity were occurring in the lung tissue itself or if they were reliant upon T cells derived from the local draining lymph node circulating via the blood. As a control we also investigated if our findings were specific to our *N. brasiliensis* model by comparing to a lung based Th1 mediated model of protective immunity.

4.1.1. Immune responses of the lung

The lung is a unique environment, heavily vascularised with a good supply of blood, circulating immune cells and also direct interaction with environmental antigen. The ability of the body to form iBALT [71, 83, 190] shows that immune responses can occur within the lung tissue, and mice that lack secondary lymphoid organs have been shown to generate lung based immune responses [70]. This data demonstrates that immune responses can occur within lung tissues, but how relevant are these immune responses to protective immunity? Moyron-Quiroz *et al* demonstrate protection in a viral model of infection, proposing that iBALT provided a niche for the maintenance of the memory T cell population [70]. The experiments contained within this chapter assess the role of immune responses mounted in the tertiary lymphoid tissues (the lung in our model) in conferring protective immunity against *N. brasiliensis*.

To assess the roles of CD4 T cell responses in the lung and lymph nodes in protective immunity against *N. brasiliensis* we needed to separate the CD4 T cell responses of the two tissues. The immunosuppressant drug FTY720 has been shown to prevent lymphocyte recirculation by preventing egress into the blood from the secondary lymphoid tissues [125]. Several studies have effectively used FTY720 to prevent lymphocyte recirculation [101, 150] and it has been used with mixed success in a variety of disease models. FTY720 has been demonstrated to delay graft rejection caused by Graft Versus Host Disease (GVHD) [145, 147, 148], prevent Th1 and Th2 mediated colitis [137, 149], suppress Th1 and Th2 induced airway inflammation [151] and FTY720 also shows promise in the

treatment of multiple sclerosis [153, 154], with the drug currently in phase III clinical trials. We sought to take advantage of the unique properties of FTY720 to separate T cell responses of the lung tissue and the corresponding draining lymph node.

To control for our findings in the *N. brasiliensis* model, we wanted to look at the effects of separating secondary and tertiary lymphoid responses in a Th1 mediated lung model of protective immunity. In the murine model of *Mycobacterium tuberculosis* infection, BCG-vaccinated mice exhibit a one $\log_{10}$ reduction in their lung bacterial burden upon subsequent exposure to aerosolised *M. tuberculosis,* compared to naïve mice [191]. The protection observed in vaccinated mice is dependent on T helper 1 (Th1) CD4 T cell responses [192, 193], and the production of IFNγ [194].

This makes the BCG model of protective immunity a good candidate to control for our findings in the Th2 mediated *N. brasiliensis* model of protective immunity.

4.1.2. Aims

The following series of experiments were undertaken to first establish the concentration of FTY720 required to sequester lymphocytes into lymph nodes, thus blocking CD4 T cell recirculation between lung and lymph node. Upon establishing this model the effects of blocking CD4 T cell recirculation between lung and lymph node during *N. brasiliensis* infection were investigated, by assessing the impact of FTY720 treatment on worm burden in both primary and secondary infections. The final experiments within this series were aimed at addressing the changes in immune response mounted against *N. brasiliensis* infection after segregation of secondary and tertiary lymphoid responses. Results obtained using the *N. brasiliensis* model were then compared to a BCG model of lung based Th1 mediated protective immunity.

Specific aims:

- To block circulation of CD4 T cells between the lymph node and lung using the drug FTY720
- To assess the effects of blocking CD4 T cell circulation between the lymph node and lung in the *N. brasiliensis* model, on both protection from reinfection and the cellular immune response to *N. brasiliensis*
- To compare our findings in the *N. brasiliensis* model with a lung based Th1 mediated model of protective immunity

4.2. Results

4.2.1. FTY720 administration in vivo sequesters CD4 T cells

To test whether CD4 T cells were sequestered to the lymph nodes by FTY720 treatment FTY720 was administered by oral gavage in a volume of 200μL to G4/IL-4 mice. Mice received one dose of FTY720 treatment at 1.0, 0.5 or 0.1mg/kg and were bled an hour after treatment for time zero. The resulting lymphopenia in the blood over time is shown in Fig 4.1. It was found that FTY720 treatment resulted in a decreased number of CD4 T cells very rapidly (within an hour of the first treatment) and at higher concentrations this effect was prolonged. Although reversible, the effects of high doses of FTY720 took between seven and fourteen days to dissipate. Figs 4.1B-D show the effects of FTY720 treatment on the cell populations in the lungs of naïve animals. Treatment with FTY720 for one, two or three days resulted in a loss of CD4 T cells from the lung and airways (Fig 4.1B), although the total numbers of CD45+ cells remained relatively unchanged (Fig 4.1C) as compared to untreated animals. In naïve FTY720 treated animals there are very few GFP+/IL-4 producing CD4 T cells as demonstrated in Fig 4.1D.

As a result of these experiments a dose of 0.5mg/kg was used, as this dose induced rapid and prolonged lymphopenia. To ensure this dose was effective over time, animals were treated daily to separate secondary and tertiary residing lymphocytes and hence the immune responses of the secondary and tertiary lymphoid tissues.

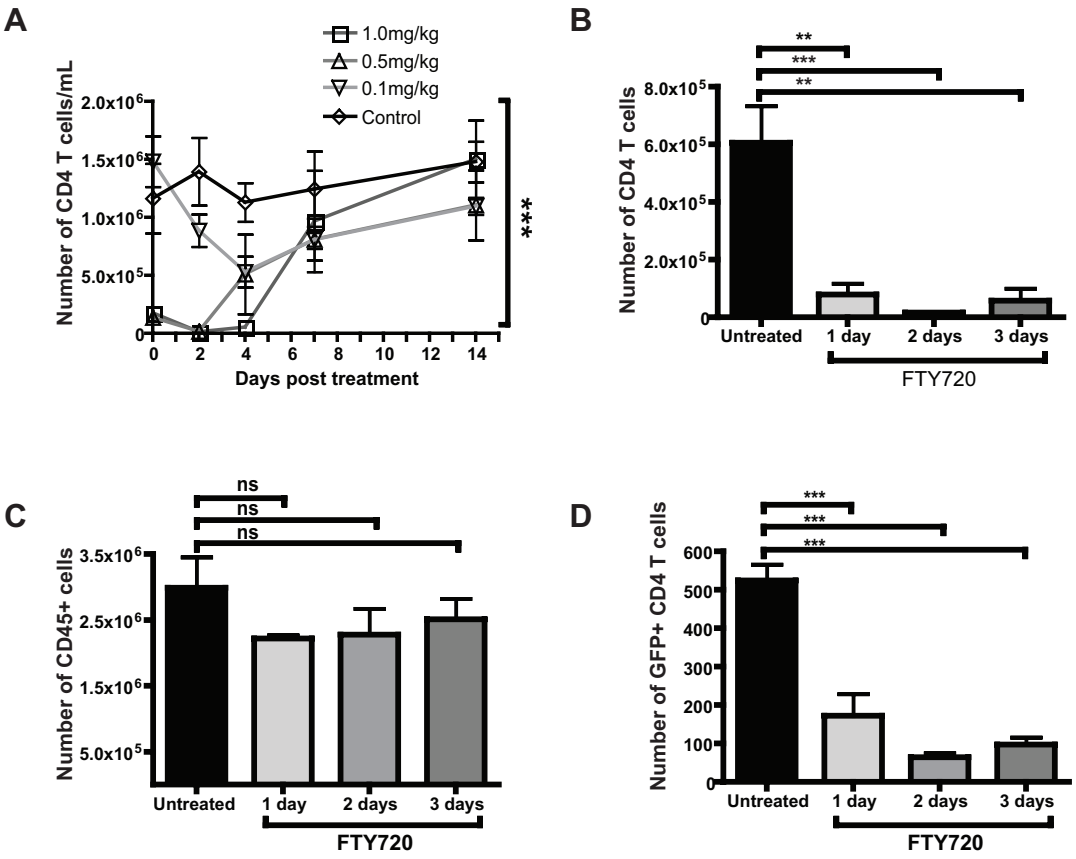

Figure 4.1 FTY720 depletes circulating and lung residing CD4 T cells.

A. Effects of FTY720 on circulating CD4 T cells. G4/IL-4 mice (n=3/group) received 1.0 (squares), 0.5 (triangles), 0.1 (inverted triangles) mg/mL FTY720 or did not receive drug (control, diamonds) at day 0. At the indicated time points mice were tail bled, the resulting cells were stained with αTCR αB220 and αCD4 and FACs analysis was performed using a four colour flow cytometer. Values represent the mean number of CD4 T cells/mL of blood± S.E.M. Statistics were calculated using repeated measures two-way ANOVA with Bonferroni post test and FTY720 treated groups had significantly fewer CD4 T cells/mL (***) compared to control mice * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Both 0.5mg/kg and 1.0mg/kg treatments significantly differed (*) from control. **B - D.** FTY720 treatment reduces the number of T cells resident in the lung. G4/IL-4 mice (n=3 per group) were treated with FTY720 (0.5mg/mL) for 0, 1, 2 or 3 days. Lungs were harvested, enriched for CD45+ cells by AutoMacs© bead separation and the resulting cells surface stained for CD4, CD62L and CD44 expression. FACs analysis was performed using a four colour flow cytometer. Values represent the number of CD4 T cells **(B)** CD45+ cells **(C)** or GFP+ CD4 T cells **(D)** in the lung tissue ± S.E.M. Statistics were calculated using one-way ANOVA with Dunnets post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.1.1. Profile of lung residing CD4 T cells after FTY720 administration in naive mice.

Once an effective dose for FTY720 treatment was established we wanted to look at the activation phenotype of any remaining tissue resident T cells. The activation profile of lung T cells in naïve mice either with or without FTY720 treatment is presented in Fig 4.2. Untreated naïve mice exhibit a large population of CD62Lhi CD44lo CD4 T cells – consistent with a naïve phenotype and also a smaller CD62Llo CD44hi population more consistent with an effector phenotype. FTY720 treatment reduces the population of CD62Lhi CD44lo naïve like cells, consistent with reports in the literature [101, 102], however the effector like CD62Llo CD44hi population does not appear similarly affected by treatment. When observing GFP+ CD4 T cells they are spread between naïve and effector phenotypes – implying background measurements of GFP expression (Fig 4.2B). These results show that FTY720 treatment reveals a population of lung resident T cells.

4.2.2. FTY720 treatment affects the number of circulating T cells during N. brasiliensis infection

With the knowledge that FTY720 treatment prevents the recirculation of CD4 T cells in naïve animals we sought to confirm this effect during *N. brasiliensis* infection. In FTY720 treated mice there is up to a 10 fold reduction in the numbers of circulating CD4 T cells during both primary and reinfection when compared to control animals (Fig 4.3B), although total numbers of circulating cells are similar between groups (Fig 4.3A). Interestingly when we looked at the proportions of CD4 T cells that were GFP+ in FTY720 treated animals receiving a secondary infection with *N. brasiliensis* (Fig 4.3C) we found an overrepresentation of GFP+ cells in drug treated animals. The proportion of GFP+ cells is almost double in FTY720 treated animals, however this finding must be assessed in respect of the fact that the numbers of GFP+ cells circulating in drug treated mice will still be significantly lower due to the reduction in the number of circulating CD4 T cells seen in Fig 4.3B.

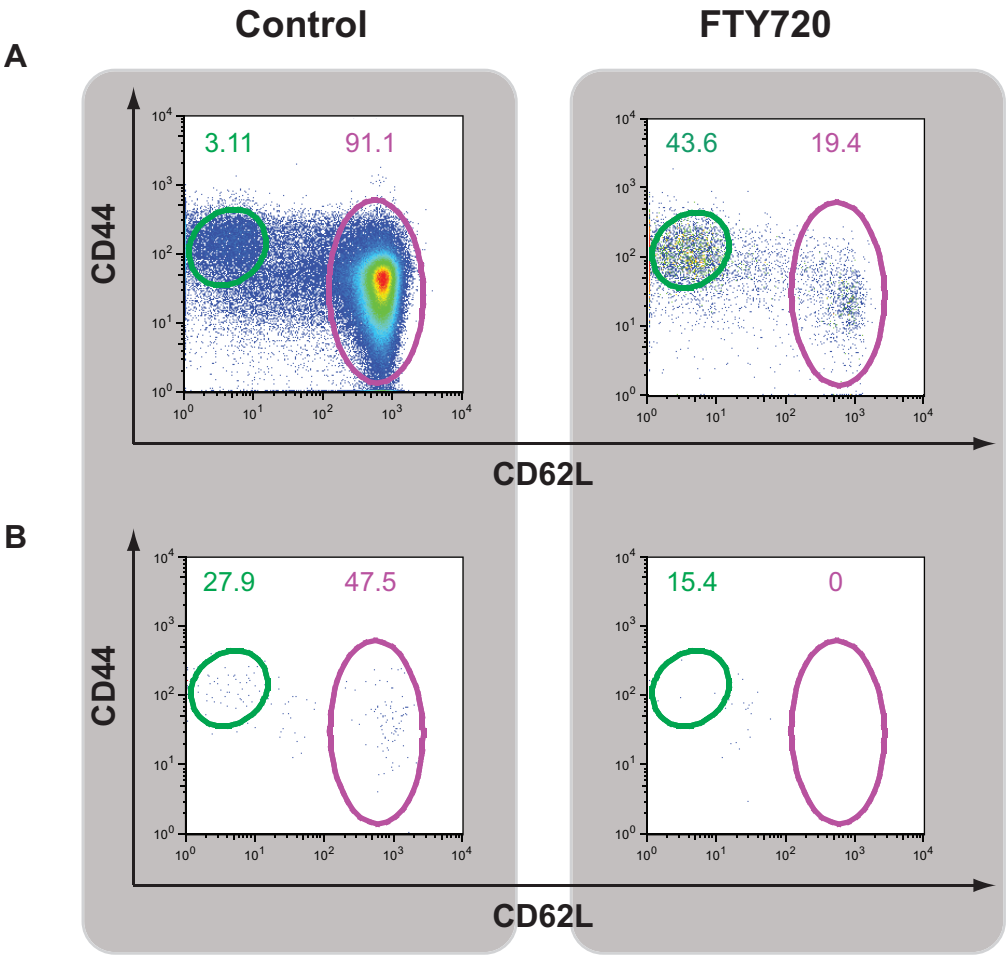

Figure 4.2 Profile of CD4 T cells in the lungs of naïve mice with and without FTY720 treatment.

G4/IL-4 mice (n=3 per group) were treated with FTY720 (0.5mg/mL) for 0 or 3 days. Lungs were harvested, enriched for CD45+ cells by AutoMacs© bead separation and the resulting cells surface stained for CD4, CD62L and CD44 expression. FACs analysis was performed using a four colour flow cytometer. FACs plots display cells from one representative animal. **A**. FTY720 treatment preferentially reduces naïve T cells in the lung tissues. Values represent proportion of CD4 T cells that are Teff/Tem like (CD44hi CD62Llo - green) or naïve (CD44lo CD62Lhi - pink). For number of events see Fig 4.1B **B**. Phenotype of GFP+ CD4 T cells in the lungs of FTY720 treated and naïve mice. Values represent proportion of CD4 T cells that are Teff/Tem like (CD44hi CD62Llo - green) or naïve (CD44lo CD62Lhi - pink). For number of events see Fig 4.1D. Data is representative of at least two independent experiments.

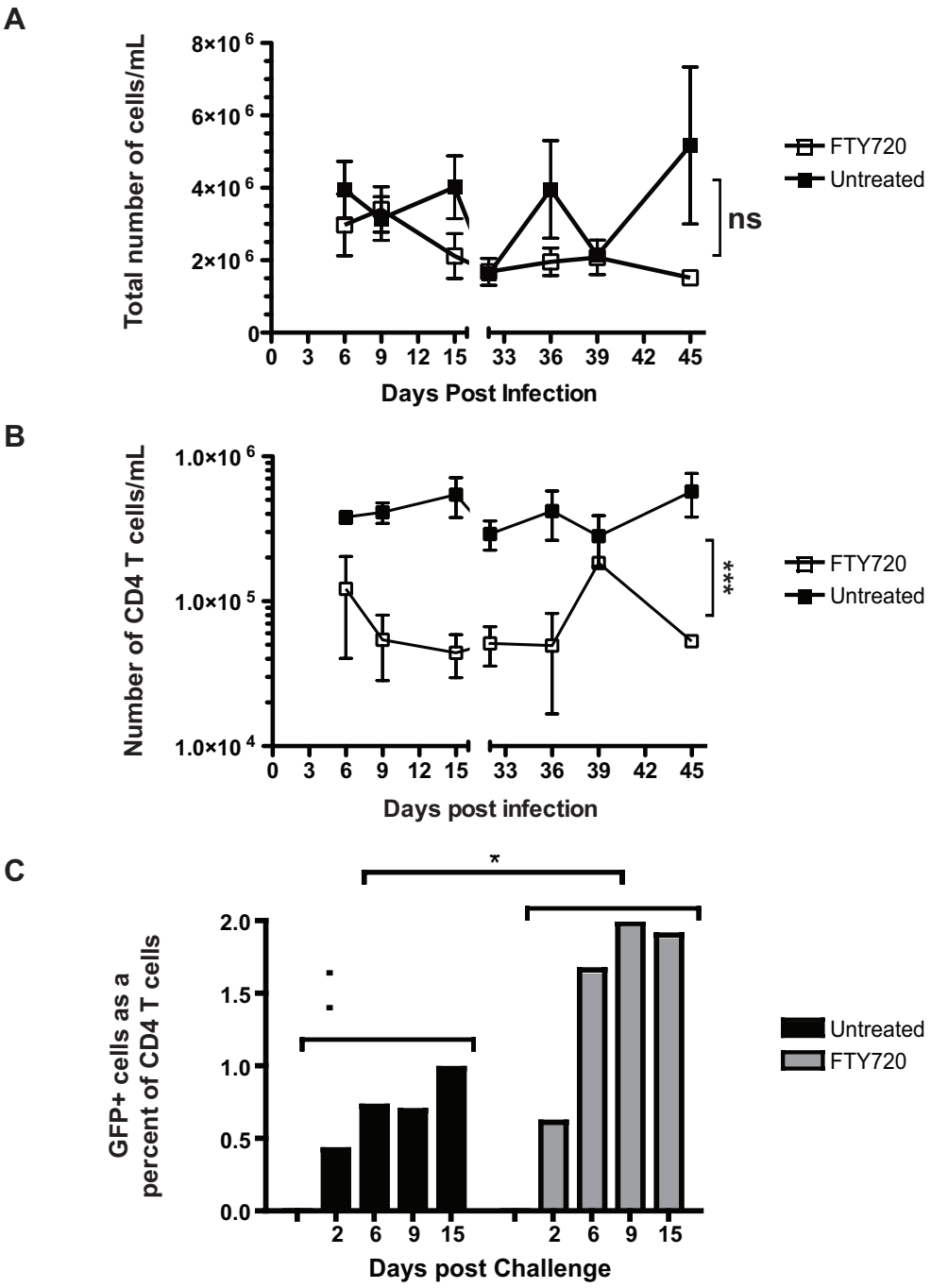

Figure 4.3 T cells in the blood during *N. brasiliensis* infection with FTY720 treatment.

G4/IL-4 mice (n=3/group, pooled) were infected with *N. brasiliensis* and also treated with FTY720 (1mg/kg) daily from one day prior to infection (open squares) or untreated (closed squares). At day 30 post infection mice were reinfected with *N. brasiliensis*. At the indicated time points blood was collected, red blood cells lysed and FACs analysis performed using a four colour flow cytometer. **A**. The total number of cells present in the blood of FTY720 treated and untreated *N. brasiliensis* infected mice is similar. Values represent the total number of live cells per mL of blood as counted under the microscope by trypan blue exclusion. Presented data is mean and S.E.M of three combined independent experiments (n=6-9/group total). Statistics were calculated using a non-parametric Mann-Whitney test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. **B**. CD4 T cell numbers are reduced in the blood of FTY720 treated *N. brasiliensis* infected mice. Values represent the number of CD4 T cells per mL of blood. Presented data is mean and S.E.M of three combined independent experiments (n=6-9/group total). Statistics were calculated using a non-parametric Mann-Whitney test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. **C**. GFP+ cells are over represented in FTY720 treated *N. brasiliensis* reinfected mice. Values represent the number of GFP+ cells as a percent of the CD4 T cell population in a pooled sample from n=3 mice. Animals receiving FTY720 treatment have a significantly higher (*) percentage of GFP+ CD4 T cells, compared to mice that remain untreated. Data are representative of two independent experiments. Statistics were calculated using paired Student's t test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.3. Worm burden in the N. brasiliensis model is unaffected by FTY720 treatment

4.2.3.1. FTY720 administration does not affect primary worm burden or clearance in *N. brasiliensis* model

In order to investigate the effect of blocking CD4 T cell recirculation between lung and lymph node on worm clearance during primary *N. brasiliensis* infection, mice were treated with 0.5mg/kg FTY720 daily (or left untreated) prior to infection with *N. brasiliensis*. At the peak time of infection in the lung (day two post infection) and gut (day six post infection) these tissues were harvested and the worms migrated out using a viable worm recovery assay. The number of viable worms recovered from the lung and gut during primary *N. brasiliensis* infection demonstrated no difference between treated and untreated mice (Fig 4.4A). In fact when a kinetic analysis of worm burden/migration was followed during FTY720 treatment no difference was observed in timing of migration or expulsion of adult worms from the host when compared to untreated control mice (Fig 4.4B).

These results indicate that blocking CD4 T cell recirculation between lung and lymph node does not alter the migration pattern or expulsion kinetics of *N. brasiliensis* worms during primary infection. This finding also suggests that the drug FTY720 does not directly affect the worms themselves.

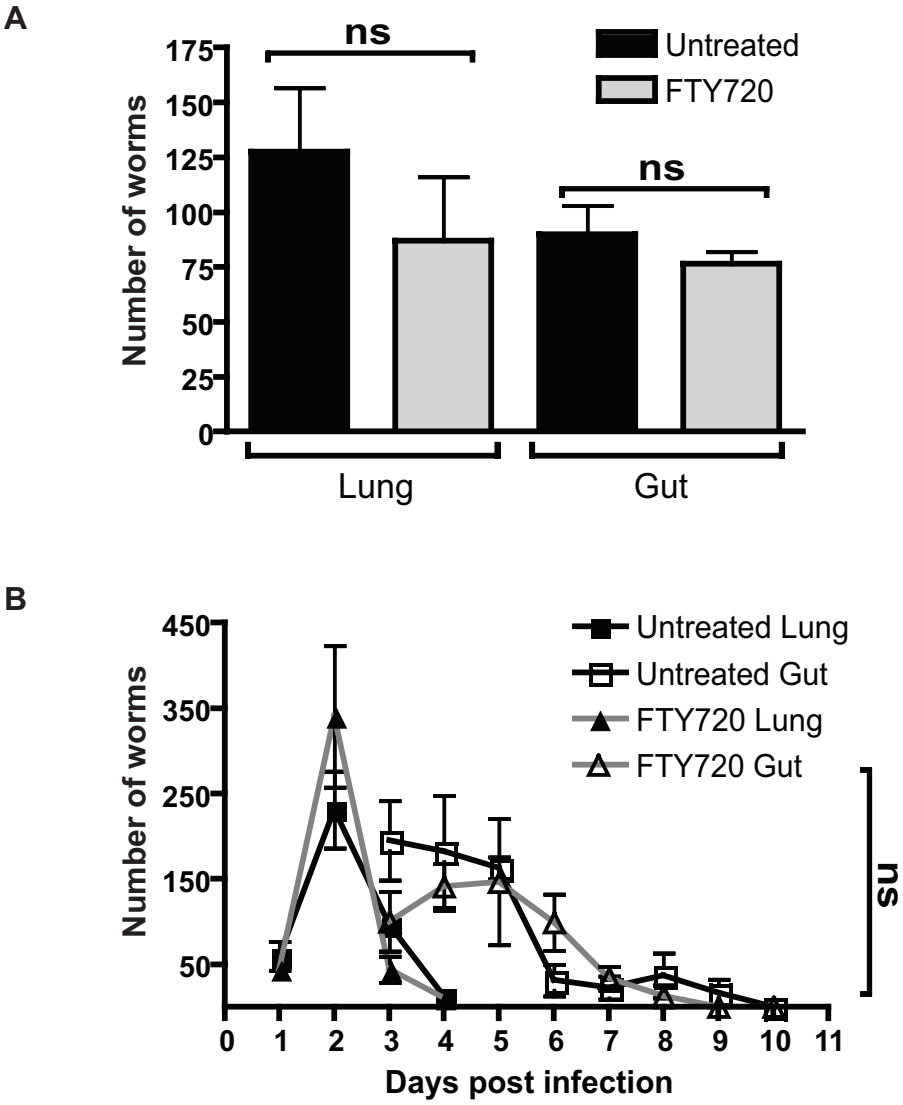

Figure 4.4 FTY720 does not affect worm burden or migration kinetics during primary *N. brasiliensis* infection.

A. No difference in worm burden of lung or gut with FTY720 treatment during primary *N. brasiliensis* infection. G4/IL-4 mice (n=3/group/time point) were infected with *N. brasiliensis* and either remained untreated (control – black bars) or were treated with FTY720 from one day prior to infection (grey bars). Lungs were harvested two days post infection and guts at day 6 post infection. Worms were recovered from the tissues using the viable worm recovery assay (described in chapter two, materials and methods) and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data are representative of at least two independent experiments. Statistics were calculated for each tissue using Student's t test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **B.** No difference in kinetics of worm migration during primary *N. brasiliensis* infection with FTY720 treatment. G4/IL-4 (n=4/group/time point) were infected with *N. brasiliensis* and either remained untreated (control – squares) or were treated with FTY720 from one day prior to infection (triangles). At the indicated time points lung (closed shapes) and gut (open shapes) tissues were harvested, worms migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Statistics were calculated for each tissue using two-way ANOVA with Bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.3.2. FTY720 administration does not affect protection against reinfection in *N. brasiliensis* model

As we observed no difference in kinetics of migration or expulsion during primary *N. brasiliensis* infection, we wanted to investigate whether prevention of T cell migration from the lymph nodes to the lung with FTY720 would affect protection from reinfection with *N. brasiliensis*. To answer this question mice were treated daily with 0.5mg/kg FTY720 from prior to primary infection until sacrificed, and 30 days after primary infection reinfected with *N. brasiliensis*. To assess levels of protection tissues were harvested at times consistent with peak worm burden (day two for lung and day six for gut) and viable worms migrated out and counted. Untreated mice with a primary *N. brasiliensis* infection were used to measure the levels of protection achieved in both FTY720 treated and untreated mice receiving a reinfection (or challenge). Lung data (Fig 4.5A) demonstrated no difference in the level of protection achieved between FTY720 treated and untreated mice when compared to primary infected control animals. A similar observation was found in the gut burden (Fig 4.5B).

These experiments demonstrate that separation of lymph node T cell responses from local lung T cell responses does not alter protective immunity against *N. brasiliensis*. The implications of this finding are that a locally developed immune response is able to confer protection against reinfection in this model of protective immunity.

4.2.3.3. Timing of FTY720 administration does not affect protection against reinfection in *N. brasiliensis* model

Experiments were undertaken to investigate whether blocking CD4 T cell recirculation between lung and lymph node at varying times during *N. brasiliensis* infection has an impact on protective immunity. *N brasiliensis* infected mice were treated with FTY720 prior to priming of an immune response, prior to challenge, or both and their protection from reinfection compared to untreated animals. Using the viable worm recovery assay worms were recovered from the lungs and guts at times of peak infection (as previously determined). As shown in Fig 4.6, mice were protected from reinfection when compared to primary *N. brasiliensis* infected controls, and this protection remained intact whether they had received FTY720 during primary infection, reinfection, both or were untreated.

This data confirms that blocking CD4 T cell recirculation between lung and lymph node does not affect protection from reinfection with *N. brasiliensis*, implying that protective responses can be initiated at the lung site.

