THE ROLE OF PULMONARY INNATE AND ADAPTIVE IMMUNE RESPONSES TO HELMINTH INFECTION

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THWSUM001

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Plagiarism Declaration

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Illustration on cover page: Female *N. brasiliensis* worm filled with eggs. Photograph by Dr. Jennifer Claire Hoving, UCT.

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Abbreviations

$^{-/-}$	Deficient
^{o}C	Degrees Celsius
AAM	Alternatively activated macrophages
Ab	Antibody
Ag	Antigen
AHR	Airway hyper-responsiveness
AM	Alveolar macrophage
AP	Alkaline Phosphatase
APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
Cre	Cre recombinase
CRD	Carbohydrate recognition domain
DC	Dendritic Cell
ELISA	Enzyme linked immune-sorbent assay
EPO	Eosinophil peroxidase
FACS	Fluorescence-Activated Cell Sorting
FTY720	Fingolimod
H&E	Haemotoxylin & Eosin
H_2O_2	Hydrogen peroxidase
HRP	Horse radish peroxidase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL-4Rα	Interleukin-4 Receptor alpha
IL-4R$\alpha^{-/lox}$	Hemizygous for IL-4Rα (Wildtype Controls).
I/N	Intra-nasal
IMDM	Iscove’s Modified Dulbecco’s Medium
KO	Knock out
L3	Third stage larvae
Lck^{cre}IL-4R$\alpha^{-/lox}$	T cell specific disrupted IL-4Rα
LN	Lymph node
LTβR	Lymphotoxin beta receptor
LTβR-Ig	Lymphotoxin beta receptor fusion protein
LysM^{cre}IL-4R$\alpha^{-/lox}$	Macrophage/ Neutrophils specific disrupted IL-4Rα
MHC	Major Histocompatibility complex
MMP	Matrix metalloproteinase
N. brasiliensis	*Nippostrongylus brasiliensis*
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PAS	Periodic Acid Schiffs
PBS	Phosphate buffered saline

P.I	Post-infection
rfhSP-D	Recombinant fragment of human SP-D
SP-D	Surfactant protein D
SLO	Secondary lymphoid organs
Teff	Effector T cells
Tem	Effector memory T cells
Tcm	Central memory T cells
TFF2	Trefoil factor 2
TGF-β	Transforming growth factor beta
Th2	T helper 2 $CD4^+$ cell
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
WT	Wild type

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Abstract

Immunity to nematode infections requires a host T helper 2 (Th2) response promoted by epithelial cell driven IL-33 induction of cytokine secretion of Interleukin (IL)-4, 5 and 13 by a range of immune cells including innate lymphoid cells type 2 (ILC2s) and $CD4^+$ T cells. This induces effector responses such as goblet cell mucus secretion and mast cell activation driving disease resolution. Finding candidate molecules and discrete cell populations that enhance these responses would provide new targets for treating infection via specific host immune-modulation and would contribute to the development of effective vaccines against nematode infections. In this study we addressed how novel components of host adaptive and innate immunity can contribute to pulmonary control of *Nippostrongylus brasiliensis* infections.

Murine reinfection studies with the parasitic nematode *N. brasiliensis* have shown development of a Th2 $CD4^+$ T cell responses in the lung to be essential for immunity to secondary *N. brasiliensis* infection. To test if T cell recruitment from secondary lymphoid tissue contributed to this immunity, we used the drug Fingolimod (FTY720) to block T cell egress from lymph nodes (LN) to peripheral tissue. T cell egress from the LN was required for resolution of a primary infection but not for secondary infection. The presence of tissue-resident IL-4Rα responsive $CD4^+$ T cells in the lung was sufficient for protective immunity to *N. brasiliensis* reinfection. These results demonstrated that effective $CD4^+$ T cell Th2 immunity can be generated at peripheral sites by pre-existing T cell populations, independently of T cell recruitment from secondary lymphoid organs (SLO).

Additionally, we identify that the pulmonary epithelial cell-secreted collectin, surfactant protein D (SP-D), is an important component of host immunity to *N. brasiliensis* infection. We demonstrate here that SP-D production is induced following *N. brasiliensis* infection in a Th2 dependent manner, it bound preferentially to lung stage L4 parasites and enhanced macrophage and ILC2 protective responses essential for controlling infection.

Taken together the data presented in this thesis provides two new important insights into pulmonary host immunity to parasitic helminth infections.

Chapter 1. Introduction

1.1 The Immune system

The immune system represents the body's host defense mechanism in fighting off various harmful pathogens. These infectious pathogens range from viruses and bacterias to fungi and parasites all of which if established in the host can cause detrimental infectious diseases (1).

The immune system is composed of various cells, tissues and secretory molecules that co-ordinate to produce a highly efficient immune response. It is primarily driven by leukocytes. These cells originate from hematopoietic stem cells of the bone marrow. While some of these cells develop and differentiate here, others migrate to the thymus and spleen to complete their development (2).

The host defense mechanism is generally divided in to the innate and adaptive immunity. The innate immunity serves as the first line of defense and consists of mechanical, chemical and cellular barriers. It normally mounts a broad and rapid response to the pathogen. The adaptive immunity on the other hand, develops more slowly and provides a more effective and antigen-specific immune response via B and T cell lymphocytes. A key feature of adaptive immunity is its ability to generate immune memory that allows for a more rapid protective response (3).

A continuous cross talk between the innate and adaptive immunity is essential for inducing an efficient immune response. In the work presented here, we will examine the role of innate and adaptive immune responses to the parasitic nematode, *N. brasiliensis*. Hence a brief introduction of the innate and adaptive immunity is first given in order to understand the fundamental concepts of the immune system.

1.1.1 The Innate Immunity

The innate immunity is the intrinsic mechanism possessed by multicellular organisms and initiates the immune responses against various foreign pathogens. It is composed of several components that include physical, chemical and cellular barriers (4).

The epithelial surface of the body contains tight junctions and provides a physical barrier between the internal environment of the body and the outer. Epithelium acts as the first line of defense and only when pathogens manage to pass through it, do they activate other components of immune system. The epithelial cells can secrete mucus that contains mucin and uses muco-ciliary mediated weep and sweep mechanism to remove pathogens. Individuals with defective secretion of mucus usually develop cystic fibrosis and are susceptible to pulmonary bacterial infections (5). Epithelium also secretes antimicrobial peptides that kill microbes. For instance, bacteriolytic lysozyme and phospholipase A are secreted in tears and saliva and have antimicrobial properties. Furthermore, the acid environment in the stomach created by the fatty acids, bile salts and digestive enzymes create unfavorable environment for the microbes (**Fig 1.1**).

The innate response is augmented by the humoral component that includes various soluble mediators that exist in biological fluids and secreted by the epithelial cells. These include complement proteins, defensins, collectins, antimicrobial peptides and Lipopolysaccharide (LPS) binding protein. These proteins can directly bind to pathogens and activate the complement cascade that aids in pathogen clearance, a process called opsonization (2–4, 6).

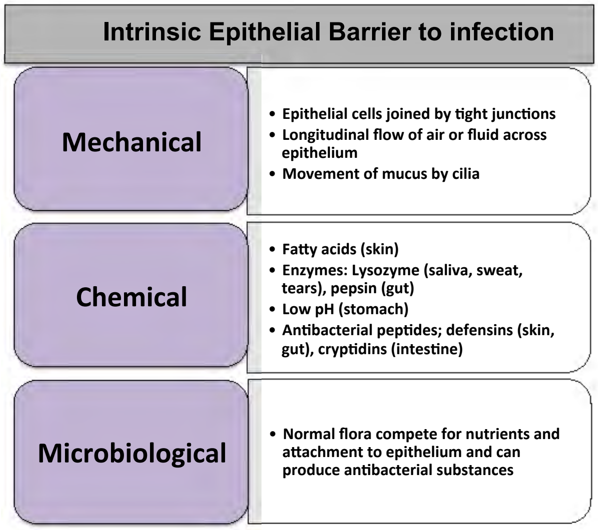


Fig 1.1: Epithelial cell-mediated host defense mechanisms. Figure adapted from Janeway's immunobiology (3).

Recognition of pathogens by the innate immunity is regulated by germline-encoded receptor system that can discriminate self from non-self. This occurs by membrane bound pathogen recognition receptors (PRR) such as toll-like receptors (TLRs) and mannose receptors that are a type of C-type lectin receptors (CLRs) that recognize conserved pathogen associated molecular patterns (PAMP) expressed on various pathogens. PRRs are widely expressed by many haemopoietic and non-haemopoietic cells of the innate system. These include epithelial cells, endothelial cells, smooth muscle cells, dendritic cells (DC) and the granulocytes; macrophages, eosinophils, neutrophils, and basophils. Recognition leads to activation of inflammatory pathways and the secretion of chemokines and cytokines that lead to pathogen elimination. Chemokines aid in attracting inflammatory leukocytes, lipid mediators and free radical species that promote tissue inflammation while cytokines aid in activating other effector cells and thereby regulating their functions (7).

The granulocytes play a prominent role in innate defense and are part of the cell-mediated component of innate immunity. When pathogens manage to penetrate the epithelium, macrophages residing in the tissue sites are activated to phagocytose the microbes and release cytokines and chemokines. This also leads to an inflammatory response that involves recruitment of effector cells to help augment pathogen killing. Neutrophils are usually among the first effector cells to be recruited to the site of infection followed by monocytes. Both these cells play a crucial role in the killing of pathogens (**Fig 1.2**) (1, 3)**.**

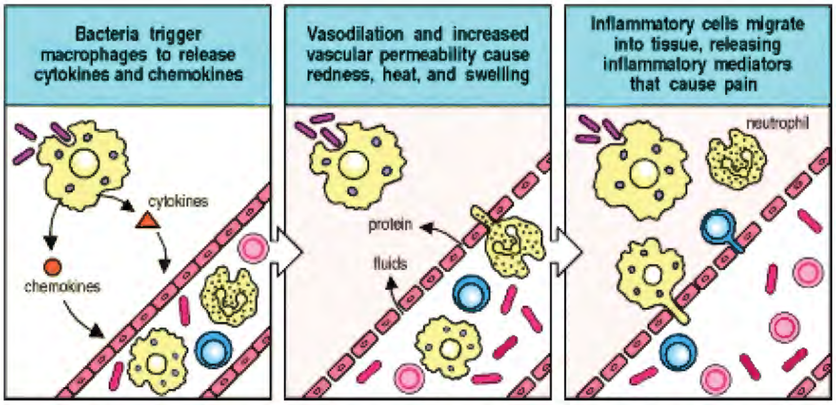


Fig 1. 2: **The inflammatory response to pathogens**. Figure adapted from Janeway's immunobiology (3).

Macrophages and neutrophils produce toxic compounds such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) that aid in killing the engulfed pathogen. Macrophages can adopt two kinds of phenotypes; the M1 and M2 depending on the environmental signals they receive. M1 are classically activated macrophages that produce large amounts of IFN-γ, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α) and therefore possess pro-inflammatory roles. On the other hand, M2 are alternative activated macrophages induced by IL-4 and IL-13 and produce IL-10 and transforming growth factor beta (TGF-β) and therefore exhibit anti-inflammatory role (8).

The inflammatory responses at the later stage also involves the lymphocytes of the adaptive immunity that interact with a range of other innate effector cells to eliminate the pathogen. These innate cells include the granulocytes such as eosinophils, basophils and mast cells that require signals from lymphocytes to sustain their effector functions.

Eosinophils are particularly activated during allergic inflammation and helminth infections (9). Their granulated cytoplasm contains toxic molecules such as eosinophil peroxidase (EPO) that is effective in killing pathogens that are too big to be engulfed. Mast cells and basophils express the receptor, FceR1 that binds to the immunoglobulin IgE with high affinity and are key initiators of hypersensitivity response. They contribute towards anti-helminth responses by secreting histamines and other soluble and lipid mediators that stimulate inflammation and smooth muscle contractility (3, 6).

The recognition of pathogens by the innate immunity leads to up-regulation of co-stimulatory receptors that serve as important linkers to the adaptive immunity. Macrophages and DCs express major histocompatibility (MHC) molecules that are important for antigen presentation and activation of lymphocytes.

1.1.2 The Adaptive Immunity

The adaptive immunity involves a fine interaction between the antigen presenting cells and lymphocytes that facilitates a pathogen-specific immune response. It is largely facilitated by T and B lymphocytes that express antigen specific receptors. T cells mature in the Thymus while antibody-producing B cells develop in the Bone marrow. During infection, the secondary lymphoid organs (SLO) provide a conducive microenvironment for activation of lymphocytes. Professional antigen presenting cells (APCs) such as DCs and macrophages migrate to the lymphoid tissue where they present antigen to the lymphocytes. This leads to their activation, differentiation and expansion and subsequent migration to the site of infection. Here effector responses by T cells and antibodies produced by B-cells help in pathogen elimination (10, 11).

1.1.2.1 T cells:

T cells express T cell receptors (TCR) that interact with antigenic peptide complexed with the co-stimulatory MHC molecules. There are two types of T cells, CD4$^+$ T helper and CD8$^+$ T cytotoxic cells. CD4$^+$ T cells normally engage with peptides presented on MHC class II while CD8$^+$ T cells engage with peptides expressed by MHC class I. Activation leads to a diverse range of effector functions that includes direct killing of infected cells, functioning as helper cells and providing signals to enhance immune responses (**Fig 1.3**) (7).

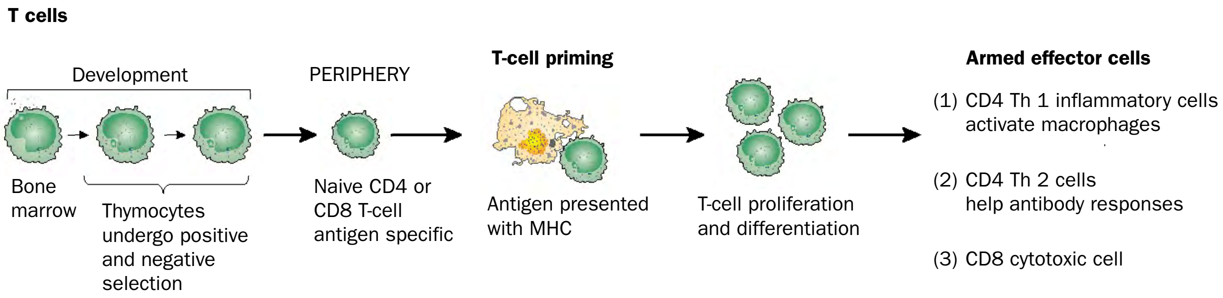


Fig 1. 3: **The role of T cells in Adaptive immunity.** Figure adapted from Parkin J & Cohen B. 2001 (7).

- CD4⁺ helper T cells:

CD4⁺ T cells are the largest group of cells and are known as T helper cells (Th) due to their helper function in stimulating antibody production by B cells and enhancing efficiency of innate responses. They are divided into two main subsets based on their cytokine profiles: Th1 and Th2. The Th1 response is induced by the cytokine IL-12 and is characterized by production of high levels of IFN-γ and TNF-α that contributes to control of bacterial and protozoan infections (12, 13). Conversely, a Th2 response is associated with the production of the cytokine IL-4 and along with production of high levels of IL-5 and IL-13 (14). Th2 immunity is critical for host immunity to helminth infection and is associated with onset of allergic inflammatory diseases. In this study we will focus on the Th2 arm of the adaptive immune system associated with parasitic nematode infection.

In addition to the Th1 and Th2 cells, other subsets of CD4⁺ T cells also exist that perform specific functions (**Fig 1.4**). These include the inflammatory IL-17 secreting Th17 cells that are found on mucosal surfaces and are involved in anti-bacterial responses, and the follicular helper T cells (Tfh) that aid B cells in producing antibodies and reside in the B cell follicles of SLO. An important subset of T cells are the CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg) cells that are induced by IL-10 and TGF-β and help in establishing a well-balanced immunity by dampening excessive inflammatory responses (11).

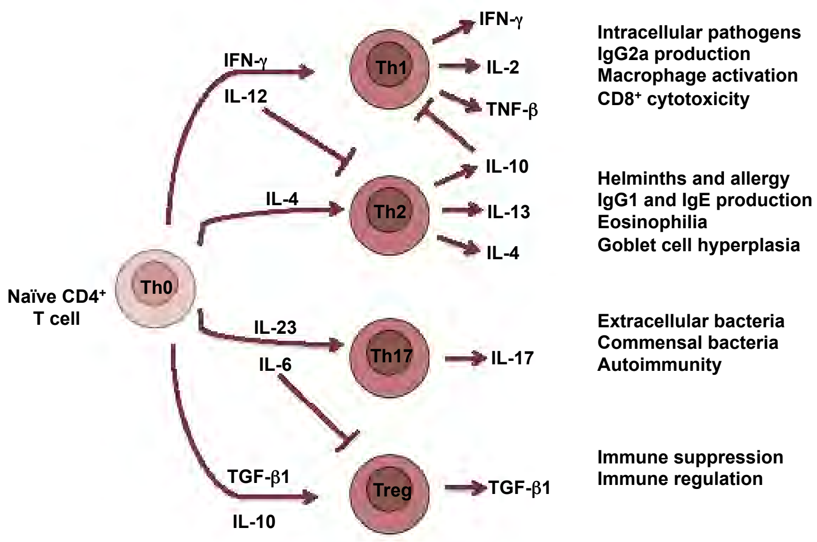


Fig 1. 4: **CD4$^+$ Th cell Subsets**. Naïve CD4$^+$ T cell can differentiate in to different subsets of T cell depending on the cytokine milieu.

- Cytotoxic CD8$^+$ T cells

Cytotoxic CD8$^+$T cells on the other hand exert cytolytic activity on virus-infected cells. These cells bind to their target cells and form pores on the cell membrane by inserting perforins. Granzymes are then released in to the target cell and this induces cell apoptosis (11).

1.1.2.2 B cells:

B cells are important in driving effective immunity against a range of pathogens and can respond in a T cell dependent or T cell independent manner (**Fig 1.5**). A T cell dependent response involves B cell receptors (BCRs), which are surface immunoglobulins that can bind to antigens directly, internalize it and present antigen on MHC class II molecules. The antigens are recognized by CD4$^+$ helper T cells that induce the activation and somatic hyper-mutation of B cells in the germinal centers to become antibody secreting plasma cells. On the contrary, some bacterial non-protein antigen such as bacterial polysaccharide can elicit antibody responses in B cells without the cognate interaction with Th cells. This response is termed as T cell independent response (3, 4, 11).

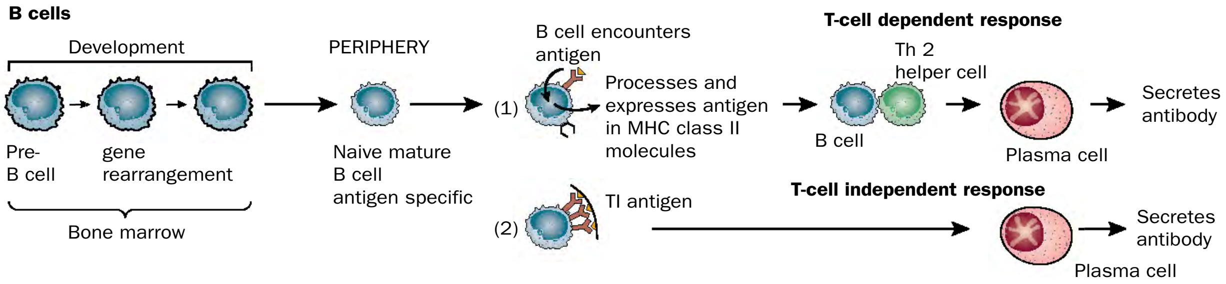


Fig 1. 5: **The role of B cells in Adaptive immunity**. Figure adapted from Parkin J & Cohen B. 2001 (7).

B-cells can function as APCs and in cytokine secretion, but their major function is to produce antigen specific antibodies of different isotypes such as IgM, IgA, IgG or IgD. Antibodies form the humoral arm of adaptive immunity and function in 3 main ways; they opsonize pathogens by binding to them directly and enhancing their recognition by phagocytes, they exert neutralizing effect on toxins secreted by the pathogens and finally they can facilitate activation of the complement pathway and subsequent pathogen clearance (**Fig 1.6**) (3).

A striking feature of adaptive immunity is its ability to establish a long-lasting memory response that is able to mount a more rapid and efficient immune response during challenge infections. Once pathogens are cleared by the adaptive immune response, unwanted effector cells are removed by apoptosis. However some of these effector cells are retained and provide memory T and B cell responses during a challenge infection (3).

In the following section, a comprehensive review is done on the innate and adaptive effector mechanisms that are at play during a typical helminth infection.

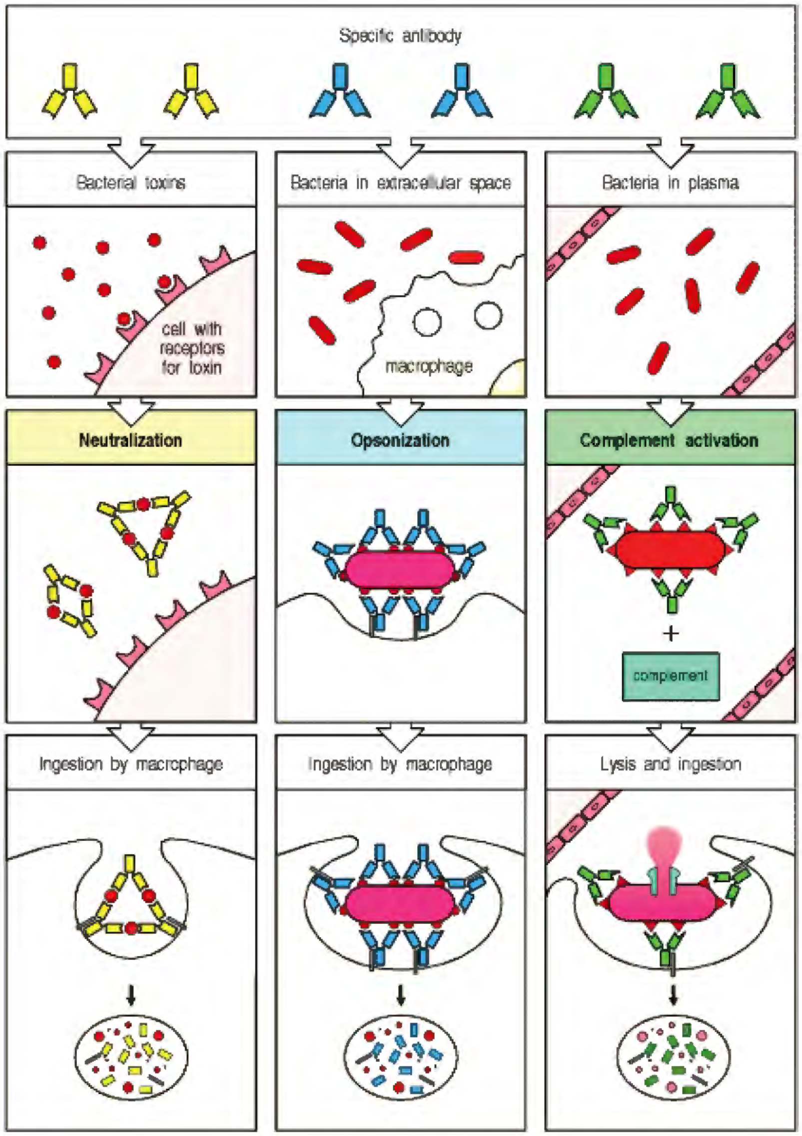


Fig 1. 6: Antibody-mediated immune defense mechanism. Figure adapted from Janeway's immunobiology (3).

1.2 Parasitic Helminth Infection

1.2.1 Significance of Helminth Infection

Parasitic helminth infections are a major global health problem; a third of all humans are infected at any one time. Infections are particularly prevalent in developing countries of sub-Saharan Africa, parts of Asia and South America (15, 16). Although infections do not result in mortality, the principle health concerns are associated with high rates of morbidity. Chronic infections can lead to anaemia and protein malnutrition in pregnant women and children and this is related to poor cognitive development in children (17–19). Strong associations have also been reported between maternal exposure to helminths and reduced efficacy of childhood vaccination and ability to control unrelated viral and bacterial infections such as salmonella and tuberculosis (20, 21). This raises new challenges before the public health community.

Although drug treatments are effective, they do not provide long-term protection, and infections do not result in robust protective immunity (22). Strategies involving mass administration of anthelmintic drugs such as albendazole are in place for the global control of these infections, however host inability to develop effective immune memory along with the rise of drug-resistance confound the efficacy of these efforts. Anthelmintic resistance affecting livestock systems is widely reported for gastrointestinal helminthes of goats, horses and sheep (23, 24). This has shifted current focus to developing effective vaccines against helminth infections. In order to achieve this, we need a developed understanding of how various components of host immunity can contribute to effective clearance of infection.

Studies using murine models infected with a range of parasitic helminthes such as *Heligmosomoides polygyrus* (25–30) *N. brasiliensis* (31–36) and *Trichuris muris* (37–39) have provided important insights in to our understanding of the immune responses that develops following primary and secondary infections. Efforts are still underway for developing successful vaccines that can reduce the likelihood of attaining severe infections. Identifying new ways to reduce parasite burdens and boost our immunity to infections is therefore critical for future vaccine design.

1.2.2 Immune Response to Helminth Infection

Th2 immune responses underlie both effective host immunity to helminth infections and the onset of allergic inflammatory diseases. Central to Th2 immunity is the IL-4 and IL-13 signaling via receptors containing an IL-4Rα subunit (40).

IL-4Rα is a key component of type I (IL-4Rα and γc) and type II receptors (IL-4Rα and IL-13Rα1) (41). While type I receptor binds only to IL-4, type II receptor binds to both IL-4 and IL-13 and is associated with activating the signal transducer and activator of transcription (STAT6) via janus kinase (JAK) and phosphoinositide 3-kinase (PI3K) signaling pathways. The involvement of other transcription factors such as GATA 3 are also important in driving Th2 polarization (40, 42, 43). Activated STAT6 dimerises and migrates to the nucleus where it binds to the promoters of the Th2 inductive genes. Ultimately this results in the physiological phenotype associated with Th2 responses (**Fig 1.7**).

IL-4Rα is widely expressed by hematopoietic and non-hematopoietic cells (44) such as macrophages (45), T cells (35), B cells (34), smooth muscle cells (33) and epithelial cells. The use of IL-4Rα deficient mice has clearly demonstrated the absolute requirement of this receptor in resolving nematode infections. Expression on non-hematopoietic cells is an essential factor for driving protection (44, 46).

Th2 immune response is promoted by epithelial cell driven induction of cytokine secretion of IL-4, IL-5 and IL-13 by a range of immune cells including innate lymphoid type 2 cells (ILC2) and $CD4^{+}$ T cells. This induces effector responses such as goblet cell mucus secretion, mast cell activation, eosinophilia and B cell IgE and IgG1 antibody production all driving disease resolution (47, 48). The effector mechanisms required for effective worm expulsion differ depending on the type of helminth infection.

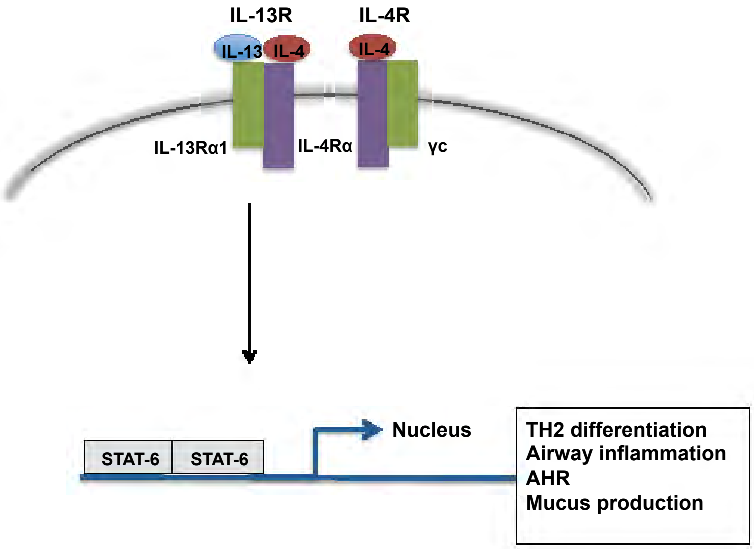


Fig 1. 7: IL-4Rα signaling pathway. The IL-4Rα is a common subunit of the type I and II receptor. Binding of IL-4 and IL-13 to their respective receptors leads to activation and dimerization of STAT6 which then migrates to the nucleus where it binds to the promoters of Th2 associated responsive genes.

1.2.3 The parasitic nematode, N. brasiliensis as a model of infection

In this study, the rodent nematode *N. brasiliensis* was used as a model of infection to examine the innate and adaptive immune-mediated protective responses that develop in the lung following helminth infection. *N. brasiliensis* serves as an excellent model to use as it is analogous to the human hookworm infections *Ancylstoma duodenale* and *Necator americanus* (49). Human hookworms as well as other parasitic nematode species such as *Ascaris lumbricoides, Toxocara canis and Strongyloides stercoralis,* that are of global health concerns, require larval migration through the lung as an obligatory phase of their life cycle. In order to gain better insights into the mechanisms underlying protective pulmonary immune responses to these nematode infections, we used *N. brasiliensis* as a model to study the immunological alterations occurring in the lung in response to these infections.

Infection with *N. brasiliensis* is initiated by penetration of the infectious free-living live third-stage (L3) larvae into the skin of the rodent from where it enters the circulation and migrates to the lung over a period of 24-48 hours. Entry into the lung leads to pulmonary inflammation and induction of a strong Th2 response that leads to eosinophilia, mucus production and airway hyper responsiveness (AHR) (36, 50–53). In the lungs the larvae mature into L4 stage before migrating via the bronchial and circulatory system to the small intestine where they establish and mature to become sexually mature L5 stage adult worms. These worms are capable of breeding and producing eggs by day 6 post-infection (P.I) and are usually cleared from the intestine by day 10 P.I (**Fig 1.8**) (54).

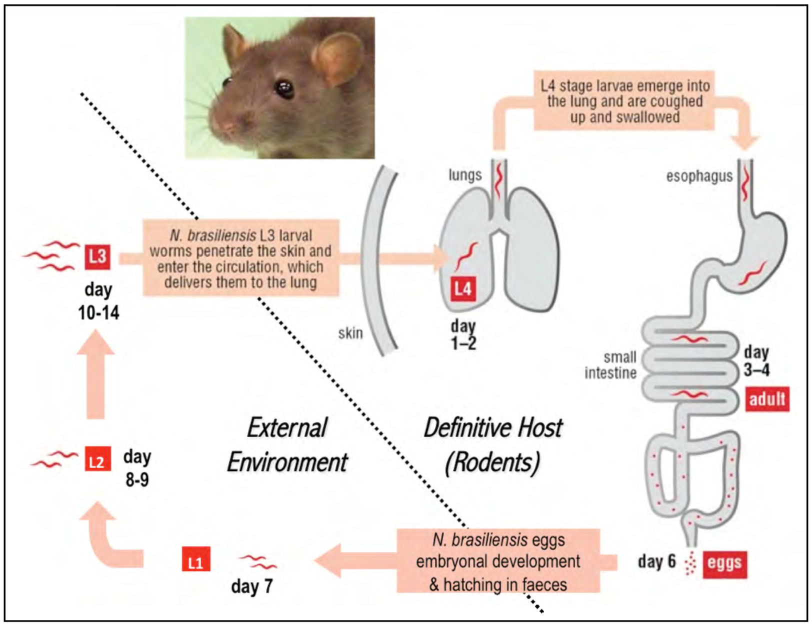


Fig 1. 8: Life cycle of *N. brasiliensis*. Figure adapted from Murray Selkirk (Imperial College London).

1.2.4 Immune responses to parasitic nematode infection

1.2.4.1 Innate immune response to nematode infection

An effective Th2 response requires cross-talk between the innate and adaptive arm of the immune system. Innate immune cells help in orchestrating and sustaining a rapid and efficient Th2 response against a range of parasites. The effector responses generated by these innate cells largely depends on the location of infection and the type of parasite species. Generally, parasitic nematode infections induce a strong Th2 immune response that is initiated by epithelial cell secretion of alarmins such as IL-25 (55, 56), IL-33 (57, 58) and Thymic stromal lymphopoietin (TSLP) (59). These cytokines in turn activate innate immune cells to secrete IL-4 and IL-13 via IL-4Rα signaling and promote Th2 differentiation and effector responses (47).