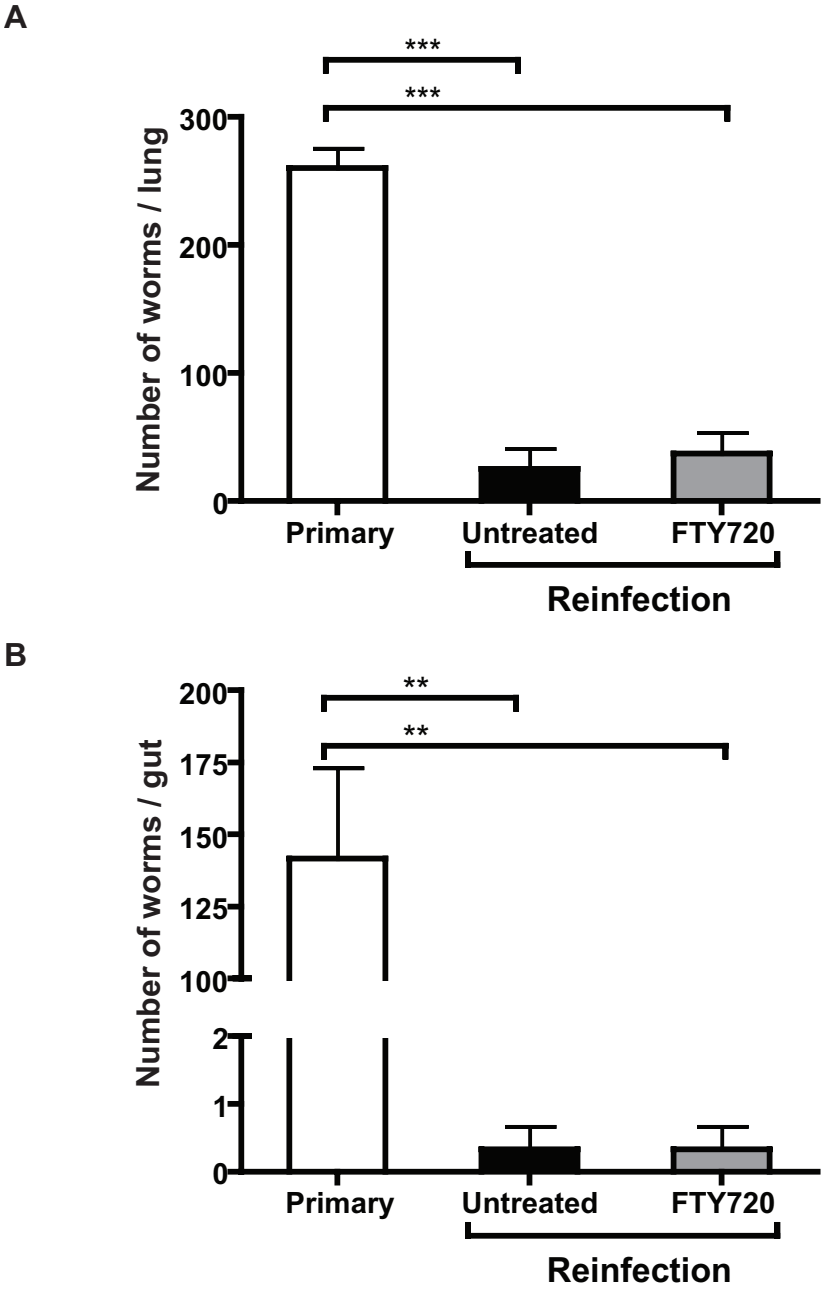

Figure 4.5 FTY720 treatment does not affect protection against reinfection with *N. brasiliensis*.

FTY720 treatment does not affect protection against reinfection with *N. brasiliensis* in the lung or gut. G4/IL-4 mice (n=3 per group per time point) were infected with ~600 iL3 *N. brasiliensis* and either remained untreated (control – black bars) or were treated with FTY720 from one day prior to infection (grey bars). After 30 days mice were reinfected with~600 *N. brasiliensis* and a further group of naïve mice were infected with ~600 iL3 *N. brasiliensis* subcutaneously at the same time to allow for measurement of protection (primary – white bars). **A.** At two days post reinfection lung tissues were harvested, worms were migrated and enumerated. **B.** At six days post reinfection gut tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data representative of at least 3 independent experiments. Statistics were calculated using one-way ANOVA with Dunnets post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

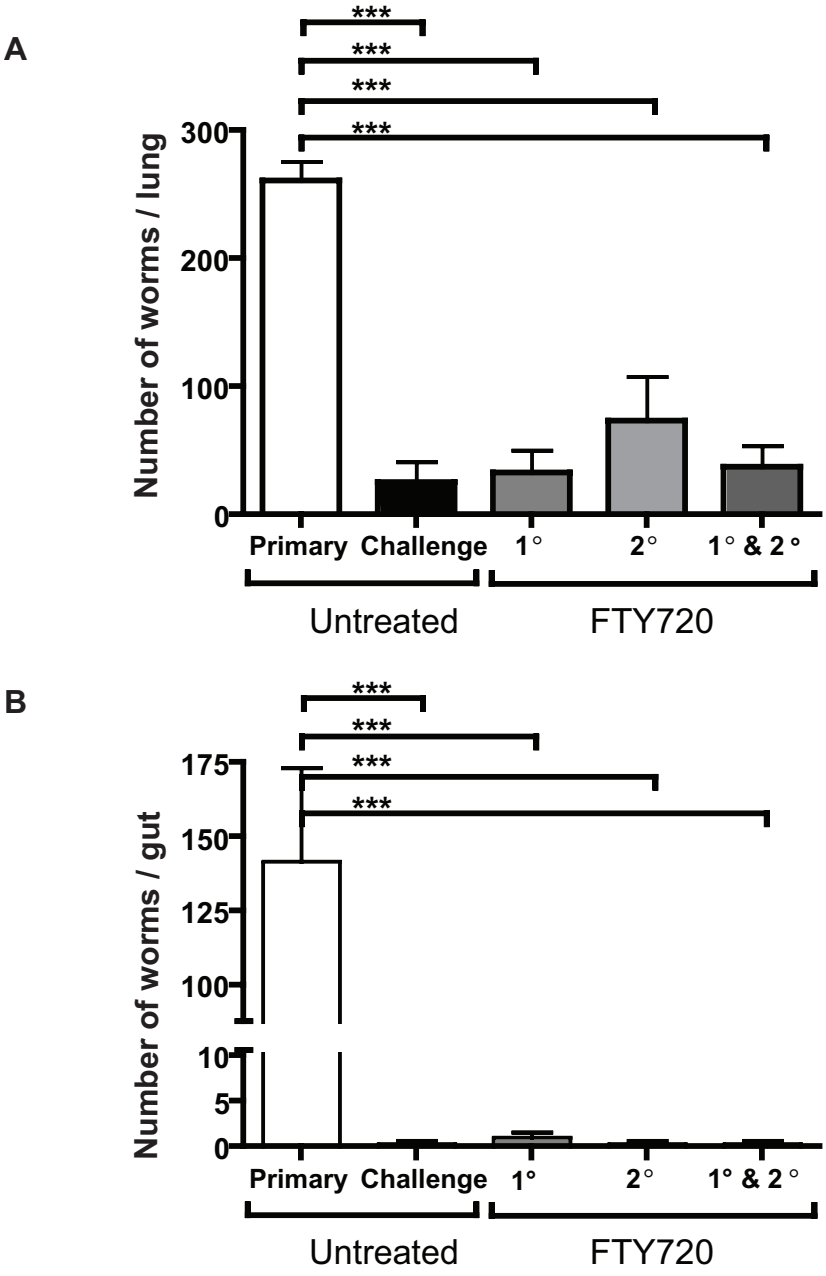

Figure 4.6 FTY720 administration does not affect protection against reinfection with *N. brasiliensis* regardless of time of treatment.

G4/IL-4 mice (n=3/group/time point) were infected with *N. brasiliensis* and either remained untreated (white and black bars) or were treated with FTY720 (grey bars). Mice receiving FTY720 had several treatment regimes; FTY720 during primary infection only (1°, day -one to day nine of infection), FTY720 during reinfection only (2°, day 29 until sacrifice) or FTY720 during entire infection (1° & 2°, FTY720 from day -one to sacrifice). At 30 days post infection mice were reinfected with *N. brasiliensis* and a further group of naïve mice were infected with *N. brasiliensis* at the same time to allow for measurement of protection (primary – white bars). **A**. FTY720 treatment does not affect protection against reinfection with *N. brasiliensis* in the lung. Two days post reinfection lung tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. **B**. FTY720 treatment does not affect protection against reinfection with *N. brasiliensis* in the gut. Six days post reinfection gut tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data are representative of at least two independent experiments. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.4. The effects of FTY720 treatment on CD4 T cell responses against N. brasiliensis

Immune responses usually follow a pattern after infection; an expansion phase characterised by the generation of effector cells from specific naïve precursors to combat infection, followed by a contraction phase where effector cells die off and memory is established. Upon reinfection an immune response tends to be faster and of greater magnitude, clearing the pathogen before it has time to establish. To build on the discovery that separation of lung and lymph node CD4 T cell responses did not alter protection against reinfection with *N. brasiliensis*, we investigated the effects of FTY720 treatment on the CD4 T cell response against *N. brasiliensis*.

4.2.4.1. FTY720 administration during *N. brasiliensis* infection reduces magnitude but not pattern of lymph node response

Investigation of the Th2 immune response against *N. brasiliensis* in the mediastinal (med) lymph node, as measured by GFP+ CD4 T cells, showed the expected trend in untreated animals (Fig 4.7A - left hand panel). An expansion of GFP+ cells was observed during the primary response, followed by a contraction to a baseline level and then upon reinfection a fast expansion of T cells up to a three fold greater magnitude than the initial primary response.

When mice were treated with FTY720 (Fig 4.7A - right hand panel) the overall magnitude of response was reduced, with the total number of GFP+ CD4 cells during both primary infection and reinfection more than four times less than that seen in the untreated animals. However the general pattern of expansion and contraction followed by an enhanced re-expansion upon reinfection remained, with a four-fold increase in GFP+ Th2 cells after reinfection when compared to primary infection.

This data shows that treatment with FTY720 decreased the magnitude of the *N. brasiliensis* induced Th2 response in the med lymph node, but did not affect the generation of primary and secondary responses.

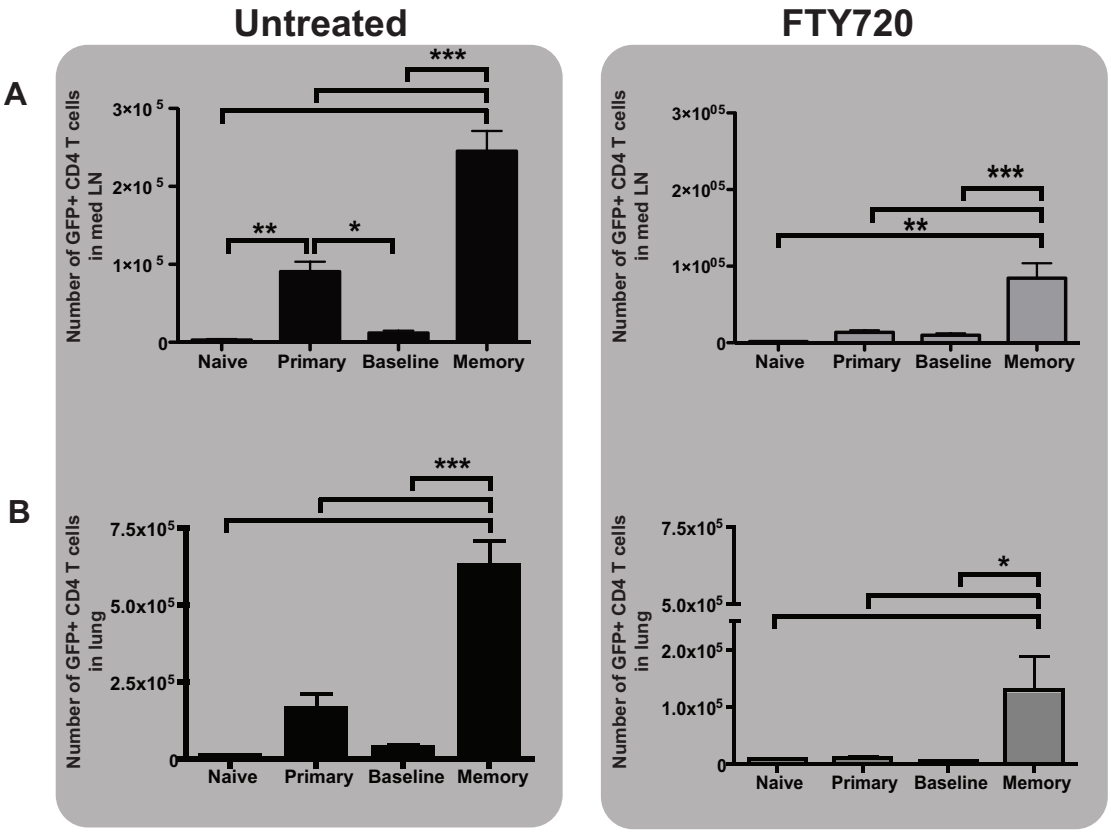

Figure 4.7 FTY720 treatment affects generation of primary immune responses in the lungs of *N. brasiliensis* infected mice.

G4/IL-4 mice (n=3/group/time point) were infected with *N. brasiliensis* and either remained untreated (black bars) or were treated with FTY720 from one day prior to infection (grey bars). At 30 days post infection mice were reinfected with *N. brasiliensis*. Mediastinal lymph nodes and lung tissues were harvested during primary infection (day nine post infection), prior to reinfection (baseline – day 39 post infection) and during reinfection (day 36 post infection) and also from naïve animals. **A**. FTY720 treatment affects the magnitude but not the pattern of Th2 response in the mediastinal lymph node. Cells were isolated from the lymph nodes by enzymatic digestion before being stained and analysed by four colour flow cytometry. Values represent the number of GFP+ cells present in the CD4 T cell population of the mediastinal lymph node ± S.E.M. Data is from two combined experiments (n=4-9/group). Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **B**. FTY720 treatment affects the magnitude and the pattern of Th2 response in the lung. Cells were isolated from the lungs by enzymatic digestion and enriched for CD45+ cells by AutoMacs© bead separation before being stained and analysed by four colour flow cytometry. Values represent the number of GFP+ cells present in the CD4 T cell population of the lungs ± S.E.M. Data representative of at least two independent experiments. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

4.2.4.2. FTY720 administration during *N. brasiliensis* affects the generation of primary CD4 T cell responses in the lung

We next assessed the effects of FTY720 treatment on the generation of a Th2 response in the relevant tertiary lymphoid tissue for this model – the lung. Fig 4.7B (left hand panel) shows the Th2 response as measured by GFP+ CD4 T cells in the lung of untreated *N. brasiliensis* infected mice. Primary infection generated an expansion of GFP+ CD4 T cells above the level of naïve animals, followed by a contraction (seen prior to challenge) and upon reinfection, an expansion of GFP+ Th2 cells almost three times greater than during primary infection.

When FTY720 treated mice were compared to the untreated *N. brasiliensis* infected mice we saw very little evidence of an expansion during the primary infection in FTY720 treated mice, as the number of GFP+ CD4 cells was very similar to that seen in naïve animals (Fig 4.7B – right hand panel). However, there does seem to be a contraction stage and post reinfection the numbers of GFP+ CD4 T cells are increased 10 fold from those seen in the lung during primary infection.

These results show that FTY720 treatment has affected the local response in the tertiary lymphoid tissue, causing a reduced primary response against *N. brasiliensis*. Interestingly, drug treatment did not prevent a more successful expansion of cells upon reinfection, suggesting that these cells were not recruited from the lymph node but expanded from an existing population of cells.

4.2.4.3. FTY720 administration during *N. brasiliensis* infection reduces magnitude but not pattern of skin CD4 T cell response

We next assessed the effect of FTY720 treatment at a tertiary site that is irrelevant for protective immunity in this model, the skin (see chapter 3). In previous experiments we had seen a difference in secondary and tertiary lymphoid responses against *N. brasiliensis*. When animals were treated with FTY720, we found secondary lymphoid responses decreased in magnitude but the pattern of expansion, contraction and re-expansion remained intact. Tertiary lymphoid responses in the lung (the site of protective priming in this model) were found to be of decreased magnitude and also deviated from the expected expansion/contraction/expansion immune response pattern, generating an expanded secondary response after a minimal primary response. In order to see whether this response

held true for all tertiary lymphoid tissues in this model, we investigated the effect of FTY720 treatment on the Th2 immune response in the skin after *N. brasiliensis* infection, as measured by GFP+ CD4 T cell numbers.

We found that FTY720 treatment during *N. brasiliensis* infection reduced the magnitude of response at the skin site during primary and secondary infection. However, the expansion of Th2 cells upon reinfection was three fold greater than that of the primary response, the same as that seen in untreated *N. brasiliensis* infected animals, in contrast to my findings in the lung where a 10 fold expansion of Th2 cells was observed in FTY720 treated animals.

These results show that the Th2 response at the skin site is decreased in magnitude but does not show a change in the overall relationship between immune cell expansion/ contraction and parasite infection/clearance with FTY720 treatment that was seen in the lung. In conjunction with the previous data this suggests that there is something unique about the lung-based immune response in this model, as prevention of lymphocytes trafficking to this tissue, although diminishing a primary Th2 immune response, does not prevent the generation of an enhanced local secondary immune response capable of protecting against reinfection with *N. brasiliensis*.

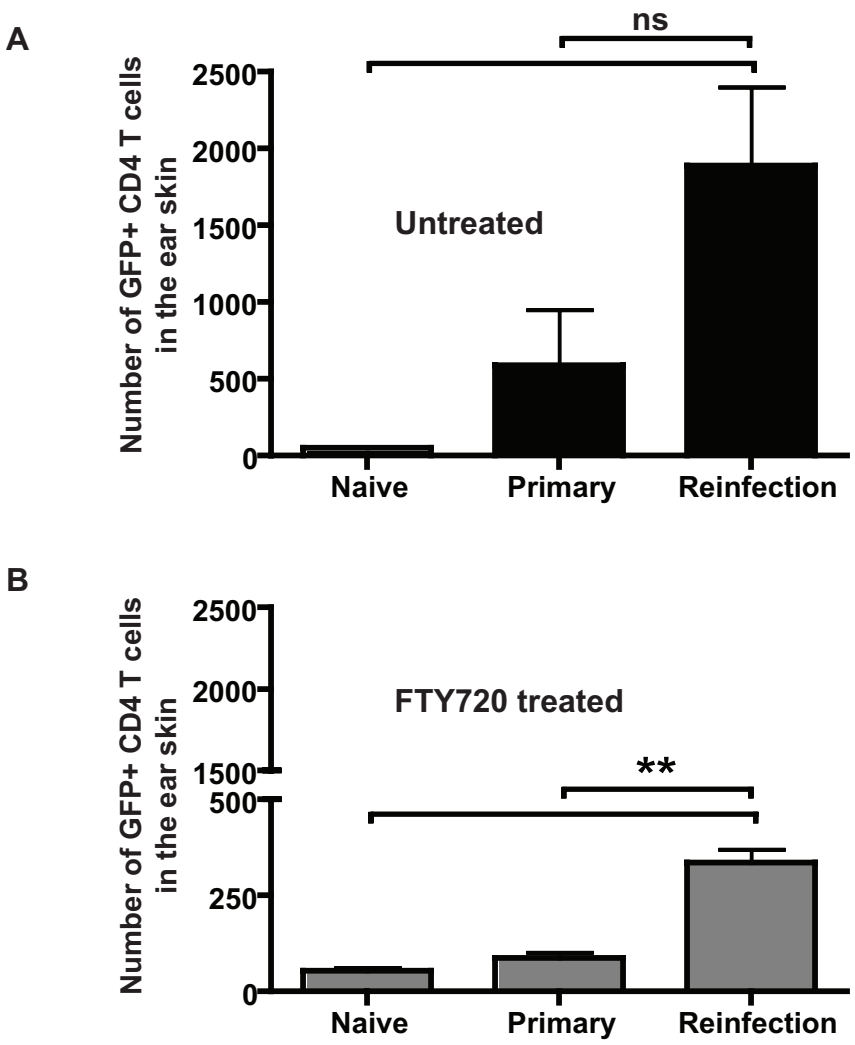

Figure 4.8 FTY720 treatment does not affect immune responses to *N. brasiliensis* in the skin.

A. Th2 immune response in the skin of *N. brasiliensis* infected mice. G4/IL-4 mice (n = 3/group/time point) were infected intradermally with *N. brasiliensis*. Ear tissues were harvested during primary infection (day 9 post infection) and also during reinfection (day 6 post reinfection) and also from naïve animals. Ears were enzymatically digested and the resulting cells stained for four colour flow cytometric analysis. Values represent the number of GFP+ CD4 T cells present in the ear ± S.E.M. **B**. Th2 immune response in the skin of *N. brasiliensis* infected FTY720 treated mice. G4/IL-4 mice (n = 3/group/time point) were treated with FTY720 from one day prior to intradermal infection with *N. brasiliensis*. Ear tissues were harvested during primary infection (day nine post infection) and also during reinfection (day six post reinfection) and also from naïve animals. Ears were enzymatically digested and the resulting cells stained for four colour flow cytometric analysis. Values represent the number of GFP+ CD4 T cells present in the ear ± S.E.M. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

4.2.5. Blocking recirculation of CD4 T cells between lung and lymph nodes in a Th1 model of lung mediated protective immunity

To see if our findings were relevant to other lung models of protective immunity we wanted to compare our findings with the *N. brasiliensis* model in a Th1 mediated model of lung protective immunity. We chose the BCG model as it is dependent on both CD4 and IFNγ. Previously we have shown the effects of FTY720 treatment on *N. brasiliensis* infection; here we show the effects of FTY720 administration during BCG vaccination and challenge.

4.2.5.1. FTY720 treatment inhibits T cell recirculation during BCG vaccination

Initially, to confirm the FTY720 mediated sequestering of lymphocytes in the lymph nodes during BCG vaccination, mice were tail bled and the proportion of CD4+ T cells in the circulation were assessed by flow cytometry (Fig 4.9). FTY720 treatment reduced CD4 T cells as a percentage of recirculating lymphocytes approximately 10 fold from 20 % to 2 %.

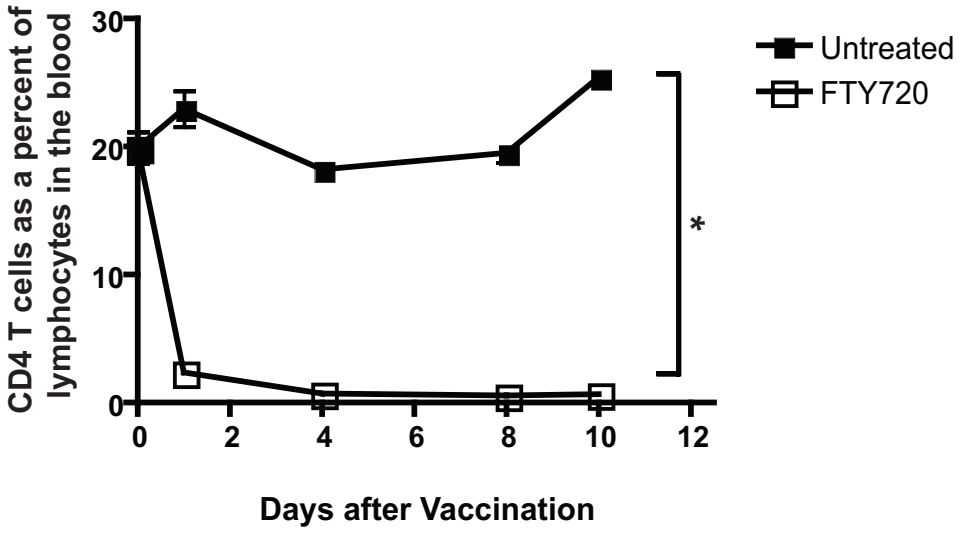

Figure 4.9 FTY720 treatment reduces circulating CD4 T cells after BCG vaccination

C57BL6 mice (n= 5/group) were untreated or treated with FTY720 (1.0mg/kg) from the day prior to subcutaneous vaccination with 1 x 10^6 CFU. Blood was collected from the tail vein at the indicated time point, red blood cells lysed and resulting cells stained for four colour flow cytometric analysis. Cells were gated on lymphocyte population using forward and side scatter and CD4 T cells were assessed as a percentage of the lymphocyte population. Values represent percentage of CD4 T cells present in the lymphocyte population of the blood ± S.E.M. Statistics were calculated using a non-parametric Mann-Whitney test* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.5.2. Blocking recirculation of CD4 T cells between lung and lymph nodes during primary BCG intranasal infection inhibits protection

To test the effects of blocking CD4 T cell recirculation between lung and lymph node during primary infection with BCG, mice were treated with FTY720 prior to intranasal infection with BCG and the resulting bacterial burden compared to that of untreated BCG infected mice.

FTY720 treated mice received 1mg/kg FTY720 daily by oral gavage from one day prior to intranasal infection with 10^6 colony forming units (CFU) BCG. At various time points post infection spleen, lung and liver were harvested, homogenised and plated onto selective 7H11 supplemented agar plates to allow growth of bacteria. Bacteria were detected in lung homogenates as early as four days post infection and from day 25 post infection bacteria were grown from the homogenised spleen and liver tissue also. Fig 4.10 shows the bacterial burden in the spleen, liver and lungs of FTY720 treated and untreated animals at day 28 post infection and it can be seen that FTY720 treated animals have a heavier bacterial burden in all tissues.

This data suggests that blocking CD4 T cell recirculation between lung and lymph node did not initially affect the bacterial load. However, over the course of the infection FTY720 treated mice that were unable to recruit lymphocytes from the secondary lymphoid organs were unable to control the BCG infection as well as control mice. This result indicates that T cell recruitment from the lymph node during primary BCG infection is important to control infection and limit dissemination.

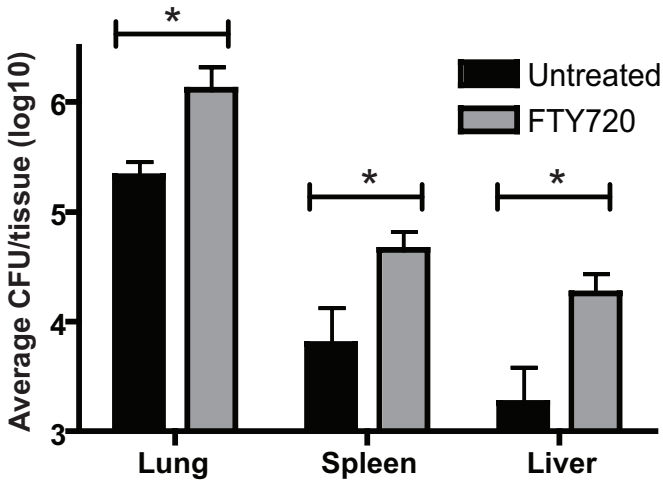

Figure 4.10 FTY720 administration during primary BCG infection leads to increased bacterial dissemination.

C57BL6 mice (n = 5/group) remained untreated or were treated with 1mg/kg FTY720 by gavage from the day prior to infection with 1 x 10^6 CFU BCG i.n. At 28 days post infection mice were sacrificed and lungs, livers and spleens harvested. Tissues were homogenised and plated on selective media to determine bacterial growth. Values represent the mean number of CFU per tissue ± S.E.M. Data are representative of at least two independent experiments. Statistics were calculated for each tissue using Student's t test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.5.3. Blocking CD4 T cell recirculation between lung and lymph node during BCG infectious challenge does not affect protection

To further investigate the contribution of lymph node and lung CD4 T cell responses to protective immunity against BCG, experiments were carried out blocking the recirculation of CD4 T cells during vaccination and challenge. Treatment with FTY720 during vaccination, challenge or both aimed to explore when T cell trafficking between lymph node and lung tissues might be required to confer protection.

Daily FTY720 treatment was established to prevent trafficking of CD4 T cells from lymph nodes to lung during vaccination, challenge or both (detailed in experimental design Fig 4.11). To allow activated T cells to enter a resting memory phase and clear chronic bacterial infection all mice were treated with the antibiotic isoniazid for a duration of four weeks, beginning four weeks post vaccination. To allow normal recirculation prior to challenge infection, FTY720 treatment was ceased five weeks post vaccination. After challenge infection with 10^4 CFU BCG, mice were sacrificed and their lungs were homogenised and plated on 7H11 selective agar plates to allow bacterial growth.

Growth of bacteria in the lungs of FTY720 treated and untreated, BCG vaccinated and challenged mice, was compared to bacterial growth in unvaccinated BCG challenged animals in order to observe protection conferred by vaccination (Fig 4.12). Mice that had been vaccinated and challenged with BCG exhibited a 4.5 fold reduction in bacterial burden compared to unvaccinated controls. Interestingly, mice that had received FTY720 treatment only during challenge had an eight fold reduction in bacteria in the lungs. However, animals that had FTY720 treatment during vaccination or during both vaccination and challenge were unprotected – exhibiting similar levels of bacterial growth to unvaccinated control mice.

These results show that the blocking CD4 T cell recirculation between lung and lymph node during vaccination can impact on the protection conferred; suggesting that a population of T cells derived from the lymph node during vaccination is important for protective immunity against BCG. This data also suggests that during challenge there is no requirement for lymph node derived T cells to confer protection, indicating that the population of protective cells has migrated to the lung tissue after vaccination but before challenge.

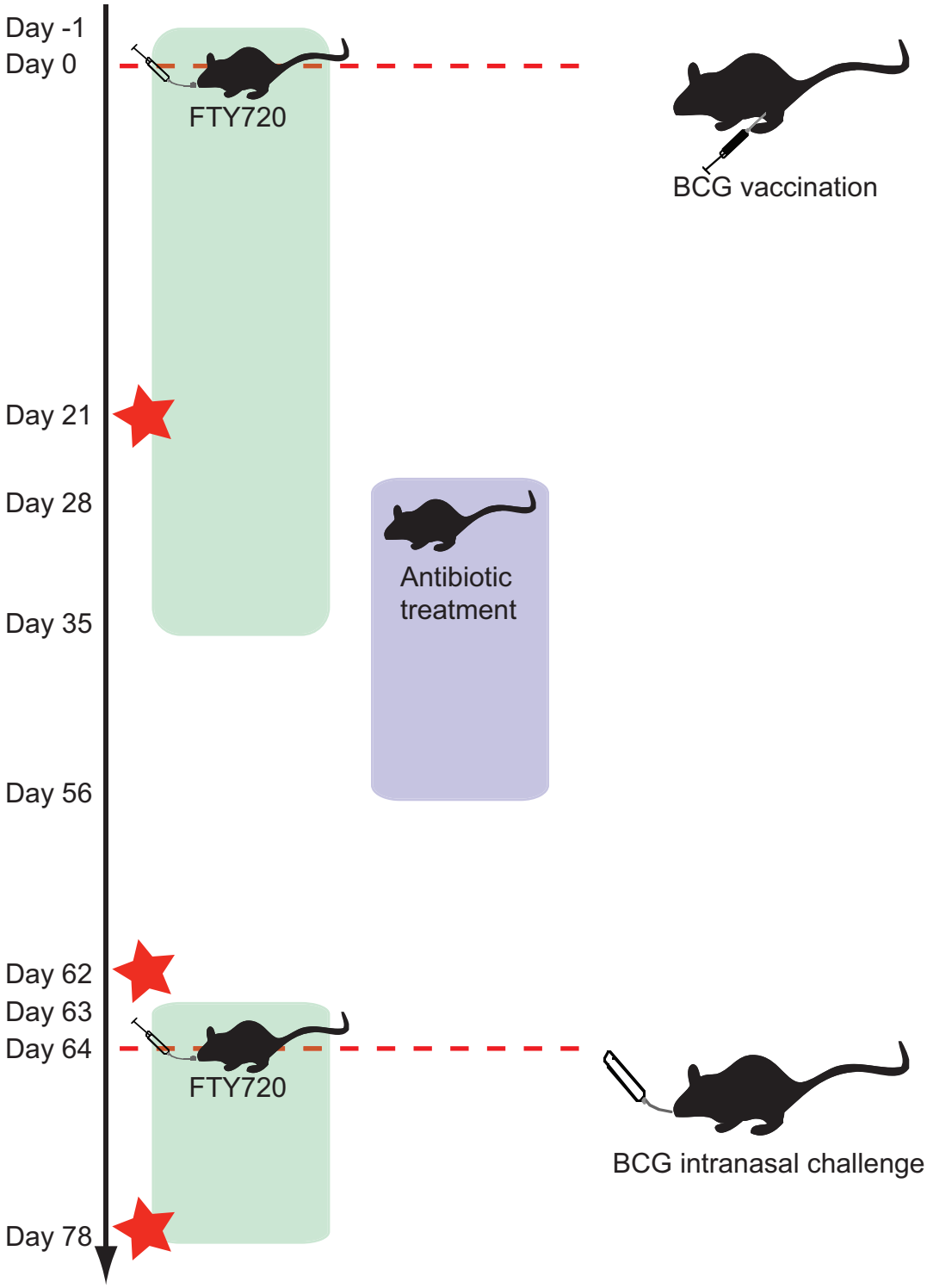

Figure 4.11 Experimental design to assess the effects of blocking CD4 T cell recirculation between lung and lymph nodes during BCG vaccination and challenge.

C57BL6 mice (n= 5/group/time point) were vaccinated s.c. with 1 x10^6 CFU BCG at day zero. Four weeks post vaccination (day 28) mice received the antibiotic isoniazid in their drinking water (1mg/ml) to clear any residual bacteria resulting from vaccination and at day 64 post vaccination mice were intranasally challenged with 1 x 10^4 CFU BCG. At the time of intranasal challenge a previously unvaccinated group was included to assess any protection conferred by BCG vaccination. Within BCG vaccinated and challenged animals several groups of mice were established to assess the effects of treatment with 1mg/kg FTY720 by daily gavage. These mice received FTY720 treatment only during primary infection (day -1 to day 35), only during challenge infection (from the day prior to challenge, day 63 to time of sacrifice), during both primary and challenge infection or not at all. At the indicated time points (red stars), mice were sacrificed and tissues harvested to assess bacterial burden or tissue immune response.

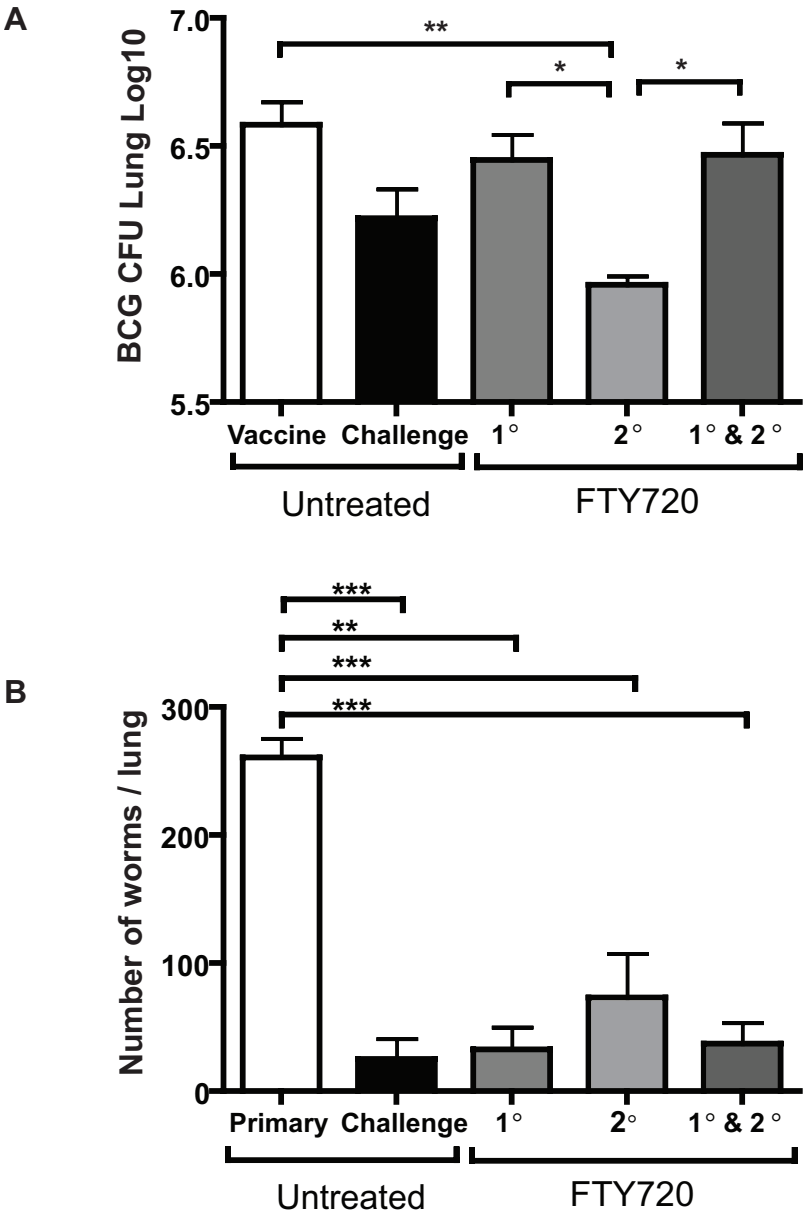

Figure 4.12 Effects of FTY720 administration on protection: a comparison of the BCG and *N. brasiliensis* models.

A. C57BL6 mice (n = 5/group/time point) were vaccinated and challenged with BCG (black bar), and treated with FTY720 as described in Fig 4.11 (grey bars). An unvaccinated group was challenged at the same time as vaccinated groups to measure protection conferred by vaccination (white bar). At day 28 post BCG challenge, lung tissues were harvested, homogenised and plated on selective agar plates to assess bacterial load. Values represent mean bacterial load in the lung tissue ± S.E.M. Data are representative of at least two independent experiments. **B.** G4/IL-4 mice (n=3/group/time point) were infected with *N. brasiliensis* and either remained untreated (black bar) or were treated with FTY720 (grey bars). Mice receiving FTY720 had several treatment regimes as described in Fig 4.6. After 30 days mice were reinfected with *N. brasiliensis* and a further group of naïve mice were infected at the same time to allow for measurement of protection (white bar). At two days post reinfection lung tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data representative of at least two independent experiments. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

4.2.5.4. The effects of blocking CD4 T cell recirculation from the lymph node on the lung immune response against BCG

Blocking CD4 T cell recirculation between lung and lymph node using FTY720 in the *N. brasiliensis* model altered the pattern of the lung but not lymph node CD4 T cell responses. Our findings in the *N. brasiliensis* model showed a decrease in the number of IL-4 producing T cells in the lung during primary infection, with a 10 fold increase upon reinfection in FTY720 treated animals (Fig 4.7B, Fig 4.13D). The following experiments assess the effects of FTY720 treatment on the lung response to BCG vaccination and challenge, showing data from mice receiving FTY720 treatment during vaccination and challenge.

Lung tissues were harvested from BCG vaccinated and challenged mice at several time points during vaccination and challenge (as described in Fig 4.11). Single cell suspensions were prepared from the collagenase digested lung tissue and the resulting cells cultured in the presence of *M. bovus* culture filtrate protein (CFP) overnight. The cultured cells were stained intracellularly to measure the antigen specific production of IFNγ (Fig 4.13A and B).

Control infected animals (Fig 4.13A) show the classic immune response pattern, with IFNγ producing CD4 T cells expanding during vaccination then contracting before expanding again following challenge infection. This result mirrors that seen in *N. brasiliensis* infected mice (Fig 4.7 and 4.13C). In the lung tissue of FTY720 treated animals (Fig 4.13B) there is an expansion of IFNγ+ CD4 T cells during primary vaccination but unusually no contraction is observed prior to challenge (baseline). This may be explained by increased recirculation due to the rest from FTY720 treatment in this model (Fig 4.11). Upon reinfection a five fold expansion of IFNγ producing T cells is observed, although this is not sufficient to protect from challenge infection (Fig 4.12). This result differs from that seen in the lungs of *N. brasiliensis* infected FTY720 treated mice where there is a minimal CD4 T cell primary immune response barely above the background observed in naïve mice followed by a ten fold increase upon reinfection (Fig 4.7 and 4.13D).

This experiment demonstrates that FTY720 treatment has different effects in the BCG model compared to our findings in the *N. brasiliensis* model, specifically when assessing antigen specific CD4 T cell responses within the lung tissue.

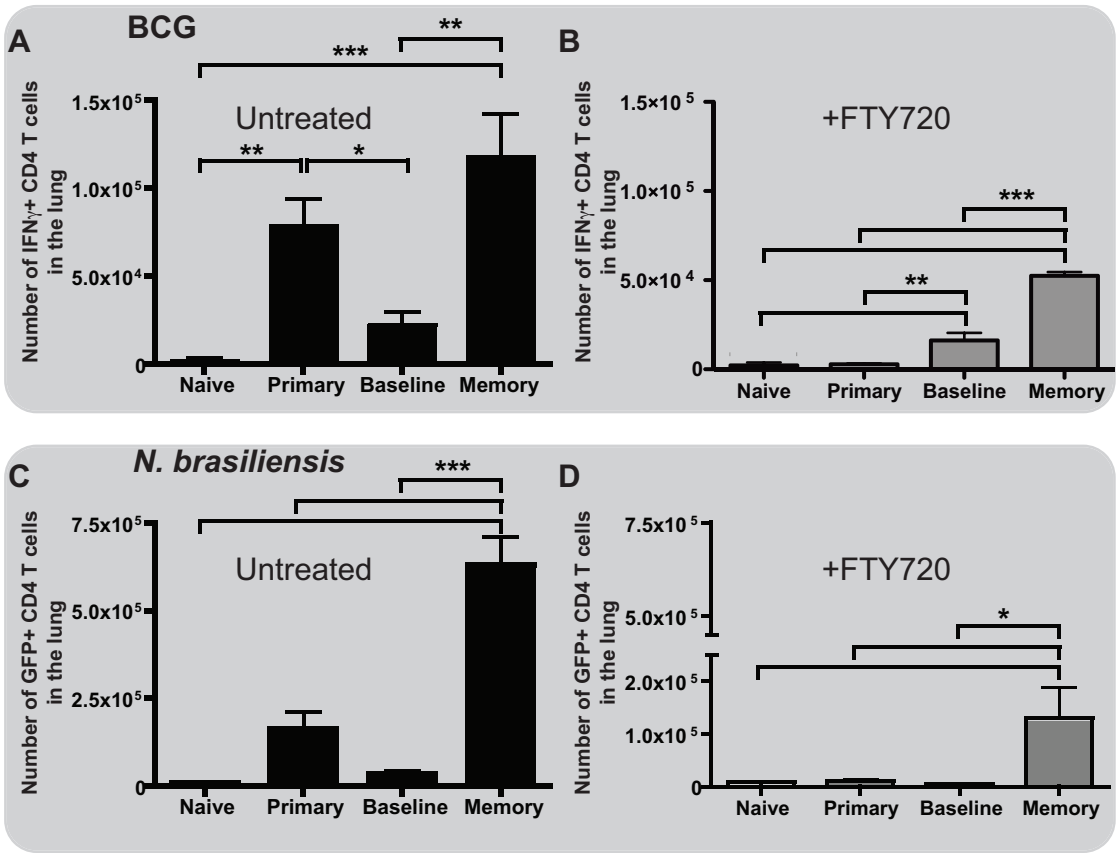

Figure 4.13 The effect of FTY720 administration on the antigen specific CD4 T cell response in the lung: A comparison of the BCG and *N. brasiliensis* models.

A and **B**. C57BL6 mice (n= 5/group/time point) were vaccinated with BCG and treated with FTY720 during vaccination and challenge infection as described in Fig 4.11. The production of IFNγ was assessed from CD4 T cells isolated from naïve animals, those at the peak immune response to primary infection (Day 21), prior to intranasal challenge (base line – day 62 post primary) and during the peak of challenge infection (day 14 post challenge). IFNγ production was measured by intracellular cytokine staining after overnight *in vitro* restimulation with CFP and analysis performed on a four colour flow cytometer. Data in **A** represents production of IFNγ by CD4 T cells isolated from the mediastinal lymph nodes, and data in **B** represents production of IFNγ by CD4 T cells isolated from the lung. Values represent the mean number of IFNγ+ CD4 T cells after overnight restimulation ± S.E.M. Data representative of three independent experiments. **C** and **D.** G4/IL-4 mice (n=3/group/timepoint) were infected with *N. brasiliensis* and were treated with FTY720 from one day prior to infection. At 30 days post infection mice were reinfected with *N. brasiliensis*. Mediastinal lymph nodes and lung tissues were harvested during primary infection (day nine post infection), prior to reinfection (baseline – day 39 post infection) and during reinfection (day six post reinfection) and also from naïve animals. **C.** Cells were isolated from the lymph nodes by enzymatic digestion before being stained and analysed by four colour flow cytometry. Values represent the mean number of GFP+ cells present in the CD4 T cell population of the mediastinal lymph node ± S.E.M. **D.** Cells were isolated from the lungs by enzymatic digestion and enriched for CD45+ cells by AutoMacs© bead separation before being stained and analysed by four colour flow cytometry. Values represent the number of GFP+ cells present in the CD4 T cell population of the lungs ± S.E.M. Data representative of at least two independent experiments. Statistics were calculated using one-way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

4.3. Discussion

The objectives of this chapter were to prevent recirculation of CD4 T cells between the lymph nodes and lung tissue using the drug FTY720, and to assess the effects on both protection from reinfection and the cellular immune response in the *N. brasiliensis* model.

We have assessed the blocking of CD4 T cell recirculation between lung and lymph node using FTY720, confirming the effects of FTY720 treatment on sequestration of circulating CD4 T cells in naïve animals, and observed a reduction in both blood and tissue dwelling T cells in treated animals. We also investigated the effects of FTY720 during *N. brasiliensis* infection and also BCG vaccination, and found a suppression of CD4 T cell recirculation in both models.

Separation of lymph node and lung CD4 T cell responses through FTY720 treatment does not affect protective immunity against *N. brasiliensis*. It is perhaps not surprising that we observed no difference in kinetics of migration or worm burden in primary *N. brasiliensis* infection, as the immune response occurs at a time after worms have already left the tissues. However, it was quite unexpected that protection against parasite reinfection was undiminished when FTY720 was administered to separate the T cell responses of secondary and tertiary lymphoid tissues. This finding suggests that the local immune response that is mounted at the lung site is sufficient to protect against reinfection, supporting the theory of effector lymphoid tissue being important in protective responses.

The expansion of antigen specific IL-4 producing CD4 T cells in the lung during reinfection was a surprising finding, particularly given the dramatic suppression seen in the lung immune response during primary infection. The ability of local lung immune responses to protect has been documented before, in a viral model using mice lacking secondary lymphoid organs [70, 90]. The appearance of iBALT after infection has been observed by several groups [71, 190], although the mechanisms behind iBALT generation remain unclear [83]. However the finding that local lung based immune responses are sufficient to protect against reinfection with *N. brasiliensis* is a novel finding. Also of interest is that the immune response at a different peripheral site, the skin, was not similarly affected.

Strikingly the result that blocking CD4 T cell recirculation throughout infection does not affect protection suggests that not only is the local tissue response important during

reinfection, but infers that potentially ELT can be generated locally during a primary response without input from the secondary lymphoid organs.

Our investigations have demonstrated that Th1 mediated lung protective immunity differs from Th2. Protection afforded by BCG vaccination was abrogated by treatment with FTY720 during vaccination, but not if FTY720 was given prior to infectious challenge. This differs from our findings with *N. brasiliensis* where we observed no difference in protection regardless of the timing of drug treatment. Worthy of note, FTY720 treated mice receiving primary intranasal BCG infection displayed increased dissemination of bacteria in lungs, liver and spleen. This finding contrasts with our data, which showed no difference in migration kinetics or worm burden in primary *N. brasiliensis* infection. This data clearly indicates that there are significant differences in the way that BCG and *N. brasiliensis* infections are controlled by the T cell response. The implications of these findings are significant in terms of vaccine development against different types of pathogens; although these results suggest that ELT in the lung tissue is important for protection in both models they also suggest that the BCG model requires T cells derived from the lymph nodes to develop ELT, which is not necessarily the case in the *N. brasiliensis* model.

Some of the observed differences between the Th1 and Th2 mediated models may stem from the inherent differences in the model pathogens. BCG is a small bacterium that can persist long term within the host, surviving within infected macrophages. In contrast, *N. brasiliensis* is a relatively large helminth with a much shorter infection of the host. The host infection cycle of *N. brasiliensis* is completed within 10 days in immune competent animals in contrast to the chronic infection established by BCG infection, which requires antibiotic clearance. These differences within the two models could lead to differences in antigen dose and persistence within the host tissues.

Interestingly the antigen specific cellular immune responses also differ between the two models. Using FTY720 throughout *N. brasiliensis* infection we see a minimal IL-4 response in the lung during primary infection that contracts before expanding upon reinfection. However in the BCG model when FTY720 treatment is received throughout, we see a low IFNγ lung response during primary infection, with numbers increasing prior to challenge and then increasing further upon reinfection. Although the differences in the FTY720 treatment regimes and cellular manipulations between the two models may account for this difference, the increase in cells capable of producing IFNγ in response to

BCG does not result in protection against reinfection. This interesting finding may be explained by the suggestion that IFNγ is not a reliable correlate of protection in the BCG model [195].

4.4. Conclusions

The aims of this chapter were to assess the ability of immune responses based in the peripheral lung tissue to confer protection in the *N. brasiliensis* model, and also to compare these finding to a Th1 mediated model of lung based protective immunity. From the above experiments we conclude that peripheral lung based immune responses are sufficient to confer protection against reinfection with *N. brasiliensis*. Furthermore we find that even if CD4 T cell recirculation is prevented throughout *N. brasiliensis* infection protection is unimpaired, suggesting an alternative development of effector lymphoid tissue through priming in lung tissue or a selective resistance of memory T cells to FTY720, allowing the development of ELT. Strikingly we see differences between the *N. brasiliensis* and BCG models of lung based protective immunity. We find that blocking CD4 T cell recirculation during BCG vaccination impedes the development of protective ELT, although blockade immediately prior to challenge infection does not. These results suggest that while the development of ELT in the lung tissue is an important component of protective immunity in both models, the generation of ELT has different requirements in the two models we assessed.

Chapter 5:
Characterisation of CD4 T cells mediating protection against N. *brasiliensis*

5.1. Introduction

5.1.1. Currently known requirements for protective immunity in N. brasiliensis model

Within our research group much data has been accumulated on the immune parameters of protection against reinfection with *N. brasiliensis*. Our model of protective immunity has been assessed in several strains of mice deficient in cytokines, signalling molecules or receptors (detailed in Table 5.1). A role for CD4 T cells in protection against reinfection in the lung is suggested by the data in Table 5.1, as MHC II deficient animals that lack CD4 T cells have reduced protection. Interestingly, IL-4 appears to be important to the protective response in the lung, but not the gut although the STAT 6 pathway has an important role in both lung and gut protection. This is likely due to redundancy between IL-4 and IL-13, with IL-13 also mediating worm expulsion through the shared IL-4Rα [158]. The result that IL-5 -/- mice retain protection against reinfection implies that eosinophils do not play a critical role in our model of *N. brasiliensis* protective immunity, in contrast to the findings of others in transgenic models over expressing IL-5 [3, 162].

Mouse Strain	Lung	Gut
WT	100	100
IL-5 -/-	97	85
Mu -/-	77	89
MHC II -/-	47	9
STAT 6 -/-	39	22
IL-4 -/-	25	93

Table 5.1 Immune requirements for protection from *N. brasiliensis* reinfection

Various strains of mice (as indicated) were infected with *N. brasiliensis*. At 30 days post infection mice were reinfected with *N. brasiliensis*. At two days post reinfection lung tissues were harvested and at six days post infection gut tissues were harvested, worms migrated out and enumerated. Values represent percent protection in the lung as compared to that seen in the tissues of corresponding wild type animals (100% = protection maintained, 0% = protection lost).

Also notable is that Mu -/- mice deficient in B cells and therefore the production of *N. brasiliensis* specific IgG1 and IgE exhibit a minor loss of protection, indicating that

although *N. brasiliensis* induces a strong IgE response, antibody is not critical in mediating protection against reinfection.

A role for CD4 T cells in gut expulsion has previously been shown [157], but the role of CD4 T cells in lung protective responses is unclear. The experiments detailed within this chapter assess the role of CD4 T cells in lung mediated protective immunity against *N. brasiliensis*. We aim to build on the work of the previous chapters by characterising the CD4 T cells resident in the lung by assessing their activation phenotype. The transmembrane glycolipid CD45 is expressed on CD4 T cells and the differential expression of the isoform CD45RB can be used to distinguish between naïve and memory CD4 T cells [196]. It is thought that CD45RB associates with CD4 and TCR complex in memory T cells, but not in naïve cells enabling faster reactivation upon antigen exposure. Interestingly it was observed that Th2 cells display an intermediate level of expression of CD45RB [196]. The adhesion molecule CD44 plays an important role in the immunological synapse, and surface expression of CD44 is often used as a measure of activation [197]. When assessed in combination with expression of CD62L (L-selectin, required for entry into secondary lymphoid organs), CD4 T cells can be categorised as naïve (Tn – CD45RB hi CD62L hi CD44 lo), effector (Teff - CD45RB lo/int CD62L lo CD44 hi), effector memory (T_{EM} - CD45RB lo CD62L lo CD44 hi) or central memory (T_{CM} – CD45RB lo CD62L hi CD44 hi). Th1 effector cells were not observed to express the CD45RB int phenotype [196], however expression of the survival marker CD127 (IL-7Rα) can also be used to distinguish between Tn, Teff, T_{EM} and T_{CM} as it is not expressed on Tn or Teff, but is present on T_{EM} and T_{CM} [198].

This array of cellular reagents have allowed the characterisation of the lung residing CD4 T cells during *N. brasiliensis* infection and reinfection, providing novel and precise mechanistic data to aid in the understanding of protective immunity in this model.

5.1.2. Aims

The following series of experiments were undertaken to investigate the CD4 T cell responses mediating protective immunity against *N. brasiliensis*. Once the relevance of CD4 T cells in this model was confirmed, the cells were studied in terms of effector function and relevance to protective immunity.

- To characterise the key CD4 T cell subtypes of the protective response against *N. brasiliensis*

- To compare these findings in a Th1 mediated CD4 model of protective immunity

5.2. Results

5.2.1. Depletion of CD4 T cells abrogates protection against N. brasiliensis

To ascertain the importance of the CD4 T cell response to protection from reinfection, CD4 T cells were depleted using an αCD4 antibody clone, GK1.5. Previous work has identified an important role for CD4 T cells in gut expulsion [157] and findings within our own research group in MHC II -/- mice (Table 5.1) have suggested that there may be a role for CD4 T cells in lung mediated protective immunity against *N. brasiliensis*. The following experiments investigate the importance of CD4 T cells in mediating protective immunity against *N. brasiliensis*.

5.2.1.1. anti CD4 administration depletes CD4 T cells

To assess depletion of CD4 T cells by αCD4 antibody, mice were injected i.p. with one dose of antibody (GK1.5) at varying concentrations and their blood was collected and monitored for the presence of CD4 T cells over time (Fig 5.1A). White blood cells were enriched by lysis of red blood cells and these cells were then stained for expression of TCRβ, CD4 and B220, before being analysed using flow cytometry.

A single dose of antibody was found to deplete circulating CD4 T cells for at least 28 days at all concentrations tested (Fig 5.1A). At this last time point mice were sacrificed and lymph node and spleen were harvested and assessed for the presence of CD4 T cells, which showed that these tissues had successfully been depleted of CD4 T cells (see appendix Fig A.2).

A further experiment investigated the effects of antibody depletion of CD4 T cells in the lungs of naïve mice. Mice received 0.5mg of αCD4 by i.p. injection and were sacrificed three days later. Lungs were perfused, harvested, collagenase digested and assessed for the presence of CD4 T cells by flow cytometry. We found that CD4 T cells in the lung were successfully depleted (99% depletion) by this method (Fig 5.1B – lower right panel).