The importance of epithelial cells in initiating Th2 immunity has come to light in recent years (60, 61). These cells are shown to increase in numbers following nematode infection and produce IL-25 and IL-33 that recruit alarm driven innate lymphocytes to release IL-4 and IL-13 and thereby promote an early Th2 response (58). IL-33 belongs to the IL-1 family of cytokine and plays a pivotal role in driving primary and secondary infection against the nematode, *N. brasiliensis* (57). On the other hand, IL-25 belongs to the IL-17 family of cytokines and is a key component of mucosal immunity (56). Gene knockout studies have provided strong evidence that support a role for these cytokines in effective worm expulsion. IL-33 deficient mice have abrogated ILC2 production of IL-13 that impairs intestinal resistin-like molecule beta (RELM-β) expression and eosinophil recruitment thereby resulting in delayed worm expulsion (62). Similarly, deficiency of IL-25 leads to impaired Th2 protective immunity that is associated with diminished intestinal smooth muscle and epithelial responses to *N. brasiliensis* (56). IL-33 and IL-25 therefore represent an efficient mechanism developed by the host to initiate Th2 responses. A fascinating evasion strategy developed by nematodes to override these Th2 initiators was recently described. The cytokine IL-1β was identified to be elicited by *H. polygyrus* and this attenuates Th2 immunity by suppressing IL-25 and IL-33 thereby maintaining helminth chronicity (63).

The newly identified ILC2 are the first to produce type 2 cytokines in response to IL-25 and IL-33 and create conditions that favor Th2 cell induction. IL-5 and IL-13 produced by these cells induces eosinophilia and goblet cell hyperplasia thereby leading to anti-helminth immune responses (64–66). IL-25 deficient studies were the first to describe this non-B non-T cell population that responded to IL-25 by secreting IL-13. These cells are negative for lineage markers and express high levels of cKit and Thy1.2. Deficiency of these populations as seen in IL-25 deficient mice, severely compromises the ability to expel worms effectively and when adoptive transferred in these mice, restores protective immunity (56).

Induction of Th2 cytokines via ILC2 stimulates a range of downstream effector mechanisms that involves the recruitment and activation of other innate immune populations required for mounting protective anti-helminth responses (**Fig 1.9**). These include basophils that are shown to play the roles of chief coordinators in clearance of secondary helminth infection. During challenge infection, the rapid secretion of IL-4 is thought to be initiated by basophils and this may promote the development of an enhanced Th2 memory response (67–73). A mechanism for basophil mediated enhanced protection during secondary infection is provided by a recent study where IgE armed basophils facilitated skin larval trapping through mechanisms that involved secretion of IL-4 which in turn enhanced AAM production of Arginase 1. This resulted in better protection and a concomitant reduction in lung tissue damage (74).

Dendritic cells (DCs) represent an important type of innate cells that prime naïve T cells to initiate the Th2 responses during helminth infection. *N. brasiliensis* secretory antigens activate DCs to induce Th2 immune responses by up regulating their expression of OX40L (75, 76). Importance of this ligand is evident in studies using mice deficient for OX40L. Such mice failed to mount appropriate primary and secondary Th2 response to *H. polygyrus* (77). Furthermore, mice depleted of CD11c^{hi} DCs show impaired ability to mount effective response to *S. mansoni* and *H. polygyrus* infections (76, 78, 79). These results clearly indicate a crucial role of these innate populations in the initiation of type 2 immunity.

Th2 cytokines also drive the alternative activation of macrophages (AAMs). AAMs are Th2 responsive macrophage populations which are associated with tissue remodeling and control of exacerbated inflammation. They are characterized by heightened expression of Arginase-1, YM-1, FIZZ1 and mannose receptor (80, 81). Experimental helminth infections have been important Th2 disease models for dissecting AAM function. *S. mansoni* infection of AAM deficient LysM^{Cre}IL-4R$\alpha^{-/lox}$ mice highlighted AAM ability to control host pathology and mortality to this Th2 polarising infection (45, 82). Related studies with *S. mansoni* and *N. brasiliensis* infection have further demonstrated important immune-modulatory roles of AAM in controlling Th2 immuno-pathologies and for acute wound healing (81, 83, 84).

One of the striking features of helminth infection is the IL-5 dependent induction of eosinophils. The protective effector mechanisms exerted by eosinophils is not completely understood but is largely attributed to their ability to accumulate around the worm and secrete granular toxic contents such as major basic protein (MBP) and eosinophil peroxidase (EPO). These toxic secretions are shown to promote clearance of *Litomosoides sigmondontis* and other parasites too (85, 86). Eosinophils may also act as amplifiers of Th2 response and function as antigen presenting cells and prime Th2 cells to secrete cytokines and chemokines (87, 88). Mice overexpressing IL-5 tend to show increased eosinophil activity at the site of infection and this is associated with restricted movement of *N. brasiliensis* larvae (86). Furthermore, IL-5$^{-/-}$ mice that have defects in eosinophilopoiesis show impaired resistance to early secondary *N. brasiliensis* infection thereby suggesting a protective role for eosinophils in development of early immunity during challenge infections (89).

In the gut, IL-4Rα signaling via epithelial cells is central for induction of innate defense mechanisms that are crucial for promoting worm expulsion (59). These include stimulation of intestinal muscle hypercontractility (31) and differentiation of intestinal epithelial cells (IECs) to goblet cells that produce a range of effector molecules such as trefoil factor 2 (TFF2) (90), mucin Muc5ac (91) and the innate protein, resistin like molecule β (Relmβ) (62, 92) all of which mediate resistance to intestinal nematode infection. Importantly, these goblet cell responses largely depend on IL-22, a cytokine that belongs to the IL-10 family and is secreted by a wide range of innate and adaptive cells (93). Mice deficient in IL-22 showed impaired worm expulsion that is related to reduced goblet cell hyperplasia and expression of goblet cell markers thereby depicting its key role in driving intestinal antihelminth responses.

A role for Mast cells in the infected gut has also been described. These cells expand in response to IL-9 and IL-18 and release proteases; monocyte chemotactic protein (MCP)-1 and MCP-9 that alters the permeability of intestinal epithelium by degrading tight junctions thereby promoting the weep and sweep response (94, 95). Furthermore, intestinal smooth muscle cells (SMC) also contribute to the control of infection. Here, SMC function in an IL-4Rα dependent manner as mice deficient in smooth muscle cell specific IL-4Rα expression (SM-MHC^{Cre}IL-4Rα$^{-/lox}$) display a delayed ability to expel *N. brasiliensis* worms. This effect was concomitant with delayed goblet cell hyperplasia, M3 muscarinic receptor expression, and IL-13 production in the intestine along with reduced intestinal contractions (31, 33).

With all these tight innate effector mechanisms in place, the nematode constantly attempts to adapt immune evasion strategies in order to survive for long periods in the host. One such mechanism is the nematode haemoglobin. Nematode haemoglobin is thought to sequester oxygen and create an anaerobic environment and participate in host NO and H_2O_2 breakdown. A recent study successfully isolated a monoclonal antibody (48Eg) to the nematode hemoglobin that enhances protective immune responses to *N. brasiliensis* (96).

Neutrophils are another type of innate players in the spectrum of Th2 immune response. They respond to IL-4 and IL-5 and may contribute to anti-helminth responses (97, 98). For instance, in *Ancylostoma caninum* vaccinated mice, neutrophils are essential for worm control during a challenge infection by attaching to and preventing the spread of L3 larvae to the host tissue (99). A similar observation was made in infections with the filarial worm *L. sigmodontis* (98). In such mice, neutrophils contributed to the formation of an inflammatory nodule around the adult filarie that was important for worm control. Following *N. brasiliensis* infection, neutrophils transiently enter the lymph node (LN) in a chemokine ligand 2 (CCL2) dependent manner and function as important effector cells for control of infection. Here, depletion of neutrophils led to increased expression of interferon-γ (IFN-γ), establishment of systemic bacterial infection, decreased Th2 response and delayed worm expulsion. This study reveals the critical requirement of neutrophils in clearing parasite-associated bacterial infection which would otherwise abrogate the Th2 response and lead to increased mortality (100).

All together, the innate immune system appears to have co-evolved with helminths by adapting effective strategies against a range of nematodes that is capable of mounting an efficient and well-regulated Th2 immune response.

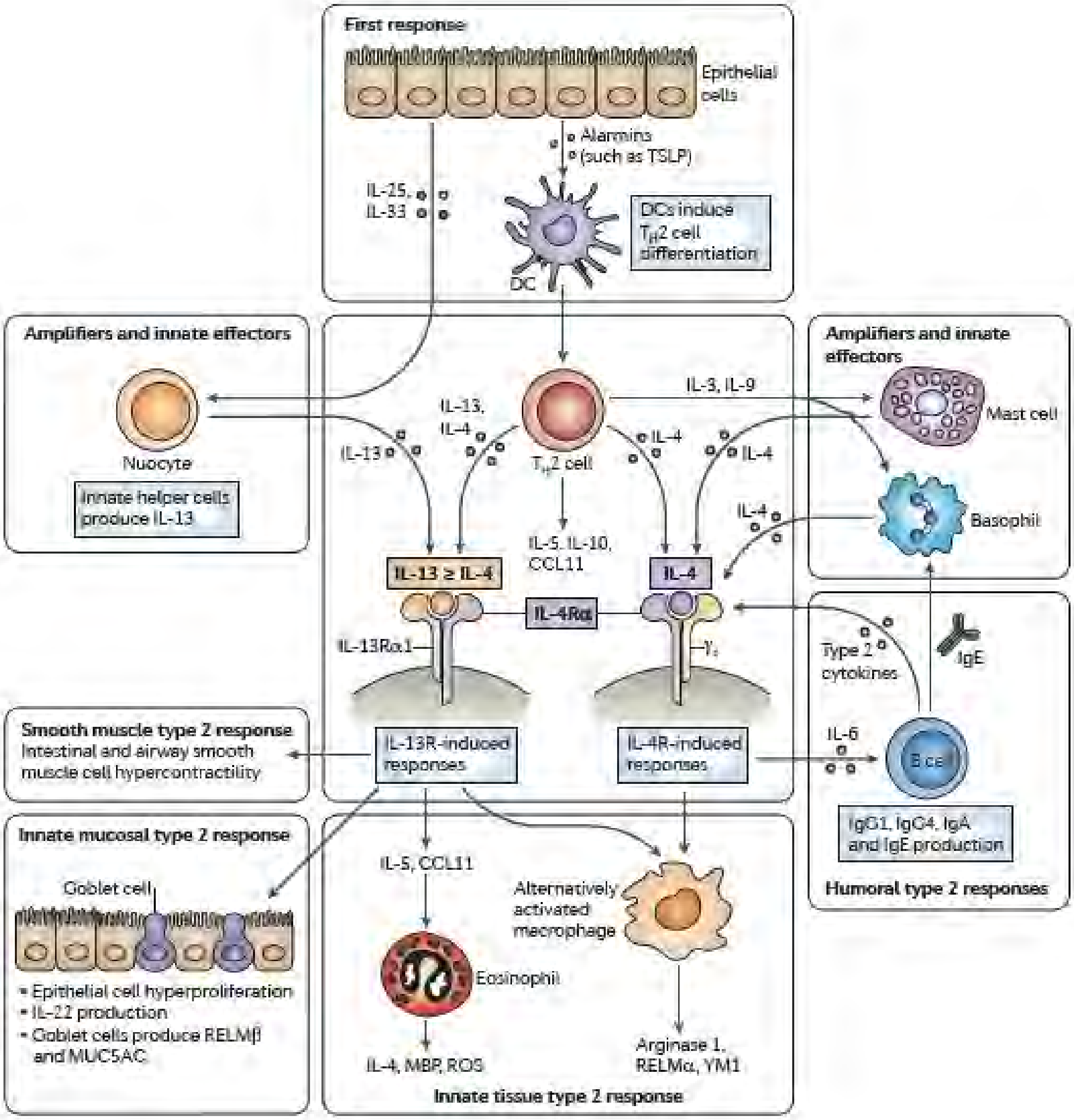


Fig 1. 9: The interplay between innate and adaptive immunity to produce a strong Th2 response to nematode infections. Figure adapted from Allen J & Maizel R. 2011 (47).

1.2.4.2 The Adaptive immune response to nematode infection

The adaptive immune response is mirrored by a range of innate helper cell responses resulting in an overall enhanced effector responses that promote targeted effector functions against a range of parasites. Adaptive response to nematodes is largely driven by $CD4^+$ T cell mediated Th2 immunity and to some extent by B-cell derived antibodies.

IL-4Rα expressing CD4$^+$ Th2 cells are central to type 2 immunity, as shown in **Fig 1.9**. They accumulate in large numbers following infections of *N. brasiliensis* and *H. Polygyrus* (36, 101, 102). They are a major source of the cytokines, IL-4 and IL-13 that are required for worm expulsion. Secretion of these cytokines provides signals for amplifying the innate effector responses. Once activated, innate cell populations help to sustain and promote expansion of the Th2 effector cell population (47). CD4$^+$ T cells are crucial for effective worm expulsion of *N. brasiliensis* infection. Depletion of these cells during primary infection blocks worm expulsion, development of mast cell hyperplasia and generation of polyclonal IgE responses (103). Although resolution of infection is independent of IL-4Rα expression on these cells, the pulmonary pathology associated with infection is greatly reduced. This is associated with disrupted development of airway mucus secretion, recruitment of eosinophil and lymphocytes and production of Th2 cytokines (35).

Re-infection studies with *N. brasiliensis* have further extended a role for CD4$^+$ T cells in generation of protective immunity during challenge infections (36, 102). This protection is strongly related to CD4$^+$ T-cell activation with effector memory T cell and central memory T cell subsets being particularly important (38). A study by Harvie *et al* 2010, showed that protective immunity against *N. brasiliensis* reinfection is largely dependent on a lung-initiated CD4$^+$ Th2 mechanism. Here the development and migration of helminth parasites through the lung appears to be arrested by mechanisms that involve CD4$^+$ T cell mediated production of cytokines and effector molecules. Compelling evidence for the role of CD4$^+$ T cells is further provided by the use of MHCII deficient mice that have significantly impaired worm expulsion (36). Importantly, this protective immunity requires the generation of a polyclonal T cell response for effective worm expulsion (104).

After development of Th2 adaptive immunity, T cells function to provide important cues for the amplification and maintenance of the innate effector cells. For instance, during *N. brasiliensis* infection, T cells are required for prolonged expansion and survival of nuocytes, a type of ILC2 that are shown to be the predominant source of IL-13 during primary infection. *N. brasiliensis* infected recombination-activation gene deficient (RAG$^-$

$^{/-}$) mice that lack mature lymphocytes, have an abrogated expansion of nuocytes that results in a failure to efficiently expel the worms (65). Hence an effective dialogue is required between the T cells and the innate arm for effective control of infection.

During Th2 responses, B cell class-switching to IgE is promoted by the cytokine IL-4. IgE serves to activate mast cells and basophils by binding to FceRI expressed on these cells and promoting degranulation and release of soluble mediators (105). Helminth specific antibodies form an essential component of immunity to secondary *H. polygyrus* infections (25, 28, 30), *T. muris* (39) and *Trichinella spiralis* (106) infections. Here B cell antibodies use mechanisms that involve antibody-mediated macrophage activation, macrophage adherence to the larvae and a resulting suppression of larval motility (107).

Until recently, no role for B cells towards *N. brasiliensis* was defined. Work by Liu and colleagues showed that in response to *N. brasiliensis* infection, B cells have no part in protective immunity, as B cell deficient mice were able to expel worms and induce Th2 immunity normally (25). However, work by Horsnell *et al* 2013, recently demonstrated that a molecular change such as IL-4Rα deletion on B cells impairs host ability to resolve secondary *N. brasiliensis* infection. The IL-4Rα responsive B cell-mediated protection was associated with B cell-IL-13 production and B cell-priming of T cells. This work suggests that B cells may be able to function by additional mechanisms that are independent of antibodies (34).

Therefore in summary, the adaptive and innate arm of the Th2 response requires constant communication in order to produce a highly intricate response that is efficient at killing the nematode and at the same time able to regulate the response in order to prevent excessive tissue damage. As mentioned previously, human hookworms as well as other parasitic nematodes require migration through the lung to complete their developmental life cycle. This therefore induces a strong Th2 immune response at this local site. Recent evidence points to the lung as a critical tissue site for mediating protective immune responses. In the following section we discuss this in detail and present work that supports a role for the lung as a site for mediating protective responses.

1.2.5 Pulmonary Immune response to Nematode infection

The lung is constantly exposed to a variety of pathogens and has therefore evolved immune mechanisms to protect us from a broad spectrum of pathogens. The defense mechanisms are highly specialized and involve innate and adaptive immune cell populations that mount appropriate effector responses to tackle the challenge encountered.

The lung serves as a pre-requisite niche for many forms of parasitic helminthes for completion of their life cycle. Migration through the lung induces a strong Th2 immune response with IL-4Rα responsive CD4$^+$ T cells driving resolution of infection (36). Larval migration through lung tissue causes extensive hemorrhage during acute infection and leads to pulmonary inflammation, airway hyper-responsiveness (AHR) and airway goblet cell hyperplasia all of which lead to a state of acute and chronic lung pathology (50, 51, 108). Moreover, helminthes have long since developed evasion strategies to escape the host defenses of the lung. They are skillful in manipulating the immune system and thereby establishing a long-term relationship with the host. In fact some parasites use molecular signals induced in the host for their development. The lung has therefore co-evolved with helminthes to adapt its effector responses to overcome these evasion strategies and at the same keep its regulatory and repair mechanisms in check to prevent excessive lung tissue injury (109). As a result, the effector cells of the lung exhibit a diverse and redundant role in order to generate alternative mechanisms and duplicate fundamental roles that are essential for resolution of infection (**Fig 1.10**).

Due to the delicate nature of the lung tissue, Th2 responses induced during helminth infections have also developed mechanisms to promote wound-healing processes in order to limit tissue injury. Indeed the absence of the receptor IL-4Rα in *N. brasiliensis* infected mice that have impaired IL-4 and IL-13 signaling results in abrogated resolution of acute lung injury (110). Here, effector cells such as ILC2, macrophages and eosinophils are shown to contribute to tissue repair and control of lung pathology (111, 112).

Alveolar epithelium serves as the first line of defense. Physical barriers such as mucus and cilia on the epithelium together with expression of a range of receptors and secretion of effector molecules produce a highly efficient innate immune response to nematodes. Activation of epithelial cells caused by larval secretions and migration leads to production of inflammatory mediators such as chemokine and cytokines that can initiate immune responses (113, 114).

Alveolar epithelial type II (ATII) cells are shown to increase in numbers following nematode infection and express high levels of IL-25 and IL-33 (58). As described previously, these cytokines are crucial in recruiting innate effector cells to release IL-4 and IL-13 and thereby promote an early Th2 response. Key innate cells that are essential to promoting pulmonary Th2 immunity are the ILC2. These cells expand locally in the lung in response to IL-33 and IL25 and produce IL-5 and IL-13 in an IL-9R-dependent manner leading to goblet cell hyperplasia and pulmonary eosinophilia as depicted in **Fig 1.10**. Lung IL-9R signaling via ILC2s is a crucial factor for their survival, maintenance and optimal function. The ILC2 derived IL-9 represents an important mechanism through which functions of ILC2s are amplified. In the absence of IL-9R, not only are the ILC2 numbers reduced but also their downstream processes of IL-5 and IL-13 production, eosinophil recruitment and AAM are abrogated thereby leading to impaired lung tissue repair. ILC2 have also been identified to be a source for Amphiregulin, a member of the epidermal growth factor family that promotes regeneration of epithelium. It is possible that ILC2 uses this to mediate tissue repair in *N. brasiliensis* infected lungs (58, 64, 112).

ATII cells also secrete the repair molecule Trefoil factor 2 (TFF2) that is necessary for rapid production of IL-33 within lung epithelia and alveolar macrophages (AMs). Trefoil factors are protease resistance mediators that are important in repair of mucosal barrier. They are released within hours of injury and promote C-X-C chemokine receptor type 4 (CXCR4) mediated epithelial cell restitution, organ regeneration and healing in various injury models. The lung has been shown to utilize TFF2 as a repair mechanism to control hookworm mediated lung injury and at the same time promote the initiation of Th2 responses (90). Early instructions of Th2 development in the lung is dependent on TFF2 while during the later stages of infection, the protein exhibits redundant roles similar to other effector cells. A striking role of this protein is its requirement for IL-33 production by inflammatory DCs that are recruited to the lung draining LN (90) and are central in the initiation of type 2 responses (75). Epithelial cell secretion of TFF2 therefore represents an important mechanism adapted by the lung tissue during hookworm infections to induce a well-balanced immune response.

Presence of TGF-β responsive myeloid cells is yet another repair mechanism existing in the lung (111). TGF-β is normally involved in regulation of cellular proliferation and differentiation and exhibit immunosuppressive properties. Although TFG-β negatively regulates Th2 immune responses to hookworm infection, it is critically involved in pulmonary repair mechanisms of infection-induced tissue damage.

The first line of host defense, the epithelium, appears to have evolved multiple strategies to mount effective Th2 responses and at the same time limit helminth induced injury in the lung. There are many other epithelial cell derived cytokines and proteins that form part of the lung innate defense mechanism. Surfactant proteins are one of them. In particular, surfactant protein D (SP-D) has been implicated in host defense against a wide range of pathogens (115). In our work shown in chapter 3, we further elucidate a role for pulmonary SP-D as an innate mechanism used by the lung for host immunity to nematode infection.

In addition to epithelial cells, lung IL-4Rα responsive smooth muscle cells (SMC) also contribute to initiation of Th2 responses and *N. brasiliensis* induced pulmonary pathology. Expression of IL-4Rα on SMC is important for airway goblet cell hyperplasia, IL-6 induced pulmonary infiltration by T cells and the induction of pulmonary cytokines, IL-5 and IL-13 and the chemokine macrophage inflammatory protein (MIP)-1α (31).

An important feature of immunity in the lung is the presence of AMs; during Th2 immune responses these are predominantly alternatively activated and are associated with control of tissue damage and immune pathology. A key role of AAM in control of pathology, in both helminth and other chronic disease models, is their role in directly regulating excessive T cell driven Th2 responses by producing arginase 1, YM1 and

Relmα. Aginase 1 can induce production of proline and polyamines that contribute to collagen deposition and cell proliferation (110). AAM adaptations to control pathology have also been highlighted by recent identification of them being lung tissue resident and to proliferate locally in response to Th2 stimuli, thereby enhancing their ability to control pathological inflammation (110, 116).

Effector cells such as basophils and eosinophils are among the major type of cellular infiltrates found around the larvae and are associated with the pulmonary pathology that develops after infection. Basophils were found to significantly increase by 50-fold in the lungs of *N. brasiliensis* infected mice and serve as important sources of IL-4 (117). Here B cell antibodies act to arm these FC-receptor expressing effector cells and mediate their effector functions and IL-4 production. Similarly, a study by Dent and colleagues has demonstrated that increasing eosinophil numbers can confer resistance to challenge infection with *N. brasiliensis,* particularly at the skin and lung sites (118).

N. brasiliensis migration through the lung induces strong adaptive immune responses at this tissue site with protective CD4$^+$ Th2 immune responses driving disease resolution. Here CD4$^+$ T cell mediated production of effector responses underlies ability of the lung to obstruct larval migration and development (36). In our work shown in chapter 4, we further investigate whether these protective responses require the recruitment of T cells or are induced by a pre-existing lung CD4$^+$ T cell population.

The role of B cells in response to *N. brasiliensis* appears to be redundant. Absence of these cells does not have any effect on the ability to resolve *N. brasiliensis* infection (25, 119). However a recent study by Horsnell *et al* shows that disruption of B cell IL-4Rα expression significantly impairs host ability to control challenge *N. brasiliensis* infection. Here a rapid IL-4Rα-dependent and antibody-independent B cell mediated protection occurs in the lung that involves enhanced B and CD4$^+$ T cell IL-13 responses. This in turn activates important effector cells such as epithelial cells, SMC cells and other innate cell populations (34).

Taken together, these studies provide strong evidence for the lung to represent an important site for mediating Th2 protective responses to hookworm infections. Since it is a focal point crossed by many nematode larvae, a consequence of this is that it induces immunological alterations and massive tissue injury in the lung. However the pulmonary environment has managed to counter this by adapting its host defense mechanism in a way that induces a strong Th2 inflammatory responses that aid in parasite killing and at the same time has wound healing properties to induce rapid tissue repair. To date, many of the helminth studies have looked at the gut to understand worm expulsion mechanisms and development of resistance to reinfection. However the current understanding of the lung immunological responses sets new targets for development of vaccine-induced immunity by suggesting that the Th2 response induced in the lung may represent a far more effective tissue site. An area that needs further insight is the requirement to discriminate between responses that are directed towards the parasite itself and the responses directed at the larval-induced mechanical tissue damage to enhance wound repair. The work presented in this thesis tries to explore novel aspects of the pulmonary innate and adaptive mechanisms that can contribute to protective immunity to *N. brasiliensis* infection.

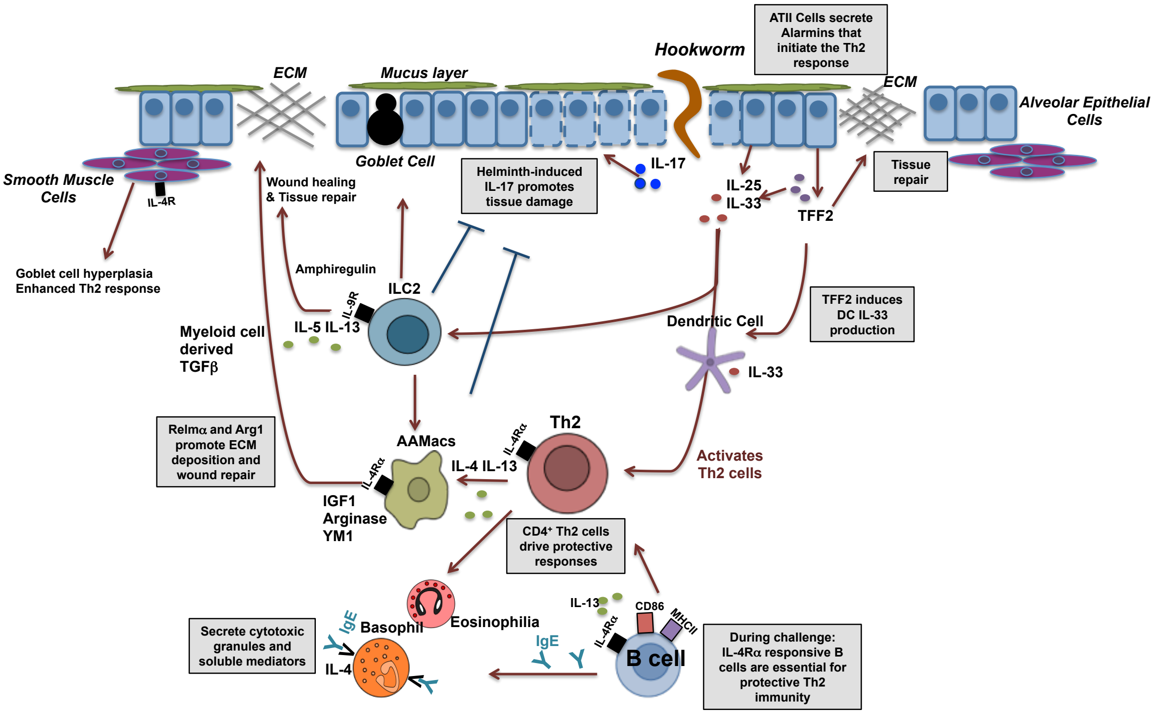


Fig 1. 10: The pulmonary Th2 immune response to hookworm infections. Penetration of hookworms in to the lung induces activation of alveolar epithelial cells to secrete the alarmins IL-33, IL-25 and TFF2. These cytokines initiate Th2 responses by activating innate and adaptive effector cells populations that enhance worm expulsion.

1.3 Objectives of study

It is known that the lung serves as a key site for priming protective CD4$^+$ T cell mediated immunity (36). Contributing to this immunity are innate effector mechanisms that are specific to and localized in the lung and are crucial for effective worm expulsion (47). This suggests that the lung has evolved effective strategies for mounting protective Th2 immune responses that induce resistance to nematode infection. The aim of this study was to identify novel molecules of pulmonary innate and adaptive immunity that further contribute to this protective immunity.

In the first part, we examined the role of the innate pulmonary collectin, surfactant protein D in immunity to *N. brasiliensis* infection. SP-D serves as a key player in host defense against various pathogens and is involved in a range of immune functions (115). Since it is known that SP-D levels are positively influenced by the cytokine levels of IL-4 and IL-13 (120) and can directly bind to the fucosylated glycol-conjugates present on the surface of *S. mansoni* (121), we hypothesized a possible role for SP-D in protective immunity to nematode infections. To test our hypothesis, we used in depth mouse studies to address the following:

1. Determine whether increase in SP-D levels in the lung correlates with protection to *N. brasiliensis* infections
2. Determine whether SP-D can bind to the surface of *N. brasiliensis* larvae and thereby prevent larval migration.
3. Determine by exogenous administration of the protein, the protective roles of SP-D

In the second part of our work, we used the parasitic nematode *N. brasiliensis* to uncover the roles of lung-resident T cells in nematode infections. It is known that development of a Th2 polarized CD4$^+$ T cell-mediated protective immune response in the lung is a key requirement for effective immunity to secondary *N. brasiliensis* infection (36). Furthermore, a role for IL-4Rα responsive CD4$^+$ T cells in promoting the *N. brasiliensis* induced pulmonary pathology has also been established. Here although worm expulsion did not require IL-4Rα responsiveness by T cells; infected CD4$^+$ T-cell IL-4Rα knockout

($\text{Lck}^{\text{cre}}\text{IL-4R}\alpha^{-/\text{lox}}$) mice showed significantly reduced Th2 responses and airway mucus production (35). This suggests that T-cell IL-4Rα responsiveness may contribute significantly to rapid adaptive response during secondary *N. brasiliensis* infection.

To test this, we first determined how T cell recruitment to the lung contributes to *N. brasiliensis* protective immunity. For this, we used the drug FTY720 to block T cell migration from LN to peripheral tissue. We examined if development of a protective Th2 response occurs in the lung in the complete absence of T cell recruitment from the LN. Secondly we examined whether this response was dependent on IL-4Rα expression on these resident T cells.

Together this body of work will provide key proof of principles for further understanding the contribution of the pulmonary immune defense mechanisms in protective control of nematode infections.

Chapter 2: Material & Methods

2.1 Animal Work

2.1.1 Generation of cell-specific IL-4Rα knockout mice

Macrophage specific IL-4Rα knock out (LysM^{cre}IL-4Rα$^{-/lox}$) and T cell-specific IL-4Rα knockout (Lck^{cre}IL-4Rα$^{-/lox}$) mice that were used in this study were generated using the Cre/loxP recombinase system.

In this system, the desired gene (IL-4Rα) to be deleted is flanked by a pair of short target sequences called LoxP sequences. These sequences are binding sites for the enzyme cyclisation recombinase (Cre). Binding of Cre to the LoxP sites results in deletion of the DNA sequence between these sites. The gene for Cre can be inserted into the genome of a cell, downstream of its promoter. Transcription of the cell promoter results in expression of Cre and this deletes the target gene from that cell. Cell specific deletion can occur when the Cre is inserted downstream of a promoter that is only active in a certain cell type.

For generation of Lck^{cre}IL-4Rα$^{-/lox}$, Floxed IL-4Rα mice were intercrossed with mice expressing Cre under the T cell specific promoter Lck. The resulting hemizygous Lck^{cre}IL-4Rα$^{-/lox}$ mice were identified by PCR genotyping. Similarly, to generate LysM^{cre}IL-4Rα$^{-/lox}$ mice, Floxed IL-4Rα mice were intercrossed with mice expressing Cre under the macrophage/neutrophil cell specific promoter LysM. Hemizygous IL-4Rα$^{-/lox}$ littermates were used as controls in all experiments (**Fig 2.1**).

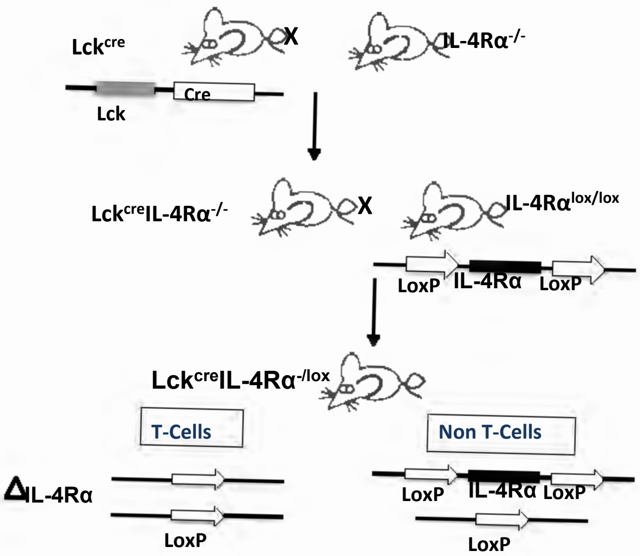


Fig 2. 1: **Generation of Lck^{cre}IL-4Rα$^{-/lox}$ mice.** IL-4Rα$^{-/-}$mice were crossed with transgenic Lck^{cre}mice that express Cre recombinase under the control of T cell–specific promoter Lck to produce Lck^{cre}IL-4Rα$^{-/-}$mice. These were intercrossed with IL-4Rα$^{lox/lox}$ mice to generate Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice (122, 123).

2.1.2 Animals used and ethics

6-10-week-old female mice were obtained from the University of Cape Town specific-pathogen-free animal facility. All experimental procedures were carried out in accordance with protocol no 011/008 and 012/054 approved by the University of Cape Town Animal Ethics Committee. BALB/c background LysM^{cre}IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice were used and hemizygous IL-4Rα$^{-/lox}$ mice were used as wild type controls.

2.2 Working with *N. brasiliensis*

2.2.1 N. brasiliensis life cycle maintenance

L3 stage larvae were maintained by passaging through Wistar rats every 2 weeks. Rats were inoculated with 5000 L3 larvae suspended in 0.5ml 0.9% sodium chloride (NaCl) and faeces were collected on day 6, 7 and 8 P.I. Faeces were softened with distilled water containing Fungizone (Gibco Life Technologies) and this was mixed with equal volumes of granulated charcoal and sphagnum moss to form a thick paste. Thereafter, this was spread on to the center of a large petri dish containing a stack of gauze and piece of filter paper, both moistened in Fungizone (Gibco Life Technologies). The fecal cultures were maintained at room temperature to allow the migration of L3 larvae to the edge of the filter paper. These are then washed in 0.65-0.9% NaCl before inoculating the mice (54).

2.2.2 N. brasiliensis infection

Sub-cutaneous infection:

Mice were inoculated subcutaneously with 500 *N. brasiliensis* L3 larvae suspended in 0.65%-0.9% NaCl using a 21G needle (Braun, Melsungen, Germany). At day 5 or 10 P.I, mice were killed.

For secondary infections, mice were treated with 10mg/ml Ivermectin from day 7 to day 14 post primary infection to clear the parasite. Thereafter, mice were shelved for 3 weeks before being re-infected with *N. brasilensis* and subsequently killed at the desired time point of infection **(Fig 2.2).**

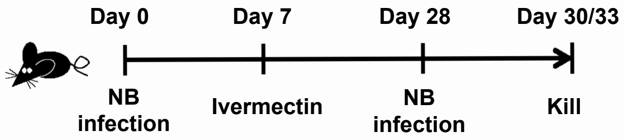


Fig 2. 2. Experimental time line for secondary *N. brasiliensis* (NB) infection

Intra-nasal infection:

For intra-nasal infections of recombinant fragment of human SP-D (rfhSP-D) coated L4 lung stage *N. brasiliensis* larvae, viable motile L4 stage larvae of *N. brasiliensis* were isolated by finely cutting 2-day post-infected lung tissues, placing on sterile gauze and suspending them in a 50ml centrifuge tube containing 1X PBS at 37°C for at least 3 hours. Viable worms migrated to the bottom of the tube and were counted under a dissecting microscope (Nikon Eclipse). The worms were then incubated with rfhSP-D or BSA control for 1 hour at 32°C. Infection of mice with L4 worms was achieved by intranasal administration of 250 viable L4 worms in a 50µl droplet to lightly anesthetized mice.

2.2.3 Quantification of worm burden

Adult worm burdens were determined by removing the small intestine and exposing the lumen by dissection. The intestines were incubated at 37ºC for 4 hours in 0.65% NaCl to allow the worms to migrate out after which the numbers of worms were counted under a dissecting microscope (Nikon Eclipse).

To quantify lung worm burdens, L4 stage larvae were isolated as described above and viable worms were counted under the microscope.

2.2.4 Generation of N. brasiliensis soluble antigen

L3 larvae were washed from the edge of the filter paper into autoclaved H_2O to which Penicillin & Streptomycin (Pen/Strep) were added at 50U/ml and 50µg/ml respectively to kill any contaminating bacteria. This mixture was left for 1 hour to allow the larvae to settle to the bottom of the container. The larvae are then concentrated into 2 ml of distilled H_2O and dipped into liquid nitrogen to snap freeze them and disrupt cellular walls and membrane in order to release the cellular contents. Following this, the larvae were homogenized and thereafter centrifuged to pellet out all the large insoluble cellular and tissue debris, which was discarded. The supernatant containing the soluble fraction of the L3 larvae proteins and glycoproteins was taken for protein quantification. Protein concentration was determined by nanodrop and adjusted to a working concentration of 500µg/ml and stored at -80ºC for future use. Antigen was used at 10µg/ml for re-stimulations of lung cells.

2.3 Administration of proteins and drugs in mice

2.3.1 Generation of rfhSP-D

The SP-D used in these experiments was kindly prepared and provided by Prof. Howard Clark of the University of Southampton, UK. They generated rfhSP-D by using the following procedures as described in Clark *et al* 2002 (124):

"A recombinant homotrimer of SP-D (rfhSP-D), composed of eight Gly- Xaa-Yaa repeats from the collagen region, the α–helical coiled-coil neck region, and the carbohydrate recognition domain (CRD) of human SP-D, was expressed in *Escherichia coli*. After solubilization and refolding of expressed protein from inclusion bodies into a functional trimeric form, the rfhSP-D trimer was purified by ion exchange, affinity, and gel filtration chromatography as described by Madan *et al* 2001 (125). The recombinant preparation was judged to be pure by using SDS-PAGE, immunoblotting, and amino- terminal sequencing. The purified trimeric recombinant protein was assessed for correct folding by disulfide mapping and by its crystallographic structure complexed with maltose in the carbohydrate-binding pockets. rfhSP-D was passed over a column of polymyxin beads to remove endotoxin. The final amount of endotoxin present in the rfhSP-D preparations was < 0.1 endotoxin units/μg protein while the native full length SP-D had endotoxin levels of < 0.0061ng/μg protein."

2.3.2 Intra-nasal administration of rfhSP-D

For administration of rfhSP-D, mice were lightly anesthetized before 20μg of rfhSP-D, native full-length SP-D or BSA control were intra-nasally applied in 50μl of PBS using sterile micropipette. Mice were held upright until all of the fluid was inhaled.

For blocking of CRD head region of rfhSP-D, 20mM Maltose was incubated with rfhSP-D in the presence of 1mM $CaCl_2$ for 1hour at 32°C.

2.3.3 Administration of Fingolimod (FTY720)

In order to block migration of lymphocytes from the lymph nodes to peripheral sites, mice received daily administration intra-peritoneally (i.p) of 0.5mg/kg of FTY720 (Enzo Life sciences) dissolved in 100µl of sterile water.

FTY720 acts as an agonist to the G protein-coupled receptor; sphingosine-1-phosphate receptor that is expressed on lymphocytes and is required for their egress from secondary lymphoid tissue to peripheral tissue sites. FTY720 is phosphorylated in vivo by sphingosine kinase 2, and the phosphorylated drug subsequently binds to sphingosine 1-phosphate receptor and blocks lymphocyte migration as shown in **Fig 2.3** (126–128).

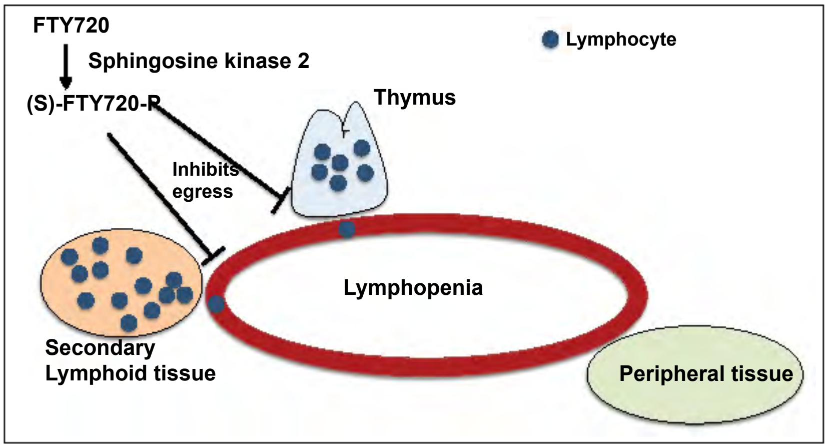


Fig 2. 3: **Mechanism of action of FTY720.** FTY720 is phosphorylated in vivo by Sphingosine kinase 2 and acts as an agonist to Sphingosine-1-phosphate receptor expressed by lymphocytes. Binding results in block of lymphocyte egress from secondary lymphoid tissue to peripheral sites.

2.3.4 Administration of LTβR-Ig/ MOPC21

The systemic antagonist Lymphotoxin beta receptor immunoglobulin (LTβR-Ig - murine IgG1 Fc) fusion protein and the mouse monoclonal IgG1 control MOPC21 were kindly provided by Biogen/Idec. 100µg of either reagent was injected intra-peritoneally once a week to block formation of ectopic lymphoid tissue following infection.

Soluble LTβR- Ig fusion protein can block the LTβR pathway. This construct binds to both the ligands LTαβ and LIGHT with high affinity, consequently blocking their interactions with the receptor LTβR. The LTβR is expressed mainly on non-hematopoietic and myeloid lineage cells such as stromal cells, epithelial cells, monocytes and DCs, while activated lymphocytes and a subset of resting B cells express LT and LIGHT. LT-LTβR interactions plays a critical role in organogenesis and maintenance of the secondary lymphoid organs as shown in **Fig 2.4.** (129–132)**.**

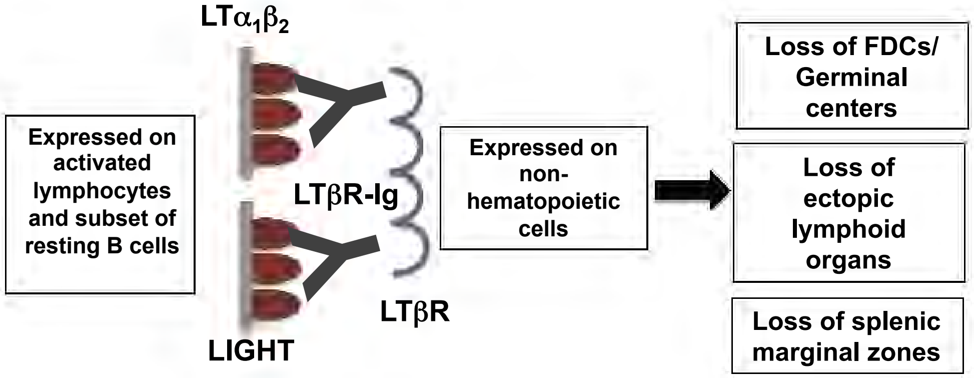


Fig 2. 4: **The mechanism of action of LTβR-Ig**. The fusion protein binds to the ligand LTαβ and LIGHT and blocks their interaction with LTβR. This results in a disruption of various lymphoid structures.

2.4 Cell and Tissue processing

2.4.1 BAL fluid, lung homogenates and serum

Mice were sacrificed at specific time points following infection. Approximately 500μl of blood was collected by cardiac puncture and the serum isolated before being stored at -80ºC until further analysis.

Mice underwent bronchoalveolar lavage (BAL) with sterile PBS containing 0.25mM Ethylenediaminetetraacetic acid (EDTA). The lungs were lavaged thrice with 1ml of PBS. BAL fluid was centrifuged at 1200 rpm for 5 mins and the supernatant was frozen at -80ºC.

The left lobe of the lung was snap frozen in liquid nitrogen and subsequently stored at -80ºC until analysis. To prepare lung homogenates, 400μl of MACs buffer containing protease inhibitor (Sigma) was added to each lung and homogenized with a homogenizer

(Polytron). The homogenates were centrifuged at 14000 rpm for 20 mins and the protein concentrations of the supernatants were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Thereafter, protein concentrations for all samples were equalized to 15mg/ml and levels of IL-4 and IL-13 were determined with enzyme-linked immunosorbent assay (ELISA).

2.4.2 Preparation of single cell-suspension of lung tissue

Whole lung was removed from individual mice, finely cut and digested in Iscove's modified Eagle medium (IMDM) (Invitrogen) containing 50U/ml collagenase type I (Invitrogen) and 13µg/ml DNase (Roche) at 37°C for 90mins. Digested lung tissue were pushed through 70 or 100µm nylon cell strainer (Becton Dickson, New Jersey) and subjected to red cell lysis. Cells were then counted using haemocytometer slide and resuspended at a concentration of 1 x 10^7 cells/ml.

2.5 Flow Cytometry

2.5.1 Staining method

Flow cytometry was used to analyze T cells, B cells, eosinophils, DCs, AMs and ILC2 populations. Approximately 1x10^6 cells per FACS sample was used. The samples were stained in 96-well V-bottom plates with 40µl antibody master mix (MACS buffer containing 2% Rat serum, 2% anti-FcγII/III and the required antibodies diluted to the correct concentration) for 30min on ice, before the cells were washed twice in MACS buffer to remove excess unbound antibodies. The cells were re-suspended in MACS buffer and read by a 4 laser Becton Dickinson FACS FORTESSA machine and the data was collected by Cell Quest© or DIVA© BD (San Diego, CA).

For intracellular cytokine staining, cells were stained with surface markers for 20 mins, fixed in 2% paraformaldehyde before being permeabilized with saponin containing permeabilization buffer for 1 hour at 4°C. Cells were subsequently stained for intracellular markers YM1 and Relmα for 1 hour, washed and stained with their respective secondary antibodies, strep Qdot and Rabbit anti-goat Alexa488 for 45mins and thereafter-excess antibody was washed off and re-suspended in MACS buffer for acquisition. FMOs were used as controls.

The data was analyzed by FlowJo© Treestar (Ashland, OR) and graphed with GraphPad Prism software. Appropriate FACS compensation beads (BD bioscience) were used to compensate between signal intensities of each fluorochrome to avoid spectral overlay. Antibodies used are summarized in **Table 2.1**.

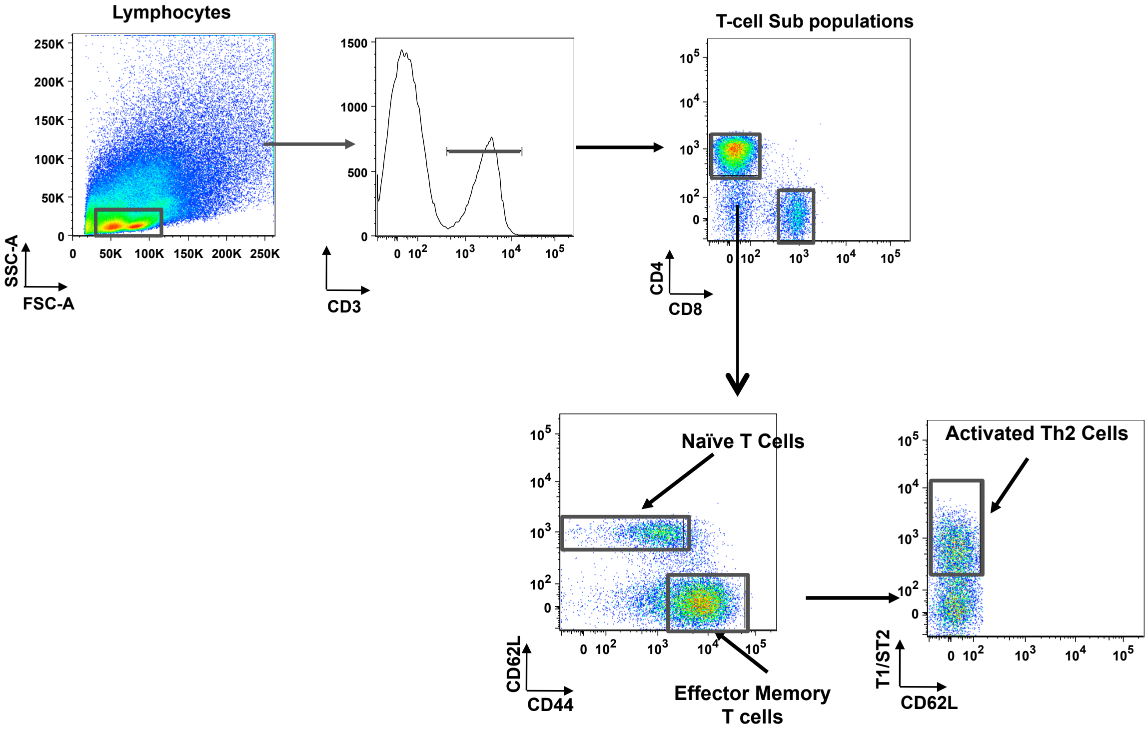
2.5.2 Antibodies used:

Table 2. 1: The monoclonal antibodies used for Flow Cytometry.

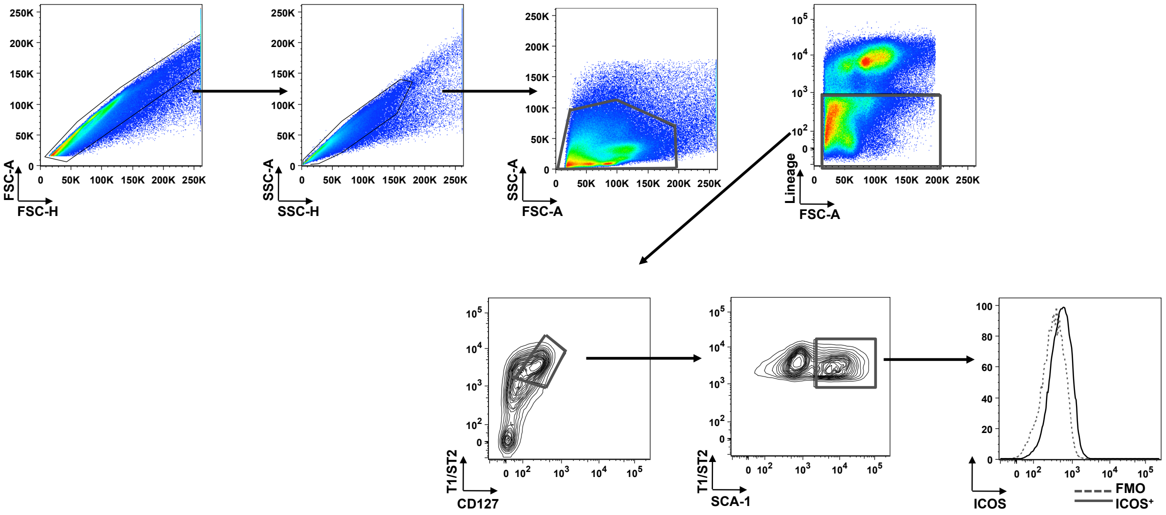
Antibody	Fluorophore	Clone	Company
CD3	Alexa 700	500A2	BD Pharmingen
CD4	PerCP	RM4-5	BD Pharmingen
CD8	V500	53-6.7	BD Pharmingen
CD62L	V450	MEL-14	BD Pharmingen
CD44	PE	IM7	BD Pharmingen
T1/ST2	FITC	DJ8	BD Pharmingen
SigLecF	PE	E50/2440	BD Pharmingen
CD11C	APC	HL3	BD Pharmingen
Ly6G	APC-Cy7	RB6-835	BD Pharmingen
MHCII	FITC	M5/114	Home grown
Lineage: CD3, CD19, CD11b, FceR1, Ter119, CD4, CD8, B220, Ly6G/6C	PE	-	Biolegend
CD127	PECy7	SB/199	BD Pharmingen
ICOS	Biotin	7E.17G9	BD Pharmingen
SCA-1	V450	D7	BD Bioscience
Strepavidin	Qdot	n/a	Life technologies
RELMα	Unconjugated	E19	Santacruz Biotech
YM1	Biotin	ECF-L	Homegrown
Rabbit anti-goat	Alexa488	n/a	
B220	FITC	RA3-6B2	BD Pharmingen
CD19	PerCP-Cy5	ID3	BD Pharmingen
CD21	APC	7G6	BD Pharmingen
CD23	PE	B3B4	BD Pharmingen

2.5.3 Gating strategies:

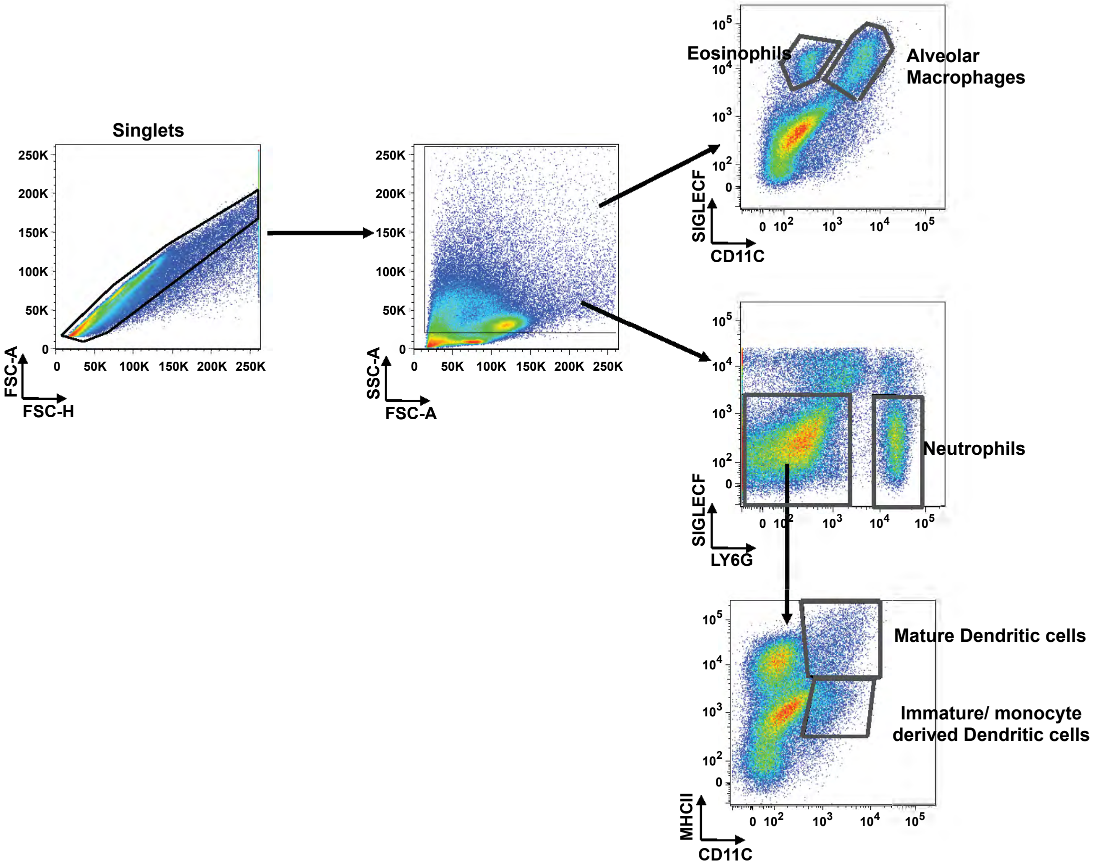
a. Lung T cells:



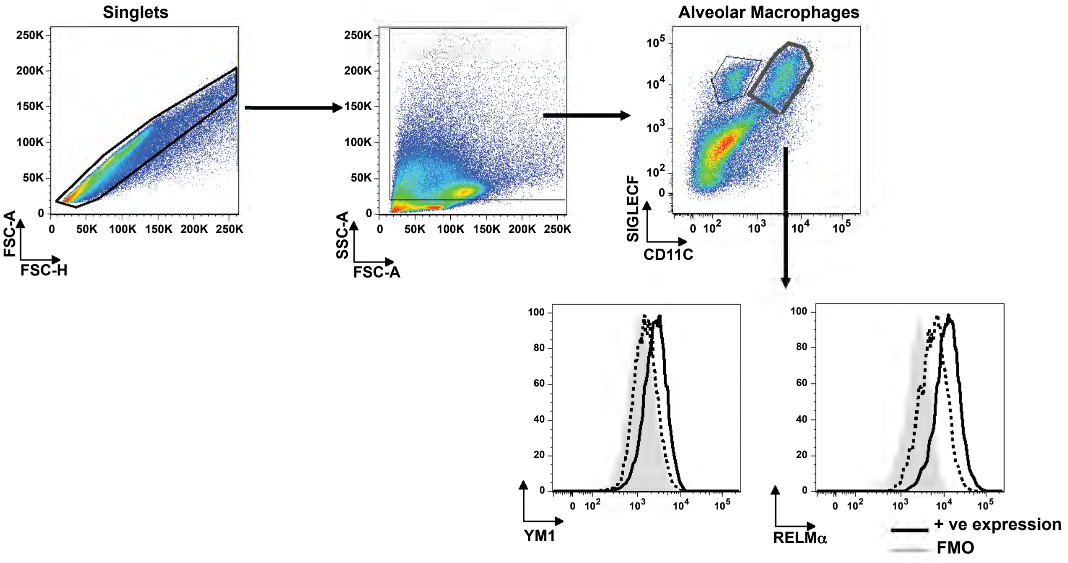
b. Lung Innate lymphoid Type 2 cells (ILC2)



c. Lung eosinophils, DCs, Neutrophils & AMs



d. Lung Alternative activation of macrophages (AAM):



e. Spleen Marginal Zone B Cells:

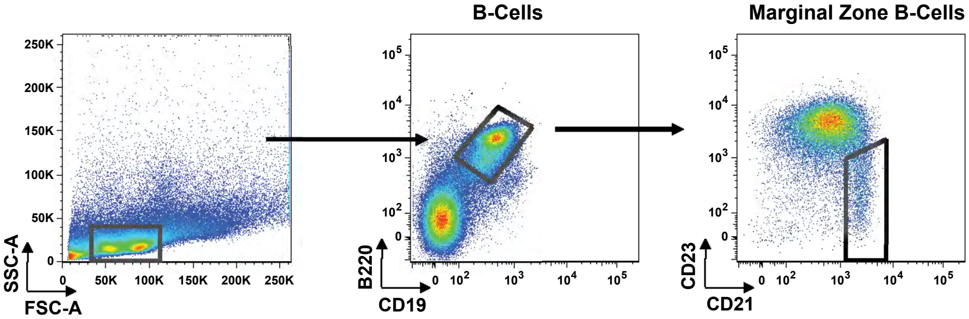


Fig 2. 5: **Gating strategy for acquisition of effector cell populations**. FACS plots demonstrate the gating strategy used to isolate the different lung T cell subset populations (a) Innate lymphoid type 2 cells (b) Macrophages, eosinophils, DCs, and neutrophils (c) alternative activation of macrophages (d) and Marginal Zone B cells (e).

2.6 Cell sorting and Adoptive transfer experiment

2.6.1 Isolation of CD4$^+$ T cell population and transfers

Single-cell suspensions of pooled lungs were prepared at day 10 P.I and stained with anti-CD3 PE-conjugated and anti-CD4 PerCP-Cy5.5 conjugated monoclonal antibody (MAb) (BD Pharmingen) before they were isolated (> 90% purity) using a FACSVantage cell sorter (Becton Dickinson). 2 x 10^4 CD4$^+$ T cells were then transferred intra-nasally in to naïve BALB/c mice 24 hours prior to *N. brasiliensis* infection.

2.6.2 Isolation of Alveolar macrophages (AM) and transfers

Mice were treated with 20μg of rfhSP-D or BSA at D0, 1, 2, 3, 6 and 7 P.I. Single-cell suspensions of pooled lungs were prepared at day 8 P.I and AMs were stained with anti-CD11c APC-conjugated and anti-Siglec F PE conjugated monoclonal antibody (MAb) (BD Pharmingen) before they were isolated (> 95% purity) as CD11c$^+$Siglec F$^+$Autoflourescent^{high} using a FACSVantage cell sorter (Becton Dickinson). 1 x 10^5 macrophages were then transferred intra-nasally in to naïve BALB/c mice 24 hours prior to *N. brasiliensis* infection.

2.7 *In vitro* Cell Cultures

2.7.1 In vitro culture of macrophages with SP-D

Naïve AMs ($CD11c^+SiglecF^+AF^{high}$) were isolated from single cell suspensions of lung tissue by FACSARIA as described above and plated in duplicates at $4x10^5$ cells per well. Cells were stimulated with either recombinant mouse IL-4/IL-13, IL-4/IL-13 + 20μg/ml of rfhSPD or left untreated. The cultures were incubated for 60 hours at 37ºC. Thereafter, cells were washed and stained for alternative activation markers, YM1 and Relmα as described above, and acquired with FORTESSA.

Average activity of the recombinant IL-4 ($1x10^5$ U/μg) and IL-13 ($2x10^4$ U/μg) were used as indicated on the manufacturer's sheet.

2.7.2 Cytospin preparations of AMs

To confirm the morphology of the isolated AMs, cytospin slides were prepared and stained using the Rapid diff staining kit (**Fig 2.6).**

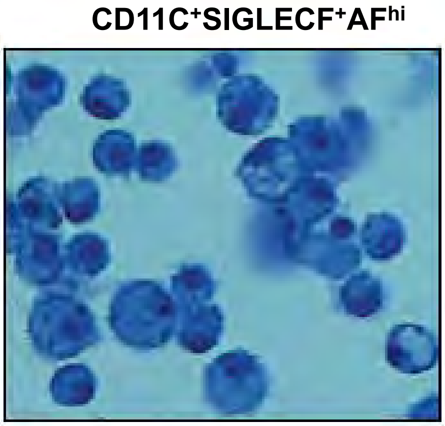


Fig 2. 6: Morphology of isolated Alveolar macrophages

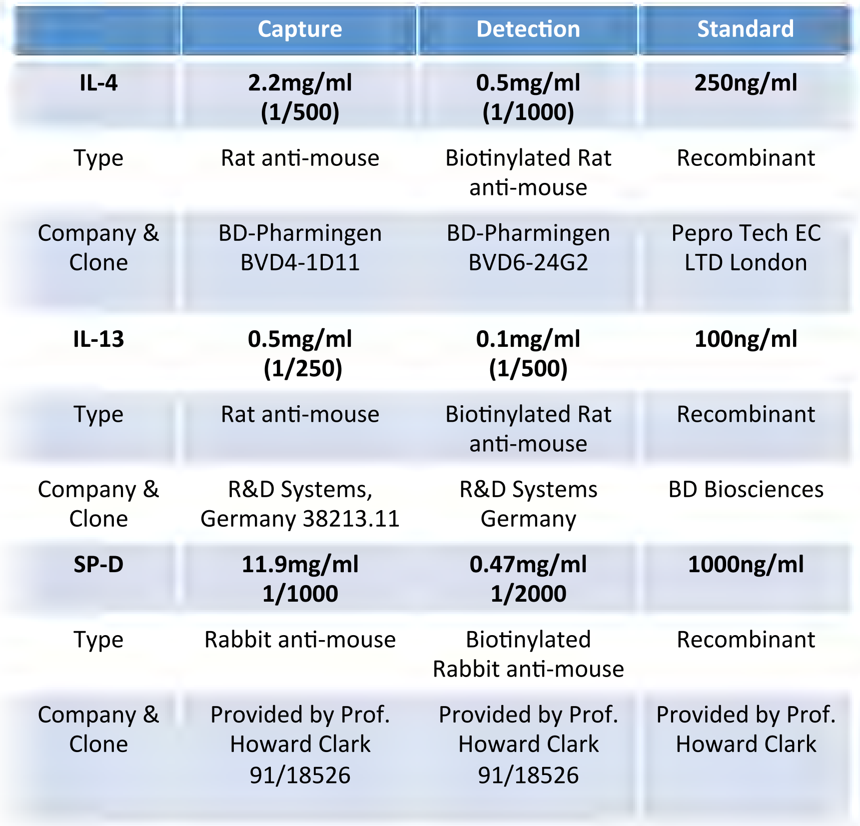
2.8 Enzyme-linked immunosorbent assay (ELISA) analysis

Whole lung single-cell preparations were re-stimulated with sub-optimal concentration of 2μg/ml of CD3 and 10μg/ml of *N. brasiliensis* secretory antigen and incubated for 120 hours at 37ºC. Supernatants from lung re-stimulations or whole lung homogenates were used to measure IL-4 and IL-13 cytokine secretion by ELISA technique.