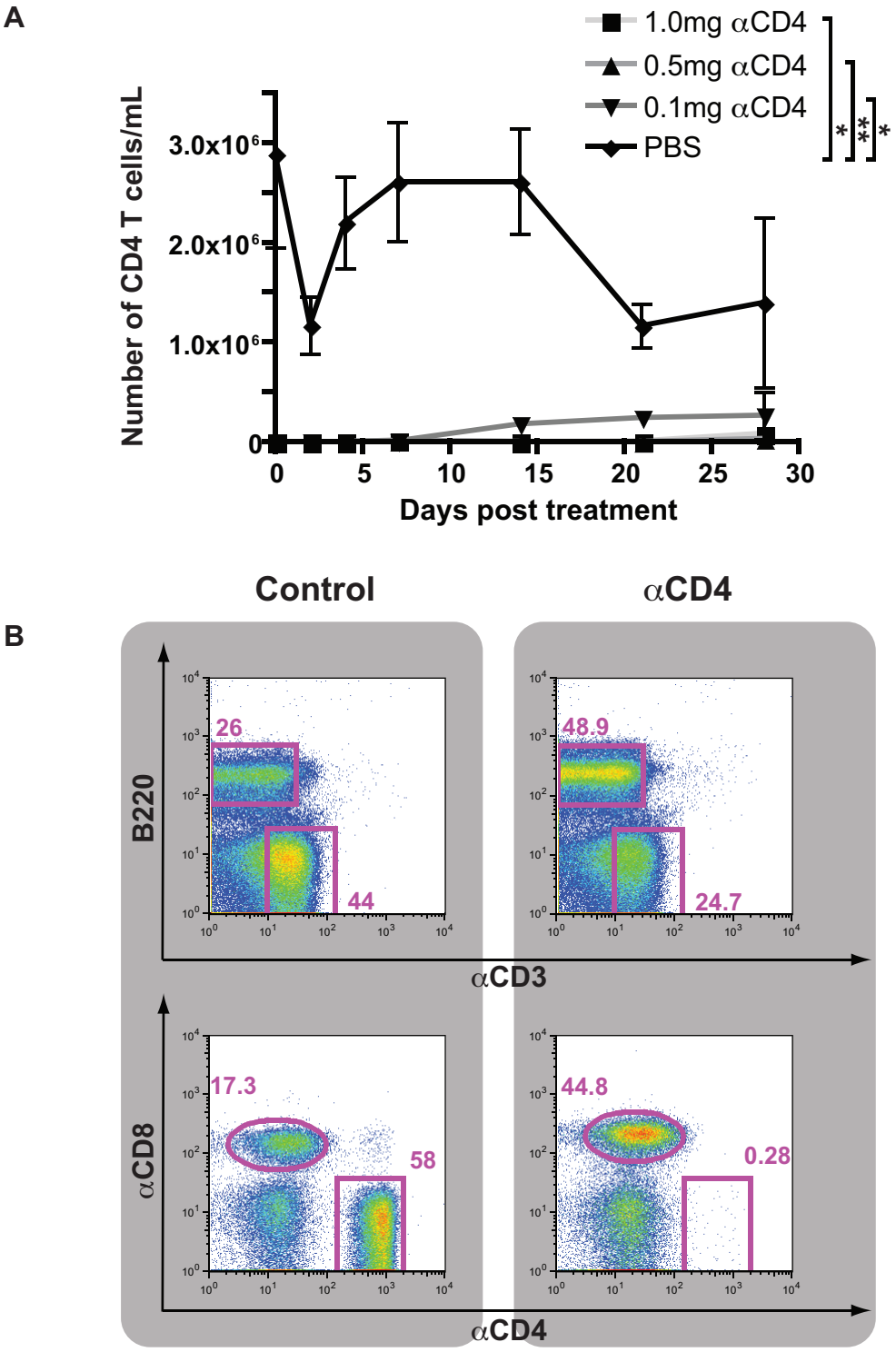

Figure 5.1 Depletion of CD4 T cells using αCD4 antibody.

A. Titration of rat IgG2b anti-CD4 depleting mAb. G4/IL-4 mice (n = 3/group) were treated with the indicated concentration of anti-CD4 antibody (clone GK1.5) by intraperitoneal injection at time zero. Mice were tail bled at the indicated time points and a known volume of blood collected. Red blood cells were lysed and circulating cells were analysed by four colour flow cytometry. The number of CD4 T cells per mL of blood was calculated by multiplying the percentage of CD4 positive lymphocytes as determined by flow cytometry with the total number of live cells as determined by trypan blue exclusion. Values represent the number of CD4 T cells per mL of blood ± S.E.M. Statistics were calculated using Kruskal Wallis analysis with Dunns post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. **B.** anti-CD4 treatment depletes CD4 T cells in the lung tissue. G4/IL-4 mice (n=3/group) were treated with 0.5mg anti-CD4 antibody (GK1.5 clone) and three days later lung tissues were harvested and enzymatically digested. CD45 positive cells were enriched by AutoMacs© bead separation before being stained and analysed by four colour flow cytometry. Upper panels show cells gated on the lymphocyte population by forward and side scatter and details gating of CD3+ and B220+ cells. Lower panels show expression of CD8 and CD4 on cells within the CD3+ gate. Facs plots are from one representative animal per group.

5.2.1.2. Depletion of CD4 T cells abrogates protection against *N. brasiliensis*

To test if CD4 T cells are required to mediate lung protective immunity against *N. brasiliensis* infections, mice were depleted of CD4 T cells either prior to reinfection or throughout priming and reinfection with *N. brasiliensis*. This depletion was carried out in *N. brasiliensis* infected mice treated with FTY720 as well as untreated *N. brasiliensis* infected control animals (experiment overview Fig 5.2). Mice were sacrificed after reinfection with *N. brasiliensis* and organs were assessed for worm burden, lungs two days post challenge and guts on six days post challenge.

Viable worms migrated from tissues were enumerated and compared to those migrated from primary infected control animals to assess protection. Depletion of CD4 T cells during reinfection or throughout infection abrogated protection in the lung and gut of FTY720 treated mice as shown in Fig 5.3A. A requirement for CD4 T cells was also observed in the gut, as depletion of CD4 T cells throughout infection or prior to reinfection abrogated the protection observed in CD4 replete animals Fig 5.3B. Similar results were observed in mice that were not treated with FTY720.

These results suggest a critical role for CD4 T cells in mediating protective immunity against *N. brasiliensis*.

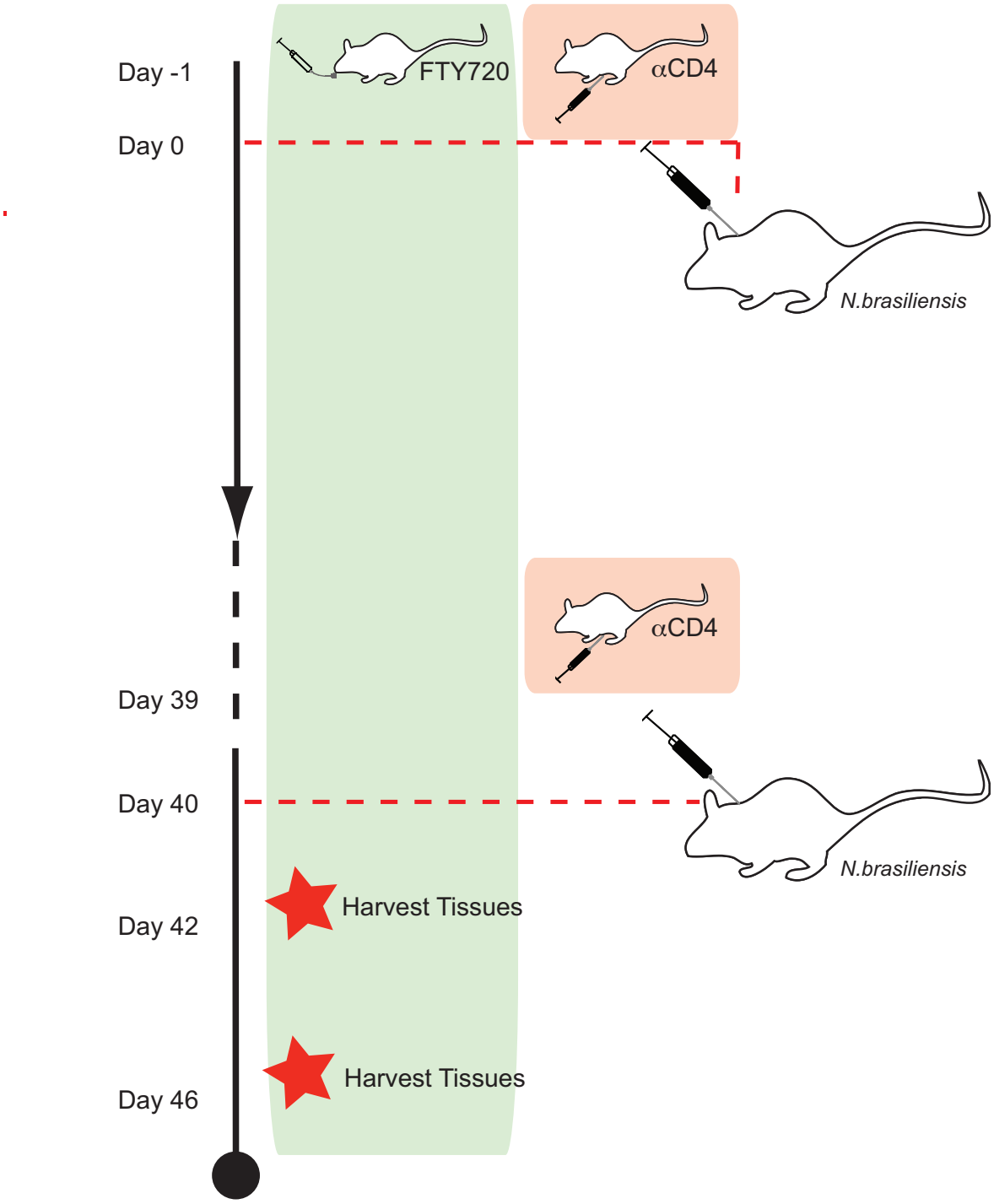

Figure 5.2 Experimental design to assess the relevance of CD4 T cells to protective immunity against *N. brasiliensis*.

On the day prior to infection G4/IL-4 mice (n=3/group/time point) were treated with FTY720 or remained as untreated controls. Within these two groups an anti-CD4 antibody depletion regime was established where mice received depleting antibody (clone GK1.5, 0.5mg/mouse i.p.) on the day prior to *N. brasiliensis* infection (at day -1) and also prior to reinfection at day 39, prior to reinfection alone, or remained untreated. At the time of reinfection a group of naïve mice received a primary *N. brasiliensis* infection in order to compare the protection attained by the reinfected mice. At two days post reinfection lung tissues were harvested and at six days post reinfection gut tissues were harvested, worms were migrated and enumerated and the effects of CD4 T cell depletion on protection against reinfection observed.

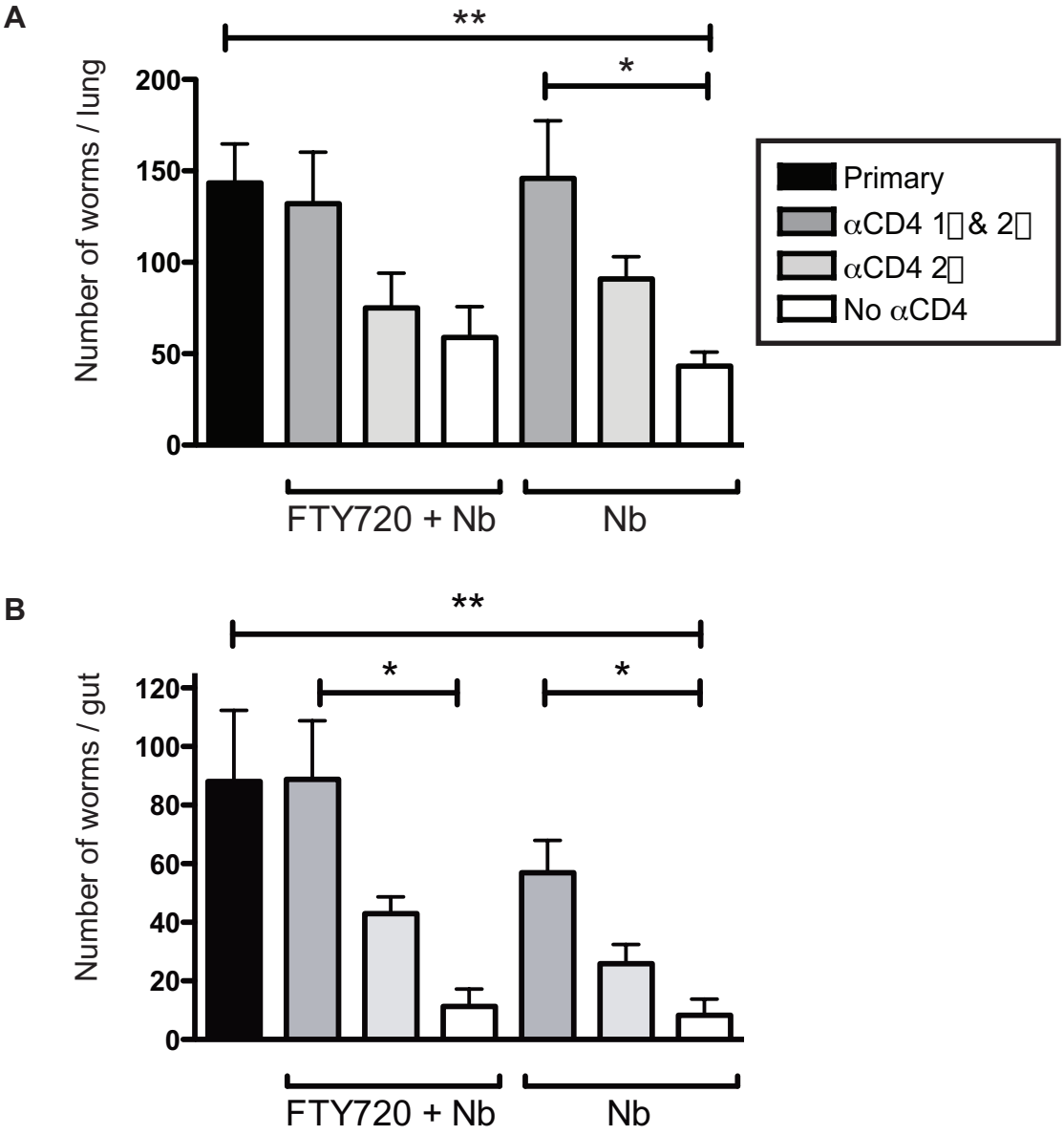

Figure 5.3 Depletion of CD4 T cells abrogates protection against reinfection with *N. brasiliensis*.

On the day prior to infection G4/IL-4 mice (n=3 per group per time point) were treated with FTY720 or remained as untreated controls. Within these two groups an anti-CD4 antibody depletion regime was established where mice received depleting antibody (clone GK1.5, 0.5mg/mouse i.p.) prior to *N. brasiliensis* infection at day -1 and also prior to reinfection at day 39 (αCD4 1° & 2°), prior to reinfection alone (αCD4 2°), or did not receive depleting antibody (No αCD4). At the time of reinfection a group of naïve mice received a primary *N. brasiliensis* infection in order to compare the protection attained by the reinfected mice. **A**. At two days post reinfection lung tissues were harvested and worms were migrated and enumerated. Data presented as combined results of three independent experiments (n=9-14/group total) **B.** At six days post reinfection gut tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data presented as combined results of two independent experiments (n=4-9/group total). Statistics were calculated using Kruskal Wallis analysis with Dunns post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

5.2.2. Adoptive transfer of CD4 T cells into naïve hosts confers protection

Having demonstrated the importance of CD4 T cells in mediating protective immunity against *N. brasiliensis*, I decided to investigate whether transfer of T cells into naïve hosts could protect them against *N. brasiliensis* infection.

5.2.2.1. Comparison of adoptive transfer techniques to establish lung localised cell transfer

Before testing whether transfer of T cells could protect against *N. brasiliensis* I first needed to optimise a method of transferring cells to the lungs of recipient mice. To determine the optimal technique I tested two methods of cell adoptive transfer, intravenous (i.v.) and intranasal (i.n.). These methods were assessed using both lung derived cells and lymph node derived cells, as "addressins" present on the cells may have influence over their recirculation – mucosal addressin $\alpha_4\beta_1$ for lung, CD62L for lymph node homing.

Lung or lymph nodes from donor mice were harvested and collagenase digested. Single cell suspensions were washed then labelled with CFSE and counted, before being transferred into recipients either i.n. or i.v. Two days later (at the time point when worms would be at peak numbers within the lung tissue) mice were sacrificed and their tissues investigated for transferred cells. Lung cells were transferred at 5×10^5 cells per animal and lymph node cells were transferred at 5×10^6 cells per animal, a 10 fold difference. Recovery of cells was assessed in terms of total numbers and also percentage of transferred cells.

Cells that were transferred i.v. could be located in the lymph nodes (Fig 5.4A) and spleens (see appendix Fig A.3) of recipient animals in high numbers. Approximately five percent of i.v. transferred cells could be recovered from the mediastinal lymph node and a similar proportion was also recovered from the spleen. However i.v. transferred cells did not home efficiently to the lung (Fig 5.4B) as less than 0.5% of transferred cells were recovered from that tissue. There was no apparent difference in homing between cells derived from donor lymph node or lung.

Cells that were transferred i.n. could be recovered from within the lung and airways with relatively good efficiency, with a 6-7% recovery (Fig 5.4B). Recovery of i.n. transferred cells from the mediastinal lymph node (Fig 5.4A) or spleen (see appendix Fig A.3) resulted

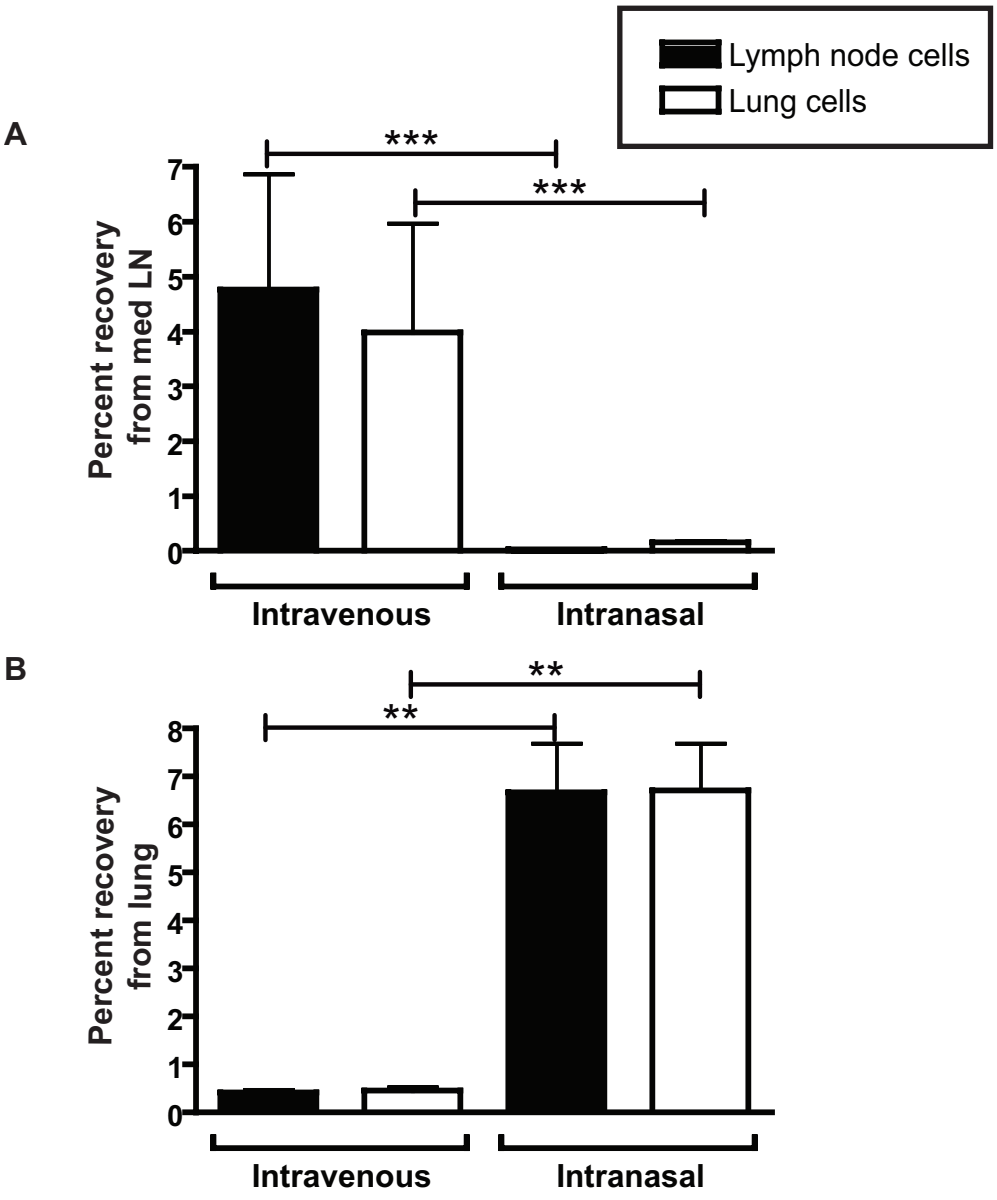

Figure 5.4 Recovery of cells from lymph node and lung tissue after adoptive transfer using different techniques.

Lymphocytes were isolated from the lungs (white bars) or lymph nodes (black bars) of Balb/c mice and labelled using CFSE before being transferred into recipient Balb/c mice (n = 3/ group) via intranasal or intraveneous route. Two days after cell transfer recipient mice were sacrificed and lungs and mediastinal lymph nodes harvested. **A**. Mediastinal lymph nodes were enzymatically digested and analysed using four colour flow cytometry for the presence of CFSE labelled CD4 T cells. **B.** Lung tissues were enzymatically digested and CD45 positive cells were enriched by AutoMacs© bead separation before being stained and analysed by four colour flow cytometry. Values represent the number of cells recovered as a percentage of adoptively transferred cells ± S.E.M. Statistics were calculated for each cell type transferred using Student's t test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

in less than 0.2% of transferred cells being recovered. There was no apparent difference in homing between cells derived from donor lymph node or lung.

So to effectively transfer cells to the lung the method we chose to use was i.n. adoptive transfer.

5.2.2.2. Transfer of *N. brasiliensis* experienced CD4 T cells into naïve mice confers protection against *N. brasiliensis.*

To investigate whether CD4 T cells are truly essential to confer protective immunity against *N. brasiliensis*, I transferred CD4 T cells from a previously primed mouse via adoptive transfer and assessed the potential to confer protection to a naïve animal. Cells were isolated from the lymph node or lung of primary infected IL-4/G4 mice by collagenase digestion day 25 post infection. Tissues were processed into single cell suspensions and enriched for CD4 T cells using positive selection with CD4 AutoMacs© magnetic beads. CD4 enriched cells were adoptively transferred i.n. into recipient Balb/c animals. One day after cell transfer recipient mice were subcutaneously infected with 600 iL3 *N. brasiliensis* and a control group received a primary infection to assess protection. Lung CD4 T cells were transferred at a higher number (4×10^6/recipient) than lymph node derived CD4 T cells (8×10^5/recipient) as we were able to recover greater numbers from donor mice. At days two and six post infection lung tissues were harvested and worm burden assessed.

Strikingly, transfer of CD4 T cells from either the lung or lymph node into naïve animals was sufficient to confer protection against reinfection with *N. brasiliensis*, as a reduced worm burden is observed in those mice which received transferred cells (Fig 5.5).

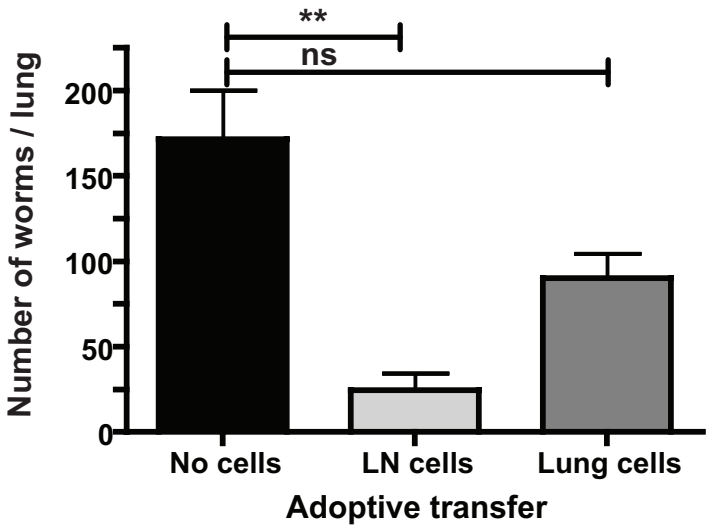

Figure 5.5 Adoptive transfer of *N. brasiliensis* experienced CD4 T cells can confer protection

Lungs or mediastinal lymph nodes were harvested from donor G4/IL-4 mice at day 25 after *N. brasiliensis* primary infection. Tissues were enzymatically digested and CD4 T cells were purified using AutoMacs© bead separation. Isolated CD4 T cells were intra nasally adoptively transferred into naïve Balb/c recipients (n = 4/group) at a final number of 4 x 10^6 lung derived cells and 8 x 10^5 lymph node derived cells. At one day post cell transfer recipient mice were infected with *N. brasiliensis*, and at this time a naïve control group was also infected to assess any protection conferred by the transferred cells. At two days post *N. brasiliensis* infection mice were sacrificed; lung tissues harvested and worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data representative of two independent experiments. Statistics were calculated using one way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

5.2.2.3. Transfer of *N. brasiliensis* experienced CD4 T cells into athymic nude mice confers protection against *N. brasiliensis.*

To build on the finding that adoptive transfer of CD4 T cells into naïve recipients conferred protection, we aimed to further define the protective CD4 T cells. Four groups of mice received i.n. adoptive transfer of approximately 4 x 10^5 CD4 T cells; one group received CD4 T cells from the lungs of FTY720 treated *N. brasiliensis* infected mice, another group received CD62L lo sorted CD4 T cells from the lungs of FTY720 treated *N. brasiliensis* infected mice, the third group received CD4 T cells from the mediastinal lymph node of *N. brasiliensis* infected mice and the fourth group received cells from the mesenteric lymph node of naïve mice. Cells in group four were initially CD4 T cell enriched, however a problem with cell death occurred and it was not possible to enrich the replacement naïve cells. Recipients in group four did however receive an equivalent number of CD4 T cells to other groups. A fifth group did not receive any cells (see Fig 5.6 for experimental plan). The recipient mice in this experiment were athymic nude mice that lack mature T cells [199]. This strain of mice was used to ensure that any protection conferred to these mice would be derived solely from the transferred T cells

One day post adoptive transfer of cells the recipient mice were infected with 600 iL3 *N. brasiliensis*, s.c. and on days two and six worm burden in the lung and gut were assessed using the viable worm recovery assay. Day six post infection the lungs and mediastinal lymph nodes of animals were also collected and assessed in terms of CD4 T cell response.

The phenotype of the adoptively transferred cell populations is shown in Fig 5.7; group one are predominantly CD62L lo, CD44 hi, group two have been sorted as CD62L lo and as such are CD62L lo and CD44 hi, group three are a mixed population of CD62L lo CD44 hi and CD62L hi CD44 lo cells, and group four are predominantly CD62L hi CD44 lo consistent with their isolation from naïve animals. An interesting point to note is that GFP expression is limited to cells that are CD62L lo and CD45RB lo. Cells of group one and two are predominantly CD45RB lo – consistent with effector/effector memory phenotype.