BAL fluid or serum from *N. brasiliensi*s infected mice were analyzed for SP-D content by ELISA.

96-well flat-bottom plates (Nunc Maxisorp; Thermo Fisher Scientifica, Roskilde, Denmark) were coated overnight at 4ºC with 50μl of primary antibody that was diluted in 1X PBS. The plates were then washed four times in wash buffer and subsequently blocked with 200μl blocking buffer at 37ºC for 3 hours. Following this, three-fold dilutions (1/160, 1/480 and 1/1440) of the BAL samples or lung homogenates (1/3, 1/9 and 1/27) or neat concentrations of serum were prepared in dilution buffer and the diluted samples and standards were loaded into wells and incubated overnight at 4ºC. The plates were further washed and 50μl of biotinylated secondary antibody diluted in dilution buffer was added and incubated at 37ºC for 3 hours. 50μl of Streptavidin-coupled horseradish peroxidase (HRP) (1/5000 (cytokine) or 1/10000 (SP-D) dilution) was added after washing the plates and left in the incubator for 1 hour at 37ºC. The plates were developed with TMB microwell peroxidase substrate system, and the reaction was stopped with 1M H_3PO_4. The plates were read at an absorbance of 450nm using a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A). Antibodies used are summarized in **Table 2.2**.

Table 2. 2: List of Antibodies used for cytokine ELISA



	Capture	Detection	Standard
IL-4	**2.2mg/ml (1/500)**	**0.5mg/ml (1/1000)**	**250ng/ml**
Type	Rat anti-mouse	Biotinylated Rat anti-mouse	Recombinant
Company & Clone	BD-Pharmingen BVD4-1D11	BD-Pharmingen BVD6-24G2	Pepro Tech EC LTD London
IL-13	**0.5mg/ml (1/250)**	**0.1mg/ml (1/500)**	**100ng/ml**
Type	Rat anti-mouse	Biotinylated Rat anti-mouse	Recombinant
Company & Clone	R&D Systems, Germany 38213.11	R&D Systems Germany	BD Biosciences
SP-D	**11.9mg/ml 1/1000**	**0.47mg/ml 1/2000**	**1000ng/ml**
Type	Rabbit anti-mouse	Biotinylated Rabbit anti-mouse	Recombinant
Company & Clone	Provided by Prof. Howard Clark 91/18526	Provided by Prof. Howard Clark 91/18526	Provided by Prof. Howard Clark

2.9 Histology

2.9.1 Immunohistochemistry (IHC)

Intestinal and lung tissue were fixed in neutral buffered formalin solution and embedded in paraffin before cutting in to 5µm section with a cryostat. Sections were stained with periodic-acid Schiff reagent (PAS) for quantification of pulmonary and intestinal mucus production.

Spleens frozen in liquid nitrogen, were embedded in OCT, cut at 7µm thickness and fixed with acetone. Sections were stained with either rat anti-mouse IgM or peanut agglutinin (PNA) coupled to HRP (B-1075) to delineate marginal zone B cells and germinal centers respectively. Follicular areas were stained using sheep anti-mouse IgD. Sections were thereafter stained with their respective secondary antibodies, rabbit anti-rat biotin and donkey anti-sheep HRP. Slides were then washed and streptavidin-complex with alkaline phosphatase (AP) was added (Dako) for 30 minutes. Slides were developed sequentially using DAB and napthol AS-MX phosphate with Fast Blue salt, respectively. All antibodies were purchased from Abcam and summarized in **Table 2.3**.

Table 2. 3: Antibodies used for IHC stains

Antibody	Chromophore Used	Raised in	Concentration	Company	Clone
Primary antibodies					
IgD	HRP	Sheep	1/1000	Abcam	Polyclonal
CD3	AP	Rat	1mg/ml 1/1000	Abcam	KT3
PNA	AP	n/a	5mg/ml 1/5000	Vector Labs	n/a
IgM	AP	Rat	1mg/ml 1/500	Serotec	LO-MM-9
Secondary antibodies					
Anti-rat	Biotin	Rabbit	2mg/ml 1/500	Abcam	Polyclonal
Anti-Sheep	HRP	Donkey	1mg/ml 1/100	Abcam	Polyclonal

2.9.2 Confocal microscopy

SP-D binding to N. brasiliensis larvae:

L3, L4 and adult stage larvae of *N. brasiliensis* were fixed overnight in 2% paraformaldehyde at 4°C. The larvae were extensively washed using 1X PBS containing 0.2% BSA and 1mM $CaCl_2$. Non-specific binding was blocked by incubation of the larvae in 0.2% BSA in PBS for 1 hour at room temperature. Thereafter, the larvae were incubated with 20µg/ml rfhSP-D in PBS containing 0.2% BSA and 1mM $CaCl_2$ for 1 hour at 32°C. After extensive washing, the larvae were incubated with biotinylated rabbit anti-rfhSP-D antibody (HYB 246.04, Antibody shop) used at 1/200 and left overnight at 4°C. To detect the SP-D binding, the organisms were subsequently incubated with Streptavidin cy3 (1/500) for 2 hours at room temperature (RT). Organisms were mounted on to slides using mowiol containing anti-fading reagent. All sections were viewed with a Zeiss Axiovert LSM 510 Meta NLO microscope.

T cell localization in the lung:

Lungs were inflated with 1:1 OCT:PBS, embedded in OCT and frozen over liquid nitrogen. Sections were cut at 9μm thickness, fixed with acetone for 20mins at 4ºC, air dried and stained for $CD3^+$ T cells. Rat anti-mouse CD3 (KT3) was purchased from Abcam. Lung sections were stained with this primary antibody (1/200) and left overnight at 4ºC. Thereafter the secondary antibody, Goat anti-rat Alexa-488 (1/500) was used for detection. Slides were stained for 3 hours at RT and counterstained with Hoechst nuclear stain. All sections were viewed with a Zeiss Axiovert LSM 510 Meta NLO microscope.

2.10 Statistics

Data were expressed as mean ± standard deviation and analyzed using Mann-Whitney nonparametric T test with a 95% confidence interval. P-Value < 0.05 were considered significant and are indicated by an asterisk.

Chapter 3: The Pulmonary Innate Immunity to *N. brasiliensis*: Role of Surfactant Protein D (SP-D) in protective immunity against *N. brasiliensis* infection

3.1 Introduction:

In this chapter, the role of pulmonary innate immune response to *N. brasiliensis* is examined. A brief introduction is first given to introduce important components of the lung innate immunity that appear to play a critical role in Parasitic Nematode infection.

3.1.1 Pulmonary innate immunity

The respiratory system of the body is composed of three major parts; the airways, lungs and muscles of respiration. The airways mainly consist of the trachea, bronchi and bronchioles that aid in taking the air to the lungs. The lung is an intricate organ of the respiratory system. It is made up of tiny air sacs called alveoli that are lined with thin simple squamous epithelium to allow gaseous exchange with the surrounding capillaries. The role of the lung constantly exposes it to a vast array of airborne pathogens present in the environment. It has therefore developed a highly specialized defense mechanism to facilitate the efficient clearance of inhaled pathogens. These mechanisms include the innate and acquired immune responses (133, 134).

The innate component of the immune response consists of mechanical barriers; anti-microbial components secreted in the airways and innate cells such as the epithelial cells, resident AMs and recruited polymorphonuclear (PMNs) leukocytes.

The airway epithelium serves as the first line of defense. It responds to environmental pathogens via Toll like receptors (TLRs) present on its surface. Activation of TLRs leads to production of inflammatory mediators such as chemokine, cytokines and anti-microbial peptides that can initiate immune responses. Epithelial cells along with other innate cells such as AMs and DCs can also recognize conserved molecular patterns present on microorganisms via PRRs. These receptors occur either in soluble form such as mannan-binding lectin (MBL) or appear as trans-membrane receptors that directly initiate immune responses (135–137).

Airway epithelium consists of two main types of cells, Alveolar epithelial type I (ATI) and type II (ATII) cells. These cells cover >99% of the internal surface area of the lung and are largely involved in initiating inflammatory immune responses by secreting molecules such as lysozyme, complement, fibronectin, defensins, cathelicidins, and collectins (134). The airway epithelium is also reported to support the production of mucosal IgA antibodies that are essential for mucosal defense (138).

Although the pulmonary innate system is composed of a broad range of effector cells, a large part of this introduction will focus on discussing lung defense mechanisms by pulmonary innate cells such as alveolar epithelial cells, AMs and the epithelial cell secreted collectins.

3.1.1.1 Alveolar Epithelial Type I cells (ATI)

ATI cells comprise 93-97% of the alveoli while the remaining 3-7% is comprised of ATII cells. ATI cells are flat elongated cells that facilitate gaseous exchange with the surrounding capillaries. The morphology of these cells and the inability to isolate them has posed limitations for dissecting the function of this population. Hence putative roles have been suggested based on the surface proteins expressed by these cells. ATI cells are proposed to be important in the regulation of alveolar fluid homeostasis and surfactant secretion by ATII cells (139). They express intercellular adhesion molecule-1 (ICAM-1) that may regulate phagocytic activity of alveolar macrophages. ICAM-1 expression has been shown to be crucial for host defense against pulmonary pathogens such as *Klebsiella pneumonia* (140, 141). Among other proteins expressed by these cells are T1-α, aquaporin 5 (AQP-5), functional ion channels, caveolins, adenosine receptors, and multidrug resistance genes (113). Expression of caveolins further supports a role for these cells in alveolar homeostasis. Caveolins are shown to mediate bulk transport across cells and participate in cell signaling, modulate reactive oxygen species, and may have tumor suppressor activities (142, 143). However, the metabolic ability of ATI cells to sustain active ion transport and alveolar homeostasis remains questionable due to the absence of mitochondria in these cells.

A recent study has suggested a role for ATI cells in mounting innate immune response during *Pneumococcal pneumonia* by inducing TLR2 and stimulator of interferon genes (STING) expression. STING mediates recognition pathways for *Pneumococcal* DNA and drives the expression of innate immunity mediators such as type I interferons (144).

Altogether, further research is essential for expanding our understanding of ATI cell function.

3.1.1.2 Alveolar Epithelial Type II cells (ATII)

ATII cells are small cuboidal cells that account for approximately 5% of the alveolar surface and perform a variety of immunological functions. They are morphologically characterized by the presence of intracellular organelle, lamellar body that synthesize, store and secrete pulmonary surfactant. Secretion of surfactant is important for reducing surface tension and preventing alveolar collapse. Surfactant contains soluble innate immune components such as surfactant protein A (SP-A) and D (SP-D) and antimicrobial peptides that play a crucial role during infections. ATII cells can also secrete cytokines, chemokines and antimicrobial molecules that mediate the recruitment of monocytes, macrophages, neutrophils and lymphocytes to the site of infection. Moreover, they aid in creating a microbicidal pulmonary microenvironment that is rich in opsonins by expressing high levels of Ig transporters and secreting complement components (113, 145). In addition, they express MHCII receptors on their surface that have been proposed to function in presenting antigens to CD4$^+$ T cells (146). ATII cells also act as progenitor cells for ATI cells which is particularly important during lung injury to facilitate alveolar repair (145). They also play a critical role in alveolar homeostasis by regulating fluid volume, ion transport and recycling of surfactant. The importance of these cells for pulmonary host defense is clearly demonstrated by the severe pulmonary diseases caused by deficiencies or gene mutations in the proteins secreted by these cells. For instance, deficiency in surfactant protein B (SP-B) has been associated with fatal neonatal respiratory diseases. Similarly, a deficiency in a-1AT, a serine protease inhibitor secreted by ATII cells for maintaining alveolar homeostasis, results in pulmonary emphysema (147). These studies suggest a critical role of ATII in pulmonary host defense.

3.1.1.3 Alveolar macrophages (AM)

AMs are phagocytic cells that constitute 95% of the cells found in the BAL. They represent the first line of immune defense cells in the lung, playing important roles in initiating immune responses, tissue remodeling, phagocytosis and homeostasis. They express PRRs that identify conserved patterns on the surface of inhaled pathogens called PAMPs. Upon recognition, they can engulf and digest the pathogen and also secrete mediators of inflammation, cytokines that initiate adaptive immune responses and chemokines that help in recruiting inflammatory cells to the site of infection (134). In steady state, AMs have suppressive functions and inhibit T cell stimulation thereby preventing excessive inflammation and tissue injury in response to airborne antigens. A recent study showed the ability of AMs to generate FoxP3$^+$ regulatory T cells and promote airway tolerance (148–150).

AMs are characterized based upon their function and cytokine profile as mentioned earlier. Two sub-types exist: M1 and M2 subsets. M1 subset, also known as classically activated macrophage, is polarized to this state by IFN-γ. It is associated with engulfment and killing of bacterial/ protozoan and fungal pathogens resulting from their ability to produce pro-inflammatory cytokines (TNF, IL-1, IL-6) and reactive nitrogen and oxygen species (NO, H_2O_2). In contrast, M2 subset also known as alternative activated macrophages (AAMs), are Th2 (IL-4, IL-13) responsive macrophage populations which manifest anti-inflammatory roles and are associated with tissue remodeling and control of exacerbated inflammation. They are characterized by heightened expression of Arginase-1, YM-1, FIZZ1 and mannose receptor (80).

3.1.1.4 Pulmonary Surfactant

Surfactant is a complex mixture of phospholipids and proteins that is synthesized and secreted by ATII cells, airway Clara cells and sub-Mucosal cells. Surfactants form a complex bilayer consisting of an outer hydrophobic lipid monolayer with an underlying hydrophilic head. It consists of 90% lipid with the remaining portion consisting of proteins. Surfactant is essential for reducing surface tension at the air-liquid interface of the lung and also plays a critical role in host defense against pathogens. About 10% of the surfactant consists of proteins of which 4 have been defined: SP-A, SP-B, SP-C and SP-D. The protein fraction of surfactant forms a critical part of the lung innate immunity. SP-B and SP-C are hydrophobic proteins found within the lipid monolayer, and contribute significantly to reducing the surface tension and preventing lung collapse. On the other hand, SP-A and SP-D are hydrophilic proteins that are members of the collectin family of proteins and have immune-modulatory functions that contribute to host-defense against pathogens (151, 152).

The importance of surfactant is highlighted in premature infants who are born before their surfactant machinery has fully developed. In the absence of surfactant, the lung tends to collapse due to the surface tension being extremely high. This results in a condition called respiratory distress syndrome where breathing becomes difficult and surfactant replacement and ventilatory support becomes necessary for survival (151). Below, we briefly provide an overview of the main functions of the pulmonary surfactants; SP-A, SP-B, SP-C and SP-D.

3.1.1.4.1 Surfactant protein A (SP-A)

SP-A is the predominant protein among the surfactant proteins. It is a 26kDa - 36kDa protein encoded by the *SFTPA* gene found on human chromosome 10. Structurally it is homologous to MBL and exists as a single monomer chain that usually assembles into an octadecamer. It consists of collagen-like amino (N)-terminal regions and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). It binds to monosaccharide via CRD in a calcium dependent manner and is therefore categorized as a collectin (153, 154).

Expression of SP-A has been shown to increase with gestational age and is suggested to be a mediator of parturition (155). SP-A is believed to play an important role in surfactant homeostasis and pulmonary immunity. It acts as an opsonin and binds to various pathogens such as viruses, bacteria, fungi and apoptotic cells. It can functionally bind to *Herpes simplex type I*, *influenza A*, human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), *Staphylococcus aureus*, *E. coli, Pseudomonas aeruginosa,* *Aspergillus fumigatus and Candida tropicalis* (154, 156)

In addition, SP-A binds to innate immune cells such as AMs and increases the expression of PRRs such as mannose receptor, scavenger receptor A and TLR2 (157, 158). It also enhances the uptake and clearance of apoptotic cells by macrophages, which can otherwise be toxic to the lung. SP-A-modulation of cellular function is suggested to occur via C1q receptors including CD91/calreticulin complex and signal regulatory protein alpha (SIRPα) (159).

Studies with SP-A knockout mice (KO) have revealed an important immune-modulatory role of this protein during infection with various pathogens. SP-A KO mice have higher levels of pro-inflammatory mediators and are more susceptible to RSV infections, *Haemophilius influenza, P. aeruginosa* and *group B streptococci* (160–162). Altogether these studies reveal a crucial requirement of SP-A for effective host defense against pathogens.

3.1.1.4.2 Surfactant protein B/ Surfactant protein C (SP-B/ SP-C)

SP-B and SP-C are small hydrophobic proteins (18 & 5kDa respectively) that have important roles in surfactant function (163). Their importance is highlighted in gene knockout studies where deficiency in their expression due to mutation in the genes encoding these proteins is associated with lung diseases. A deficiency in SP-B results in fatal neonatal lung disease whereas mutations in SP-C, results in chronic interstitial lung disease in both children and adults (152, 163). SP-B is important in the surfactant biosynthetic pathway and contributes to the packaging of surfactant into lamellar bodies within ATII (163, 164). Both SP-B and SP-C play a critical part in surfactant homeostasis by promoting uptake of vesicles by ATII cells and adsorption of lipid molecules to the air-liquid interface of surfactant thereby enhancing their stability during respiration (164). In addition, SP-B has also been shown to induce immunoglobulin M (IgM) immune response to antigens (165).

3.1.1.4.3 Surfactant protein D (SP-D):

3.1.1.4.3.1 SP-D discovery and classification:

In 1988, SP-D was first described as a basic surfactant associated protein. It was originally isolated from rat ATII cells as collagenous protein 4 (CP4) and later termed as surfactant protein D in accordance with the accepted nomenclature (166, 167).

SP-D is 46kDa hydrophilic calcium dependent, carbohydrate binding protein and is therefore classified under the collectin family of proteins (168). It is encoded by the long arm of human chromosome 10, which also encodes SP-A and MBP-C (169).

3.1.1.4.3.2 SP-D Secretion:

Like other surfactant proteins, SP-D is also secreted by ATII cells, sub mucosal cells and Clara cells (170, 171). It has its own secretory vesicle that extrudes from ATII cells into the alveolar lumen and associates with the underlying hydrophilic layer (172). Although the majority of SP-D is expressed in the lung, transcripts of SP-D have also been detected in other parts of the body. A study by Madsen showed expression to be localized in the intestine, lungs, thymus, prostrate, brain, testes, salivary gland, lachrymal gland and heart (173).

3.1.1.4.3.3 SP-D Structure and structural requirements:

The basic structure of SP-D is organized into four regions: a cysteine containing N-terminal region, a triple-helical collagen region composed of Gly-X-Y triplets, an a-helical coiled coil neck region and a globular head region at the C-terminus consisting of a homotrimeric carbohydrate recognition domain (**Fig 3.1**) (154). The SP-D is assembled as trimeric subunits of basic polypeptide chain which multimerizes to varying degrees of oligomers but typically is found as a dodecamer. They are formed from linking of four trimers by disulphide bonds at the N termini (174).

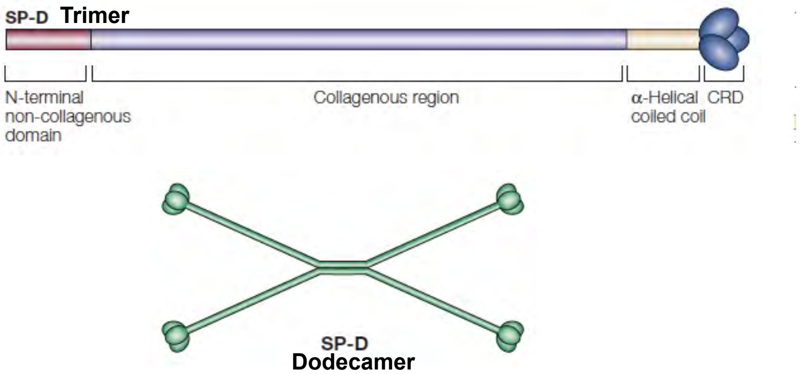


Fig 3. 1: Structure of SP-D. Figure adapted from wright *et al* 2005 (115)**.**

The carboxy-terminal domains have C-type (calcium-dependent) lectin activity that mediates the interaction of collectins with a wide variety of pathogens. This results in pathogen opsonization and enhanced uptake by phagocytes. The neck region has disulphide binding sites that form inter-chain bonds that is required for assembling the SP-D into trimers. The N-terminal domain confers structural stability on the protein, owing to its disulphide-bonding pattern and dictates the degree of multimerization of the single trimeric subunits of both SPA and SP-D (154, 175).

Although the full fragment of SP-D is required for the full range of functions, studies have demonstrated that some functions can be achieved successfully *in vivo* via a truncated form of SP-D that exists as a trimeric subunit consisting of carbohydrate binding domain (CRD) and part of the collagenous region (176).

3.1.1.4.3.4 SP-D Functions ***(Figure 3.2)****:*

- Immunomodulatory Roles:

In a steady state, SP-D has important functions in maintaining the surfactant homeostasis and normal physiology of the lung. It enhances clearance and uptake of apoptotic cells by binding to cell debris and cell-surface DNA thereby controlling inflammation (124). It regulates surfactant homeostasis by influencing the surfactant structure and its uptake by ATII cells and AMs (177–179).

SP-D knockout (KO) mice have been useful models in highlighting the importance of this protein in maintaining the physiology of the lung. These mice have chronic inflammation, show extensive pulmonary lesions and emphysema, have foamy activated macrophages, secrete higher levels of hydrogen peroxide, have hypertrophic ATII cells, increased peribronchial lymphoid tissue along with increased phospholipid content of alveolar fluids (180). The expression of the pro-inflammatory cytokines IL-12 and IL-6 was also up regulated in the lungs of these KO mice, suggesting that lack of pulmonary SP-D leads to persistent T-cell activation, possibly in response to exogenous antigens (120, 181).

SP-D also controls inflammation within the lung by inhibiting IL-2 production from lymphocytes thereby preventing proliferation of T cells (182, 183), diminishing lipid peroxidation and oxidative cellular lung injury (184), decreasing TNF-α and NO production by AMs (185, 186) and decreasing MHCII presentation by lung DCs along with inhibiting maturation and activation of pro-inflammatory DCs by inhibiting TNF-α production (185, 187).

During allergen-induced airway inflammation, SP-D mediates immunomodulatory roles that include binding to aero-allergens, inhibiting mast cell degranulation and histamine release and modulating the activation of AMs and DCs during the acute hypersensitive phase of allergic response (188).

These facts provide evidence supporting that SP-D plays an essential role for maintaining immunological homeostasis in the lung and thereby prevents excessive lung injuries.

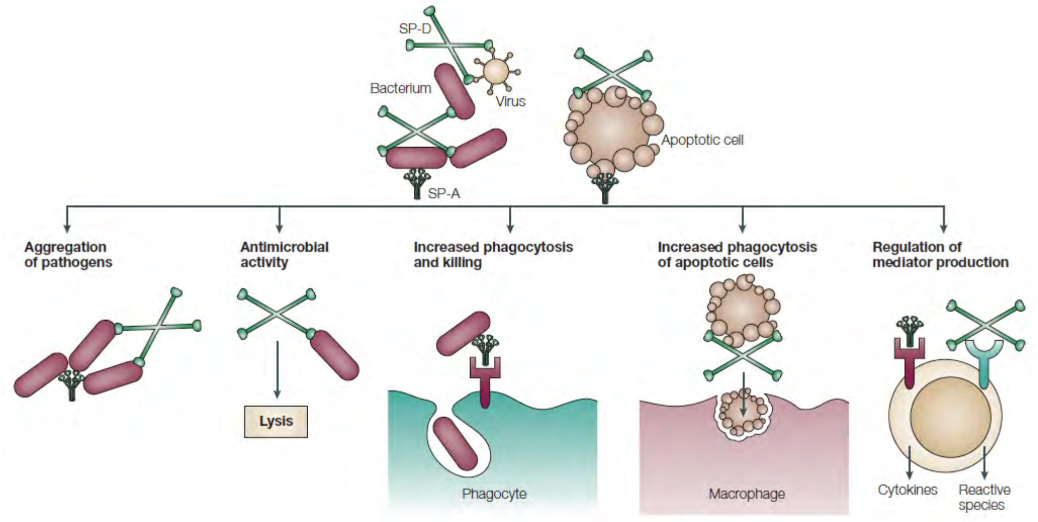


Fig 3. 2: **Functions of SP-D**. Surfactant protein D can bind to various pathogens and function as an opsonin to promote uptake by phagocytes. Figure adapted from Wright *et al* 2005 (115).

- Host-defense functions:

i. Binding and opsonization of pathogens:

SP-D binds to various pathogens such as fungi, bacteria and viruses via the CRD binding domain. Binding occurs through the various carbohydrate moieties expressed on the surface of the pathogen.

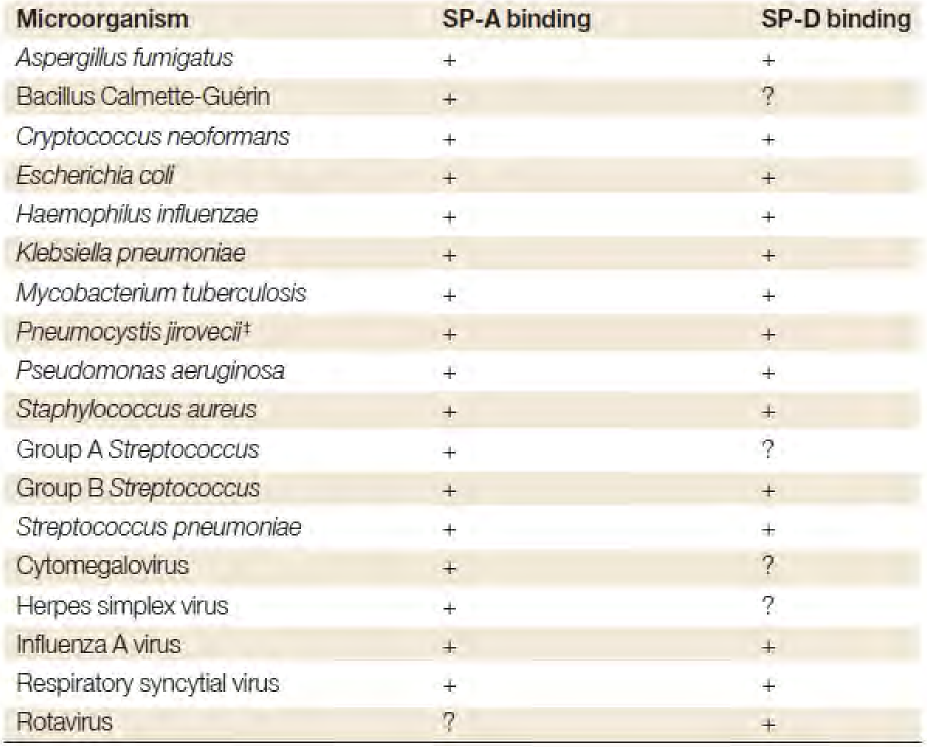
SP-D is an effective host-defense molecule against bacteria and neutralizes inhaled endotoxins as summarized in **Table 3.1**. Lipopolysaccharide (LPS) isolated from various gram-negative organisms such as *E. coli, Klebsiella pneumonia* and *P. aeruginosa* serves as a ligand for SP-D (189–191). SP-D also interacts with cellular LPS receptors CD14 (192), TLR4 (193) and MD2 (194) and can exert direct anti-bacterial activity by increasing membrane permeability (195). SP-D binds to di-mannose containing O-antigens expressed by a subset of *Klebsiella* serotypes. It can also bind to the lipoarabinomannan of the virulent Edman strain of *Mycobacterium tuberculosis*. Interaction with Gram-positive organisms such as *Streptococcus pneumonia* and *Staphylococcus aureus* has also been reported to occur (196).

SP-D is also protective against viruses and can specifically interact with influenza virus, HIV, Herpes simplex virus and RSV. Binding to influenza virus occurs through interaction with viral haemagglutinin protein and neuraminidase envelope glycoproteins thereby preventing inactivation of innate immune cells (197–199). In fact, SP-D KO mice show increased susceptibility to influenza virus infection (200). SP-D can also recognize RSV through the respiratory G (attachment)-protein in a Ca^{2+} dependent manner that results in decreased viral infectivity (161, 201).

Furthermore, SP-D is protective against fungal pathogens by binding to the sugar moieties present on the cell wall and enhancing their agglutination. It mediates host defense against various fungi such as invasive pulmonary aspergillosis, *Candida albicans, Cryptococcus neoformans* and *Pneumocystis carinii* (202–205).

Taken together, these studies indicate that SP-D can recognize various binding motifs present on a diverse range of pathogens that are usually targets for the CRD region.

Table 3. 1: **Interaction of SP-A and SP-D with various pathogens.** Table adapted from Wright *et al* 2005 (115).



Microorganism	SP-A binding	SP-D binding
Aspergillus fumigatus	+	+
Bacillus Calmette-Guérin	+	?
Cryptococcus neoformans	+	+
Escherichia coli	+	+
Haemophilus influenzae	+	+
Klebsiella pneumoniae	+	+
Mycobacterium tuberculosis	+	+
Pneumocystis jirovecii‡	+	+
Pseudomonas aeruginosa	+	+
Staphylococcus aureus	+	+
Group A *Streptococcus*	+	?
Group B *Streptococcus*	+	+
Streptococcus pneumoniae	+	+
Cytomegalovirus	+	?
Herpes simplex virus	+	?
Influenza A virus	+	+
Respiratory syncytial virus	+	+
Rotavirus	?	+

ii. SP-D receptors and modulation of response from immune cells

Opsonization of pathogens almost always leads to varying degrees of agglutination, subsequent uptake by phagocytes such as AMs, monocytes, DCs and neutrophils, and a regulated release of inflammatory mediators thereby decreasing pathogen survival and protecting the lung from injury. The higher the order of multimers, the more effective is the agglutination of pathogens (176).

SP-D can directly bind to host immune cells and influence their response and phagocytic activity. Recent studies have suggested a role for surfactant proteins in linking innate and adaptive immunity in the lung by modulating functions of AMs, DCs, and T cells (115). SP-D displays chemotactic activity on neutrophils and certain mononuclear phagocytes and can induce directional actin polymerization in AMs in a concentration dependent manner (206–209). It also modulates the production of cytokines and inflammatory mediators in a pathogen dependent manner (210).

Recent studies by Gardai and co-workers show that SP-A and SP-D modulate their cellular functions depending on the cellular receptor that is bound, which in turn depends on whether the proteins are interacting with a host cell or with a pathogen, a concept termed "Head or Tail Hypothesis" **(Fig 3.3)** (210).