The effects of cell transfer on protective immunity are detailed in Fig 5.8. It can be seen that mice were most effectively protected when they had received T cells that were derived from the lungs of FTY720 treated *N. brasiliensis* infected animals when compared to control animals that did not receive any transfer of cells (Fig 5.6). The cells transferred to these most efficiently protected groups were CD62L lo CD44 hi and CD45RB lo,

consistent with effector/effector memory phenotype as seen in Fig 5.7. Animals that received cells derived from the lymph node of *N. brasiliensis* infected animals were also protected to some degree, however animals receiving transfer of naïve cells were

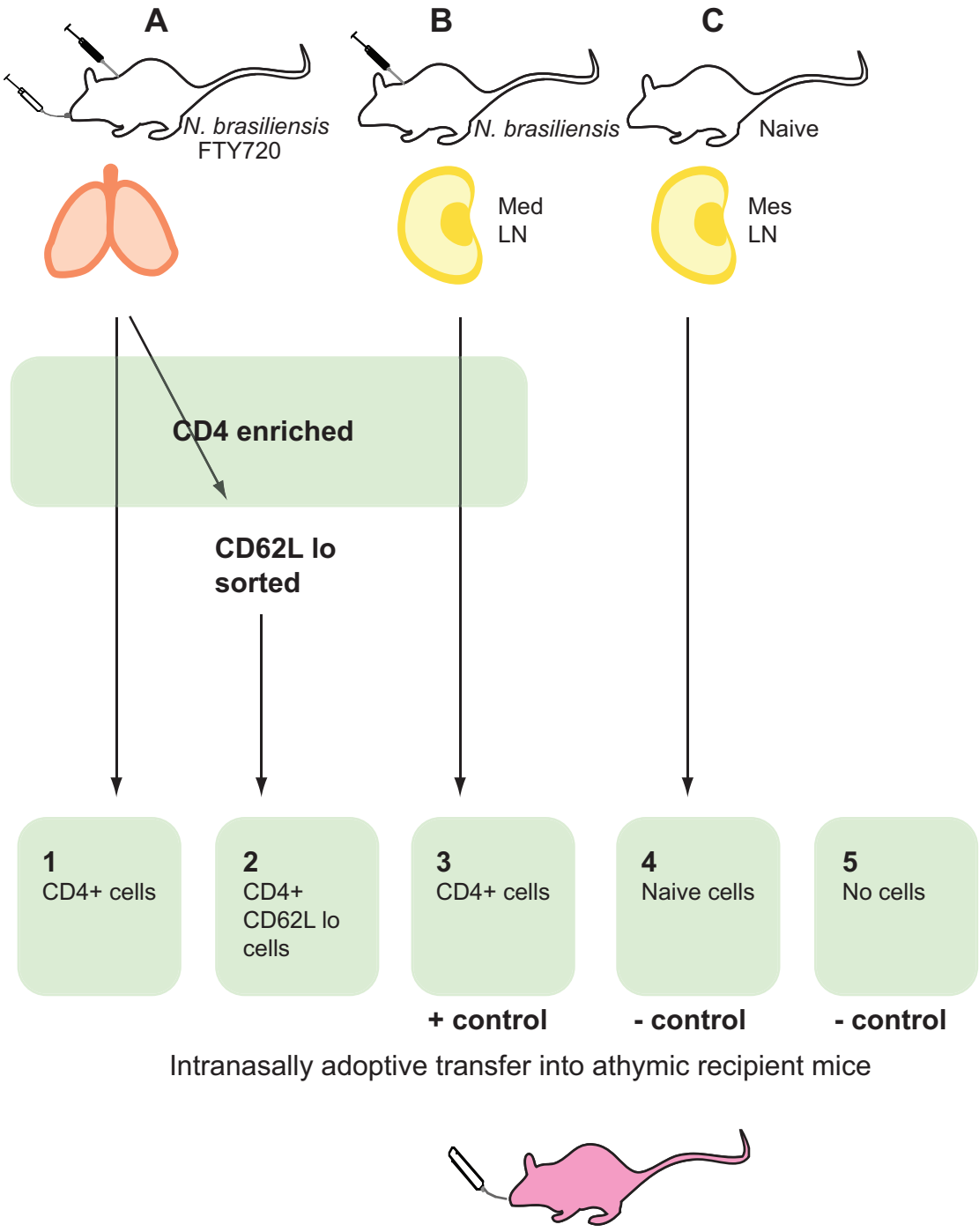

Figure 5.6 Experimental design to assess whether effector lymphoid tissue can protect against reinfection with *N. brasiliensis*.

Donor G4/IL-4 mice were treated with FTY720 from one day prior to *N. brasiliensis* to generate lung effector lymphoid tissue (A), infected with *N. brasiliensis* (B) or remained naïve (C). At day 25 post infection lung tissues were harvested from donor group A, mediastinal lymph nodes were harvested from donor group B and mesenteric lymph nodes were harvested from donor group C. Both lung and lymph nodes were enzymatically digested and enriched for CD4 T cells using AutoMacs© bead separation, with the exception of group C which was not enriched. CD4 T cells were further sorted from group A donors into a CD62L lo population. Recipient athymic nu/nu mice (n = 5/group) could be classified into five groups; those receiving CD4 T cells isolated from antigen experienced lung tissue (group 1), those receiving CD62L lo CD4 T cells isolated from antigen experienced lung tissue (group 2), those receiving CD4 T cells isolated from antigen experienced lymph node tissue (group 3 – positive control), those receiving lymphocytes isolated from naïve lymph node tissue (group 4 – negative control) and those that did not receive cell transfer (group 5). Cells were adoptively transferred in approximately equal numbers (~4 x 10^5) using an intranasal transfer method. One day after cell transfer, recipient mice and control mice (group 5 – negative control) received *N. brasiliensis* infection. Lung tissues were harvested two days post infection and worms were migrated and enumerated to measure any protection conferred by cellular transfer.

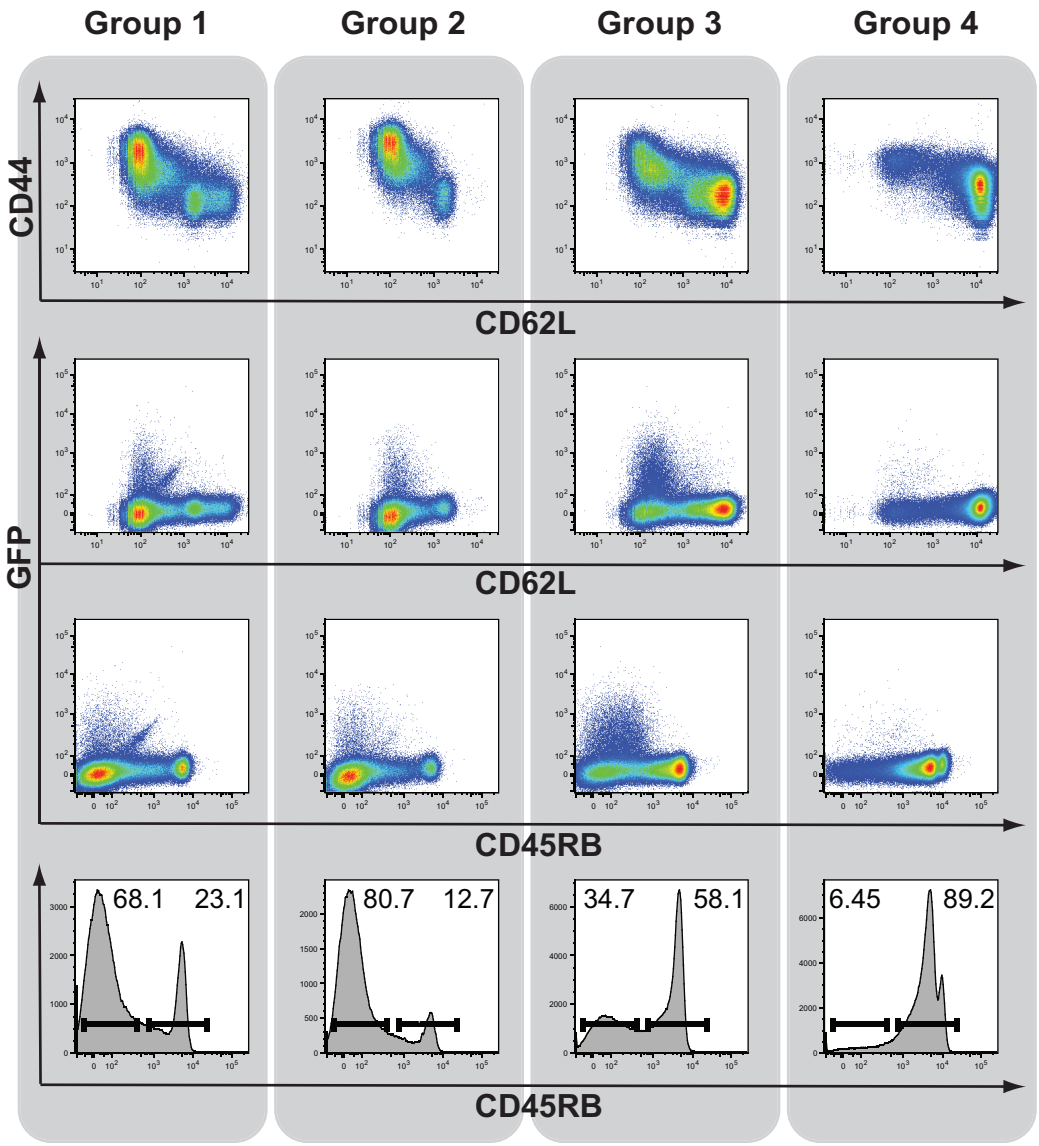

Figure 5.7 Phenotypic profile of CD4 T cells prior to adoptive transfer.

A sample of cells isolated from donor G4/IL-4 mice as previously described in Fig 5.6 were analysed using multicolour FACs analysis on a LSR II flow cytometer to assess their activation phenotype at the time of adoptive transfer. Cells were isolated from tissues as previously described in Fig 5.6 and stained with antibodies against CD45RB, CD62L, CD44, CD3 and CD4. DAPI was used to exclude dead cells and GFP reporter was also present as donor mice were from a G4/IL-4 background. Plots are gated on DAPI negative CD3/CD4 positive cells and are representative of the transferred cell population.

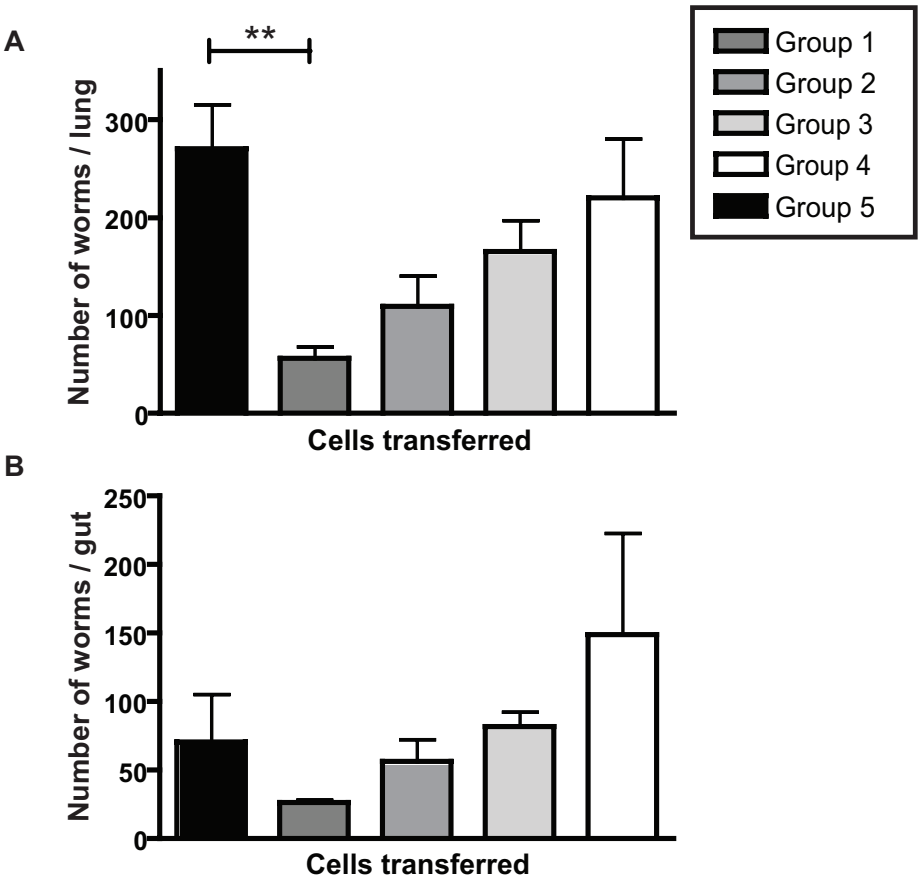

Figure 5.8 Adoptive transfer of CD45RB lo CD62L lo CD44 hi CD4 T cells (ELT) can confer protection to *N. brasiliensis* in athymic nude mice.

Athymic nu/nu mice (n = 5/group/time point) received adoptive cell transfer as described in Fig 5.6. One day post cell transfer mice were infected with *N. brasiliensis*. **A.** At two days post transfer lung tissues were harvested, worms were migrated and enumerated. **B.** At six days post reinfection gut tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Statistics were calculated using one way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. This experiment was performed once.

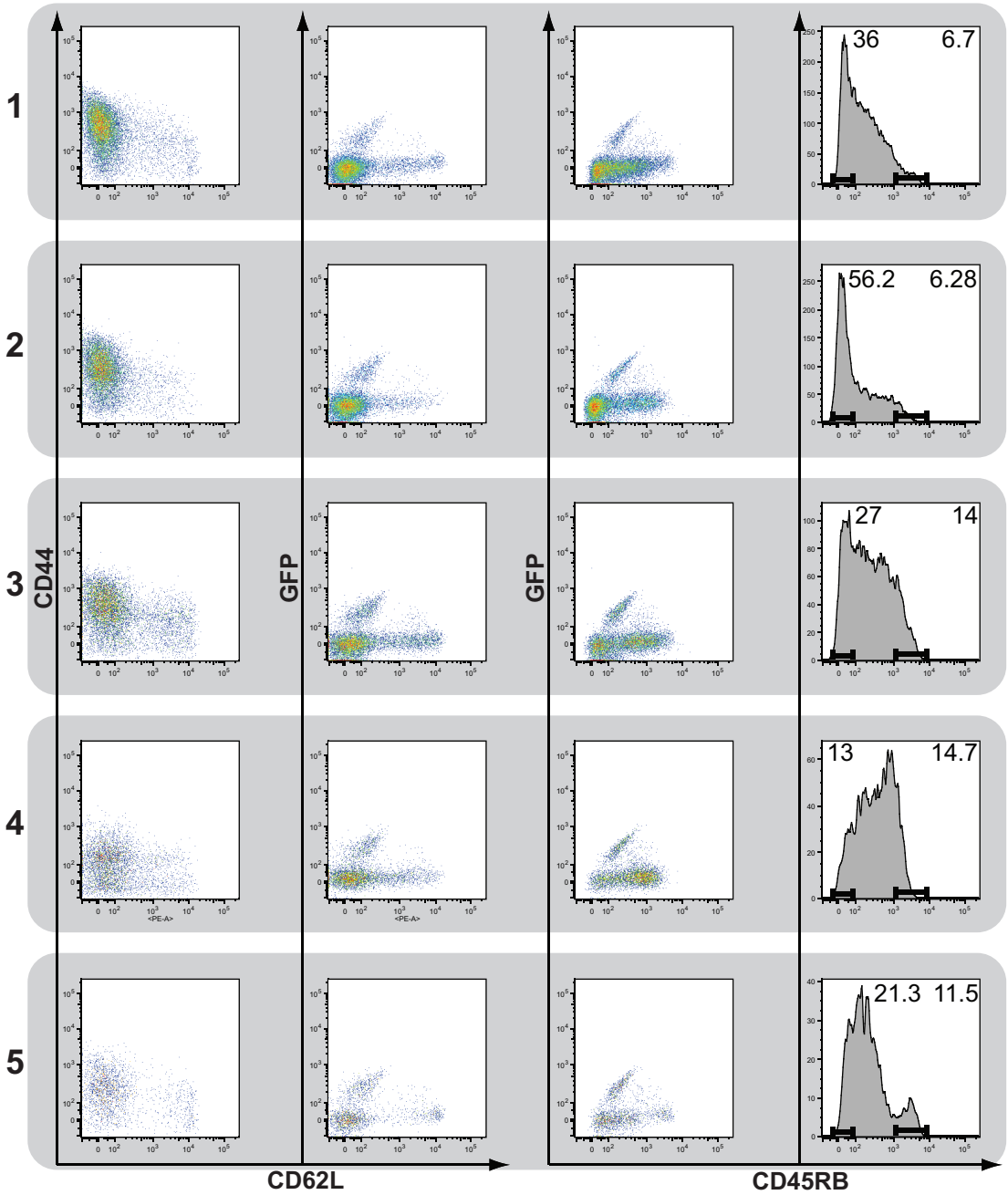

Figure 5.9 Phenotype of lung isolated CD4 T cells from *N. brasiliensis* infected athymic mice.

Athymic nu/nu mice (n = 5/group/time point) received adoptive cell transfer as described in Fig 5.6. One day post cell transfer mice were infected with *N. brasiliensis*. At day six post adoptive transfer lung tissues were harvested, enzymatically digested and CD45 positive cells were enriched by AutoMacs© bead separation. Isolated cells were stained with antibodies against CD45RB, CD62L, CD44, CD3 and CD4 and analysed by flow cytometry using an LSR II flow cytometer. DAPI was included to exclude dead cells and GFP reporter was also present as donor mice were from a G4/IL-4 background. Facs plots are gated on DAPI negative CD3/CD4 positive cells and are from one representative animal per group. A table with mean and S.E.M. from all recipients is included in appendix as Table A1.

unprotected. This protection result was observed in the lung and gut (Fig 5.8A and B). The worm burden in the gut of control mice was unexpectedly low, however it can be seen that the pattern of protection observed in the lung tissue is repeated.

The expression of surface markers on CD4 T cells in the lungs of the recipient mice were examined by flow cytometry on day six post *N. brasiliensis* infection. Similarly to before transfer (Fig 5.7), lung isolated CD4 T cells from groups one and two were predominantly CD45RB lo, CD62L lo and CD44 hi, CD4 T cells isolated from group three had a mixed population of CD62L lo CD44 hi CD45RB lo cells and CD62L hi CD44 lo and CD45RB hi cells, while CD4 T cells isolated from the lungs of group four displayed a predominantly CD62L hi, CD44 lo, CD45RB hi phenotype (Fig 5.9, also Table A1 in appendix). The observed CD4 T cells present in group five reflect the fact that T cell precursors are still present in nude mice, and that as the mice age it is possible some (few) T cells mature in the spleen [200]. The few CD4 T cells that were isolated from group five, which did not receive cell transfer, resemble the cells isolated from group four which received naïve cells.

The results of this experiment show that lung derived effector memory like cells (effector lymphoid tissue) are capable of mediating protection in otherwise naïve T cell deficient hosts.

5.2.3. Kinetic profile of lung CD4 T cells during N. brasiliensis *infection and reinfection*

To observe the generation of protective effector lymphoid tissue in the lung the CD4 T cell response in the lungs of FTY720 treated and untreated *N. brasiliensis* infected mice was profiled throughout the course of infection. CD4 T cells were assessed in terms of activation by expression of CD62L, CD44 and CD45RB and also by assessing function in terms of IL-4 production as measured by GFP expression.

Mice were treated with 0.5mg/kg FTY720 daily from day -1 (if required) and animals were infected subcutaneously with 600 iL3 *N. brasiliensis* at day zero and reinfected at day 30. At various time points during the course of infection animals were sacrificed and the immune response in the lung and mediastinal lymph node assessed using flow cytometry (experimental design Fig 5.10).

Expression of CD45RB is high in naïve cells and upon activation is down regulated and therefore expression of CD45RB in effector and memory cells is low, with Th2 cells

described as intermediate [196]. We also found that during primary *N. brasiliensis* infection the expression of CD45RB on CD45RBlo cells was intermediate when compared to CD45RBlo in secondary infection (see appendix Fig A.4). In mice receiving a control

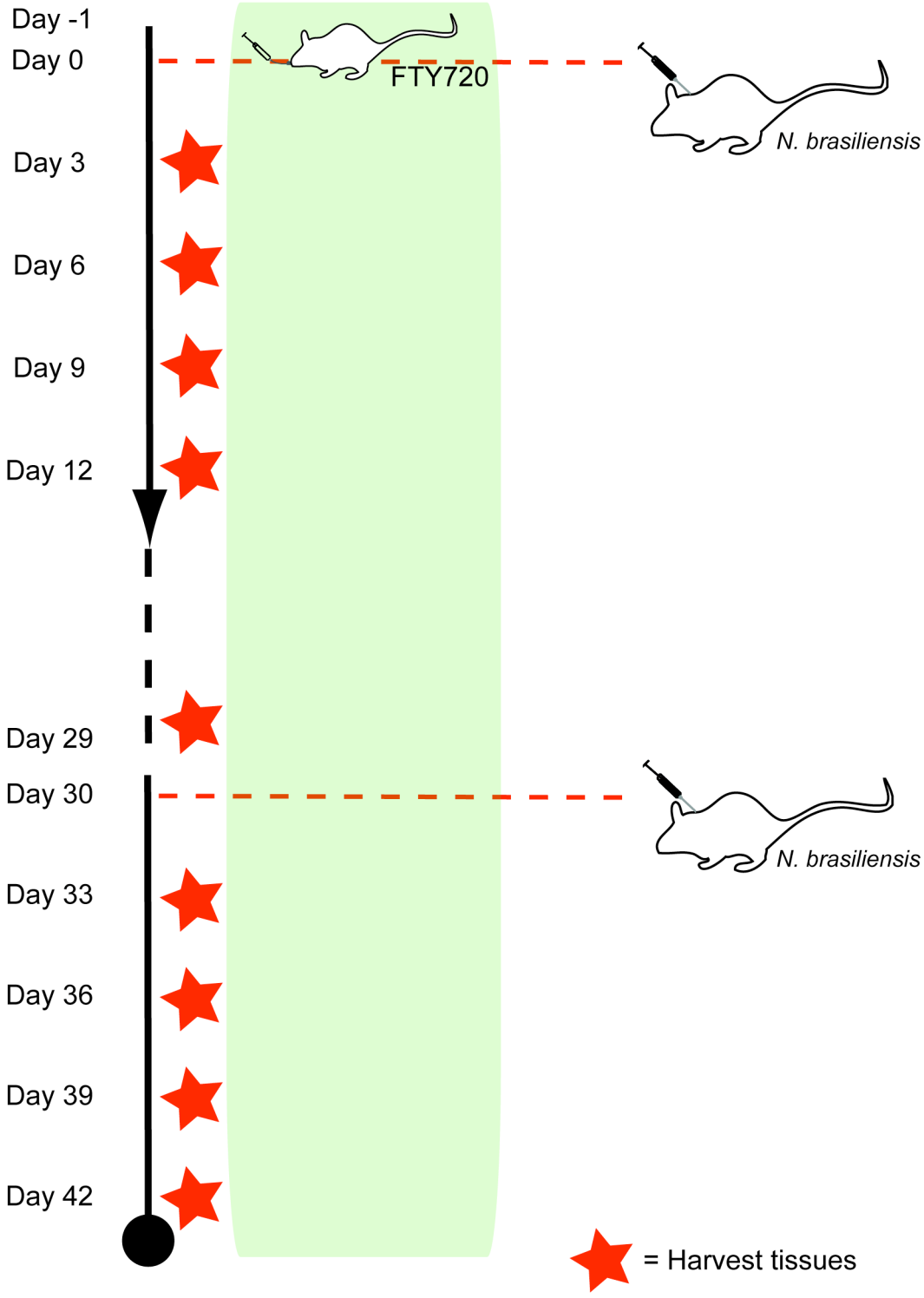

Figure 5.10 Experimental design for following lung based CD4 T cell response.

infection there are two CD4 populations present in the lung, CD45RB hi and CD45RBlo (Fig 5.11 – left hand panel). In naïve mice the majority of the CD4 T cells present within the lungs are CD45RB hi, consistent with a naïve phenotype. Upon infection with *N. brasiliensis* the CD45RB lo population begins to increase, until at day 12 post infection there are relatively equal proportions of high and lo cells present.

Prior to reinfection this balance of CD45RB hi and lo cells is preserved, however upon reinfection the CD45RB lo population increases (Fig 5.12 – left hand panel). It can also be seen that this CD45RB lo population is responsible for the production of IL-4, as measured by GFP expression. This is the case both primary and secondary infection and would be consistent with cytokine production from Teff or T_{EM} cells. Further investigation of the CD45RB hi and lo populations shows that CD45RBhi cells are CD62Lhi, CD44lo, consistent with a naïve phenotype (Fig 5.13 – left hand panel). In addition the CD45RBlo population demonstrates low expression of CD62L and high expression of CD44, consistent with an effector or effector memory phenotype.

In FTY720 treated naïve mice reduced numbers of CD4 T cells are present and those that are present represent a mixed population of both CD45RB hi and lo cells, in contrast to the mainly CD45RB hi population seen in untreated animals (Fig 5.11 – right hand panel). This finding is consistent with reports that naïve cells are preferentially depleted from peripheral organs with FTY720 treatment [101, 102]. Upon infection with *N. brasiliensis*, the ratio of CD45RB hi and lo cells begins to alter with the CD45RB lo population increasing, and the majority of CD4 T cells being CD45RBlo by day 12 post infection.

Prior to reinfection in FTY720 treated mice (Fig 5.12 – right hand panel) there is one population of CD4 T cells present in the lung that are CD45RB lo, suggesting they have been previously activated. These cells are functionally active as they are responsible for the production of IL-4 as measured by GFP expression. The measured GFP expression in primary infection is negligible in FTY720 treated animals, however the cells producing IL-4 are CD45RB lo – consistent with an activated phenotype. Furthermore these CD45RB lo cells express low levels of CD62L and high levels of CD44 (Fig 5.13 – right hand panel), similarly to the CD45RB lo population in untreated mice.

Treatment with FTY720 during *N. brasiliensis* infection has revealed the development of effector lymphoid tissue in the lung. This data suggests that a population of CD44hi, CD62Llo CD45RBlo cells has been selectively generated within the lungs of FTY720

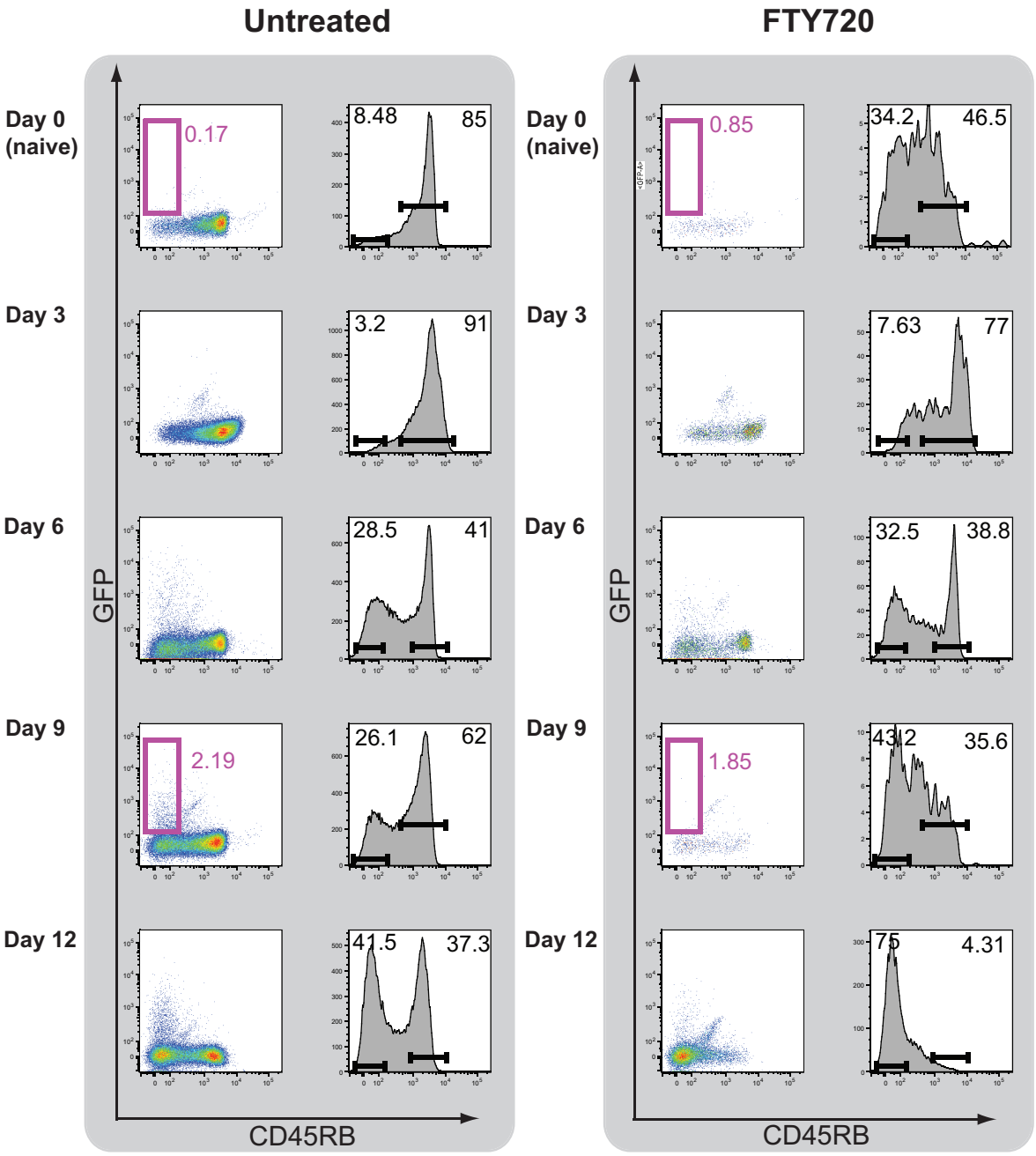

Figure 5.11 Kinetics of the lung CD4 T cell response against primary *N. brasiliensis* infection.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. Facs plots are gated on DAPI negative CD3/CD4 positive cells and are from one representative animal per group. A table with mean and S.E.M. from all individuals is included in appendix as Table A2.

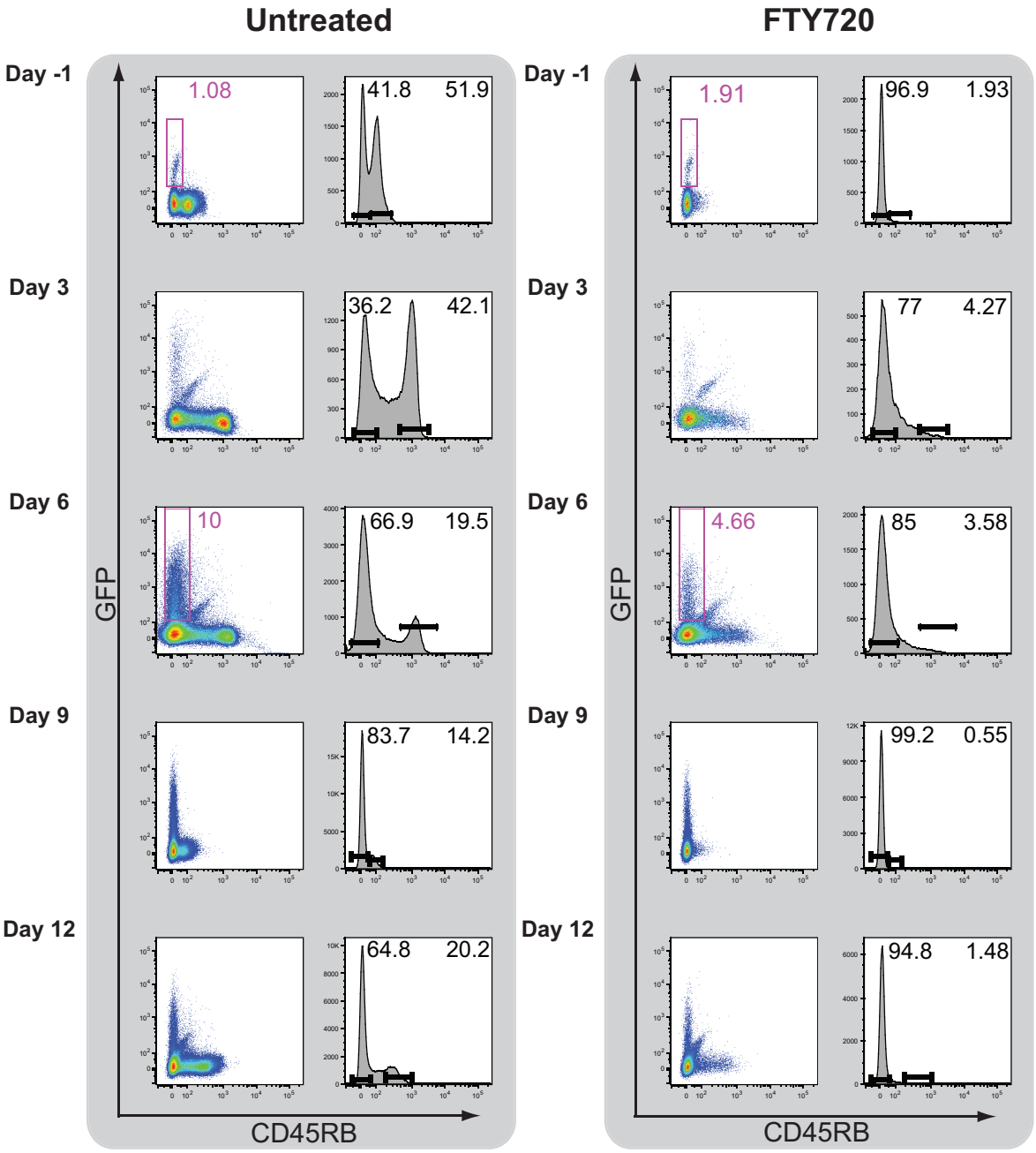

Figure 5.12 Kinetics of the lung CD4 T cell response against *N. brasiliensis* reinfection.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At 30 days post infection mice were reinfected with *N. brasiliensis*. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. Facs plots are gated on DAPI negative CD3/CD4 positive cells and are from one representative animal per group. A table with mean and S.E.M. from all individuals is included in appendix as Table A3.

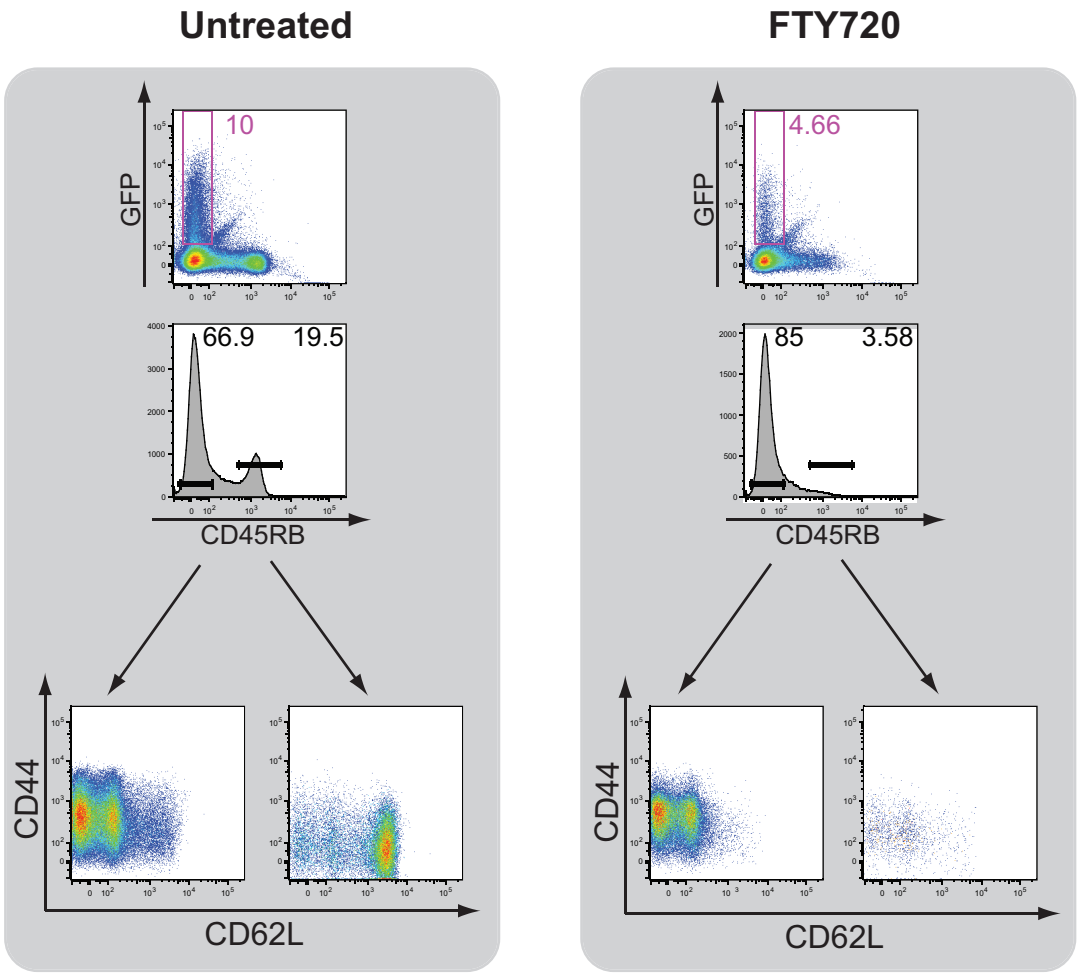

Figure 5.13 Phenotype of CD4 T cell CD45RB hi and lo populations isolated from the lung.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At 30 days post infection mice were reinfected with *N. brasiliensis*. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. The FACs plots shown in this figure represent cells from the lungs of mice from day six post reinfection gated on DAPI negative CD3/CD4 positive cells, showing the activation phenotype of CD45RB hi and lo cells in terms of CD44 and CD62L expression. FACs plots are from one representative animal per group.

treated mice. When this data is evaluated in respect to the work presented in chapter 4 the observed population of Teff/ T_{EM} cells appear to mediate protection from reinfection with *N. brasiliensis*.

5.2.4. T regulatory cells do not appear to have a central role in protection against reinfection in N. brasiliensis model.

The diminished primary response and enhanced response to reinfection observed in the lungs of FTY720 treated animals (Fig 4.7) raised the possibility that FTY720 administration may have affected regulation by Tregs. To address this we determined if there was any difference in the number and phenotype of FoxP3+ Tregs in the lungs of FTY720 treated mice. Using Foxp3^{gfp} reporter mice [201] we investigated the number of FoxP3+ cells in FTY720 treated and untreated *N. brasiliensis* infected mice.

Expression of CD103 has been shown to be a marker of effector or memory Tregs [202] and may maintain Tregs at the infection site [203]. We measured CD103 expression to indirectly assess functionality, as in our *N. brasiliensis* model there is no specific antigen to directly measure suppressive activity using *in vitro* assays.

We found no significant difference in Tregs as a proportion of CD4 cells in the lung (Fig 5.14A). Both FTY720 treated and control mice had elevated proportions of Tregs when compared to naïve mice, as might be expected in an infection situation. Interestingly there was also no difference in CD103 expression as measured by median fluorescence intensity (Fig 5.14B), indicating equal functionality of Tregs in FTY720 treated or control animals. These results suggest that the protection observed in FTY720 treated mice is not due to an alteration in the immune regulation by Tregs.

5.2.5. NKT cells are irrelevant to confer protection against N. brasiliensis reinfection

NKT cells are a lineage of T cells expressing CD4 that differ from conventional CD4 T cells as they recognise glycolipid antigen presented on CD1 molecules. As NKT cells can express CD4, we sought to assess if there was a role for NKT cells in protection against reinfection with *N. brasiliensis*. Mice deficient in CD1 lack NKT cells [176], so *N. brasiliensis* infections were established in CD1 -/- mice and corresponding C57BL6 control animals. Upon reinfection worm burdens in the lung and gut of reinfected animals were assessed and compared to primary infected controls.

Worm burdens in both the lungs and guts of CD1 -/- mice were diminished after reinfection with *N. brasiliensis* (Fig 5.15 A and B). The protection observed in CD1 -/- animals was comparable to that of the C57BL6 controls, suggesting that NKT cells do not play an important role in protection against reinfection with *N. brasiliensis*.

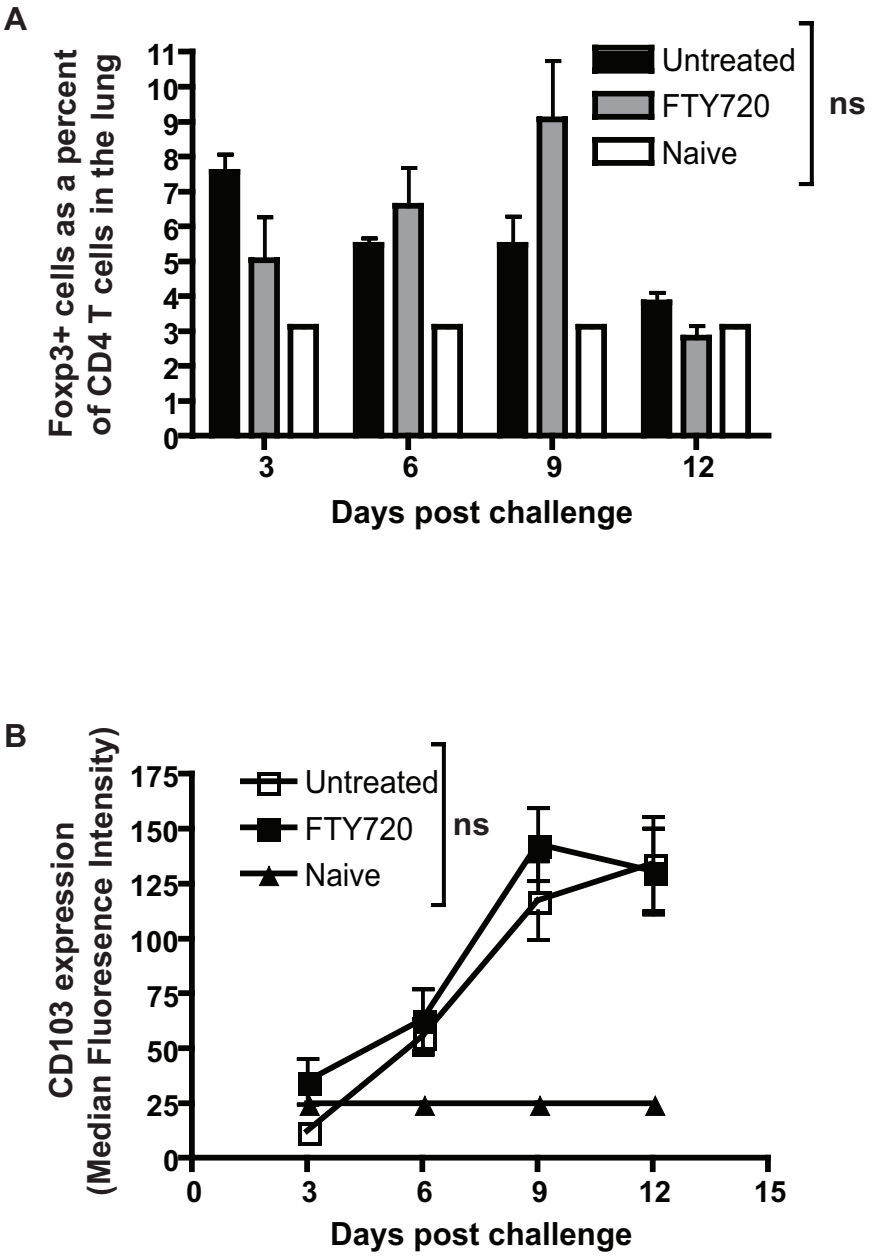

Figure 5.14 Effect of FTY720 treatment on Treg number and activation profile in *N. brasiliensis* reinfection model.

Foxp3^{gfp} reporter mice (n = 4/group/time point) treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. After 30 days mice were reinfected with *N. brasiliensis*. A naïve group was also included to establish the background levels of Treg number and activation. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed a four colour flow cytometer. **A.** The percentage of CD4 T cells that were Tregs was calculated by measuring the percentage of GFP+ cells in the CD4 T cell population. Values represent percentage of GFP+ cells in the CD4 T cell population ± S.E.M. **B.** Activation of Foxp3 positive cells was assessed by measuring the relative expression of CD103 on GFP positive cells. Values represent median fluorescence intensity of CD103 expression on GFP+ cells in the CD4 T cell population ± S.E.M. Data representative of two independent experiments. Statistics were calculated using Kruskall Wallis with Dunns post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

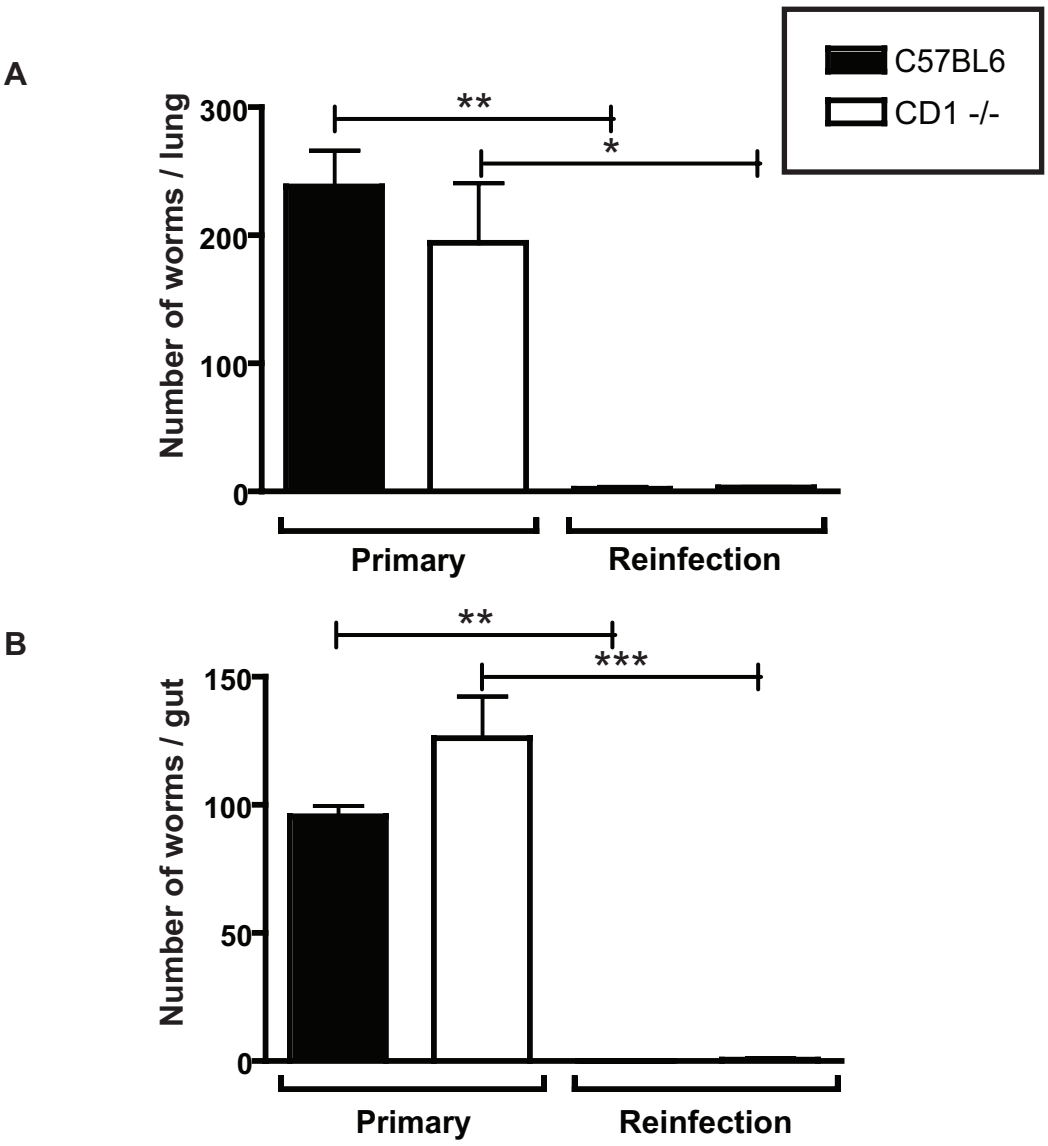

Figure 5.15 CD1 -/- mice are protected from reinfection with *N. brasiliensis*.

CD1 -/- (white bars) and C57BL6 mice (black bars) (n=3/group/time point) were infected with *N. brasiliensis*. At 30 days post infection mice were reinfected with *N. brasiliensis* and at this time a further group of naïve mice were infected with *N. brasiliensis* to allow for measurement of protection (primary). **A**. At two days post reinfection lung tissues were harvested, worms were migrated and enumerated. **B**. At six days post reinfection gut tissues were harvested, worms were migrated and enumerated. Values represent mean number of larvae present in the tissue ± S.E.M. Data representative of at least two independent experiments. Statistics were calculated for each mouse strain using Student's t test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

5.2.6. T_{EM} CD4 T cells in the lungs of BCG vaccinated and challenged mice do not correlate with protection

The phenotype of lung CD4 T cells was investigated in the lungs of FTY720 treated and control mice in the BCG model of protective immunity after infectious challenge to compare with our findings in the *N. brasiliensis* model. Expression of the activation markers CD62L and CD44 and the survival marker CD127 (IL-7Rα) was used to discriminate between Tn (CD62Lhi CD44lo CD127hi), Teff (CD62Llo CD44hi CD127lo), T_{EM} (CD62Llo CD44hi CD127hi) and T_{CM} (CD62Lhi CD44hi CD127hi) CD4 T cells.

The major population of CD4 T cells present within the lungs of mice after BCG infectious challenge is T_{EM} cells, in both FTY720 treated and control mice (Fig 5.16). FTY720 treatment during challenge infection resulted in reduced numbers of Tn and T_{CM}. However mice receiving FTY720 only during primary vaccination had restored levels of both Tn and T_{CM} after challenge infection.

Our findings in the *N. brasiliensis* model identified a protective population of T_{EM} like cells within the lung tissues. In the BCG model T_{EM} cells dominate the CD4 T cell response to challenge infection in FTY720 treated and control treated mice (Fig 5.16). In contrast to our findings in the *N. brasiliensis* model, T_{EM} cells do not correlate with protection in the BCG model.

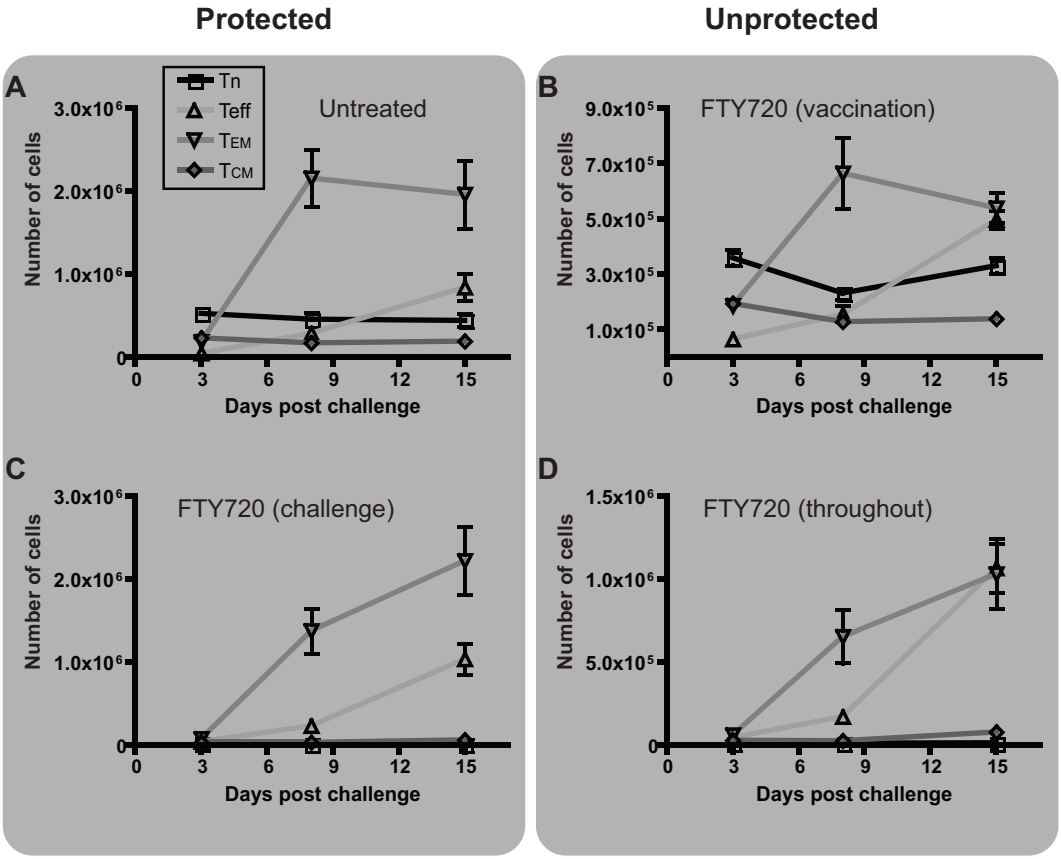

Figure 5.16 T_{EM} cells do not correlate with protection in the BCG model

C57BL6 mice (n = 5/group/time point) were vaccinated and challenged with BCG and remained untreated or were treated daily with 1mg/kg FTY720 as described in Fig 4.11. At the indicated time points post BCG infectious challenge lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed a four colour flow cytometer. Expression of the activation markers CD62L and CD44 and the survival marker CD127 (IL-7Rα) was used to discriminate between Tn (CD62Lhi CD44lo CD127hi), Teff (CD62Llo CD44hi CD127lo), T_{EM} (CD62Llo CD44hi CD127hi) and T_{CM} (CD62Lhi CD44hi CD127hi) CD4 T cells. Data showing bacterial counts from lung tissue is found in Figure 4.12 A. **A.** Displays data collected from mice with No FTY720 treatment **B.** Displays data collected from mice with FTY720 treatment during vaccination only **C.** Displays data collected from mice with FTY720 treatment during challenge infection only **D.** Displays data collected from mice with FTY720 treatment during vaccination and challenge infection. Values represent mean number of CD4 T cells displaying indicated activation phenotype ± S.E.M. Data representative of at least two independent experiments.

5.3. Discussion

The results in this chapter attempt to clarify the role and phenotype of CD4 T cells in protective immunity against *N. brasiliensis.* The loss of protection against reinfection resulting from the antibody-mediated depletion suggests that CD4 T cells mediate protective immunity against *N. brasiliensis*. Although the work of Katona *et al* [157] shows a requirement for CD4 T cells in gut expulsion of *N. brasiliensis* worms, the role of CD4 T cells in relation to immunity at the lung site has not previously been investigated.

Thus, the ability to confer protection in naïve animals through the adoptive transfer of *N. brasiliensis* experienced CD4 T cells is a novel finding. This result suggests that not only are CD4 T cells the key mediators of protective immunity against *N. brasiliensis*, but protection against reinfection occurs rapidly, as the lung burden of protected mice is reduced when assessed at 24 hours. Furthermore CD4 T cells isolated from the lungs of FTY720 treated mice were sufficient to protect against reinfection. This finding supports the ability of peripheral T cell responses to confer protective immunity.

The ability of adoptively transferred T cells to confer protection rules out tissue remodelling as being critical for protection against reinfection. The physiological changes in the lung observed after *N. brasiliensis* infection have been documented by Reece *et al* who describe an environment altered at the cellular and molecular level [173]. The authors propose that these changes play a role in modulating subsequent allergen induced inflammatory responses. However the data presented here demonstrates that CD4 T cells alone are sufficient to confer protection against *N. brasiliensis* infection in naïve mice.

Exploring the surface activation markers of CD4 T cells in the lungs of FTY720 treated and control mice revealed interesting results. The cell surface marker CD45RB was used to distinguish CD45RB lo effector and memory from CD45RB hi naïve cells. During primary *N. brasiliensis* infection we observed that the CD4 T cell population in the lungs of FTY720 treated animals progressed from a mixed population of both CD45RB hi and lo cells to a population dominated by CD45RB lo CD4 T cells. Production of GFP (representative of effector cytokine IL-4) during primary infection was restricted to cells that were CD45RB lo. Prior to challenge almost all CD4 T cells were CD45RB lo but produced very little GFP, suggesting that they were a population of resting memory cells. Upon reinfection the population of CD4 T cells expanded, remained CD45RB lo and rapidly began to express GFP to greater levels than observed in primary infection. The

phenotype of CD45RB hi cells was CD62L hi and CD44 lo, consistent with naïve cells and the CD45RB lo cells were CD62L lo CD44 hi, consistent with Teff or T_{EM}.

We explored the activation phenotype of lung resident CD4 T cells in the BCG model of protective immunity to compare with our findings in the *N. brasiliensis* model. We found that the T_{EM} subset dominated the lung response in all groups, but as not all groups were protected against infectious challenge, this did not correlate with protection. FTY720 treatment during challenge infection prevented recirculation of Tn and T_{CM} from the lymphoid tissues as reported by others [101, 102]. Our findings with the *N. brasiliensis* model identified a protective T_{EM} population of cells, however the T_{EM} populations in the BCG model do not appear to correlate with protection. This implies that T_{EM} are not the protective CD4 cell type in the BCG model, or alternatively that there are differences in the quality or threshold of T_{EM} required in protected versus unprotected animals.

CD4 is expressed on subsets of Tregs and also NKT cells and as such these cells may have been transferred within our CD4 purified cell population during adoptive transfer in our *N. brasiliensis* protection model. To confirm that protection was not due to disregulation of the immune response we investigated Treg number and function in the *N. brasiliensis* model. Although we found differences in the total number of Tregs FTY720 treated animals displayed a trend towards increased numbers of Tregs and as such, would be subjected to more regulation. Expression of the integrin CD103 has been associated with Tregs in parasitic infection [167] and has also been associated with effector/memory function [202] and tissue retention [203]. Our data showed no differences in the expression of CD103 in FTY720 treated compared with control animals as measured by median fluorescence intensity. Together these results would suggest that reduced Treg function does not play a role in the protection against *N. brasiliensis* observed in FTY720 treated mice.