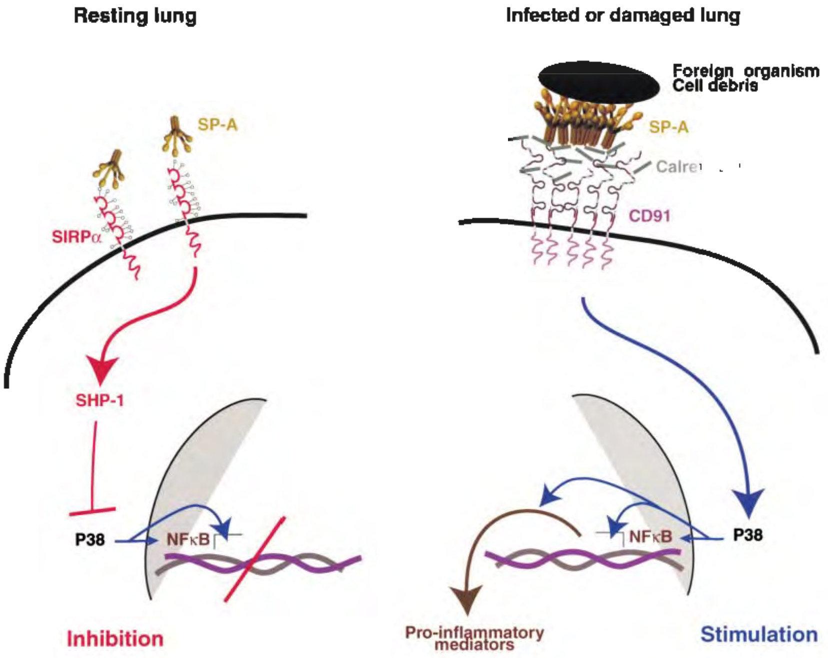


Fig 3. 3: **Head or Tail hypothesis.** During Steady-state conditions, SP-A and SP-D bind to SIRPα via their CRD head region and this inhibits NFκB pathway. However when the CRD head region bind to a foreign pathogen, the N-terminal domain is exposed and this binds to CD91/ Calreticulin and induces activation of NFκB signaling. Figure adapted from Gardai *et al* 2003 (159).

SP-D is capable of differentially binding to cell receptors through either the CRD or the collagen domain, which dictates whether the immune response elicited will be anti- or pro- inflammatory. Binding of CRD region to the receptor signal regulatory protein alpha (SIRPα) that is mostly expressed on DCs and macrophages inhibits pro-inflammatory cytokine production and NFK-β signaling by mediating an inhibitory signal transduction pathway. *In vitro* and *in vivo* studies by Gardai and colleagues showed that SP-D binding to SIRPα on macrophages led to inhibition of phagocytosis and cytokine production stimulated by H_2O_2 and LPS (159, 211). During this state, the dodecameric structure of SP-D tends to hide the N-terminal collagen domain.

However when the CRD region is bound to a foreign pathogen, the oxidative changes and pro-inflammatory environment can alter the structure of the SP-D such that it de-oligomerises to trimeric forms thereby exposing the N-terminal collagen domain. This allows for the free collagen domain to bind to the cellular receptor calreticulin/CD91 and induce the activation of immune cells via the p38 and nuclear factor kappa-light-chain-enhancer of activated B cells (NFK-β) signaling molecules (159, 210, 212).

SP-D can also bind to various other cellular receptors such as TLR2, TLR4 and CD14 via its CRD region and modulate their function (192, 193, 213). SP-D and SP-A both were shown to inhibit production of TNF-α and other pro-inflammatory cytokines and chemokines by AMs when stimulated with LPS (115, 210). SP-D also binds to Gp340 in a Ca^{2+} dependent manner, a pulmonary SP-D binding protein that is expressed on macrophages and functions as an opsonin receptor for various pathogens (214).

Binding of SP-D through the collagen receptor can also up regulate PRRs, induce cytokine and reactive molecular species production, induce chemotactic activity and also influence the intracellular signaling pathways in the innate cells thereby leading to phagocytosis and removal of apoptotic cells (115, 175, 210).

During allergic airway challenge, SP-D can modulate effector cell functions of AMs, DCs, mast cells and eosinophils. It increases production of IL-10, IL-12 and IFN-γ by AMs and inhibits NO release from macrophages. It also inhibits allergen induced histamine release from mast cells thereby regulating airway inflammation (215). Both SP-D and SP-A are shown to inhibit DC activation and maturation and its release of TNF-α. Eosinophilia is a hallmark of allergic airway inflammation. SP-D can bind to eosinophils and inhibit their chemotaxis and eosinophil cationic protein (ECP) degranulation of activated eosinophils (215).

Taken together, these studies suggest a dual role for SP-D that is dependent on the presence or absence of pathogens, which then dictates the anti or pro-inflammatory role of SP-D. Pathogen sensing directly modulates the function of innate immune cells by regulating the release of inflammatory mediators and resulting in aggregation and phagocytosis of the pathogen. Whether SP-D plays any role in host-defense against parasitic infection is unknown. Our work is focused on extending and dissecting the role of SP-D in immunity to helminth infections.

3.1.1.4.3.5 Dependency of SP-D on IL-4/ IL-13

It has been shown that the levels of SP-A and SP-D can be influenced by various cytokines and other mediators present in the lung environment (216, 217). For instance, mice overexpressing IL-4 (216), IL-5 (218), and IL-13 (219) have markedly elevated levels of SP-D expression in the lung. In cultured type II alveolar epithelial cells, expression of SP-D was enhanced by IL-4 (217) and IL-13, but not by IFN-γ (120) thereby demonstrating the direct effects of these cytokines on SP-D level. During a Th2-type allergic inflammation in rats, there was significant production of cytokines from ATII cells as well as Clara cells and goblet cells (220). Gene knockout studies using IL-4/IL-13 double knockout mice and STAT-6-deficient mice showed a failure to increase SP-D production upon allergen challenge (120), suggesting SP-D transcription to be dependent on IL-4 and IL-13 signaling via IL-4 receptors and STAT6.

Since helminth infections are strong inducer of Th2 inflammatory pathway, we deduced a possible protective role of SP-D against helminth infections.

3.1.1.4.3.5 Therapeutic potential of SP-D

The host defense functions of SP-D and its role in modulating immune responses of various innate immune cells have clinical implications for various lung diseases. The ability of truncated recombinant form of SP-D (rfhSP-D), that contains the CRD head region and a short part of the collagen stalk (**Fig 3.4**), to correct various disease states in mouse models of allergic and inflammatory disease raises the prospects of using it as a therapeutic agent.

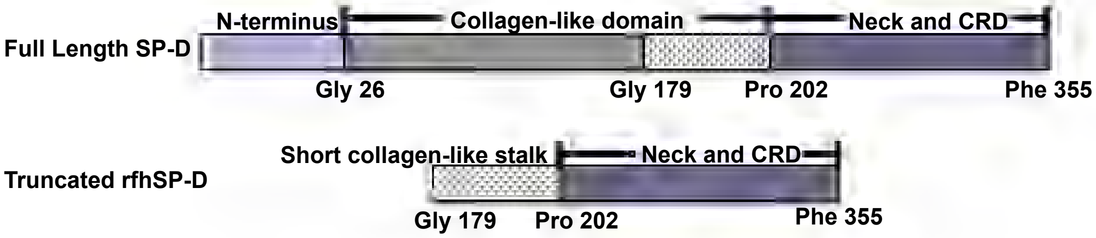


Fig 3. 4: **Structure of rfhSP-D**. The truncated fragment of SP-D lacks the N-terminal region and part of the collagen stalk.

During allergen induced airway inflammation, intranasal administration of rfhSP-D in mice was shown to be effective in dampening the allergic airway response by reducing eosinophilia, goblet cell hyperplasia, IgE production and bronchial hyper-responsiveness along with induction of regulatory cytokines such as IL-10, IL-12 and IFN-γ by macrophages (205, 215, 221–223). In fact, a study by Erpenbeck and colleagues showed that treatment with rfhSP-D before allergen challenge with *Aspergillus fumigatus* inhibits the early airway response and airway hyperresponsivness (224). This suggests a potential therapeutic role for SP-D in patients suffering from asthma.

The usefulness of SP-D to control lung inflammation was also illustrated recently where full length SP-D was shown to be effective at preventing endotoxic shock induced by LPS in premature newborn lambs during ventilation (225). Patients suffering from cystic fibrosis have low levels of BAL SP-D and are more susceptible to colonization with *Staphylococcus aureus, Aspergillus fumigatus* and *Pseudomonas aeriginosa*. SP-D is protective against these pathogens and promotes phagocytosis and pathogen killing. This supports the therapeutic potential of SP-D for cystic fibrosis (226)

Artificial pulmonary surfactant therapies that lack SP-A and SP-D have been successful in reducing RDS induced neonatal mortality rates that results from surfactant deficiency. However 40% of the infants that survive after birth develop neonatal chronic lung disease due to oxygen therapy and ventilation. Associated risk factors include oxidative lung injury, prolonged positive pressure ventilation and recurrent infections. These infants also have low levels of SP-A and SP-D and this correlated with an increased risk of chronic lung disease (176, 226). SP-D functions as an anti-oxidant and has important immunomodulatory role in maintaining a normal physiology of the lung. SP-D deficiency in mice and baboon models contributed to the development of emphysema and chronic lung disease (227, 228).

Moreover, studies have suggested a correlation between the emphysemous lungs of smokers and AM apoptosis (229). Such patients also have low SP-D levels in their BAL (230). Since SP-D is an important mediator of apoptotic cell clearance and preventing emphysema, and that the ability of exogenous rfhSP-D in correcting the abnormal lung phenotype in SP-D deficient mice has been explicitly shown, it suggests a possible beneficial role for SP-D therapy during neonatal RDS in reducing the risk of chronic lung disease development and in preventing smoking associated emphysema (176, 226).

Altogether, this suggests a therapeutic potential for the use of recombinant forms of SP-D as supplements for the treatment of lung-associated diseases.

3.1.2 A Role for SP-D in Parasitic Nematode infections:

In helminth infections, a range of non-hematopoietic and innate immune cells contribute significantly to orchestrating and sustaining a rapid and efficient host protective Th2 responses during infection (47).

As described in detail in chapter 1, helminth infections induce polarized Th2 adaptive immune responses characterized by eosinophilia, mucosal mastocytosis, goblet cell hyperplasia and elevations in IgE levels. Non-hematopoietic and innate immune cells are essential in the initiation of these adaptive immune responses. Therefore understanding their contribution to the development of host immunity to helminth infections is important for enhancing vaccine strategies and understanding helminth regulation immune-pathologies (47, 48).

An important anatomical location for controlling these immune responses following *N. brasiliensis* infection is the lung; responses here form an important component of the host protective immune response to re-infection. In particular, pulmonary epithelial cell secretion of cytokines such as IL-25 (56) and IL-33 (57, 58) drive the initiation of these protective Th2 immune responses by enhancing production of classic Th2 cytokines such as IL-4, 5 and 13 by a range of immune cells such as ILC2s and CD4$^+$ T cells. This ultimately leads to the induction of effector responses that drive disease resolution.

In our study, we for the first time demonstrated that the pulmonary epithelial cell-secreted collectin, SP-D is an important component of host immunity to *N. brasiliensis* infection. SP-D plays a critical role in the lung, it can directly bind carbohydrate residues on allergens, viruses, bacteria and other fungal pathogens. Binding to pathogens results in opsonisation and subsequent uptake by phagocytes (115). Binding of SP-D to fucose residues of *S. mansoni* has also been demonstrated (121).

We demonstrate here that SP-D production is induced following *N. brasiliensis* infection in a Th2 dependent manner, it bound preferentially to lung stage L4 parasites and enhanced macrophage and ILC2 protective responses essential for controlling infection.

3.2 Results:

3.2.1 SP-D levels are increased following N. brasiliensis infection.

SP-D levels have previously been shown to be increased in BAL and serum of mice following challenge with a range of antigens and pathogens (115, 154, 156, 196). As the lung is the site of control of secondary *N. brasiliensis* infection (36), we examined if *N. brasiliensis* infection also resulted in infection associated increased pulmonary and systemic levels of SP-D. Analysis of SP-D levels in the BAL (**Fig 3.5a**) and serum (**Fig 3.5b**) of *N. brasiliensis* infected mice showed SP-D levels to correlate with the kinetics of *N. brasiliensis* infection. Highest levels of SP-D were found at peak of infection; namely day 7 post primary infection in both BAL and serum. Following secondary infection, SP-D levels were also enhanced. This data associates SP-D concentration with host protective immunity to infection.

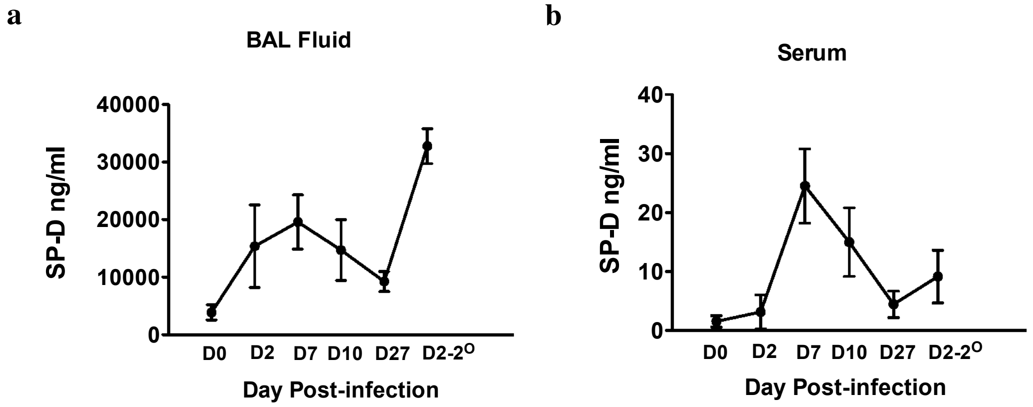


Fig 3. 5: **SP-D is induced in the bronchoalveolar lavage fluid following *N. brasiliensis* infection**. Kinetics of SP-D levels were measured by ELISA in BAL fluid (**a**) and Serum (**b**) following *N. brasiliensis* infection. Data are representative of two individual experiments. N= 5 mice per group.

3.2.2 SP-D expression is modulated by IL-4 and IL-13 cytokine levels

SP-D production has been shown to be dependent on IL-4 and IL-13 signaling via IL-4 receptors and STAT6 (120, 216–219)**.** Immunity to *N. brasiliensis* associates with both enhanced IL-4 and IL-13 host secretion, with IL-13 being essential for resolution of infection (44, 46, 231). We investigated requirements of IL-4 and IL-13 for SP-D production in response to *N. brasiliensis* infection. WT and IL-4/IL-13 double KO mice were exposed to primary (1°) and secondary (2°) *N. brasiliensis* infection and at 5 days P.I, SP-D levels in BAL fluid (**Fig 3.6a**) and serum was quantified (**Fig 3.6b**). WT mice had significantly higher SP-D levels compared to KO mice, thereby suggesting that SP-D production following infection was indeed dependent on IL-4 and IL-13.

Together, these findings suggest a direct correlation between SP-D and IL-4 and IL-13 cytokine levels. SP-D induction therefore associates with host protective immunity to *N. brasiliensis* infection.

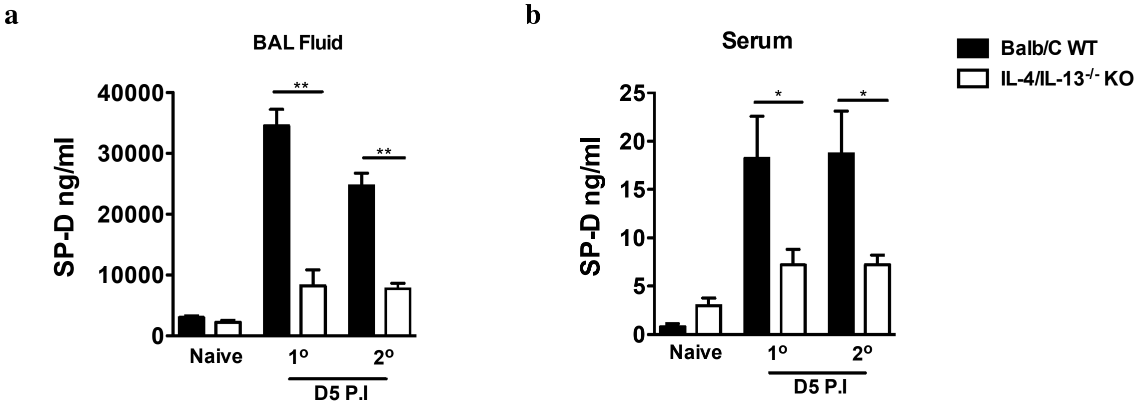


Fig 3. 6: SP-D expression is modulated by IL-4 and IL-13 cytokine levels. SP-D levels of WT and IL-4/IL-13$^{-/-}$ KO mice were measured in BAL fluid (**a**) and Serum (**b**) of naïve mice and infected mice at day 5 post primary (1°) and secondary (2°) infection. Data are representative of two individual experiments. N= 5 mice per group. $*P<0.05$, $**P<0.01$.

3.2.3 Intra-nasal administration of rfhSP-D enhances protective immunity to N. brasiliensis

Our initial findings show that heightened SP-D levels correspond with host control of *N. brasiliensis* infection. This suggests that increased SP-D levels in the lung could confer enhanced host immunity to *N. brasiliensis*.

We tested this by intra-nasal administration of recombinant fragment of human SP-D (rfhSP-D) prior to *N. brasiliensis* infection (**Fig 3.7a**). This manipulation of pulmonary SP-D levels resulted in enhanced host ability to control infection as demonstrated by reduced intestinal burdens of adult *N. brasiliensis* in rfhSP-D treated mice compared to BSA treated controls (**Fig 3.7b**). We found protection correlated with an increase in levels of *N. brasiliensis* protective pulmonary Th2 cytokines IL-4 and IL-13 (**Fig 3.7c**).

This was associated with increased proportions and total numbers of ILC2 in rfhSP-D treated mice (**Fig 3.7d**). ILC2s are newly identified innate cells that have been shown to play a crucial role in protection against helminth infections by inducing IL-13 cytokine responses (58, 65).

Indeed, rfhSP-D treated mice had elevated pulmonary SP-D levels that enhanced host ability to control *N. brasiliensis* infection and this relates to SP-D associated enhancement in host Th2 immunity to *N. brasiliensis*.

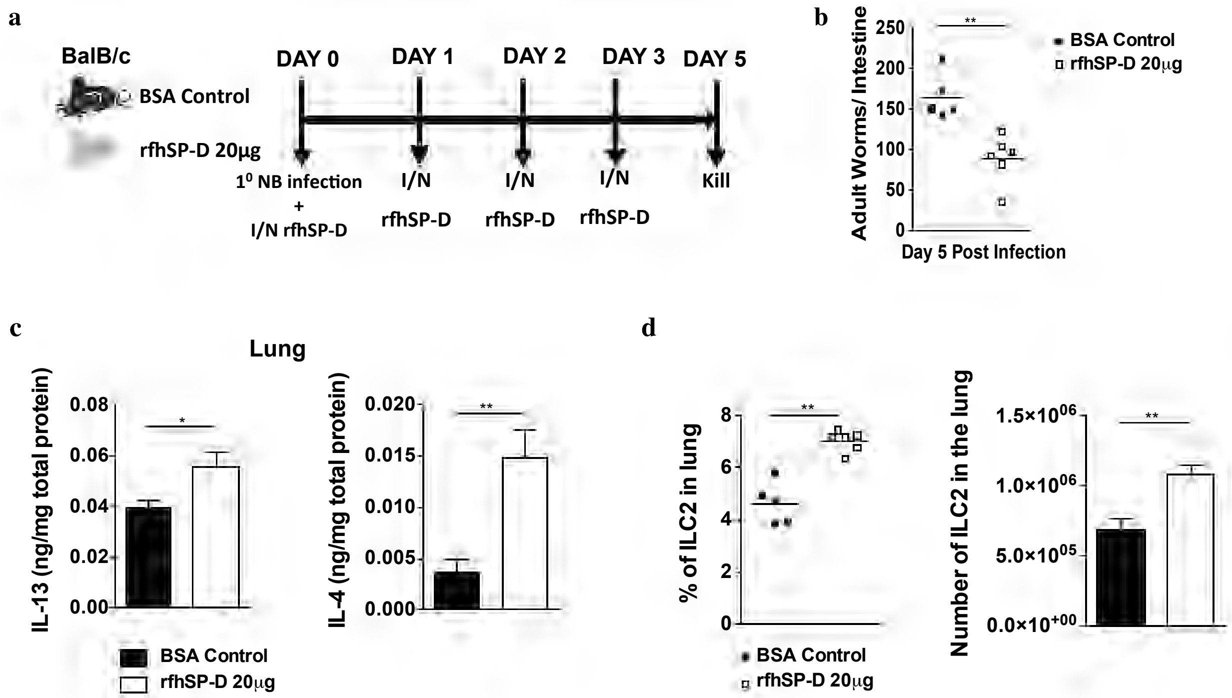


Fig 3. 7: **Intra-nasal administration of rfhSP-D enhances protection to *N. brasiliensis***. rfhSP-D treated or untreated mice were given *N. brasiliensis* infection and killed at day 5 P.I. (**a**). Enhanced protection in rfhSP-D treated mice was established by quantification of intestinal worm burdens at day 5 P.I. (**b**). IL-4 and IL-13 cytokine levels in lung homogenates was detected by ELISA (**c**). Flow cytometric analysis of cell suspension of whole lung stained for innate lymphoid type 2 cells (lin$^-$CD127$^+$T1/ST2$^+$SCA-1$^+$ICOS$^+$) (**d**). Data are representative of two individual experiments. N= 5-6 mice per group. *P<0.05, **P<0.01.

3.2.4 SP-D requires carbohydrate binding head region for its optimal function

SP-D binds preferentially to inositol, maltose and glucose. Previous studies have shown the dependency of SP-D's function on it head region, which can bind directly to pathogens and mediate their clearance via opsonisation and neutralization (176). In order to better understand the mechanisms underlying the ability of SP-D to confer protection against *N. brasiliensis*, we asked whether SP-D-induced protection occurred through mechanisms that required the head region. We used 20mM maltose to block CRD head region of rfhSP-D (**Fig 3.8a**). Mice treated with maltose-blocked rfhSP-D had higher worm burdens than mice treated with rfhSP-D alone (**Fig 3.8b**) thereby indicating the requirement of the CRD head region for its optimal function.

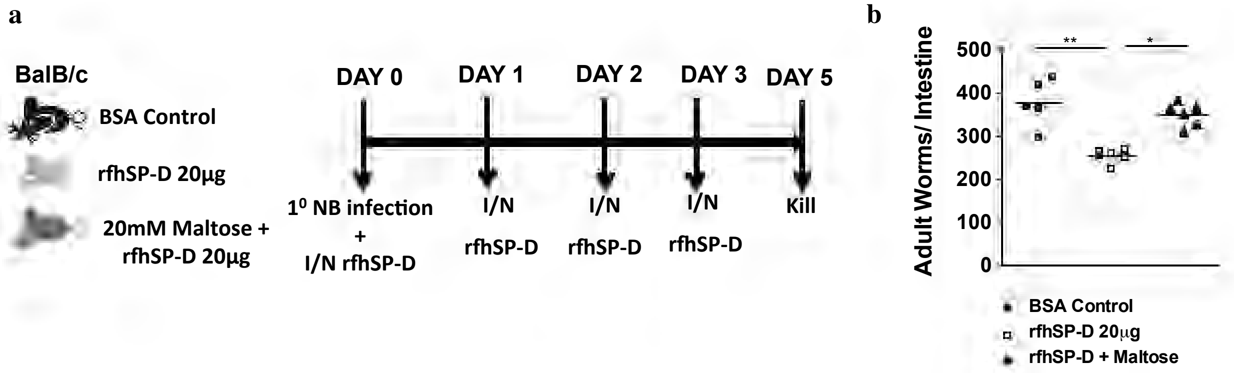


Fig 3. 8: **SP-D requires carbohydrate binding head region for its optimal function**. rfhSP-D was incubated with 20mM maltose to block the CRD head region before being intra-nasally administered. Mice were treated with BSA, rfhSP-D or maltose bound rfhSP-D (**a**). Intestinal worm burden was quantified at day 5 P.I. (**b**). Data are representative of two individual experiments. N= 5-6 mice per group. $^{*}P<0.05$, $^{**}P<0.01$.

3.2.5 rfhSP-D treated AMs show increased alternative activation

The rapid nature of rfhSP-D-enhanced protection suggested improved host innate cell mediated immunity to *N. brasiliensis* infection. SP-D is associated to interact with AMs (159, 185, 186, 211, 232–234). Moreover, *N. brasiliensis* infection drives macrophage polarization to the IL-4Rα dependent alternatively activated phenotype (235, 236). AAMs have been shown to be required for recall immunity against *H. polygyrus* (27) and also for survival during *Schistosomiasis* (45, 82). We found this was also the case for recall immunity to *N. brasiliensis*; mice deficient for IL-4Rα on macrophages ($\text{LysM}^{\text{Cre}}\text{IL-4R}\alpha^{-/\text{lox}}$) had an impaired ability to control secondary *N. brasiliensis* infection (**Fig 3.9a**). This suggested that enhanced SP-D mediated immunity to *N. brasiliensis* maybe a result of SP-D interaction with AMs.

We examined if SP-D enhanced alternative activation of AMs. This was indeed the case; intranasal rfhSP-D treatment increased expression of the AAM markers YM1 and Relmα in CD11c$^+$SiglecF$^+$ AMs when compared to BSA treated control mice (**Fig 3.9b**).

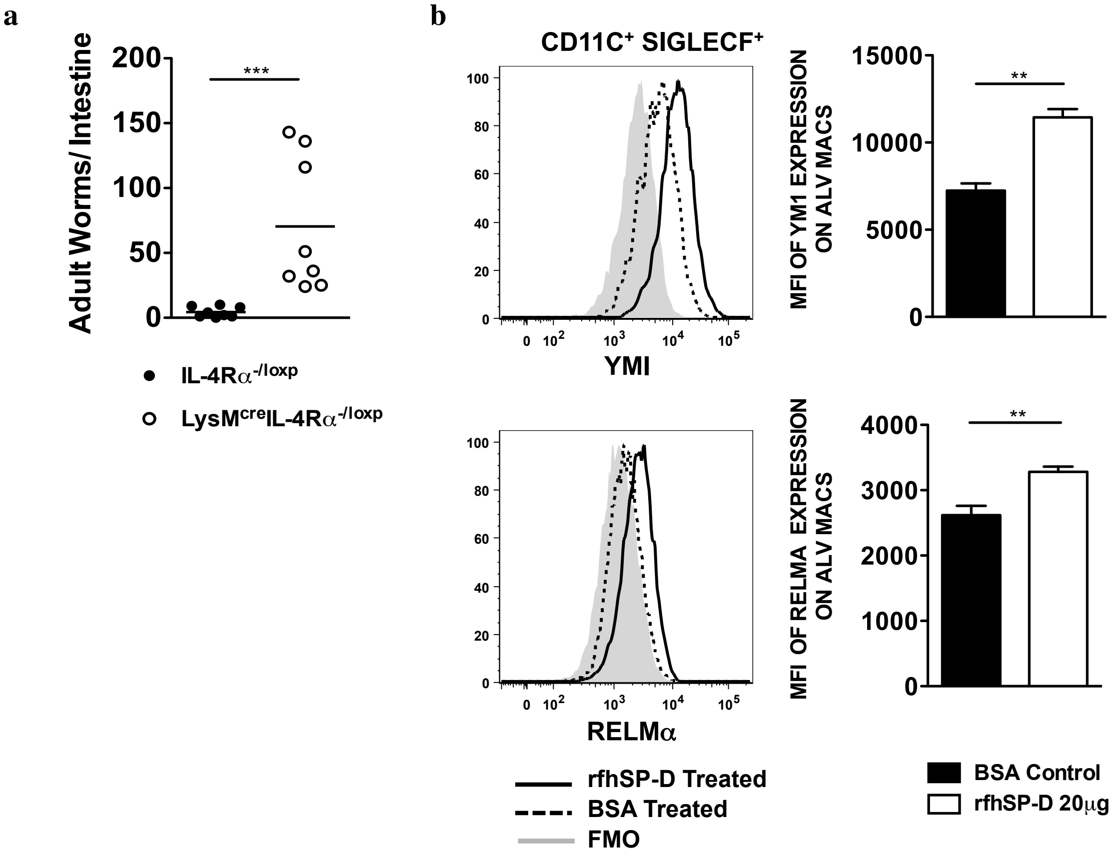


Fig 3. 9: rfhSP-D enhances alternative activation of alveolar macrophages. WT or LysM^{cre}IL-4Ra$^{-/lox}$ were given secondary *N. brasiliensis* infection and at day 5 P.I, intestinal worm burdens were quantified (**a**). Mean fluorescence intensity (MFI) of YM1 and Relmα expression on CD11c$^+$SiglecF$^+$ AMs from lungs of D5 post-infected rfhSP-D treated or untreated mice was established by FACS analysis (**b**). Data are representative of two individual experiments. N= 5-6 mice per group. $^{**}P<0.01$, $^{***}P<0.001$

We then directly tested whether SP-D could enhance alternative activation of AMs isolated from naïve mice. Naïve AMs were artificially polarized to AAM by *ex vivo* culture with recombinant IL-4/IL-13 in the presence or absence of rfhSP-D. Co-culture with rfhSP-D resulted in increased YM1 and Relmα expression when compared to macrophages treated with only IL-4/IL-13 (**Fig 3.10**).

Together these data suggest that increased SP-D levels in *N. brasiliensis* infected mice may enhance host ability to control infection by driving AAM activation.

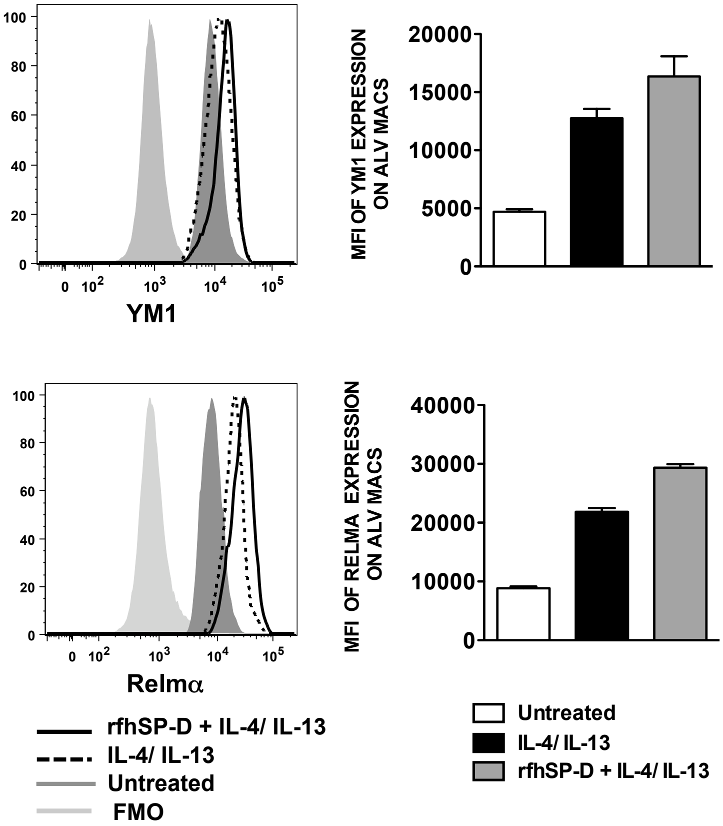


Fig 3. 10: rfhSP-D enhances macrophage activation *in vitro* in the presence of IL-4/ IL-13. 4 x 10^5 macrophages sorted from naïve lungs of mice were cultured in duplicates for 60hrs with either IL-4/IL-13, rfhSP-D + IL-4/IL-13 or left untreated before staining for Relmα and YM1. MFI was measured by flow cytometry. Data are representative of two individual experiments.

3.2.6 rfhSP-D decreases numbers of lung DCs but does not alter numbers of eosinophils and AMs.

Having shown that rfhSP-D increases the alternative activation of macrophages; we next tested the influence of rfhSP-D on other innate effector cell populations. Intranasal administration of rfhSP-D did not affect the numbers of lung AMs **(Fig 3.11a**) and eosinophils (**Fig 3.11b**). However interestingly, we found that it significantly decreased the numbers of both immature and mature DCs in the lung (**Fig 3.11c**). A few reports describe the ability of SP-D in up regulating the expression of C-C chemokine receptor 7 (CCR7) on activated mature DCs and enhance their migration to LN where they exert their antigen presentation function (210).