The role of NKT cells in protective immunity against *N. brasiliensis* was assessed using CD1 -/- mice, which are unable to present antigen to NKT cells. As *N. brasiliensis* infection progresses the immune system is exposed to many antigens from the worms themselves to their secreted products, which potentially include glycolipid antigen. NKT cells are known to produce large amounts of cytokine upon activation, including IL-4, suggesting these cells as a relevant candidate to confer the observed protective immunity against *N. brasiliensis*. The infection of CD1 -/- mice allowed us to investigate the role for NKT cells in conferring protective immunity in our *N. brasiliensis* infection model. We

found no difference in protection against *N. brasiliensis* in CD1 -/- mice when compared to C57BL6 control animals. Worm burdens of both the lung and gut tissues were significantly reduced when compared to animals receiving primary infection. This would suggest that NKT cells do not play a critical role in protection against reinfection with *N. brasiliensis*.

5.4. Conclusion

The aim of this chapter was to confirm the role of CD4 T cells in *N. brasiliensis* protective immunity and characterise the phenotype of the protection conferring cells. Depletion studies with αCD4 and adoptive transfer of CD4 T cells with T_{EM} phenotype confirmed their critical role in conferring protection against reinfection with *N. brasiliensis*. Furthermore, treatment with FTY720 revealed the development of a distinct CD45RB lo population of CD4 T cells in the lungs of infected mice during primary *N. brasiliensis* infection. These CD45RB lo cells also expressed low levels of CD62L and high CD44 consistent with a Teff or T_{EM} phenotype. The CD45RB lo cells persisted and upon reinfection rapidly expressed GFP at levels higher than those seen in primary infection. These CD45RB lo cells were able to confer protection when adoptively transferred into naïve athymic mice. Studies employing transgenic and knock out animals demonstrate that CD4 expressing Tregs or NKT cells do not play an important role in the protection against reinfection with *N. brasiliensis*.

Chapter 6:
General Discussion

The work detailed within this thesis sought to investigate the mechanisms of protective immunity in the *N. brasiliensis* infection model. Initially we aimed to identify at which peripheral tissue sites immune priming occurred which led to protection against reinfection with *N. brasiliensis*. We developed and used a partial infection assay where primary worm infection was restricted to specific tissue sites to identify the lung as an important site in the generation of protective immunity against *N. brasiliensis*. The use of G4/IL-4 reporter mice allowed us to sensitively measure the production of effector cytokine IL-4 in response to *N. brasiliensis* infection, revealing a correlation between increased numbers of IL-4 producing CD4 T cells in the mediastinal lymph node and protection at the lung site.

The second key aim of this work was to investigate the contribution of lymph node and lung based CD4 T cell responses to the protective immune response against *N. brasiliensis*. Manipulation of CD4 T cell recirculation between lymph node and lung was achieved using the lymphocyte sequestering drug FTY720. The approach of using FTY720 treatment in the GFP reporter mice revealed that protection was unimpeded by drug treatment, although changes in the CD4 Th2 cell response within the lung tissue were observed. The finding that lymphocyte recruitment from the lymph node was not required to confer protection implicated a role for effector lymphoid tissue in protective immunity against *N. brasiliensis*.

To build on this finding we aimed to characterise the protective CD4 T cell phenotype within the lung. Studies confirmed the crucial role of CD4 T cells in the protective immune response as depletion of these cells abrogated protection and strikingly, localised adoptive transfer of *N. brasiliensis* experienced CD4 T cells into lung tissues of naïve hosts was sufficient to confer protection against reinfection. The development of the lung resident CD4 T cell response was observed in FTY720 treated and control mice. In the lungs of FTY720 treated mice a population of Tem like cells developed over the course of primary infection that was maintained up to 30 days post infection. Upon reinfection these cells expanded and rapidly produced effector cytokine; furthermore, transfer of these Tem like cells into athymic recipients conferred protection. A role for NKT cells potentially transferred within the CD4 T cell population was ruled out by the finding that CD1 -/- mice retained protection against reinfection to the same level as control mice.

A central aim for this thesis was to formulate a hypothesis on the protective mechanism generated during *N. brasiliensis* infection. Our findings indicate an important role for ELT in the conferring of protective immunity against *N. brasiliensis*. Interestingly our results

also reveal a potential alternative mechanism of development for ELT. Lastly it was aimed to compare our findings in the *N. brasiliensis* model with a Th1 mediated model of lung based protective immunity. This aim was achieved by using the BCG model of protection conferred by vaccination. Although the findings with the BCG model confirmed an important role for ELT, the generation of protective ELT has different requirements within this model.

The following chapter discusses our findings in the context of both our aims and the findings of others within the scientific community.

6.1. The lung is a key site for the generation of protective immunity against N. brasiliensis

The extensive migration of *N. brasiliensis* through its host presents a number of peripheral locations where protective immunity could occur. During an infection with *N. brasiliensis*, parasitic larvae traverse the skin, entering into the circulation and bursting into the parenchyma of the lung as early as 14 hours post infection. A brief period of maturation in the lung is followed by migration to the gastrointestinal tract where worms fully mature, mate and lay eggs. Reinfection results in a reduced worm burden in the lung and gut tissues. The peripheral tissues of skin, lung and gut all experience localised infection during primary worm infection and the initial question addressed in this thesis asked which of these three peripheral tissues is responsible for the protective immune response against *N. brasiliensis*.

We found no role for the skin in priming for protective immunity. *In vitro* experiments investigated the ability of worms to physically migrate through the skin (Fig 3.2), finding that worms could traverse naïve and immune skin with equal efficiency. Further *in vivo* experiments (Fig 3.3) confirmed that parasitic larvae were able to migrate away from the infection site in the skin as early as five minutes post infection in both naïve and immune animals – suggesting any protective response observed in *N. brasiliensis* infection was not due to an immune response mounted at the skin site of infection. This contrasts with the findings of others who have reported that iL3 can be trapped at the infection site, unable to migrate to the lung [3]. This difference may be explained by the different model systems used as these experiments were performed in transgenic mice over-expressing IL-5 where we have used Balb/c background animals.

To assess at which site primary infection was important to protect against reinfection we generated a model that isolated primary *N. brasiliensis* infection to specific tissue sites (Fig 3.4). Using this model we showed that when mice were primed in the lung, or skin and lung they were protected against reinfection in the lung and gut tissues. This finding suggests the lung is an important site for when conferring protective immunity, especially when considered with the results of earlier experiments that showed no difference in penetration of and migration away from naïve and immune skin (Figs 3.2, 3.3). Further evidence for the importance of lung priming in the protective response was the striking finding that lung restricted primary infection could confer protection against gavaged L5 worms (Fig 3.8).

The protective response at the gut site has been a focus of research in the *N. brasiliensis* field with evidence to show that CD4 T cells, IL-4, IL-13 and STAT 6 play a major role in expulsion [157-159]. Although we have identified the lung as an important site for priming of protective responses, we also found that priming at the gut site was able to generate local protection from reinfection. Evidence collected within our group and also within this thesis shows there are differences in the parameters of lung and gut immunity against *N. brasiliensis*. A requirement for IL-4 in lung protection that is not necessary for protection within the gut is shown in Table 5.1. This may be a reflection of the redundancy in the roles of IL-4 and IL-13 in gut expulsion of worms, where the as yet unknown mechanisms of protection within the lung must be more dependent on IL-4.

The studies included within this thesis clearly demonstrate an important role for the lung in protective immunity against *N. brasiliensis*. Not only have we found that the lung is an important site for reducing the numbers of migrating larvae but that it is the key site for priming the protective CD4 Th2 response that occurs in the lung. Furthermore it was found that priming in the lung could confer protective immunity against gut dwelling adult worms. The importance of the lung in the *N. brasiliensis* model has not been previously reported, although a recent paper hints at the importance of targeting the worms early in the protective response [160]. These unique findings create a new focus in the study of protective immunity in the *N. brasiliensis* model.

6.2. CD4 T cell effector responses in the lung can confer protective immunity against N. brasiliensis

To determine the location of protective cells in the context of immune system structure we separated peripheral tertiary and secondary lymphoid tissues. Initially we developed a model using FTY720 to separate CD4 immune events of the secondary and tertiary lymphoid tissues. After titration of the drug in naïve mice confirmed the lymphocyte sequestering properties observed by others, we assessed drug function during *N. brasiliensis* infection (Fig 4.3). We found a dramatic decrease in circulating CD4 T cells and a significant drop in CD4 T cell numbers in the peripheral lung tissue.

We found that the confinement of T cell responses to either secondary or peripheral lymphoid tissues with FTY720 did not affect the immune response against *N. brasiliensis,* despite a reduction in magnitude. Worm burdens in primary infection were comparable between FTY720 treated and control animals, with no difference observed in the migration or expulsion of the parasites. Reinfection of FTY720 treated animals confirmed the same level of protection as that of control animals. This result suggests that the localised T cell response occurring in the lungs is sufficient to confer protection against *N. brasiliensis*. The discovery that lung localised adoptive transfer of CD4 T cells can confer protection in naïve animals further confirmed this finding (Fig 5.5).

Our results support a role for effector lymphoid tissue in protective immunity. Effector lymphoid tissue describes the retention of antigen specific memory T cells within peripheral tissues, where they are poised to respond immediately to a reinfection conferring protective immunity. The original hypothesis of ELT postulated that after primary infection memory T cells migrated to peripheral tissues where they are maintained as peripheral resident ELT. Our finding that preventing T cell recirculation using FTY720 treatment did not affect protection against *N. brasiliensis* raises the intriguing possibility of an alternative mechanism of ELT generation where the localised peripheral primary T cell response can give rise to Tem like cells that can persist in the peripheral location to become ELT (Fig 6.1).

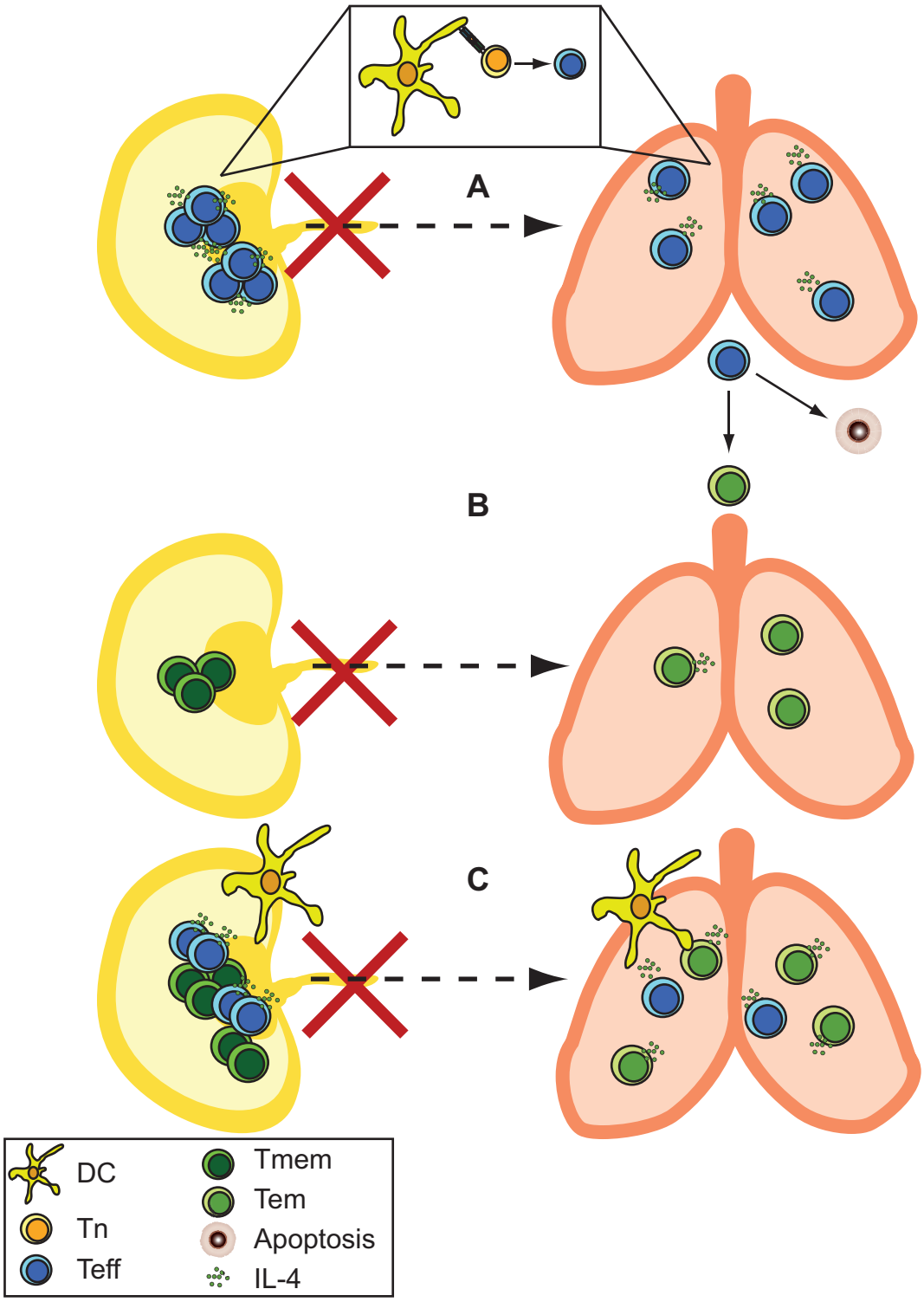

Figure 6.1 Hypothesis for the alternative generation of ELT.

During primary *N. brasiliensis* infection in the presence of FTY720 treatment antigen bearing APC can activate effector T cell responses in the lung and also in the draining lymph node, however drug treatment prevents recirculation of CD4 T cells between the two tissues **(A)**. The resulting lung residing Teff either apoptose or persist in the lung tissue, becoming T_{EM} like cells **(B)**. Cells within the lymph node also go on to become T_{MEM}, however FTY720 treatment prevents recirculation to the lung tissue. Upon reinfection with *N. brasiliensis* **(C)** T_{EM} cells residing in the lung that were generated during primary infection are re-activated, leading to pathogen clearance and protective immunity. New Teff may also be generated. In the lymph node T_{MEM} are re-activated and new Teff are also generated, however they do not contribute to protection from reinfection as they can not recirculate to the lung tissue due to FTY720 treatment.

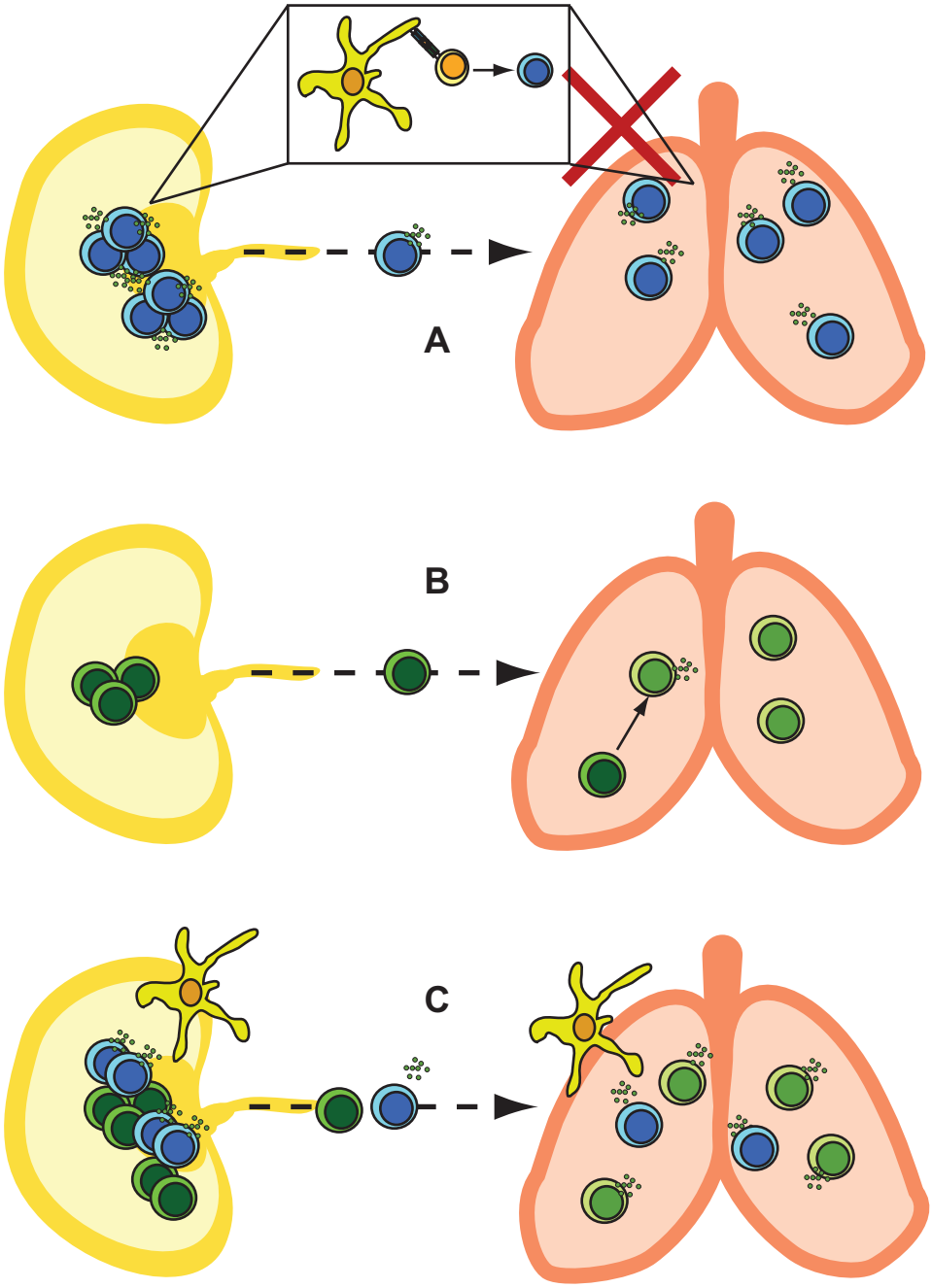

Figure 6.2 Hypothesis of selective escape from FTY720 treatment.

During primary *N. brasiliensis* infection in the presence of FTY720 treatment antigen bearing APC can activate effector T cell responses in the draining lymph node, but not the lung tissue. Drug treatment prevents recirculation of CD4 T cells between the two tissues, however some antigen specific Teff cells escape FTY720 treatment and recirculate to lung tissue **(A)**. Cells within the lymph node go on to become T_{MEM} **(B)**, and despite FTY720 treatment some T_{MEM} recirculate to the lung tissue. Upon reinfection with *N. brasiliensis* **(C)** T_{EM} cells residing in the lymph node that were generated during primary infection are re-activated, new Teff may also be generated. Some antigen specific Teff and T_{MEM} cells escape FTY720 treatment and recirculate to the lung tissue where they and other previously escaped T_{MEM} cells mediate pathogen clearance and protective immunity.

Recirculation of T cells is prevented by FTY720 treatment, however there is a possibility that some T cells are resistant to FTY720 treatment and are able to recirculate to the periphery and establish ELT (Fig 6.2). We observed that in the circulating CD4 T cells that remained after FTY720 treatment GFP+ cells were over-represented. However, the number of blood circulating CD4 T cells post FTY720 treatment is very low, and although FTY720 treated animals had a higher percentage of GFP+ cells than of control animals, the actual numbers were quite different. Untreated animals had 10x more circulating CD4 T cells per mL of blood, of which 0.5 – 1% were GFP+; FTY720 treated animals had fewer circulating CD4 T cells of which 0.5 – 2% were GFP+ (Fig 4.3), still quantitatively much fewer but not disproving the hypothesis of targeted resistance from FTY720. The number of GFP+ cells circulating is at its lowest at day 2 post challenge, yet this is when protection is occurring. We know worms (and therefore antigen) arrive as early as 14 hours post infection in the lung. This means the T cells are able to orchestrate protection between 14 hours and 48 hours post infection (when we measure lung worm burdens). If T cells were being recruited from the lymph node by selective escape from FTY720 treatment, these cells would need to have been exposed to antigen in the lymph node, activated, possibly proliferate and also traffic to the lung site and orchestrate protection all in a very short time window. If certain T cells are able to selectively escape FTY720 treatment to establish ELT and confer protection they are unable to do it during BCG vaccination, as FTY720 treatment during vaccination abrogates protection. This may be due to experimental limitations (as discussed later in 6.4 Th1 vs Th2) but could also support that selective escape of protective cells is unlikely.

6.3. Characterising the CD4 T cell protective response

An important aim of this body of work was to characterise the key CD4 T cell lineage responsible for mediating protective immunity against *N. brasiliensis*. Following the immune response and phenotyping the CD4 T cells mediating protective immunity against *N. brasiliensis* in the lung has revealed the development of a T_{EM} like population of CD4 T cells that can swiftly produce cytokine upon reinfection (Figs 5.11, 5.12, 5.13). We used expression of CD45RB on the cell surface to distinguish Teff/ T_{MEM} from naïve cells [196], as naïve cells express high levels of CD45RB compared to the lower levels expressed on Teff and T_{MEM} (see appendix Fig A.4). The CD4 T cells in the lungs of FTY720 treated animals develop from a mixed population of CD45RB hi and lo cells into

a predominantly CD45RB lo population during primary *N. brasiliensis* infection in FTY720 treated mice, suggesting a transition from a mixed population of naïve and T_{MEM}/Teff cells into a predominantly T_{MEM}/Teff phenotype. This suggests activation and also possible expansion of CD4 T cells in the lung tissue as numbers increase over time within the tissue, yet FTY720 treatment prevents the recirculation of CD4 T cells. This raises several questions that need to be further addressed.

Firstly, do the lung resident T cells proliferate within lung tissues during primary and also reinfection with *N. brasiliensis*? Evidence from Harris *et al* would suggest not, as their experiments with *in vitro* cultured and activated transgenic CD4 T cell adoptive transfers found that Th2 cells would not proliferate within the lung, although they could be activated to produce cytokines [204]. However these experiments differ from ours as they use experimentally manipulated *in vitro* grown T cells, transferred i.v. rather than i.n. and also used a very different model antigen. Our experimental model allows sensitive tracking of *in vivo* generated antigen specific CD4 T cells, responding to a parasite infection with a potentially vast array of antigens. Obhrai *et al* show that maintenance and antigen specific response of existing peripheral memory CD4 T cells does not require secondary lymphoid tissues, however development of CD4 T_{MEM} from Teff does. This would dispute the proposed alternative hypothesis of development of ELT in the periphery. This study used a Th1 mediated model of immunity and this may account for the requirement for secondary lymphoid organs in memory development as IFNγ is a toxic compound a that can kill Th1 effectors through activation induced cell death (AICD). We use a Th2 model of protective immunity and IL-4 does not display the same toxicity as IFNγ, and there is evidence to suggest Th2 cells do not undergo AICD [205]. This may make an alternative method of ELT development a possibility in our model. The work of Marc Jenkins looks at the precursor frequencies of antigen specific T cells in an OVA model, showing that there are very few naïve precursors for OVA [206]. This work would imply that it is unlikely that we would find *N. brasiliensis* specific naïve precursor cells within the lung tissue to become activated and develop ELT. The model used for these experiments looks for T cells specific for only one antigen. The protective epitopes of *N. brasiliensis* have yet to be identified but there is a strong likelihood that a polyclonal T cell response is mounted against *N. brasiliensis*, further increasing the chance of having naïve antigen specific precursors within the lung residing CD4 T cell population.

As reviewed in the introductory chapter there is increasing evidence to suggest that T cells can be both primed for activation and proliferation within peripheral tissues [88, 89], including the lungs [90]. Wakim *et al* demonstrate the ability of DC to present antigen in the periphery with their transplant experiments using an HSV model [13]. They also show the antigen specific proliferation of tissue residing T cells. Mice lacking secondary lymphoid organs are able to mount lung based immune responses through the generation of iBALT. It is possible that we are generating a similar situation chemically using FTY720. Further experiments should be performed to assess proliferation within the lung tissues during FTY720 treatment to investigate the possibility of proliferation within the lung versus the potential selective escape of T cells from the sequestering effects of FTY720 (chapter 4).

A second question raised by our findings is how do these protective T_{EM} like cells develop? When we take CD4 T cells from the lungs of naïve, primary infected and reinfected mice and compare the expression of CD45RB between the groups we see differences in expression. CD4 T cells from naïve mice are CD45RB hi and cells from mice with primary or reinfections are CD45RB lo, interestingly although the CD4 T cells from primary infected mice exhibit low expression of CD45RB it is not as low as the expression on CD4 T cells during reinfection (see appendix Fig A.4). In FTY720 treated mice CD45RB expression becomes lowered during primary infection, remaining low prior to reinfection, when CD45RB lo cells begin to make GFP/IL-4 and mediate protection (Figs 5.11, 5.12, 5.13). These results indicate that T_{EM} like cells have developed from the small number of Teff cells generated during primary infection. In support of this theory several groups have published findings showing the generation of memory T cells from T effector cells.

Lastly, can these T_{EM} like cells confer protection after longer periods of time? Our experiments look at reinfections occurring 30 days after primary infection as we have previously seen the primary immune response has contracted at this point (see appendix Fig A.1). Experiments within our group have looked at longer term protection in the *N. brasiliensis* model and although gut protection was robust, there did seem to be some decline in lung protection (see appendix FigA.5). The experiments of Ely *et al* show in their viral model that in the antigen specific memory population T_{EM} cells are dominant in the first 1-3 months but at later times (13-14 months post infection) T_{CM} like cells dominate the memory population[207]. It would be of interest to observe the protection

conferred by ELT at longer times after primary infection to see if there is perhaps a requirement for secondary lymphoid derived T_{MEM} in the long term maintenance of ELT in the *N. brasiliensis* model.

The finding that NKT cells are not required for protection against reinfection provides us with information about the nature of the *N. brasiliensis* antigen(s) protective immunity is mounted against. Glycolipid antigens presented on CD1 molecules have been shown to activate NKT cells and the finding that CD1-/- mice remained protected to the same degree as control animals suggests that the *N. brasiliensis* antigens stimulating the immune response are not glycolipids. There remains the question of whether carbohydrates play a role in the induction of the strong *N. brasiliensis* induced Th2 immune responses, however the evidence presented within this thesis shows a clear role for CD4 T cells that are activated specifically by peptides presented on MHC II.

6.4. A comparison of Th1 vs Th2 mediated lung immunity

Most studies of peripheral memory T cells (and by proxy ELT) are conducted in models of CD8 or Th1 mediated protection so we compared our findings in the *N. brasiliensis* model to a Th1 model of lung immunity, BCG. Vaccination with BCG can confer protection against challenge infection with aerosolised *M. bovus* of up to one log10. IFNγ plays a critical role in BCG protective immunity, as in animals where IFNγ has been neutralised the protection afforded by BCG vaccination is abrogated [194].

Our initial investigations into primary BCG infection showed that FTY720 treatment prevented effective control of the infection and we observed increased dissemination to spleen and liver in FTY720 treated animals. This contrasted with our finding that FTY720 treatment had no effect on worm burden or migration kinetics in primary *N. brasiliensis* infected animals.

Although lung based T cells (ELT) were able to confer protection in both the *N. brasiliensis* and BCG models, the generation of the ELT differs. We found in the *N. brasiliensis* model, protection occurs regardless of when FTY720 is present, however in the BCG model timing of drug treatment is critical and FTY720 treatment during vaccination can prevent protective ELT from forming. Although mice receiving FTY720 during BCG vaccination lost the protection afforded by the vaccine, animals receiving drug treatment immediately prior to BCG challenge infection retained their protection (Fig 4.12). This result indicates that there is a requirement for lymphocyte trafficking to the

lung during or after primary infection to establish ELT in the BCG model that we do not see in our *N. brasiliensis* model.

The requirement for lymphocyte trafficking during/after vaccination in the BCG model implies that lymph node derived T cells are required to "seed" the lung tissue with protective memory cells to form ELT. This also suggests that the localised antigen specific T cell responses observed within the lung during primary vaccination in FTY720 treated mice are insufficient to establish a protective population of memory cells that are retained within the lung. The need for lymph node derived T cells to form peripheral memory tissue is supported by the work of David Woodlands group who propose that peripheral memory CD8 T cells are maintained by continual recruitment in their sendai virus model of protective immunity [107]. The difference observed in the BCG model may be due to the toxicity of IFNγ [208-210], with the IFNγ producing cells dying after performing their effector function during primary infection; therefore requiring additional cells to become established resident memory T cells. This would potentially not be an issue for Th2 cells as IL-4 is a growth/survival factor and previous studies have shown that although Th1 effectors die from activation induced cell death, Th2 cells do not [106, 205].