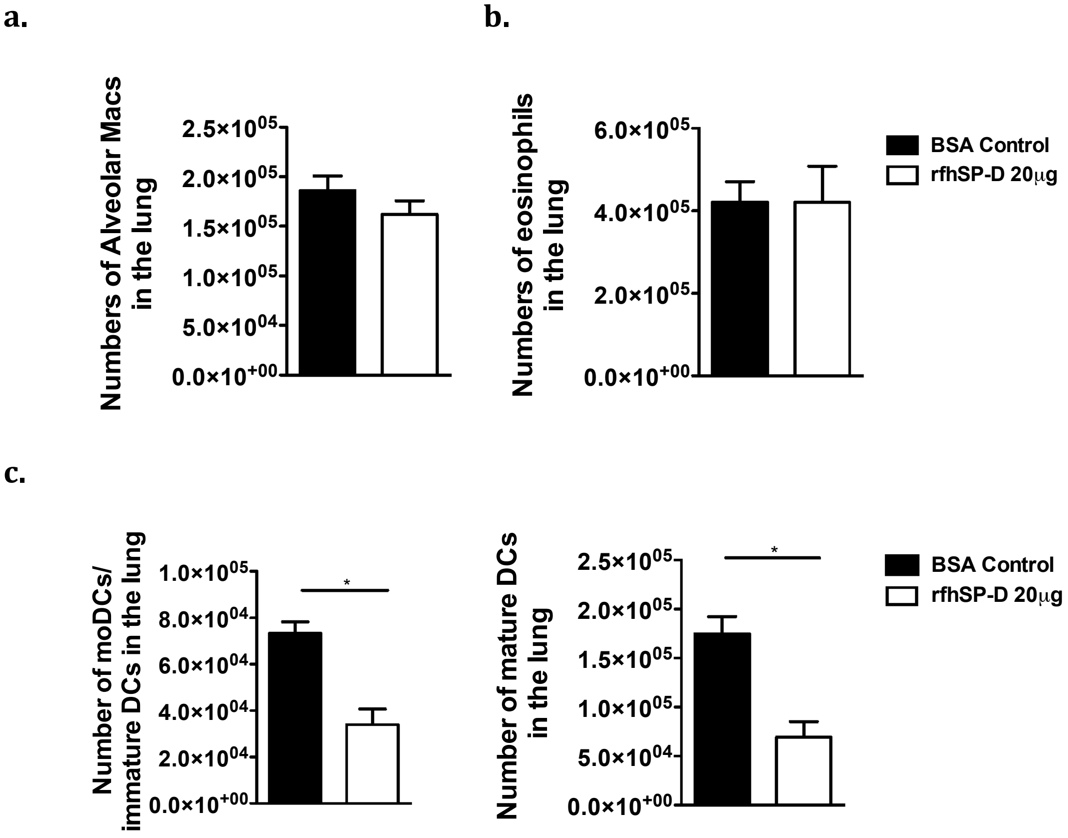


Fig 3. 11: Effect of rfhSP-D on numbers of innate effector cells. Quantification of cell numbers of CD11c$^+$ SigLecF$^+$ AMs (**a**), CD11c$^-$SigLecF$^-$ eosinophils (**b**) and SigLecF$^-$ CD11c$^+$MHCII^{lo} monocyte derived (moDCs)/ immature and SigLecF$^-$CD11c$^+$MHCII^{hi} mature DCs (**c**) by flow cytometric analysis of cell suspension of whole lung. Data are representative of three individual experiments. N= 5-6 mice per group. *P<0.05

3.2.7 rfhSP-D treated macrophages confer enhanced protection to N. brasiliensis infection

To test whether rfhSP-D treated macrophages conferred protection against *N. brasiliensis*, we isolated AMs from *N. brasiliensis* infected rfhSP-D treated or untreated mice and intra-nasally transferred in to naïve mice. Recipients of rfhSP-D treated macrophages had reduced intestinal worm burdens following *N. brasiliensis* infection when compared to recipients of untreated macrophages (**Fig 3.12**). This suggests that SP-D-enhanced protection against *N. brasiliensis* infection is partly at least mediated by an enhanced AAM protective response.

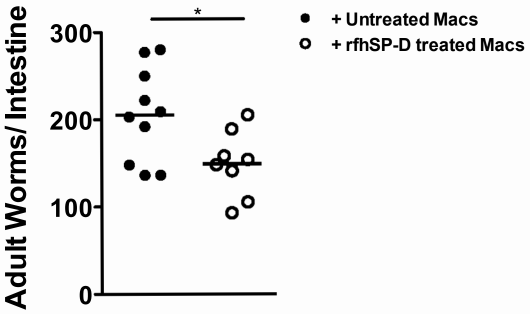


Fig 3. 12: rfhSP-D treated macrophages confer enhanced protection to *N. brasiliensis* infection. Macrophages isolated from lungs of infected rfhSP-D treated or untreated mice were intra-nasally transferred into naïve BALB/c mice. Mice were thereafter infected with *N. brasiliensis* and worm burdens were quantified at Day 5 P.I. Data are representative of two individual experiments.

3.2.8 rfhSP-D binds to L4 larval stage of N. brasiliensis

In addition to interacting with AMs, SP-D also functions as an opsonin of pathogens (115). SP-D has high affinity for the monosaccharides mannose, glucose and fucose and can bind to the fucosylated glycoconjugates present on the surface of *Schistosoma mansoni* (121). It is unknown if SP-D can bind to parasitic nematodes. We examined if SP-D could directly interact with *N. brasiliensis*.

We used confocal microscopy to demonstrate SP-D's ability to directly bind *N. brasiliensis* L3, L4 larvae and also to the adult worm. SP-D binding was restricted to the surface of *N. brasiliensis* L4 larval stage (**Fig 3.13a**). Secondary only controls were used to ensure the specificity of binding (**Appendix Fig 1**). *N. brasiliensis* L4 are the lung associated stage of the parasite life cycle. This data shows that, in addition to contributing to the Th2 associated pulmonary immunity to *N. brasiliensis*, SP-D also acts as an interface between *N. brasiliensis* L4 and cells of the pulmonary immune system. To test this, we intra-nasally infected naïve mice with *N. brasiliensis* rfhSP-D coated L4 or uncoated L4. Analysis of host intestinal parasite burdens at day 5 P.I revealed significantly reduced parasite numbers in mice intra-nasally infected with rfhSP-D coated L4 stage larvae when compared to mice infected with uncoated L4 larvae (**Fig 3.13b**). Furthermore, the macrophages from mice infected with rfhSP-D coated L4 showed increased expression of RELMα and YM1 when compared to control (**Fig 3.13c**). These results suggest that SP-D binding to *N. brasiliensis* L4 enhances host ability to control infection.

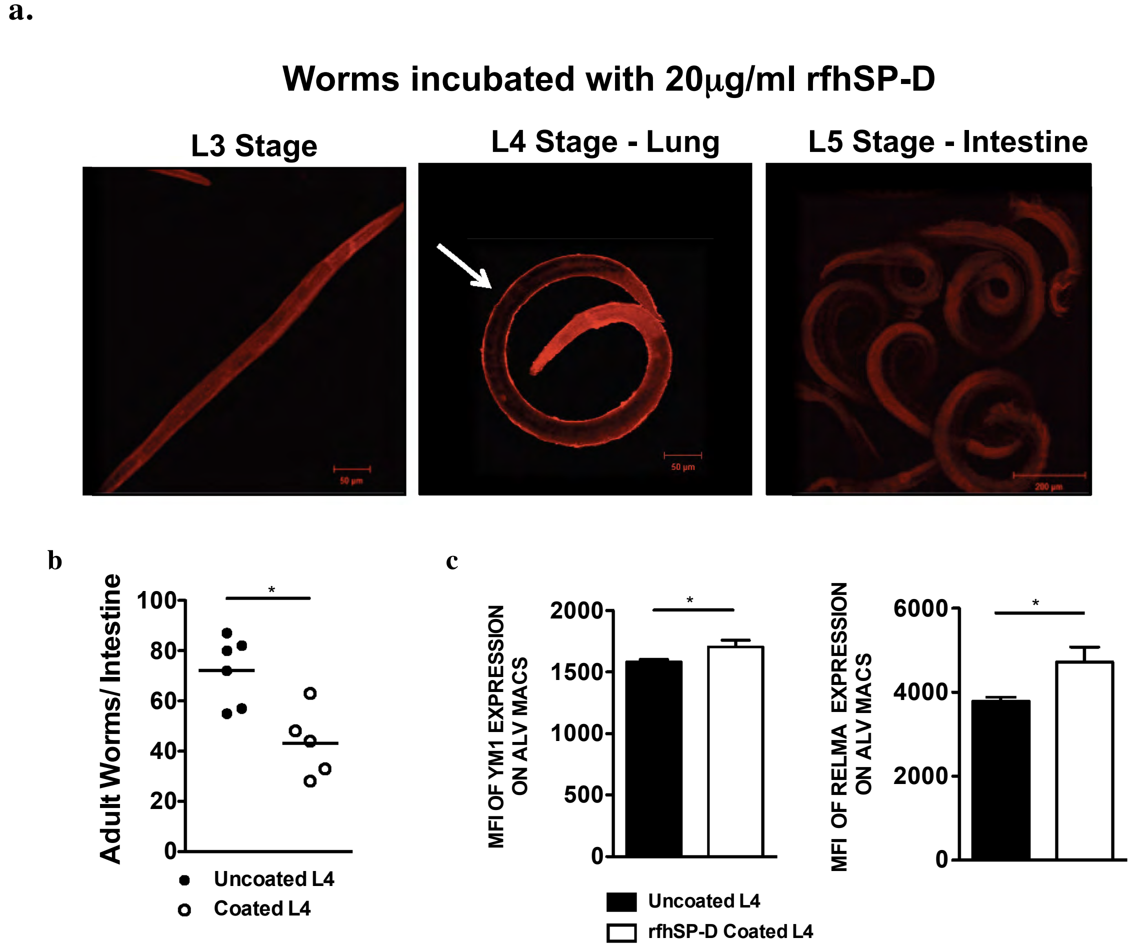


Fig 3. 13: rfhSP-D binds to L4 stage of *N. brasiliensis*. Confocal microscopic images of L3, L4 and L5 stage of *N. brasiliensis* that were incubated with 20μg/ml of rfhSP-D followed by staining with antibody to rfhSP-D (**a**). 250 L4 stage larvae, coated or uncoated with rfhSP-D were intra-nasally administered into naïve mice. Intestinal worm burden were quantified at day 4 P.I. (**b**). Flow cytometric analysis of YM1 and RELMα expression on AMs isolated from the lungs of infected mice (**c**). Data are representative of two individual experiments. N= 5 mice per group. *P<0.05.

3.2.9 Intra-nasal administration of native SP-D confers increased protective immunity to N. brasiliensis

The rfhSP-D used in our study contained a short fragment of the collagen stalk, neck region followed by the CRD head region as shown previously in Fig 3.4. This truncated fragment was able to enhance protection to *N. brasiliensis* infection as shown in fig 3.7.

In order to examine if structural differences in SP-D affected the quality of protective immune responses to *N. brasiliensis*, we intra-nasally administered the full length or truncated fragment of SP-D in infected mice and quantified intestinal worm burden as a measure of protection **(Fig 3.14a)**. Mice treated with native SP-D displayed slightly enhanced protection when compared to rfhSP-D treated mice (**Fig 3.14b**). This suggests that the collagen and N-terminal region present in full length SP-D may also be important for contributing to immune responses to *N. brasiliensis*. A difference in the effect of rfhSP-D is observed between experiments due to the possible variation in the protein stock preparation.

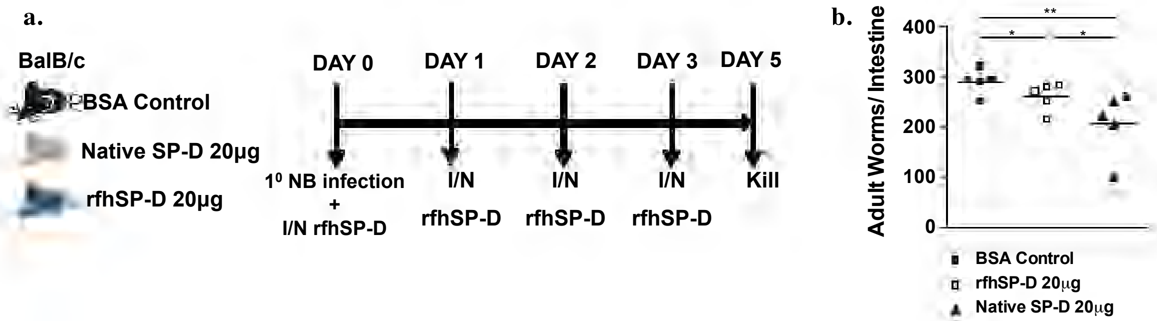


Fig 3. 14: Native SP-D confers slightly increased protection to *N. brasiliensis*. Mice were intra-nasally treated with BSA, rfhSP-D or native SP-D (**a**). Intestinal worm burden was quantified at day 5 P.I. (**b**). Data are representative of one individual experiments. N= 5 mice per group. *P<0.05, **P<0.01.

3.3 Discussion:

In this study we addressed how a component of host innate immunity (SP-D) could contribute to pulmonary control of *N. brasiliensis* infections. We demonstrated that *N. brasiliensis* exposure increased host production of SP-D. The increased BAL and serum SP-D levels correlated with the course of *N. brasiliensis* infection; peak SP-D levels coincided with acute phases (D2 – D7) of infection.

Elevated levels of SP-D have been associated with protection against asthma/ allergic inflammation in both clinical and basic studies (220, 237, 238). In interstitial lung disease, increased serum SP-D levels are a biomarker of inflammation (239). Importantly these studies point to SP-D being induced in a Th2 immune environment. SP-D production has indeed been demonstrated to be negatively regulated in the absence of IL-4 and IL-13 (120). As Th2 immunity evolves to control parasitic helminth infections, it could be hypothesized that SP-D may well be playing a role in controlling these infections. In support of this we found that enhanced SP-D production following *N. brasiliensis* infection was overwhelmingly dependent on Th2 cytokine levels; *N. brasiliensis* infected IL-4/IL-13 double knockout mice failed to increase SP-D titers as significantly as seen in WT *N. brasiliensis* infected mice.

SP-D can mediate protection from infection by binding to carbohydrate motifs on a range of allergens, viruses, bacteria and fungal pathogens and then acting as an interface between the antigen and the immune system (115). We found that SP-D bound preferentially to lung stage L4 *N. brasiliensis*, suggesting that any role for SP-D in controlling infection may be limited to the lung. Murine reinfection studies with *N. brasiliensis* have shown that pulmonary Th2 responses play a key role in controlling secondary *N. brasiliensis* infection (36). An important driver of this is pulmonary epithelial cells. These secrete cytokines such as IL-33, which are essential for enhancing the production of classic Th2 cytokines such as IL-4, 5 and 13 by a range of immune cells such as ILC2s and CD4$^+$ T cells. This ultimately leads to the induction effector responses such as goblet cell mucus secretion and mast cell activation driving disease resolution (58, 90). Epithelial cell control of this immunity is not restricted to the secretion of cytokines. For example work by Wills-Karp *et al* showed epithelial secretion of TFF2 to also be important in IL-33 initiation of Th2 immunity (90). Together this suggests that epithelial cells are the source of a complex range of mediators of host Th2 immunity. Our findings suggest that, in addition to inducing Th2 immunity by lung resident immune cells, epithelial cells also respond to this enhanced Th2 environment and a function of this is enhanced SP-D production.

To establish if SP-D can directly influence the outcome of an *N. brasiliensis* infection, we artificially enhanced pulmonary levels of SP-D by intra-nasal administration of rfhSP-D one day before infection. This manipulation of SP-D levels significantly reduced intestinal worm burdens and increased the levels of Th2 immunity in the lung. Levels of lung IL-4 and IL-13, alternative activation of macrophages (as demonstrated by up-regulation of RELMα and YM1 expression) and expansion of ILC2 were all increased in rfhSP-D treated mice. Other studies have also demonstrated that therapeutic administration of rfhSP-D can also be effective against allergy, respiratory syncytial virus (201) and the fungal pathogen, *Aspergillus fumigatus* (221, 240). These observation are further supported by studies using SP-D$^{-/-}$ mice where control of these infections is abrogated and the onset of chronic emphysema-like abnormal lung pathology is exacerbated (180, 241). Importantly our findings indicate that purely via the SP-D CRD domain protection against *N. brasiliensis* is mediated by a simultaneous SP-D interaction between the parasite and the immune system.

The rfhSP-D used in the study is composed only of CRD head region, α-helical neck region and a short stalk of collagen chain. This short fragment of SP-D poses advantages over the native SP-D in that it is easily expressed in *E. Coli* and can be produced in large quantities. Although intra-nasal administration of native SP-D in mice showed slightly better protection to *N. brasiliensis* compared to administration of rfhSP-D, the truncated fragment retains significant biological activity and therapeutic potential; being effective in correcting the emphysematous lung phenotype of SP-D deficient mice and improving host defense functions in various disease models (176, 201, 221, 240, 241). This unexpected maintenance of biological function by rfhSP-D can in part be explained by structural study of the fragment. Here high-resolution structural analysis of rfhSP-D has revealed that the fragment maintains a potential to simultaneously interact with both antigenic ligands and immune cells by holding binding sites located on the trimeric axis in a pore at the bottom of the funnel formed by the three CRDs and close to the neck–CRD interface (242). Presence of these recognition sites would allow rfhSP-D to recognize pathogens via its CRD head region and at the same time recruit immune cells to the site of infection. Additionally, the short collagen stalk of rfhSP-D has also been shown to play a role in the regulation of pulmonary macrophage activation, airspace remodeling, and surfactant lipid homeostasis as lack of this truncated region failed to correct the lung phenotype of SP-D deficient mice (243).

In our study, we demonstrated that rfhSP-D binds directly to the surface of the parasite via the CRD head region, as our *in vivo* blocking of CRD binding site with maltose ablated the ability of SP-D to enhance resolution of infection. We also demonstrate that rfhSP-D enhances two key Th2 associated innate immune cell populations; namely ILC2 and AAMs.

Macrophages are major targets of SP-D and an intricate relationship between ATII epithelial cells and macrophages is a feature of SP-D biology (124, 159, 185, 186, 211, 232, 233). AAM have important roles in controlling helminth infections and associated pathology. *S. mansoni* infection of AAM deficient LysM^{Cre}IL-4R$\alpha^{-/lox}$ mice highlighted AAM ability to control host pathology and mortality to this Th2 polarising infection. Related studies with *S. mansoni* and *N. brasiliensis* infection have further demonstrated important immune-modulatory roles of AAM in controlling Th2 immuno-pathologies (81, 83, 84). As enhanced SP-D concentrations and induction of AAM are strongly associated with Th2 immunity we explored further the relationship between SP-D and induction of AAM. We found that SP-D can directly promote induction of AAM and that this in part at least contributes to SP-D mediated protection against *N. brasiliensis*. These findings represent an important extension in our understanding of the biology of AAM in controlling *N. brasiliensis* infection; namely we show that AAM directly contribute to recall immunity against *N. brasiliensis* and this is driven in part by SP-D interactions with macrophages.

Although we found no effect of SP-D on the numbers of eosinophils and AMs in the lung, we speculate that SP-D may alter the functional immune responses of these effector cells. This however needs to be further examined. Our observation of decreased numbers of DCs in the lungs of SP-D treated mice is consistent with earlier reports that show the ability of SP-D in up-regulating the chemokine receptor, CCR7 on activated DCs. Expression of this receptor on DCs is required for homing of these cells to the LN where they exert their functions of T cell stimulation and antigen presentation (210). The significance of enhancing DC migration by SP-D and its role in promoting *N. brasiliensis* specific Th2 immune responses will need to be further investigated.

In conclusion, our data provides evidence for the biological activity of rfhSP-D in contributing to protective immune response to *N. brasiliensis*. Our study proposes the following insights into the SP-D-associated mechanism that is at play (**Fig 3.15**): 1) It is known that infection with *N. brasiliensis* leads to increased numbers of ATII cells and levels of IL-33 (58) 2) This increased ATII cell numbers increases levels of pulmonary SP-D 3) The SP-D directly binds to surface of parasite via its CRD head region and recruits immune cells to the site of infection resulting in effective Th2 immunity 4) SP-D leads to expansion of ILC2 that responds to the epithelial cell secreted IL-33 and subsequently releases IL-4/IL-13 5) SP-D also binds and activates AMs that further contribute to effective worm expulsion.

Taken together, our data provides important insights into novel mechanisms for controlling helminth infection. Current knowledge of the mode of action of rfhSP-D provides strong prospects of using it as an effective therapeutic agent.

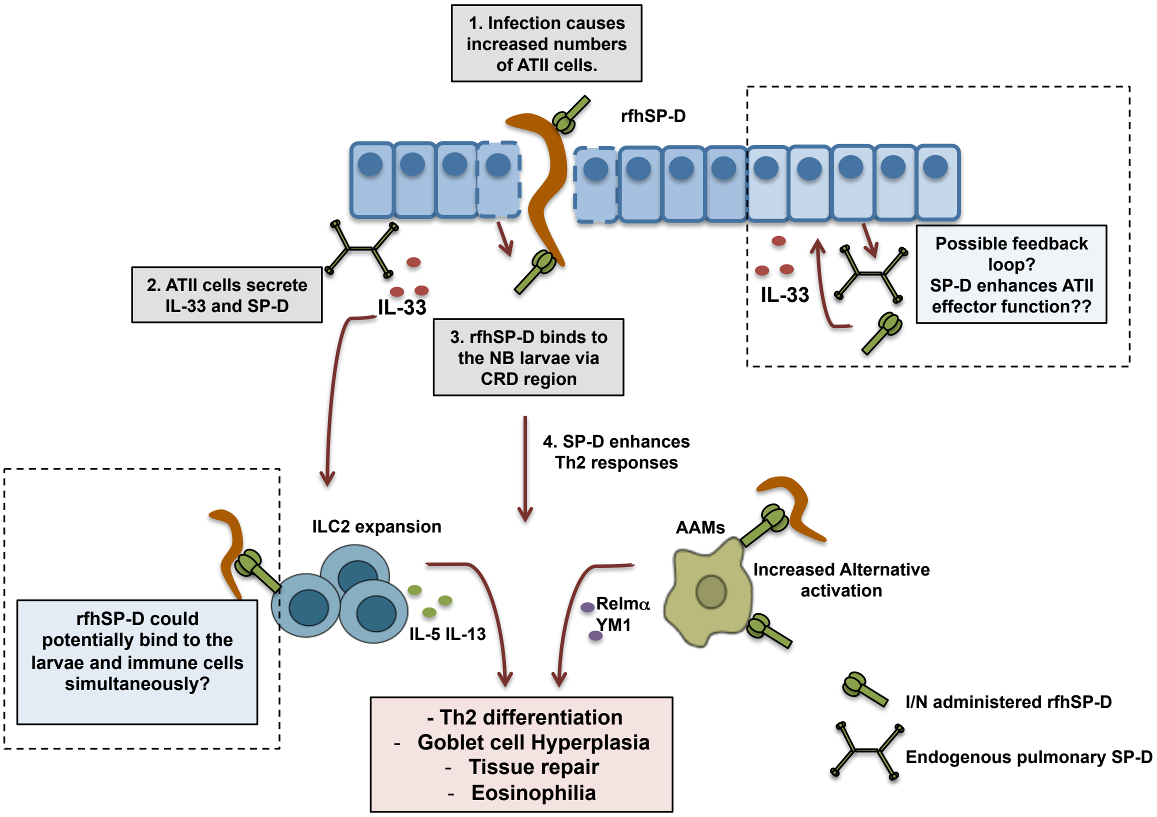


Fig 3. 15: Proposed model for rfhSP-D mediated protective Th2 immune response to Nematode infections.

Chapter 4: The Pulmonary Adaptive Immunity to *N. brasiliensis:* Role of lung-resident T cells in protective immunity to *N. brasiliensis* infection

(Thawer S & Horsnell W et al 2013)

4.1 Introduction

In the previous chapter, a crucial role for pulmonary innate immunity to *N. brasiliensis* was demonstrated. In this chapter, we will further look at the adaptive arm of immunity and examine its role in host defense to *N. brasiliensis*. A brief introduction is first given to introduce the lung adaptive immunity with a particular focus on the role of lung-resident T cells.

4.1.1 Pulmonary Adaptive immunity

Pathogens that are not cleared by the innate immunity are brought into contact with the adaptive arm of immunity that aid in eliminating the pathogen. The lung offers a well-regulated specific immune response that involves subsets of T cell populations and antibody-producing B cells that protect the lung from a wide range of pulmonary pathogens. This response is highly specific and has the ability to build an efficient memory response.

The lung is surrounded by various lung draining lymph nodes (LN) that aid in initiating the adaptive immune response. APCs such as DCs migrate to lymphoid organs and present antigens to naïve T cells, thereby facilitating the activation, expansion and differentiation of T cells. Activated T cells subsequently drain to the local site of infection in the lung where they aid in clearing the infection (133).

A large part of this discussion will center on the role of lung-resident T cells in mounting protective responses to various respiratory pathogens.

4.1.1.1 Lung resident T cell immunity

A well-established concept of immunology is that primary adaptive responses are initiated in the secondary lymphoid organs such as LN, spleen and peyer's patches. Lymphoid organs contain organized areas of T cell and B cell zone that allows for molecular interactions between B cells, T cells and APCs. Here, naïve T cells are activated by APCs to differentiate in to effector T cells, which then migrate to the site of infection where they aid in clearing the pathogen. While infections occur locally in the peripheral sites, immune responses are initiated in the secondary lymphoid organs (SLO) (244).

Importance of lymphoid organs in initiating primary immune responses have been established in several studies using alymphoplastic (*aly*) mice that lack peyer's patches and lymph nodes and have disrupted architecture of the spleen and thymus (245, 246). Such mice were unable to reject allogeneic skin graft (245), cardiac allografts (247) or fight off infections of lymphocytic choriomeningitis virus (LCMV) (248) due to lack of appropriate T cell responses. Similarly, *aly* mice were unable to reject tumors due to lack of effective cytotoxic CD8^{+} T cells (CTL) responses resulting from the inability of tumor antigens to migrate to LNs (249). Immunization of lymphotoxin alpha deficient (Lt$\alpha^{-/-}$) mice that share a similar phenotype with *aly* mice with epicutaneous hapten failed to mount contact-hypersensitivity response (250). Such mice also had reduced CTL responses towards LCMV and herpes simplex virus (HSV) (251, 252). All together, these results provide evidence for the requirement of lymphoid organs in initiating primary immune responses.

Once primary infections with microbial pathogens are cleared, effector T cells (Teff) are retained as effector memory T cells (Tem). Repeat infections are normally encountered with an enhanced and rapid immune response by the effector memory T cells which get activated to become Teff. Central memory T cells (Tcm) in the LN aid in maintaining a continuous supply of effector T cells **(Fig 4.1)** (244, 251).

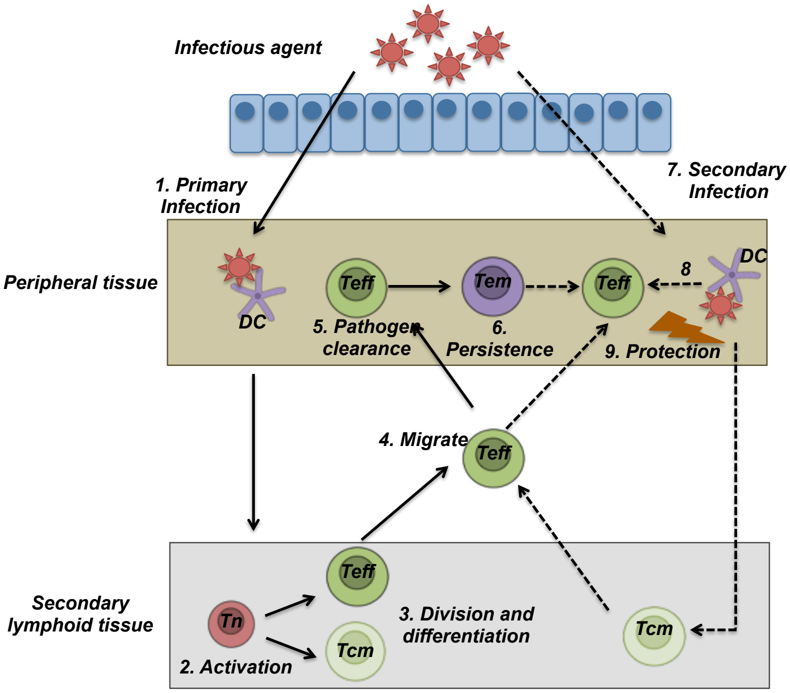
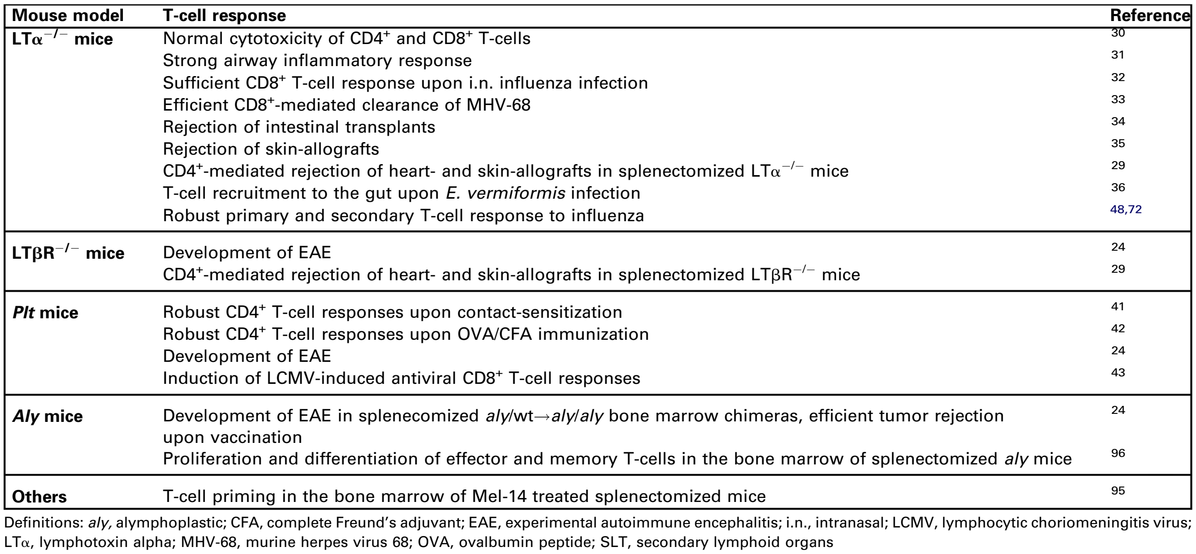


Fig 4. 1: Effector T cell responses during primary and secondary infection. During primary infections, DCs migrate to SLO where they present antigen and activate naïve T cells to differentiate to Effector T cells (Teff) or Central memory T cell (Tcm). Teff cells migrate to the site of infection where they aid in clearing the infections. These then persist as effector memory T cells (Tem). Repeat infections are normally encountered with an enhanced and rapid immune response by the effector memory T cells which get activated to become Teff. Central memory T cells (Tcm) in the lymph nodes aid in maintaining a continuous supply of effector T cells.

Contrary to this belief that the accelerated immune responses are generated in the SLOs, new evidence now points to the role of resident effector memory T cells localized at peripheral tissues in inducing these protective responses independently of SLO as summarized in **Table 4.1**. The importance of these resident Tem cells in inducing protective immunity is demonstrated in studies that have shown a direct correlation between numbers of Tem in peripheral tissue and ability to clear challenge viral infections (253). By contrast, no correlation was observed between numbers of Tcm in LN and ability to mount protective responses (254). Further supporting evidence was provided by a study carried out by Ray *et al* where the location of Tem cells was demonstrated to effect the level of protective immunity against influenza infection (255). Here the peripheral tissue-specific adhesion molecule very late antigen-1 (VLA-1) that is expressed by tissue resident T cells was deleted genetically or by blocking antibodies.

Deletion prevented influenza specific memory T cells from accumulating in the lung and such mice were unable to mount protective immunity towards influenza re-infection in spite of unchanged numbers of memory T cells in SLO.

Table 4. 1: **Efficient T cell responses in mouse models that lack SLO**. Figure adapted from Hofmann *et al* 2010 (256).



Mouse model	**T-cell response**	**Reference**
LTα$^{-/-}$ mice	Normal cytotoxicity of CD4$^+$ and CD8$^+$ T-cells	30
	Strong airway inflammatory response	31
	Sufficient CD8$^+$ T-cell response upon i.n. influenza infection	32
	Efficient CD8$^+$-mediated clearance of MHV-68	33
	Rejection of intestinal transplants	34
	Rejection of skin-allografts	35
	CD4$^+$-mediated rejection of heart- and skin-allografts in splenectomized LTα$^{-/-}$ mice	29
	T-cell recruitment to the gut upon *E. vermiformis* infection	36
	Robust primary and secondary T-cell response to influenza	48,72
LTβR$^{-/-}$ mice	Development of EAE	24
	CD4$^+$-mediated rejection of heart- and skin-allografts in splenectomized LTβR$^{-/-}$ mice	29
***Plt* mice**	Robust CD4$^+$ T-cell responses upon contact-sensitization	41
	Robust CD4$^+$ T-cell responses upon OVA/CFA immunization	42
	Development of EAE	24
	Induction of LCMV-induced antiviral CD8$^+$ T-cell responses	43
***Aly* mice**	Development of EAE in splenecomized *aly*/wt→*aly*/*aly* bone marrow chimeras, efficient tumor rejection upon vaccination	24
	Proliferation and differentiation of effector and memory T-cells in the bone marrow of splenectomized *aly* mice	96
Others	T-cell priming in the bone marrow of Mel-14 treated splenectomized mice	95

Definitions: *aly,* alymphoplastic; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalitis; i.n., intranasal; LCMV, lymphocytic choriomeningitis virus; LTα, lymphotoxin alpha; MHV-68, murine herpes virus 68; OVA, ovalbumin peptide; SLT, secondary lymphoid organs

Additional studies using gene KO studies and pharmacological agents have also demonstrated a role for lung-resident T cells in coordinating host pulmonary immunity. For instance, using the drug Fingolimod (FTY720), that prevents lymphocyte egress from secondary lymphoid tissue to peripheral sites, Connor *et al* showed that protective immune responses in response to BCG challenge was generated by lung resident multi-functional CD4$^+$ Tem cells independent of recruitment from SLO (127). Parabiosis experiments in mice infected with influenza revealed resident memory CD4$^+$ T cells that were localized in the lung to be responsible for protection against influenza (257). In experimental respiratory viral infections of LTα$^{-/-}$ mice, effective immune response was shown to be generated in response to gammaherpesvirus68 (258). Moreover, influenza specific lung-resident CD8$^+$ T cells in splenectomized LTα$^{-/-}$ mice were able to clear challenge influenza infection in the complete absence of SLO. These mice were also able to generate neutralizing antibodies and maintenance of these resident lymphocytes was shown to occur in structures such as iBALT (259).

These studies suggest the possible presence of a suitable microenvironment in the lung capable of maintaining the survival and proliferation of effector memory T cells following a challenge infection. The presence of ‘effector lymphoid tissue’ (ELT) such as iBALT, containing Tem and Teff at the site of pathogen entry has been suggested to form in tissues that have had earlier antigen-stimulated processes. These structures have been shown to contain organized B and T cell areas that supports lymphocyte proliferation and are dependent on DCs to sustain their presence and maintenance (259, 260).

The ability to prime immune responses at these local sites can be explained by the presence of resident lung APCs that are capable of mounting effective immune responses in situ (261). Indeed, T cells have been shown to respond to presented antigen in a LN-free environment (256). DCs largely serve as the main APCs in the lung. Intranasal delivery of antigens demonstrated preferential processing by lung-resident CD11c^{hi} APC that was able to present it to antigen specific resident T cells and promote Th2 differentiation in situ (262). Furthermore, lung CD11b^{+} DCs have been shown to initiate and maintain Th2 mediated immunity following exposure to house dust mite (HDM) (263, 264). In another study, resident DCs were shown to induce CD8^{+} Tem responses to HSV (265). Hence there is accumulating evidence of the presence of lung resident APCs in promoting the local pulmonary response.

An important requirement of the immune response mounted in the lung is the need for it to be appropriately regulated to prevent the delicate lung tissue from damage. Presence of a regulatory mechanism has been well reported. During steady-state conditions, lung-resident AMs can generate FoxP3^{+} regulatory T cells (Treg) and promote airway tolerance. Treg cells express FoxP3 and IL-10 that are associated with suppression of inflammation (148). Furthermore, alveolar epithelial cell-secreted surfactant protein A and D can control the allergic airway inflammation within the lung by inhibiting IL-2 production from lymphocytes thereby preventing proliferation of T cells (115).

All together, there is compelling evidence that suggests a crucial role for lung-resident T cells in mediating protective immune responses against various respiratory pathogens. The lung provides a fine environmental niche for supporting the proliferation and maintenance of these resident memory T cells. It has also developed a regulatory mechanism for preventing the delicate tissue of the lung from excessive damage thereby maintaining its physiology.

4.1.2 The role of lung resident T cells in immunity to nematode infections

Th2 immune responses underlie both effective host immunity to helminth infections (14, 47) and the onset of allergic inflammatory diseases (266). Development of Th2 immunity typically requires IL-4 and IL-13 signaling via receptors containing an IL-4Rα subunit. This activates effector responses that include goblet cell hyperplasia, Type 2 immunoglobulin production and secretion of the cytokines IL-4, IL-5, IL-9 and IL-13 (48). In primary infections, IL-4Rα expression on non-hematopoietic cells is required to drive protection (31, 33, 44). Reinfection studies with *N. brasiliensis* (36) and *H. polygyrus* (27) have demonstrated enhanced Th2 immunity rapidly clearing infection. This protection is strongly related to CD4$^+$ T cell activation with Tem and Tcm cell subsets being particularly important (267). Additionally, depletion of CD4$^+$ T cells significantly impaired host antibody responses to reinfection (103). Any specific hematopoietic cellular requirements for IL-4Rα expression following *N. brasiliensis* re-infection have not been demonstrated.

CD4$^+$ T cell populations resident in peripheral sites can profoundly influence the coordination of host immune responses (127, 268–270). Such peripheral CD4$^+$ T cell populations play important roles in immunity to parasitic nematodes. In *T. muris* infection, protective immunity is driven by gut-associated lymphoid tissue (GALT) CD4$^+$ Tcm and Tem cells (38). Host responses to pulmonary infections can also result in the formation of peripheral lymphoid like structures/effector lymphoid tissues (ELTs), which can effectively co-ordinate host immunity at the site of infection (244).

Recall immunity to *N. brasiliensis* has been associated with lung initiated CD4$^+$ T cell responses (36). Whether this is mediated by a pre-existing pulmonary IL-4Rα expressing CD4$^+$ T cell population is unknown. In this study, we inhibited lymphocyte movement with Fingolimod (FTY720), an immunomodulator that acts as an agonist to the Sphingosine-1-Phosphate (S1P) receptor, inducing lymphopenia by blocking lymphocyte egress from SLO to peripheral sites (126, 127, 271). This allowed us to test if a pre-existing pulmonary $CD4^+$ T cell population were able to co-ordinate protective secondary Th2 immunity.

We show that immunity to primary *N. brasiliensis* infection requires T cell movement but Th2 protective responses to secondary infection are independent of T cell migration. We demonstrate a lung resident $CD4^+$ T cell population to be sufficient to confer an IL-4Rα dependent protection against *N. brasiliensis* re-infection. Furthermore, we show that the protective lung-resident T cell population is independent of formation of tertiary lymphoid structures in the lung and is therefore likely to be residing in the lung interstitium.

4.2 Results

4.2.1 Migration of lymphocytes is required for protection against primary N. brasiliensis infection.

T cell lymphopenia was induced in mice by daily FTY720 treatment. Lymphopenia was confirmed by tail bleeding and staining whole blood with anti-CD3 and anti-CD4 antibodies. At 24 hours post initial FTY720 treatment, circulating $CD3^+CD4^+$ T cells decreased from approximately 8.5% to 0.1% of total cells in blood (**Fig 4.2a**). This reduction was maintained following *N. brasiliensis* infection (**Fig 4.2b**).

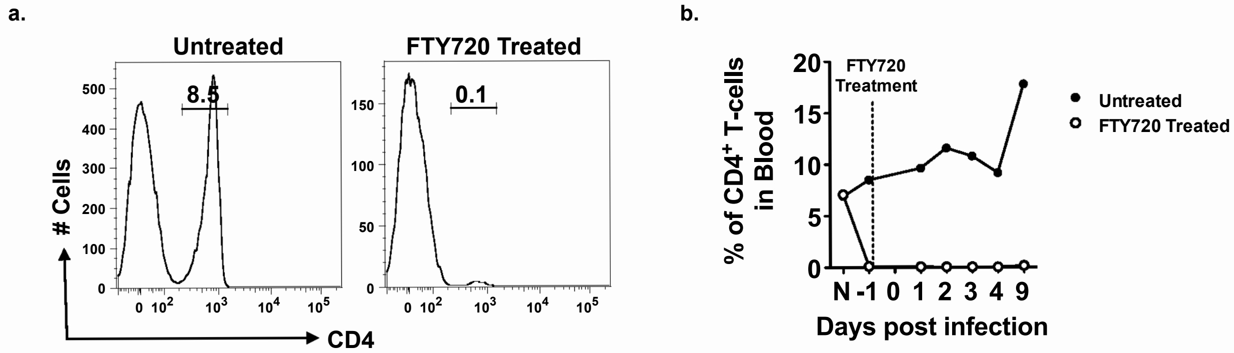
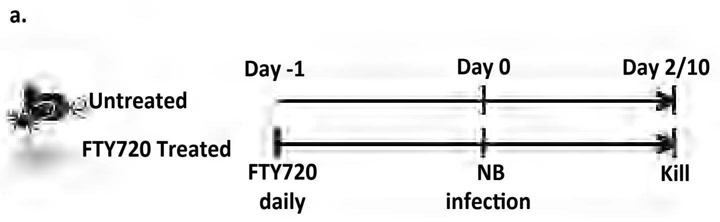
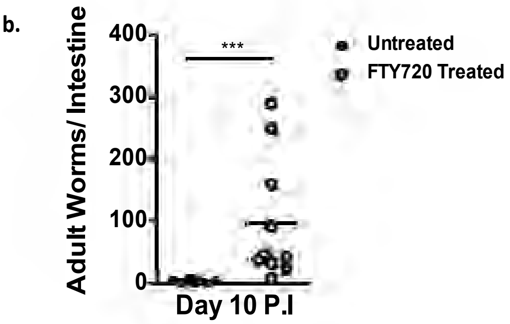


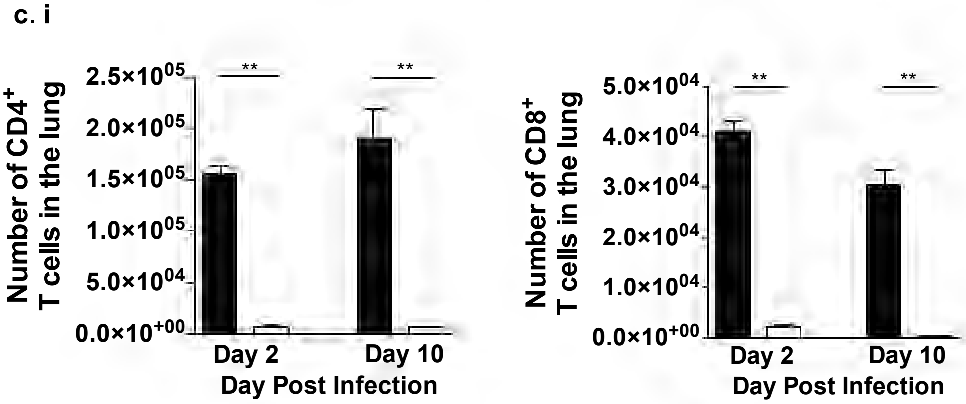
Fig 4. 2: Treatment with FTY720 induces lymphopenia. Lymphopenia was established by flow cytometric analysis of $CD3^+CD4^+$ T cells in whole blood 24 hours post FTY720 treatment (**a**). Acute lymphopenia was rapidly induced by FTY720 and maintained following *N. brasiliensis* infection (**b**). N=6 mice per group.

To demonstrate requirements for T cell egress from immune organs in the resolution of primary *N. brasiliensis* infection, control and FTY720 treated mice were infected with *N. brasiliensis*. Mice were killed at either 2 or 10-days P.I and intestinal worm burdens were quantified at day 10 P.I (**Fig 4.3a**). Control mice had resolved infection, whereas FTY720 treated mice showed high intestinal worm burdens, indicative of a severe impairment in their ability to resolve infection (**Fig 4.3b**). It has previously been demonstrated that pulmonary $CD4^+$ T cell responses closely relate to resolution of primary *N. brasiliensis* infection (35). To confirm whether impaired protection in FTY720 treated mice related to reduced peripheral T cell numbers, we quantified T cell subpopulations in the lung at day 2 and 10 P.I. Significantly reduced numbers of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells **(Fig 4.3ci)** and $CD4^{+}CD44^{lo}CD62L^{hi}$ naïve, $CD4^{+}CD44^{hi}CD62L^{lo}$ effector memory T cells were found in the FTY720 treated group compared to control (**Fig 4.3cii**). A feature of induction of Th2 immunity is up regulation of the IL-1 receptor-related molecule T1/ST2 on effector $CD4^{+}$ T cells (270). Untreated mice showed significantly increased numbers and a 10 fold increase in the proportions of pulmonary $CD4^{+}CD62L^{lo}T1/ST2^{+}$ T cell populations (**Fig 4.3d**) when compared to FTY720 treated group. Untreated groups showed increased numbers of $CD4^{+}$ effector T cell populations at day 10 when compared to day 2 P.I. This was associated with increased T1/ST2 expression and IL-13 cytokine levels.

Analysis of pulmonary cytokine production in response to *N. brasiliensis* infection by *N. brasiliensis* secretory antigen-stimulated whole-cell preparations revealed significantly impaired IL-13 secretion in FTY720 treated mice at day 2 and 10 P.I **(Fig 4.3e)**. These findings show a requirement for $CD4^{+}$ T cell migration from SLO to be important for host resolution of primary *N. brasiliensis* infection.







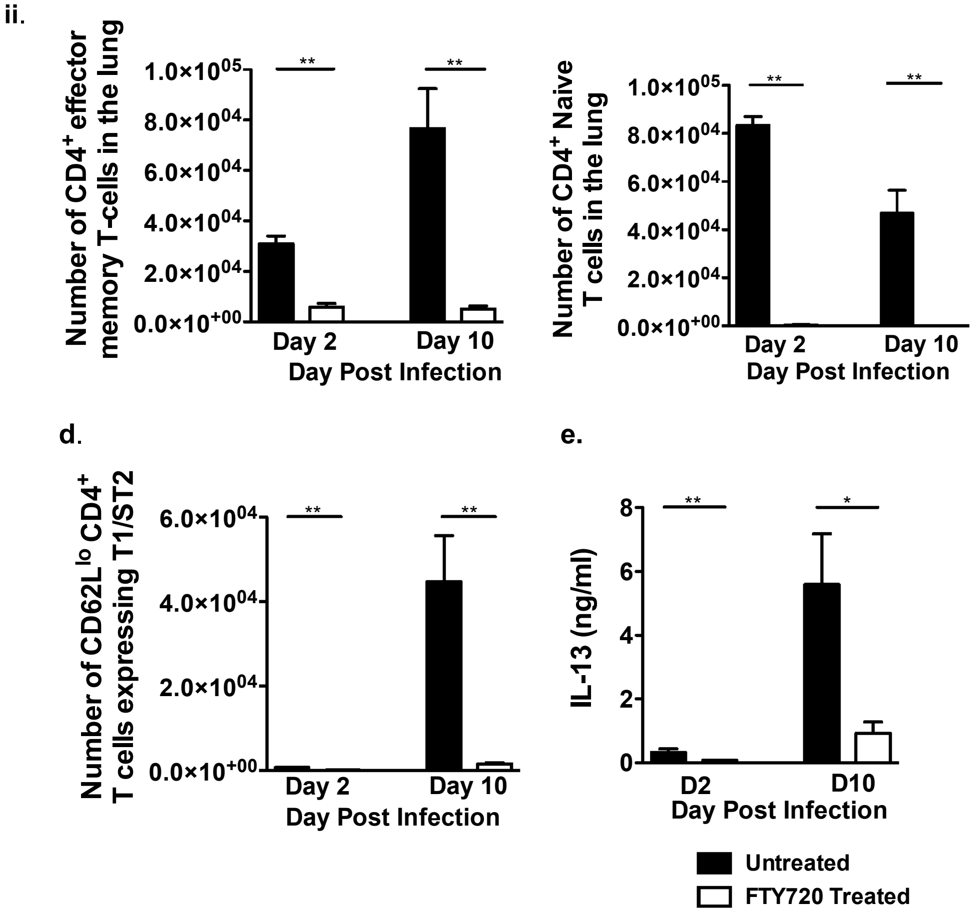


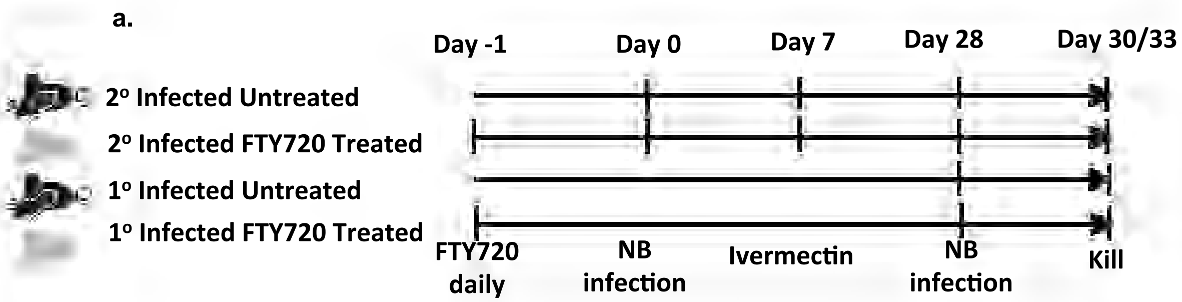
Fig 4. 3: Migration of T cells to the lung is required for protection against primary *N. brasiliensis* infection. Mice were treated daily with FTY720 beginning 1 day prior to infection and killed either at day 2 or 10 P.I (**a**). Impaired resolution of infection in FTY720 treated mice was established by quantification of intestinal adult worm burdens at day 10 P.I (**b**). Flow cytometric analysis of cell suspension of whole lung stained for $CD3^+CD4^+$, $CD3^+CD8^+$ (**ci**), $CD4^+CD44^{lo}CD62L^{hi}$ naïve, $CD4^+CD44^{hi}CD62L^{lo}$ effector memory (**cii**) and $CD4^+CD62L^{lo}T1/ST2^+$ T cells (**d**). Lung cells were re-stimulated with *N. brasiliensis* secretory antigen for 5 days, and secretion of IL-13 was detected by ELISA (**e**). Data are representative of 2 individual experiment. N=6-10 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

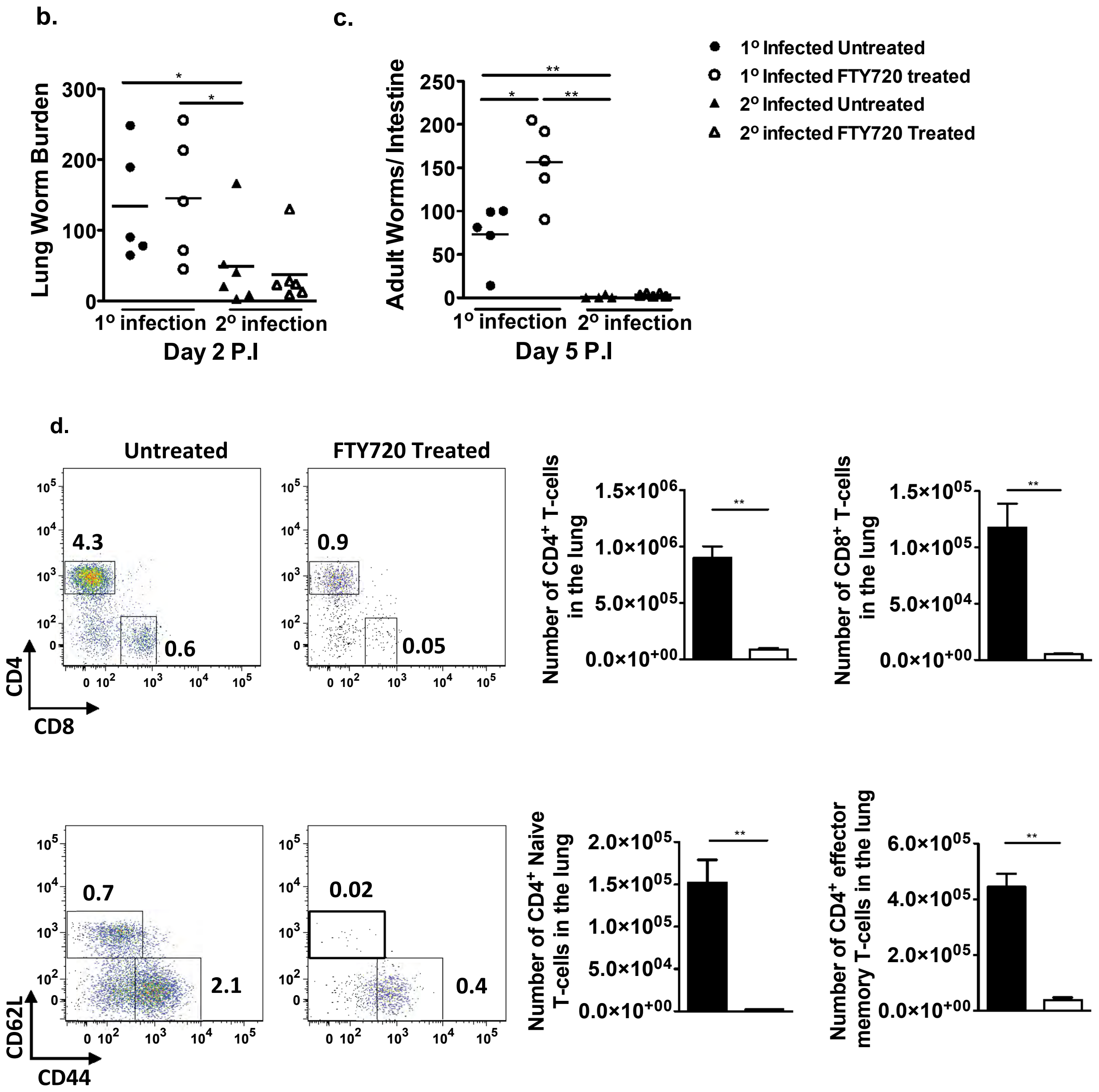
4.2.2 Lung-resident $CD4^+$ T cells are sufficient for protection against secondary N. brasiliensis infection.

$CD4^+$ T cells coordinate control of *N. brasiliensis* reinfection in the lung (36) and effective basophil IL-4 production in the lung is required by interaction with $CD4^+$ T cells following primary *N. brasiliensis* infection (73). We investigated if T cell recruitment to lung was required for this protective immunity to secondary *N. brasiliensis* infection. Mice were treated daily with FTY720 during the period of primary infection only

(**Appendix Fig 2**) or secondary infection only (**Appendix Fig 3**), as well as throughout primary and secondary *N. brasiliensis* infection (**Fig 4.4a**). This allowed us to dissect whether T cell recruitment was required at specific time periods for control of secondary infection. At 2 and 5 days post primary and secondary infection, respective lung (**Fig 4.4b**) and intestinal worm burdens (**Fig 4.4c**) were quantified. Interestingly, equivalent numbers of worms were found in FTY720 treated and untreated mice following secondary infection as opposed to primary infection, where higher worm burdens were observed in FTY720 treated group. Protection occurred irrespective of the significantly reduced T cell numbers, including $CD4^+$ effector memory T cells **(Fig 4.4d**) and reduced pulmonary IL-4 and IL-13 cytokine production (**Fig 4.4e**) in FTY720 treated mice at day 5 post secondary infection. Immuno-histological analysis of $CD3^+$ cell distribution in the lungs of untreated mice following secondary infection showed foci of $CD3^+$ cells at peri-bronchiolar sites and a uniform $CD3^+$ distribution in interstitial tissue. However in FTY720 treated mice, a significantly reduced localization of $CD3^+$ cells were observed around the peri-bronchiolar and interstitial regions when compared to untreated group **(Fig 4.4f).**

These results indicate that a small interstitially residing lung $CD4^+$ T cell population is sufficient to mount effective protective immunity to secondary *N. brasiliensis* infection.




b.
c.
d.
Lung Worm Burden
300
200
100
0
*
*
1° infection
2° infection
Day 2 P.I
Adult Worms/ Intestine
250
200
150
100
50
0
**
*
**
1° infection
2° infection
Day 5 P.I
● 1° Infected Untreated
○ 1° Infected FTY720 treated
▲ 2° Infected Untreated
△ 2° infected FTY720 Treated
Untreated
FTY720 Treated
4.3
0.6
0.9
0.05
CD4
CD8
Number of CD4⁺ T-cells in the lung
1.5×10⁰⁶
1.0×10⁰⁶
5.0×10⁰⁵
0.0×10⁺⁰⁰
**
Number of CD8⁺ T-cells in the lung
1.5×10⁰⁵
1.0×10⁰⁵
5.0×10⁰⁴
0.0×10⁺⁰⁰
**
0.7
2.1
0.02
0.4
CD62L
CD44
Number of CD4⁺ Naive T-cells in the lung
2.0×10⁰⁵
1.5×10⁰⁵
1.0×10⁰⁵
5.0×10⁰⁴
0.0×10⁺⁰⁰
**
Number of CD4⁺ effector memory T-cells in the lung
6.0×10⁰⁵
4.0×10⁰⁵
2.0×10⁰⁵
0.0×10⁺⁰⁰
**

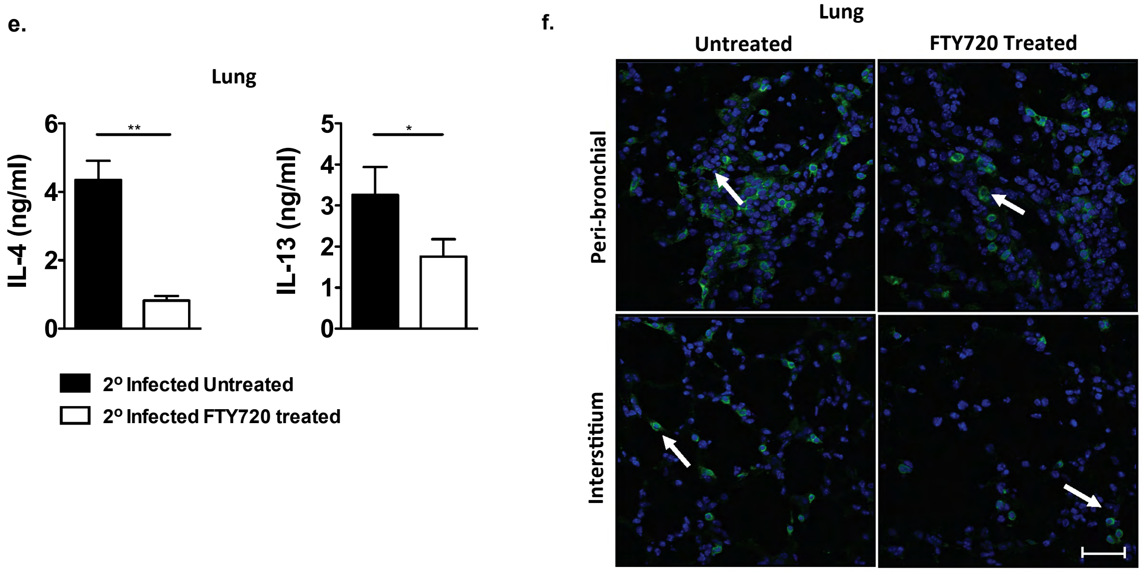


Fig 4. 4: Lung-resident CD4$^+$ T cell population are sufficient to confer protection against secondary *N. brasiliensis* infection. FTY720 treated and untreated mice were given a primary (1^o) or secondary (2^o) *N. brasiliensis* infection. Primary infections were cleared at day 7 by ivermectin treatment and mice were subsequently reinfected at day 28 and killed at day 2 or 5 P.I (**a**). Ability to resolve infection was established by quantification of lung worm burdens (**b**) and intestinal worm burdens (**c**) at day 2 and 5 P.I respectively. Flow cytometric analysis of cell suspension of whole lung stained for CD3$^+$CD4$^+$, CD3$^+$CD8$^+$, CD4$^+$CD44^{lo}CD62L^{hi} naïve and CD4$^+$CD44^{hi}CD62L^{lo} effector memory T cells at 5 days post secondary infection (**d**). Whole-cell preparations of lung were re-stimulated with *N. brasiliensis* secretory antigen for 5 days, and cytokine secretion for IL-4 and IL-13 was detected by ELISA (**e**). Confocal microscopy images of frozen sections of the lung stained with antibody to CD3 (green) and counterstained with Hoechst (blue) around the peri-bronchial and interstitial areas of the lung. White arrows indicate CD3$^+$ T cells. Scale bar is equivalent to 50μm (Data is representive of 1 individual experiment, N=8 mice per group) (**f**). Numbers in the flow cytograms indicate the percentage of T cells present relative to total cell numbers. Data are representative of 2 individual experiment. N=6-10 mice per group. * $P < 0.05$, ** $P < 0.01$.

4.2.3 Lung-resident CD4$^+$ T cell driven immunity to N. brasiliensis is independent of tertiary lymphoid structures

If CD4$^+$ T cell mediated protection is mediated by interstitially residing T cells, this would suggest that localization of these cells in an organized immune structure such as bronchial associated lymphoid tissue (BALT) is not required to mediate protection. We therefore blocked the formation of BALT like structures by treatment with the soluble decoy receptor lymphotoxin beta-receptor fusion protein (LTβR-Ig). LTβR signaling has been shown to be critical for development of not only lymphoid organs but also in the maintenance of tertiary lymphoid tissue structures at peripheral sites of inflammation

(129, 131, 132). *N. brasiliensis* infected mice were treated weekly with the antagonist LTβR-Ig or the isotype control MOPC21 (**Fig 4.5a**). Efficiency of treatment was confirmed by the depletion of splenic marginal zone B cells in the LTβR-Ig treated group (**Fig 4.5b**) and abrogated germinal center and marginal zone development in spleens of LTβR-Ig treated mice as shown previously (130, 131) (**Fig 4.5c**). Adult worm burdens were quantified at 5 days P.I. Equivalent numbers of worms were found in vehicle control and LTβR-Ig treated mice (**Fig 4.4d**). These results indicate that tertiary lymphoid structures are not required for lung-resident T cell mediated immunity to *N. brasiliensis* re-infection.