A further implication of the requirement for lymph node derived T cells in the BCG model is support for an alternative generation of ELT during primary lung *N. brasiliensis* infection. Our two hypotheses are that ELT can be generated within the lung tissue without lymph node derived T cells in the *N. brasiliensis* model or, alternatively, that memory T cells destined to become ELT are resistant to FTY720 treatment and able to traffic to the periphery regardless of drug treatment. Data generated with the BCG model suggests that T cell trafficking is effectively blocked by FTY720 treatment as primary infection is inadequately controlled without circulating T cells and more importantly, blocking T cell trafficking during primary infection prevents the establishment of protective ELT in the lung. This also suggests that BCG falls within the more classical definition of ELT generation with ELT becoming established after T cell migration following a primary infection, where *N. brasiliensis* clearly does not.

Interestingly we observed differences in lung based antigen specific T cell responses between the *N. brasiliensis* and BCG models. *N. brasiliensis* exhibits a reduced primary Th2 response in the lung followed by a contraction and expansion upon reinfection. In the BCG model we see reduced primary response but prior to challenge there are more antigen specific IFNγ producers than during primary infection. This is most likely occurring due to

the break in FTY720 treatment in the BCG model allowing recirculation of T cells; however this finding is interesting in the respect that the reduced population of Th2 cells are able to confer protection, yet the increased number of antigen specific IFNγ cells are unable to confer protection in the BCG model. This implies that it is not necessarily the magnitude of effector response, but the quality that confers protection – although this may also have to do with the differences between IL-4 and IFNγ.

Another difference between the BCG and *N. brasiliensis* models was the importance of T_{EM} like cells in conferring protective immunity. We show in chapter five that T_{EM} like cells correlate with protective immunity in *N. brasiliensis* models; the results with the BCG model were less clear. The majority of CD4 T cells during the secondary immune response displayed a Tem phenotype in both protected and unprotected mice. This finding that T_{EM} are important in peripheral immunity is not surprising given previous data showing the preferential location of T_{EM} cells in peripheral tissues [211].

The BCG model was chosen to compare with our Th2 mediated *N. brasiliensis* model, as it is a CD4 T cell mediated lung based model of protective immunity, however there are differences between the two models. The BCG model differs from *N. brasiliensis* infection model in several respects;

Firstly BCG infection is bacterial and usually chronic. This may in some way account for the different immune strategies in the generation of ELT against BCG and *N. brasiliensis* infection. T cells may need to be recruited to the site of BCG infection to replace cells that die via IFNγ toxicity or exhaustion. This would not be the case in *N. brasiliensis* infection as the worms are in the lung only transiently (days one to three of infection) so an on going immune response would not need to be supported. Furthermore, IL-4 does not share the toxicity of IFNγ - perhaps supporting the development of effectors into T_{EM} like cells. This would be supported by previous findings within our group showing that IL-4 deficient mice are 75% less protected at the lung site upon reinfection (Table 5.1). The chronicity of bacterial infection also caused some variations within our infection model, we have affected a “cure” between primary and re-infection by clearing the bacteria with antibiotic treatment (Fig 4.11), to ensure neither model has persisting antigen between primary and reinfection. FTY720 treatment also differs, as there is a break in treatment in the BCG model. This lapse in FTY720 treatment had the potential to allow recirculation after bacterial clearance via antibiotics, however we found mice treated with FTY720 during

vaccination until day 35 failed to develop ELT. This indicates T cell recirculation to develop ELT must occur earlier – most likely during primary infection. The finding that a T_{CM} like population can be derived during vaccination and confer protection via adoptive transfer (L. Connor, "Dissecting the protective memory immune response against Tuberculosis") supports a role for lymph node generated T_{CM} in ELT generation in this model.

Secondly, vaccination with BCG does not occur directly in the lung; although bacterial antigen specific lung based IFNγ producing CD4 T cells were found there. This difference may in some way contribute to the necessity for further CD4 T cell recruitment to generate protective ELT in this model. Differences between *N. brasiliensis* and BCG in the generation of ELT – was this due to systemic vaccination of BCG rather than lung based vaccination priming?

Thirdly, the bacterial infection caused by BCG causes a different type of physical damage to the lung tissue than the tissue burrowing *N. brasiliensis*. Granulomatous lesions are characteristic of the immune response to BCG, whereas *N. brasiliensis* cause a lot of tissue damage with their movement, and tissue remodelling follows infection. Our finding that CD4 T cells can transfer protection to naïve hosts would suggest that the physical changes to the structure of the lung by *N. brasiliensis* infection and the subsequent tissue remodelling are not required for protection as recently suggested by Reece *et al* [173]. This does not rule out a role for alternate activation of macrophages playing an important role in protection against reinfection with *N. brasiliensis*, however if this is indeed the case then this response in some way requires CD4 T cells for initiation or maintenance.

Lastly, measurement of IL-4 during *N. brasiliensis* infection is possible directly *ex vivo* due to the use of GFP reporter mice, however in the BCG model T cells had to be restimulated *ex vivo* with BCG antigens and intracellularly stained to measure IFNγ production. The *ex vivo* restimulation method of measuring antigen specific cells does not provide an exact picture of *in vivo* events, due to the further manipulation required. However as the experimental results were compared to appropriate controls, this should not be an impediment to a comparison of the immune responses between the two models.

The several differences between the BCG and *N. brasiliensis* models are important to be considered when interpreting the results within this thesis. However there are important similarities too; both models are CD4 mediated and also have an important role for

macrophages – BCG with the development of granulomatous lesions and engulfment and killing of bacteria with ROI, and *N. brasiliensis* with the recruitment and activation of alternately activated macrophages [171, 173] which are thought to play a role in tissue repair and remodelling [19]. Regardless of the differences between the BCG and *N. brasiliensis* models our findings provide evidence for the importance of ELT in protective immunity in both models. Furthermore a comparison of the two models has revealed potential differences in the mechanisms of generation of ELT – a striking finding.

6.5. The role of effector lymphoid tissue in protective immunity

The findings within this thesis combined with recent cutting edge research [70, 108] strongly suggest that ELT is an important part of the memory response that is capable of protecting during reinfection without the additional support of secondary lymphoid derived T cells. The mechanisms behind generation of ELT are less clear. Experiments in CD8 and Th1 models suggest that there is a requirement for lymph node derived T cells trafficking to the periphery [107] to establish ELT as proposed in the original opinion paper [104]. However the experiments within this thesis suggest there may be an alternative method of ELT generation, through the priming and maintenance of T cell responses in peripheral tissues.

There is a potential for qualitative differences in the responses of lymph node based memory T cells and those cells based in peripheral tissues. It is possible that the peripheral environment influences the responses of memory T cells allowing for faster activation and effector function [212]. Studies by Bertolino *et al* [88] shows that adoptively transferred naïve auto reactive CD8 T cells can be primed in liver and that these cells divide up to four times in 24 hours, where those in the lymph node take two to three days to recirculate to peripheral tissues. The T cells within the liver were activated very quickly, up regulating activation markers within two hours of transfer. In the instance of lung tissue, endothelial cells secrete cytokines and other factors contributing to the immune response. These environmental differences may contribute to the effectiveness of ELT.

The possibility of development and maintenance of protective memory cells in the lung tissue during *N. brasiliensis* infection, forming ELT has been proposed (Fig 6.1). The finding that FTY720 separation of tertiary and secondary lymphoid tissues did not inhibit protection (chapter 4), combined with the finding that adoptive transfer of T_{EM} like lung CD4 T cells from FTY720 treated mice could confer protection (Fig 5.8) would appear to

support an alternative method of ELT development. This alternative development is not necessarily exclusive of the classic mechanism, lymph node derived ELT development could occur simultaneously with the alternative mechanism being revealed only when secondary lymphoid responses are inhibited. This mechanism may be restricted to Th2, helminth or even specifically *N. brasiliensis* infection. The experiments of Moyron-Quiroz *et al* explore the role of ELT in a viral model using mice lacking secondary lymphoid organs [70, 71]. They found that iBALT developed in the lungs of previously infected mice and suggested that this provided a niche for maintenance of a local memory T cell population. These results support the ability of lung localised tertiary lymphoid responses to generate and maintain ELT.

Lastly, treatment with FTY720 may support the generation of ELT. Ledgerwood *et al* [130] propose that FTY720 treatment can trap T cells within peripheral tissues. We notice that FTY720 treatment reveals a lung residing population of CD4 T cells although the reduced number of CD4 T cells we observed in the lung suggests this may be only a small subset of CD4 T cells. The research of Idzko *et al* suggests that FTY720 traps DC within the lung tissue, preventing their trafficking to the lymph nodes [140]. These experiments used a local intratracheal administration of FTY720 and it remains to be seen if the systemic gavaged delivery method would have a similar effect. Data collected within our own group suggests that local FTY720 administration within the skin can result in impaired DC trafficking to the draining lymph node, however not all subsets of DC are affected equally (R. Hunter, unpublished data). The trapping of antigen loaded mature DC within peripheral tissues alongside a similarly confined CD4 T cell population could potentially result in an environment that is conducive to the establishment of ELT through the proposed alternative mechanism. Furthermore, drawing on the theories of Booki Min [69], T cell sequestration caused by FTY720 treatment may be helping to create “space” in the TCR diversity of lung residing memory T cells. Booki’s hypothesis is that if the repertoire of memory TCR diversity is limited, spontaneous proliferation of naïve T cells with different TCR specificity occurs to expand the TCR repertoire – independently of homeostatic proliferation. FTY720 treatment reducing the number of lung resident T cells could also affect TCR diversity in our model, causing the proliferation and enhanced retention of *N. brasiliensis* antigen specific cells that add to the diversity of the T cell repertoire. It would be interesting to immunoscope the profile of lung CD4 T cells in

FTY720 treated animals before during and after priming and reinfection to examine TCR diversity of the lung resident T cells.

6.6. Conclusions

My thesis sought to identify which tissues are important sites for priming protective immunity against *N. brasiliensis*. The contribution of lung tissues in the protective response was assessed by blocking CD4 T cell recirculation between lymph nodes and lung tissue using FTY720. We clearly identified that lung based immune responses were sufficient to protect against reinfection with *N. brasiliensis*, although it may reflect the escape of T_{EM} cells from FTY720 treatment. Lung localised adoptive transfer of CD4 T cells into naïve animals conferred protection, confirming that CD4 T cells can mediate protection. The finding that depletion of CD4 T cells abrogated protective immunity indicated the key role CD4 T cells play in protection against reinfection with *N. brasiliensis*.

A characterisation of the CD4 T cell response ruled out a role for NKT cells as the protective CD4 lineage as CD1 -/- animals were completely protected against reinfection. Kinetic analysis of the development of CD4 T cells FTY720 treated and untreated animals during *N. brasiliensis* infection revealed a T_{EM} like population of cells in the lung during primary infection. The T_{EM} -like population of cells expanded upon reinfection and produced the Th2 associated cytokine IL-4. Localised lung adoptive transfer of these T_{EM} like cells into naïve athymic recipients was sufficient to confer immunity against *N. brasiliensis*. Collectively these results demonstrate that a peripheral based CD4 immune response is sufficient to confer protection in the *N. brasiliensis* model of Th2 protective immunity. This finding supports the theory of effector lymphoid tissue and may demonstrate an alternative process for the development of effector lymphoid tissue in the periphery.

6.7. Future Directions

The experiment within this thesis have led to the development of two hypotheses to explain our findings; the alternative development of ELT hypothesis and the selective escape of protective CD4 T cells from FTY720 treatment. Future experiments would aim to determine which of these two hypotheses is the most likely.

Experiments investigating whether proliferation of CD4 T cells occurs within the lung tissue would be an important question to address. Labelling of lung tissue with coloured dye and incorporation of BrDU into the DNA of proliferating cells would determine if CD4 T cells proliferated within lung tissues, or "escaped" from the draining lymph node during reinfection with *N. brasiliensis* in FTY720 mice.

The experiments of Roberts *et al* suggest that T_{EM} cells are important at early times post infection, however T_{CM} dominate at later time points [106]. Investigating the effects of FTY720 treatment on ELT mediated protection in the *N. brasiliensis* model at later times following infection (more than 30 days) may reveal an additional requirement for T cell recruitment from the lymph nodes.

Appendix

Appendix

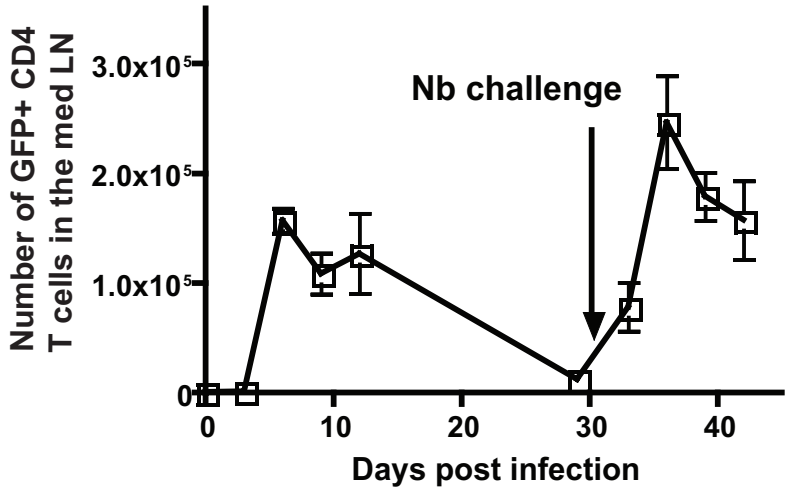

Figure A.1 Kinetics of the GFP+ CD4 T cell response following primary and secondary infection with *N. brasiliensis*.

G4/IL-4 reporter mice (n=4) were infected with *N. brasiliensis*, rested for 30 days before reinfection. At the indicated time points mice were sacrificed, mediastinal lymph nodes were harvested and the number of GFP+ CD4 T cells assessed using flow cytometry. Values represent mean number of GFP+ CD4 T cells ± SEM and are representative of at least three independent experiments.

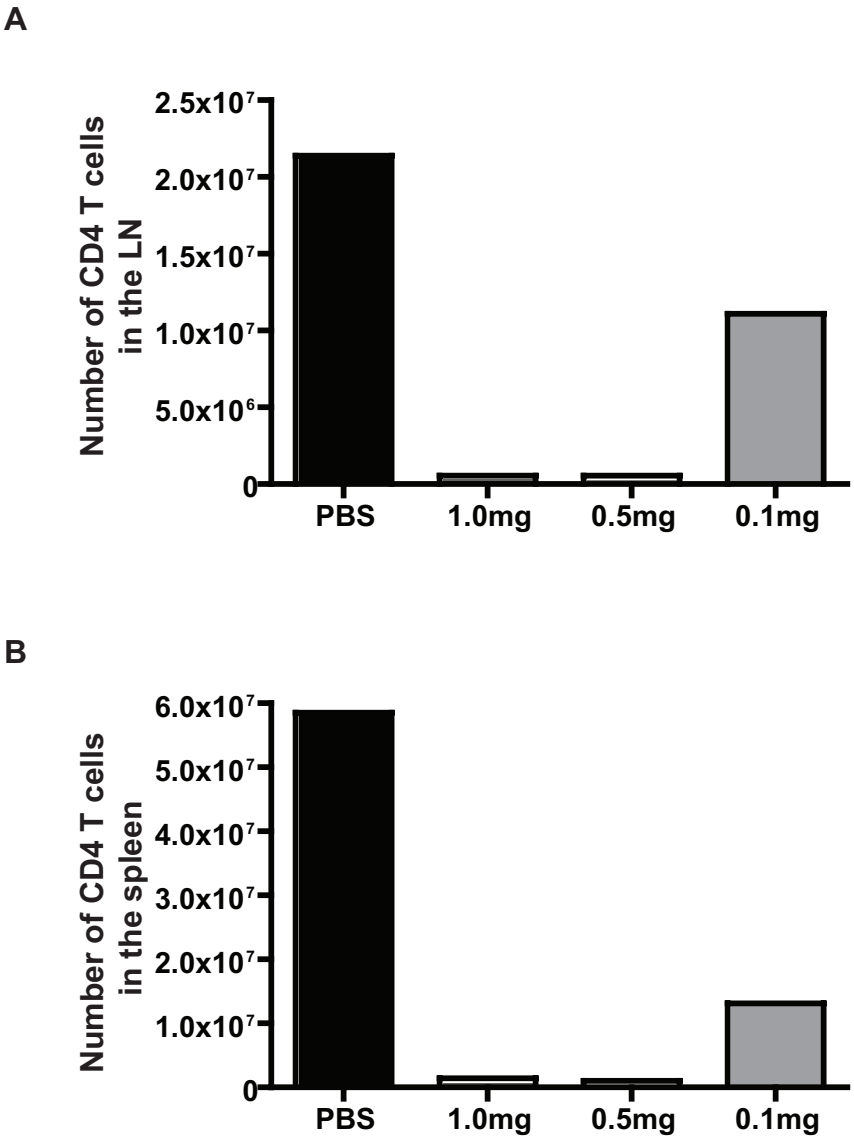

Figure A.2 Depletion of CD4 T cells using an αCD4 antibody - titration of anti-CD4 antibody

G4/IL-4 mice (n = 3/group, pooled) were treated with the indicated concentration of anti-CD4 antibody (clone GK1.5) by intraperitoneal injection at time zero. At day 28 post depletion lymph nodes **(A)** and spleens **(B)** were harvested and the number of CD4 T cells assessed using four colour flow cytometry. CD4 T cells were identified as lymphocytes based on forward and side scatter properties and were CD4 (clone RM4-5)/TCRβ positive. Values represent the mean number of CD4 T cells per tissue.

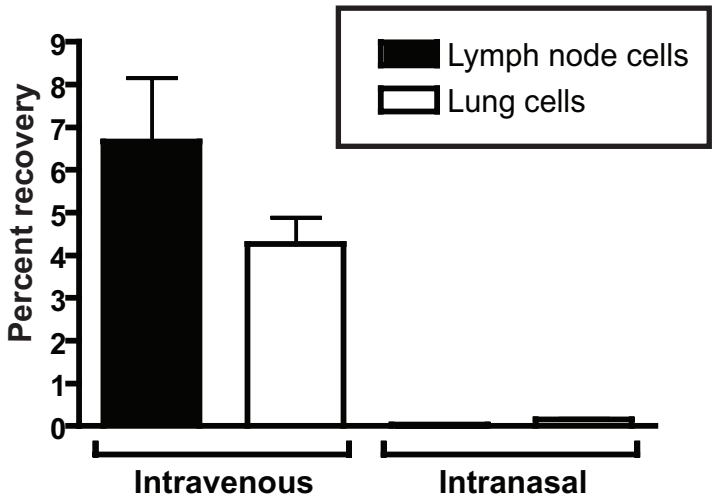

Figure A.3 Recovery of CD4 T cells from spleen after adoptive transfer using different techniques.

Lymphocytes were isolated from the lungs (white bars) or lymph nodes (black bars) of Balb/c mice and labelled using CFSE before being transferred into recipient Balb/c mice (n = 3/ group) via intranasal or intraveneous route. Two days after cell transfer recipient mice were sacrificed and spleens harvested. Spleens were enzymatically digested and analysed using four colour flow cytometry for the presence of CFSE labelled CD4 T cells. Values represent the number of cells recovered as a percentage of adoptively transferred cells ± S.E.M.

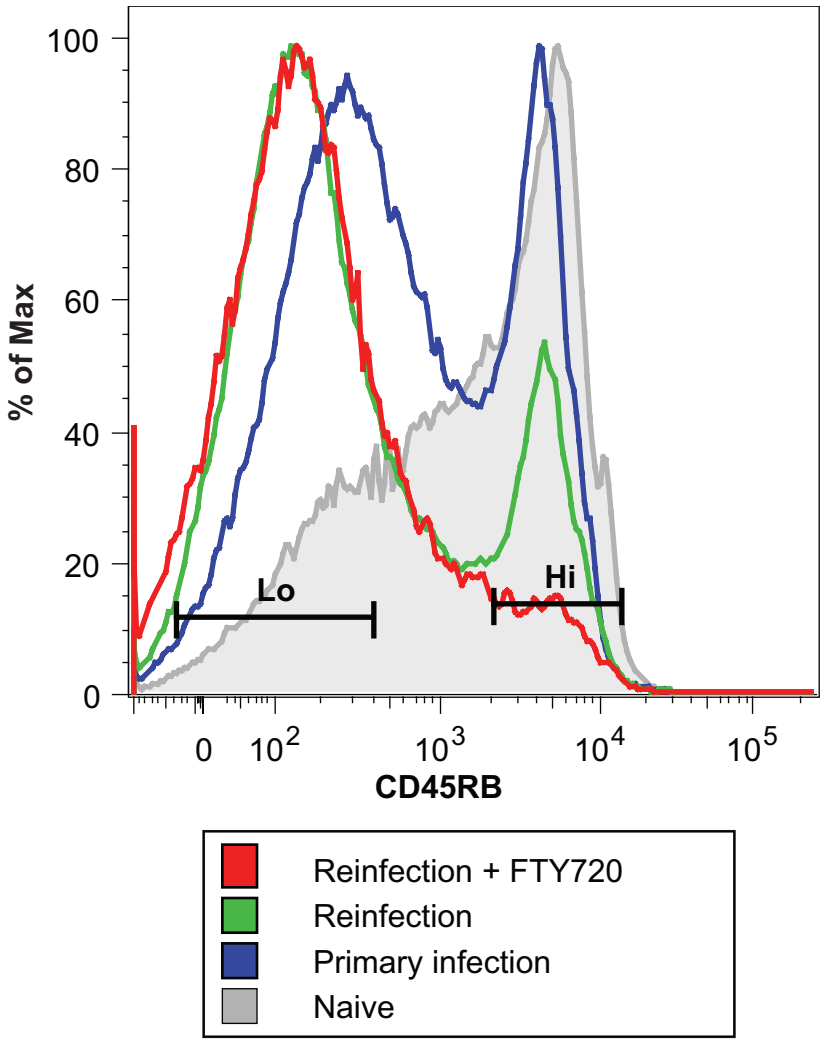

Figure A.4 CD45RB expression on CD4 T cells isolated from mice naïve, infected or reinfected with *N. brasiliensis*.

G4/IL-4 mice (n=3/group/time point) were treated with FTY720 from one day prior to *N. brasiliensis* infection or remained untreated. Mice were reinfected with *N. brasiliensis* at day 39 At six days post reinfection lung tissues were harvested, enzymatically digested and CD45 positive cells were enriched by AutoMacs© bead separation. Isolated cells were stained with antibodies against CD45RB, CD62L, CD44, CD3 and CD4 and analysed by flow cytometry using an LSR II flow cytometer. DAPI was included to exclude dead cells and GFP reporter was also present as donor mice were from a G4/IL-4 background. Facs plots are gated on DAPI negative CD3/CD4 positive cells and are from one representative animal per group.

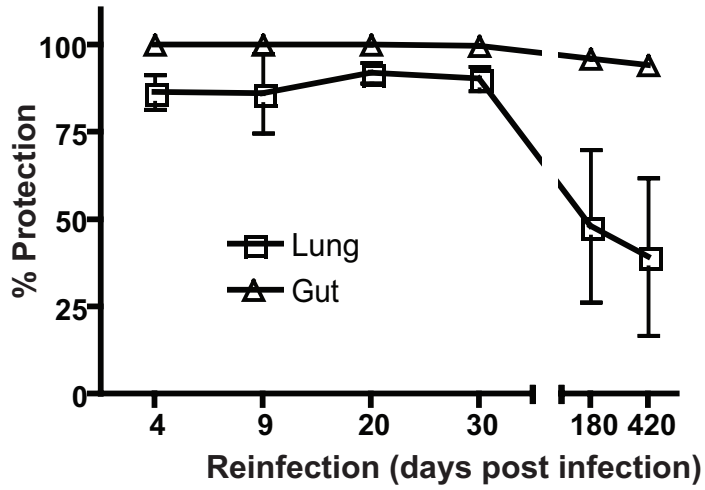

Figure A.5 Duration of protective immunity, reinfection at various time points after infection.

Balb/c mice (n=3/timepoint) were infected with *N. brasiliensis* and then reinfected at the indicated time points. Following reinfection, lungs & guts were excised on days two and six post reinfection respectively and the number of viable parasites in the tissues were recovered by migration and enumerated. For each time point aged matched naïve mice were given the identical challenge infection with 600 L3 and the numbers of viable worms recovered from tissues. The degree of protection for each time point was determined from the ratio of reduced worm burden in reinfected versus naïve mice, with reinfected mice typically resulting in a ten fold reduction in worm numbers. If reinfected mice were able to achieve ten fold reduction in worm numbers when compared to aged match naïve mice receiving primary infection then we defined that time point as maintaining 100 % protection. If reinfected mice failed to reduce worm burdens when compared to the burden in similarly infected, aged matched, naïve mice then we defined it as 0% protection.

Group	CD45RB hi ± S.E.M.	CD45RB lo ± S.E.M.
1	11.65 ±1.73	34.13 ± 2.84
2	6.98 ± 0.60	42.98 ± 1.75
3	15.34 ± 1.32	21.68 ± 1.40
4	13.54 ± 1.56	14.60 ± 1.27
5	9.64 ± 0.74	20.71 ± 1.98

Table A.1 Phenotype of lung isolated CD4 T cells from *N. brasiliensis* infected athymic mice.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. The CD45RB hi populations of groups 1-5 werenot found to be statistically significant (ns). The CD45RB lo populations exhibited significant differences with group 1 having significantly more CD45RB lo cells than group 4 (*), and group 2 having significantly more CD45RB lo cells than groups 3 (**), 4 (***) and 5 (**). Statistics were calculated using one way ANOVA with Tukey post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

	Control		FTY720	
Days post Infection	**CD45RB lo**	**CD45RB hi**	**CD45RB lo**	**CD45RB hi**
Naive	9.91 ± 0.23	81.66 ± 0.48	34.82 ± 1.25	46.06 ± 2.03
3	3.12 ± 0.07	90.36 ± 0.25	8.21 ± 0.73	76.54 ± 1.56
6	28.54 ± 0.56	37.02 ± 0.94	33.08 ± 0.31	28.7 ± 2.14
9	28.92 ± 0.61	58.6 ± 0.87	45.58 ± 0.41	33.42 ± 0.39
12	41 ± 1.32	36.32 ± 1.21	69.7 ± 2.74	5.19 ± 0.81

Table A.2 Kinetics of the lung CD4 T cell response against primary *N. brasiliensis* infection.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. FTY720 treated mice had significantly less CD45RB hi cells than control mice (***) and also significantly more CD45RB lo cells than control mice (***). Statistics were calculated using two way ANOVA with bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

	Control		FTY720	
Days post Challenge	**CD45RB lo**	**CD45RB hi**	**CD45RB lo**	**CD45RB hi**
-1	53.28 ± 2.37	40.43 ± 2.35	95.94 ± 0.25	2.47 ± 0.15
3	36.45 ± 1.18	41.55 ± 1.28	63.16 ± 3.59	11.87 ± 2.81
6	66.48 ± 0.39	20 ± 0.38	78.8 ± 1.7	4.26 ± 0.38
9	84.84 ± 0.48	13.05 ± 0.44	98.56 ± 0.21	1.01 ± 0.16
12	68.26 ± 1.71	17.29 ± 1.52	94.2 ± 0.35	1.5 ± 0.1

Table A.3 Kinetics of the lung CD4 T cell response against *N. brasiliensis* reinfection.

G4/IL-4 mice (n = 5/group/time point) were treated with FTY720 from the day prior to infection with *N. brasiliensis* or remained untreated. At 30 days post infection mice were reinfected with *N. brasiliensis*. At the indicated time points lung tissues were harvested and enzymatically digested before being enriched for CD45+ cells using AutoMacs© magnetic bead separation. The enriched cells were stained and analysed using multicolour FACs analysis on a LSR II flow cytometer. FTY720 treated mice had significantly less CD45RB hi cells than control mice (***) and also significantly more CD45RB lo cells than control mice (***). Statistics were calculated using two way ANOVA with bonferroni post test * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

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Publications