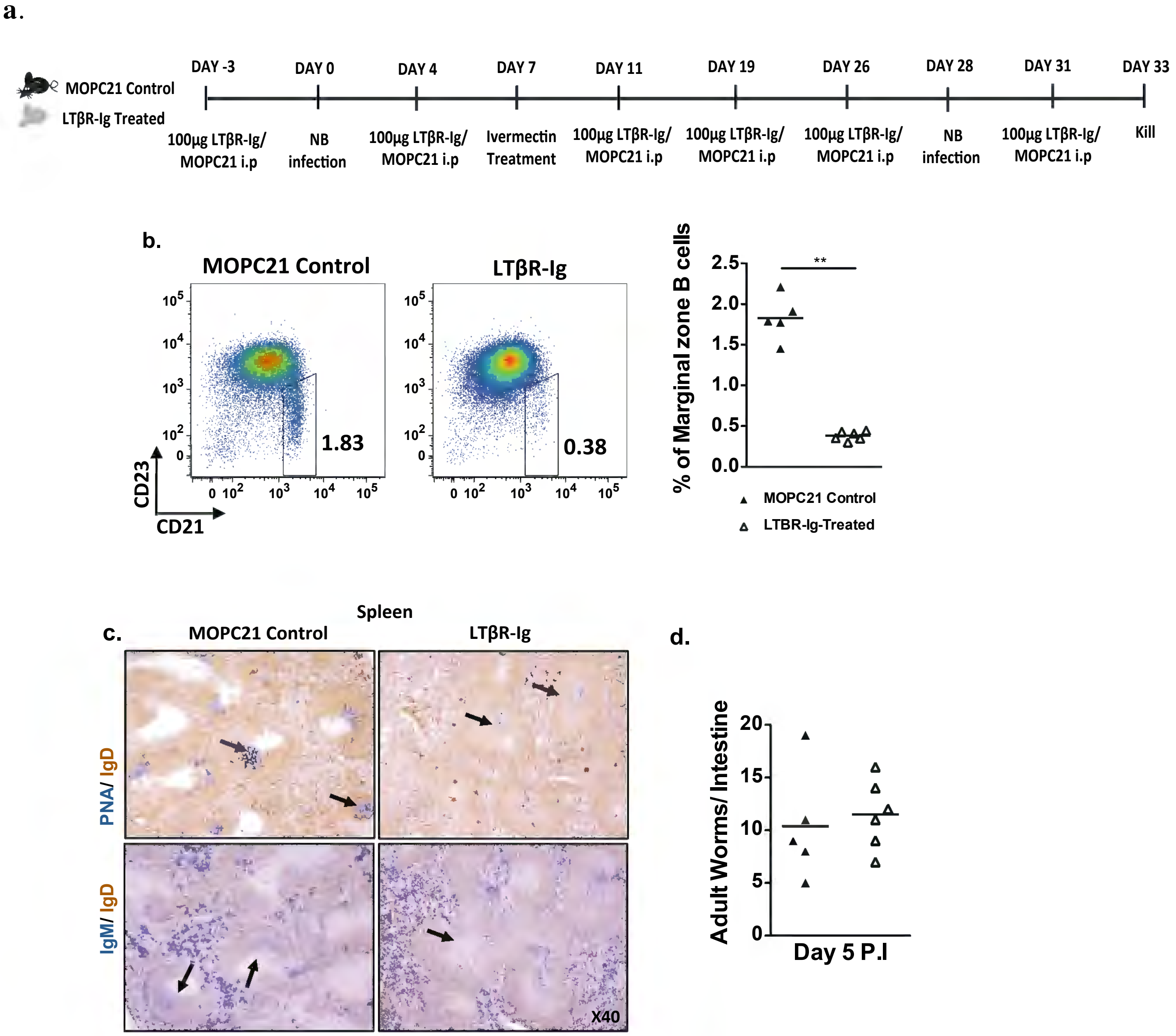
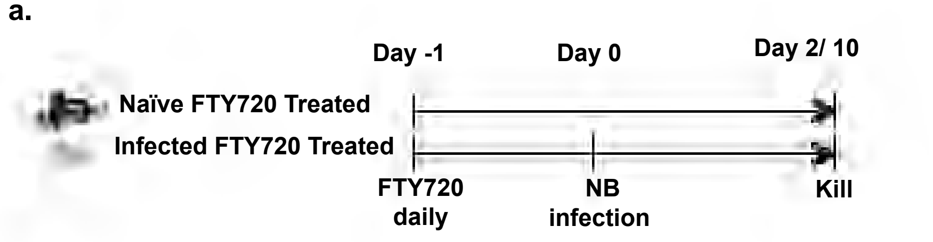


Fig 4. 5: Lung resident T cells are independent of tertiary lymphoid structures. Secondary *N. brasiliensis* infected mice were treated weekly with 100μg MOPC21 isotype or LTβR-Ig beginning 3 days prior to primary infection. Primary infections were cleared at day 7 by ivermectin treatment and mice were subsequently reinfected at day 28 (**a**). Efficiency of the treatment was confirmed by flow cytometric analysis of B220$^+$CD23^{lo}CD21^{hi} marginal zone B cells (**b**). This was further confirmed by staining spleens with either CD3 (blue)/ IgD (brown) or PNA (blue)/ IgD (brown) to look at marginal zone B cells and germinal centers respectively. Arrows indicate location of these structures. (**c**). Adult worm burdens were quantified at day 5 post secondary infection to evaluate ability to control infection (**d**). Numbers in the flow cytograms indicate the percentage of marginal zone B cells cells relative to lymphocytes. Data are representative of 1 individual experiment. N=6 mice per group.** P< 0.01.

4.2.4 Expansion of lung resident T cells occurs in absence of T cell recruitment from lymph node

To further define development of *N. brasiliensis* responsive lung resident CD4$^+$ T cells, we compared T cell immunity in FTY720 treated naïve and *N. brasiliensis* infected mice (**Fig 4.6a**). Goblet cell mucus production, required for parasite expulsion (91) was only apparent in infected mice (**Fig 4.6b**). Re-stimulation of lung cells showed infected mice to secrete higher levels of IL-13 when compared to naïve mice (**Fig 4.6c**). Quantification of pulmonary T cell populations at day 2 and 10 P.I showed significantly increased numbers of CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ (**Fig 4.6di**), effector memory and naïve CD4$^+$ T cells (**Fig 4.6dii**) in infected mice when compared to naïve mice. Infected mice showed significantly increased numbers and proportions of lung resident activated CD4$^+$CD62L^{lo} T cells expressing T1/ST2 (**Fig 4.6diii**), indicative of an increased Th2 potential.

Together these data suggest that in acutely lymphopenic mice, a pre-existing CD4$^+$ T cell population in the lung expands in response to *N. brasiliensis* infection to induce a local and protective Th2 immune response to *N. brasiliensis*.



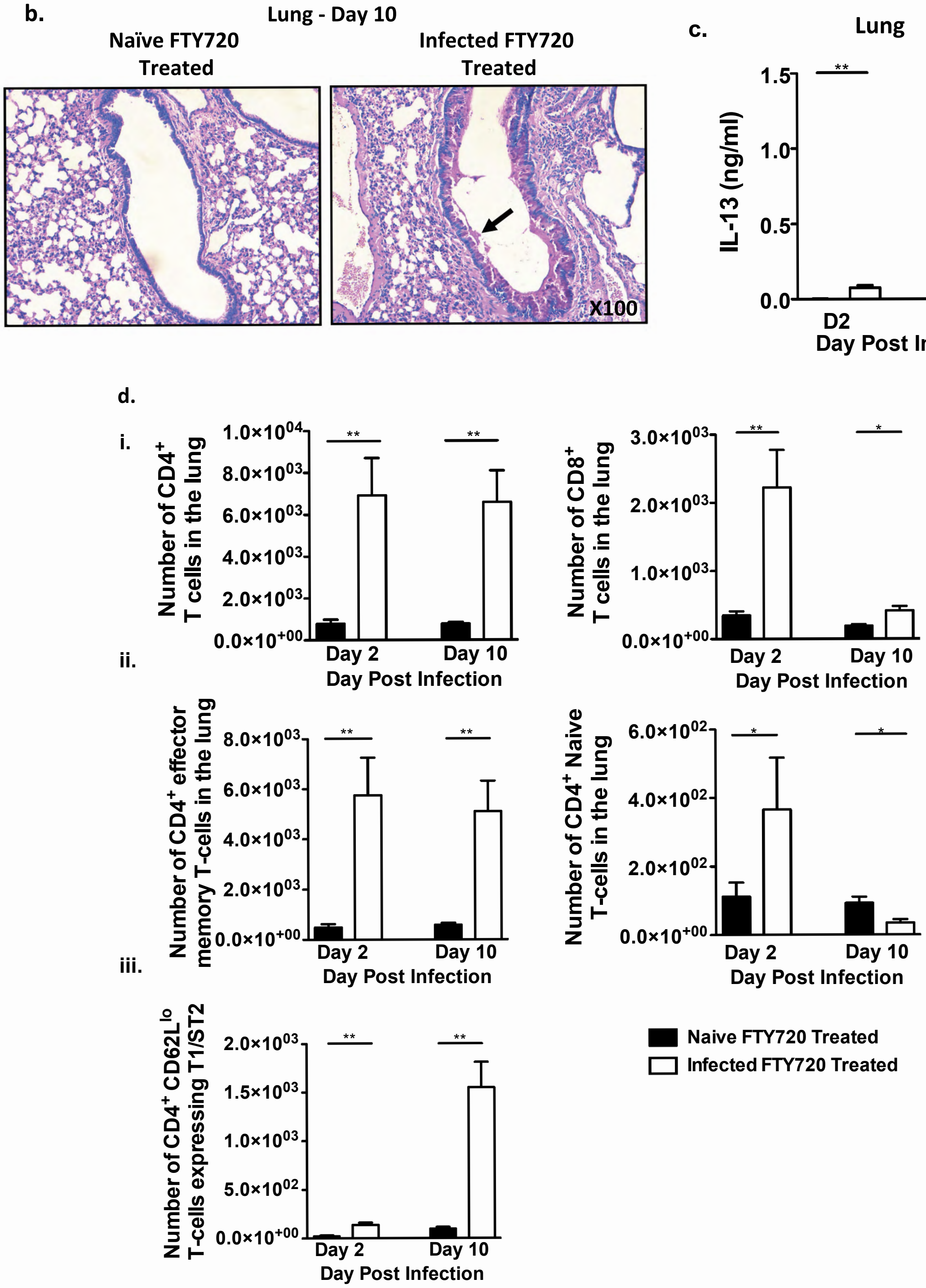


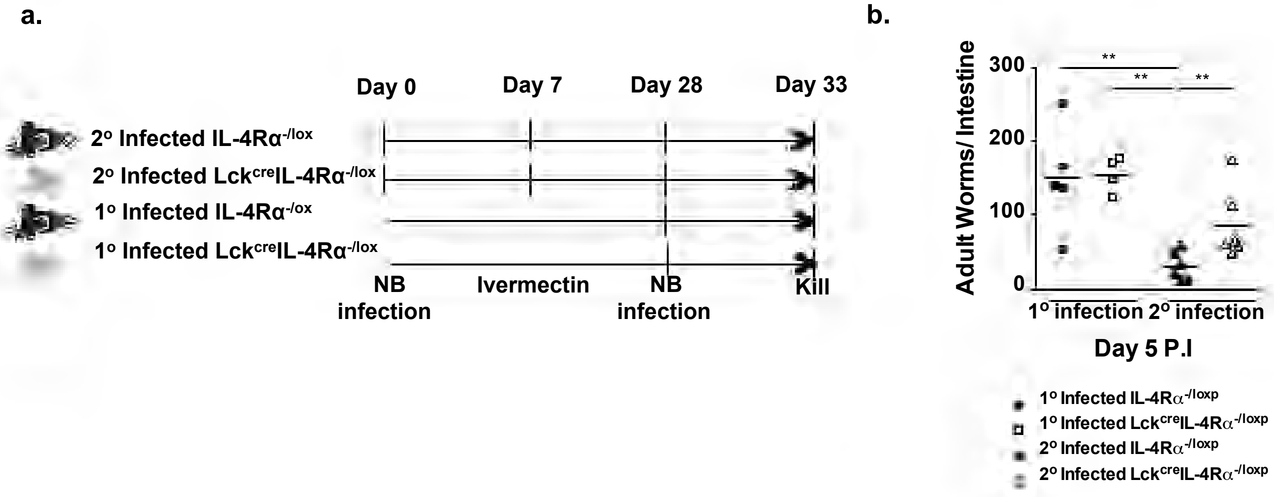
Fig 4. 6: Expansion of lung-resident CD4$^+$ T cells occurs in the absence of T cell recruitment. Naïve and *N. brasiliensis* infected mice were treated daily with FTY720 and killed at either 2 or 10 days P.I (**a**). Pulmonary tissue was removed, fixed in formalin and stained with PAS to examine mucus production, 100X magnification. (**b**). Whole-cell preparations of lung were re-stimulated with *N. brasiliensis* secretory antigen for 5 days, and cytokine secretion for IL-13 was detected by ELISA (**c**) Single-cell suspensions of whole lung was stained for CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ (**di**), CD4$^+$CD44^{lo}CD62L^{hi} naïve, CD4$^+$CD44^{hi}CD62L^{lo} effector memory

T cells (**dii**) and CD3$^+$CD4$^+$CD62L^{lo}T1/ST2$^+$ T cells and analyzed by flow cytometry (**diii**). Data are representative of 2 individual experiment. N=5-6 mice per group. * $P < 0.05$, ** $P < 0.01$.

4.2.5 Protective immunity to N. brasiliensis re-infection is dependent on T cell IL-4Rα expression

IL-4Rα signaling is essential for immunity to a range of helminth infections (44–46, 264). In primary *N. brasiliensis* infection, worm expulsion does not require IL-4Rα responsiveness by T cells, however infected CD4$^+$ T cell IL-4Rα KO (Lck^{cre}IL-4Rα$^{-/lox}$) mice do have significantly reduced Th2 responses and airway mucus production (35). This indicates that T cell IL-4Rα responsiveness may contribute significantly to rapid adaptive response which controls an *N. brasiliensis* re-infection.

N. brasiliensis re-infection studies comparing primary and secondary infected IL-4Rα$^{-/lox}$ and T cell specific IL-4Rα KO (Lck^{cre}IL-4Rα$^{-/lox}$) mice at 5 days P.I (Fig **4.7a**), demonstrated that effective control of secondary *N. brasiliensis* infection required expression of IL-4Rα on T cells. Here secondary infected Lck^{cre}IL-4Rα$^{-/lox}$ mice showed significantly increased intestinal worm burden at day 5 P.I when compared to IL-4Rα$^{-/lox}$ control mice (**Fig 4.7b**). This reduced protection was associated with decreased numbers of pulmonary T cell populations (**Fig 4.7c**) and IL-13 cytokine production in the lung (**Fig 4.7d**). These data further support a role for Th2 CD4$^+$ T cells in coordinating optimal host immunity to *N. brasiliensis*.



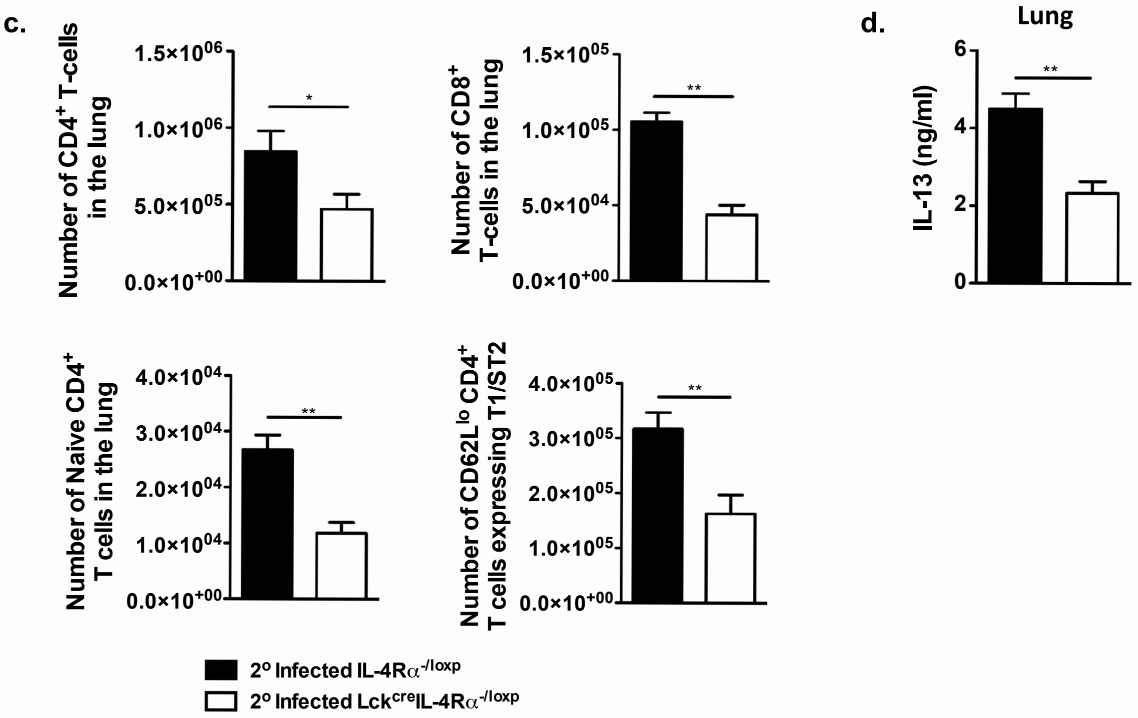


Fig 4. 7: T cell IL-4Rα expression is required for optimal immunity to *N. brasiliensis* reinfection. IL-4Rα$^{-/loxp}$ and Lck^{cre}IL-4Rα$^{-/loxp}$ were given a primary (1^{o}) or secondary (2^{o}) *N. brasiliensis* infection. Primary infections were cleared at day 7 by ivermectin treatment and mice were subsequently reinfected at day 28 and killed at 5 days P.I (**a**). Intestinal worm burdens were assessed to determine protection kinetics (**b**). CD3^{+}CD4^{+}, CD3^{+}CD8^{+}, CD3^{+}CD4^{+}CD44^{lo}CD62L^{hi} naïve and CD3^{+}CD4^{+}CD62L^{lo}T1/ST2^{+} T cell numbers were quantified by flow cytometric analysis of single cell suspensions of whole-lung (**c**). Whole-cell preparations of lung were re-stimulated with *N. brasiliensis* secretory antigen for 5 days and cytokine secretion for IL-13 was detected by ELISA (**d**). Data are representative of 2 individual experiments. N=6 mice per group. * $P < 0.05$, ** $P < 0.01$.

4.2.6 Lung-resident T cells require IL-4Rα expression for optimal immunity to N. brasiliensis reinfection

FTY720 treatment of secondary infected IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice (**Fig 4.8a**) showed that control of secondary infection by the lung-resident T cells was also IL-4Rα dependent. FTY720 treated Lck^{cre}IL-4Rα$^{-/lox}$ mice showed significantly increased intestinal worm burdens at day 5 P.I when compared to IL-4Rα$^{-/lox}$ control mice (**Fig 4.8b**). Reduced protection was associated with a decrease in IL-13 cytokine production in the lung of Lck^{cre}IL-4Rα$^{-/lox}$ mice (**Fig 4.8c**). No differences were observed in the proportions of T cell subsets between the FTY720 treated IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice (**Fig 4.8d**).

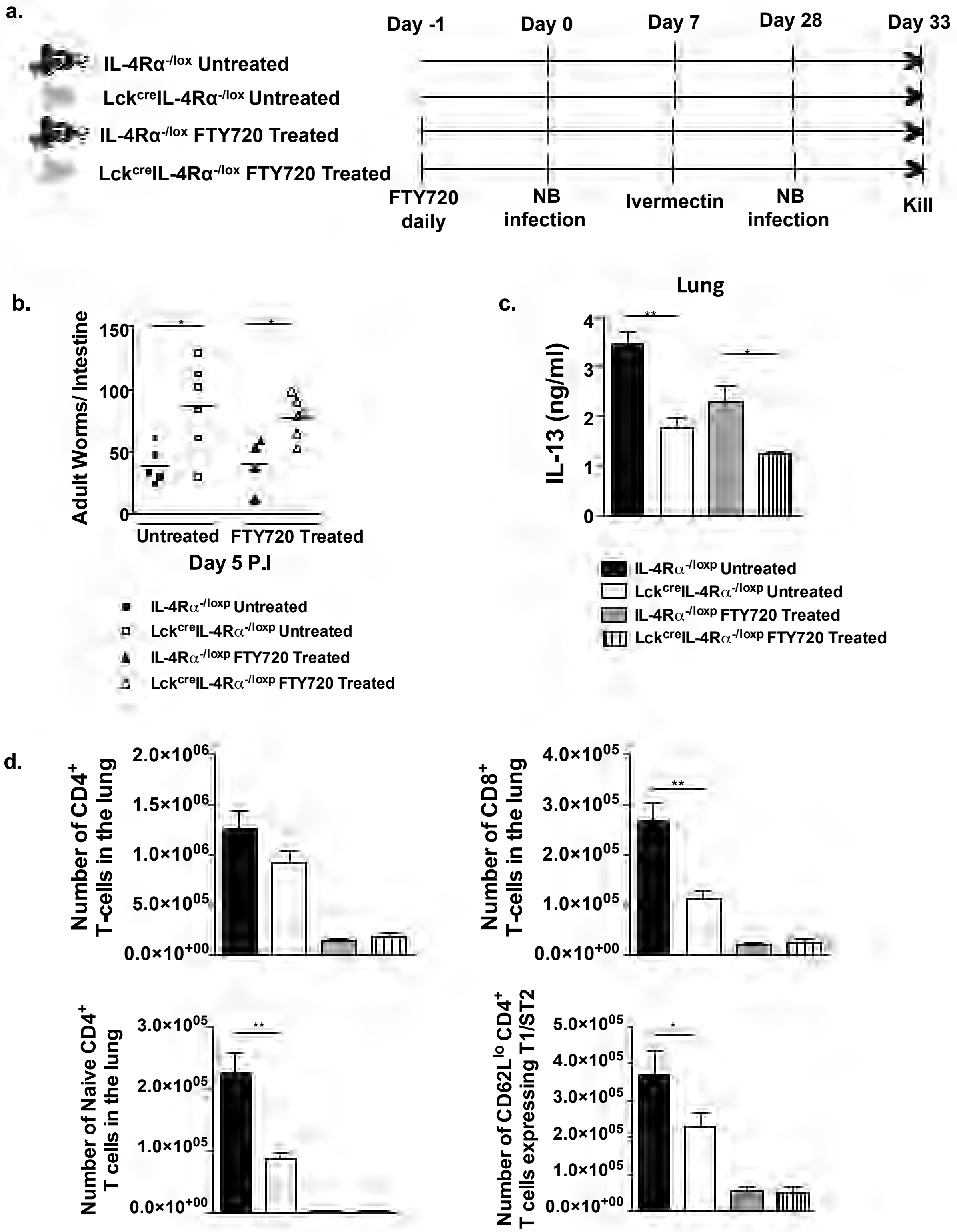


Fig 4. 8: Lung-resident T cells require IL-4Rα expression for optimal immunity to *N. brasiliensis* reinfection. IL-4Rα$^{-/loxp}$ and Lck^{cre}IL-4Rα$^{-/loxp}$ were treated with FTY720 daily or left untreated. Primary infections were cleared at day 7 by ivermectin treatment and mice were given

a secondary *N. brasiliensis* infection at day 28 and killed at 5 days P.I (**a**). Intestinal worm burdens were assessed to determine protection kinetics (**b**). Whole-cell preparations of lung were re-stimulated with *N. brasiliensis* secretory antigen for 5 days and cytokine secretion for IL-13 was detected by ELISA (**c**). CD3$^+$CD4$^+$,CD3$^+$CD8$^+$, CD3$^+$CD4$^+$CD44^{lo}CD62L^{hi} naïve and CD3$^+$CD4$^+$CD62L^{lo}T1/ST2$^+$ T cell numbers were quantified by flow cytometric analysis of single cell suspensions of whole-lung (**d**). Data are representative of 2 individual experiments. N=6 mice per group. * $P < 0.05$, ** $P < 0.01$.

4.2.7 IL-4Rα expressing lung-resident CD4$^+$ T cells enhance protection to N. brasiliensis reinfection.

To demonstrate if lung resident CD4$^+$ T cells conferred IL-4Rα dependent protection against *N. brasiliensis* reinfection, we isolated CD4$^+$ T cells from the lungs of *N. brasiliensis* infected IL-4Rα$^{-/lox}$ or Lck^{cre}IL-4Rα$^{-/lox}$ mice treated daily with FTY720. Cells were adoptively transferred intra-nasally into naïve BALB/c mice and subsequently infected with *N. brasiliensis* (**Fig 4.9a**). Transfer of primed IL-4Rα-responsive lung derived CD4$^+$ T cells into naïve BALB/c mice resulted in reduced intestinal worm burdens when compared to recipients of lung derived Lck^{cre}IL-4Rα$^{-/lox}$ CD4$^+$ T cells (**Fig 4.9b**). These data confirm that lung-derived CD4$^+$ T cells from lymphopenic mice are important for protection and show that IL-4Rα responsiveness by these lung CD4$^+$ T cells significantly contribute to effective recall immunity to *N. brasiliensis* infection.

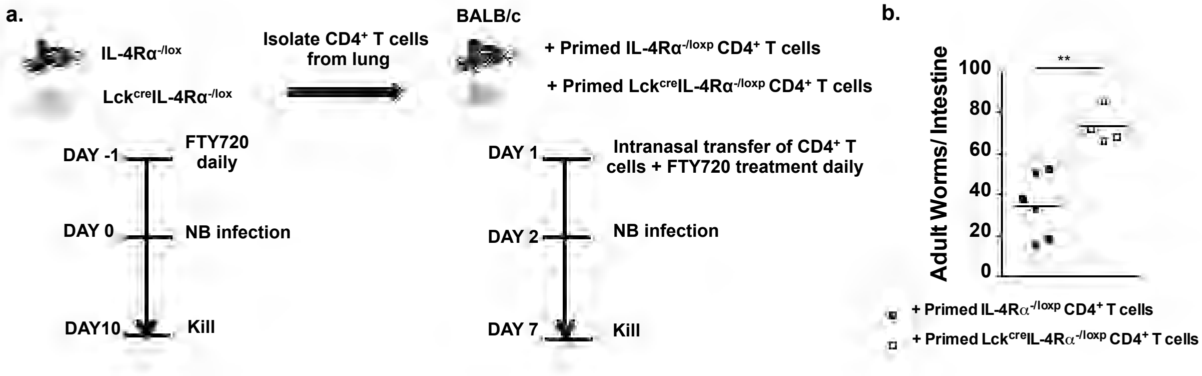


Fig 4. 9: IL-4Rα expressing lung-resident CD4$^+$ T cells enhance protection to *N. brasiliensis* infection. CD4$^+$ T cells were isolated from the lungs of FTY720 treated *N. brasiliensis* infected IL-4Rα$^{-/lox}$ or Lck^{cre}IL-4Rα$^{-/lox}$ mice and intra-nasally transferred into FTY720 treated naïve BALB/c mice (**a**). Naïve mice were then infected with 500 L3 *N. brasiliensis* larvae and worm burdens were quantified at 5 days P.I (**b**). Data are representative of 2 individual experiments. N=4-6 mice per group. ** $P < 0.01$.

4.3 Discussion

The key finding in this study demonstrates that in acutely lymphopenic mice a pre-existing CD4$^+$ T cell population in the lung can expand in response to *N. brasiliensis* infection. Expansion of this population was associated with induction of a local and protective Th2 immune response to *N. brasiliensis* infection. Importantly this indicates that CD4$^+$ T cell immunity to *N. brasiliensis* re-infection is independent of CD4$^+$ T cell recruitment from the LN.

Our data supports and builds on previous studies that indicate the importance of peripheral tissue effector T cells in coordinating host pulmonary immunity (127, 253, 259, 273–276). As discussed earlier, these cells can mount an effective secondary T cell response at the site of infection leading to increased pathogen clearance. For example, in response to BCG challenge, protective immune responses have been associated with development of a lung resident multi-functional CD4$^+$ memory T cell population independent of recruitment from secondary lymphoid tissue (127, 277). Similarly, in experimental respiratory virus infection, virus specific memory CD4$^+$ and CD8$^+$ T cells that persist in the lung enable control of challenge infections (253, 266, 271). This protective immunity could be associated with effector lymphoid tissue like structures such as bronchial associated lymphoid tissue (BALT), which contain organized B and T cell areas and support local lymphocyte proliferation (278, 279). However, T cells have been shown to respond to presented antigen in a LN free environment (127, 256, 263). The use of LTβR-Ig in our study shows that protective immunity was generated in an environment with impaired stromal cell organization of immune cells in the lung. This suggests that lung interstitial residing T cell populations were responsible for the protective immunity we demonstrate in this study.

Our study also shows that CD4$^+$ T cells contribute significantly to control of a secondary *N. brasiliensis* infection. Previous studies have shown that depletion of CD4$^+$ T cells during secondary infection does not impair recall immunity to *N. brasiliensis* infection (103). However,depletion of CD4$^+$ T cells in both primary and secondary infection does impair recall immunity (36). Taken together, these studies suggest that $CD4^+$ T cells are required for host immune priming after initial infection, but not after secondary infection. This suggests that in the initial infection, these resident $CD4^+$ T cells are activating resident effector cells.

The work we present here builds on these findings by demonstrating that IL-4Rα expression on T cells is required for optimal immunity to secondary *N. brasiliensis* infection. Our adoptive transfer studies clearly confirm that this protection can be driven by lung resident T cell population responses to Th2 cytokines. This represents an important expansion in our understanding of cell specific IL-4Rα contributions to immunity against helminth infection. This is, to the best of our knowledge, the first demonstration of IL-4Rα expression on a hematopoietic cell population contributing to the control of *N. brasiliensis* infection.

These findings are of importance to our understanding and control of analogous helminth infections such as hookworms. We suggest that effective control by vaccination may be most effective where the protective challenge manipulates host immunity to the parasite in the lung.

The most striking finding of this study is however a redundancy for SLO derived $CD4^+$ T cells in recall immunity to *N. brasiliensis*. This finding has important implications for understanding related Th2 pathologies, such as allergic airway disease and other chronic pulmonary diseases. Furthermore, it sets new targets for vaccine-induced immunity by suggesting that adaptive immune responses at peripheral sites of infection are independent of SLO.

Chapter 5: Concluding remarks/ Future work

Helminth infections are currently a major global health problem. The principle health concerns are associated with high rates of morbidity (15, 16). Although strategies involving mass administration of anthelmintic drugs such as albendazole are in place for the global control of these infections, host inability to develop effective immune memory along with the rise of drug-resistance confound the efficacy of these efforts. Therefore, finding candidate molecules and discrete cell populations that enhance these responses would provide new targets for treating infection via specific host immune-modulation and would contribute to the development of effective vaccines against nematode infections.

An important anatomical location for controlling the immune responses following *N. brasiliensis* infection is the lung; responses here form an important component of the host protective immune response to re-infection (36, 102). The human hookworm *A. duodenale* and infections of *A. lumbricoides, T. canis and S. stercoralis* migrate through the lung as an obligatory part of their developmental life cycle. This makes it imperative to examine the immunological alterations that occur at this tissue site during infection.

In this study we have addressed how 2 novel components of host innate and adaptive immunity can contribute to pulmonary control of the parasitic nematode, *N. brasiliensis*. We have shown that the pulmonary epithelial cell secreted protein SP-D has an important host defense function during *N. brasiliensis* infection. It can directly bind to the lung L4 larvae and mediate Th2 protective responses that are essential for controlling infection. We further show that the lung can mount a protective Th2 response independently of T cell recruitment from the LN and this response is driven by a lung-resident IL-4Rα responsive $CD4^+$ T cell population (32). Our data augments other studies that have highlighted the role for the lung as an effective tissue for resolving parasitic infection.

As more studies are beginning to unravel the helminth induced Th2 responses, many new effector cell types and effector mechanisms are now being identified. This illustrates how broad and complex the Th2 immunity is. The diversification and redundancy existing in

Th2 immunity suggests that host defenses appear to be constantly adapting new mechanisms to counter helminth evasion strategies (47). Helminthes can survive in the host for long periods of time. A key strategy used by them to establish chronicity is immuneregulation and the induction of a strong regulatory T cell response that inhibits effective Th2 responses. In the event that the host is successful in mounting helminth resistance, this often occurs at the cost of establishing pathology and therefore greatly confounds development of effective Th2 immunity (109).

During helminth infection, strong FoxP3$^+$ Treg responses are elicited. These cells are essential for immune-regulation and control of helminth-induced pathology by dampening excessive inflammatory responses to prevent tissue damage. However helminth parasites tend to have exploited the induction of strong Treg responses as a mechanism to suppress development of Th2 protective immunity. The ability of helminths to induce strong regulatory T cell responses have raised new prospects of harnessing it as a therapeutic for reducing allergy associated inflammation and control of autoimmune diseases. However it has also posed serious challenges for the design of effective anti-helminth vaccines that is able to overcome the induced immunesuppression (109, 280, 281). Therefore, an understanding of this regulatory responses will allow us not only to enhance Th2 immune responses but also explore the therapeutic potential of using helminth regulatory strategies as a new approach in controlling exacerbated inflammatory conditions.

The work presented in chapter 3 of the finding that SP-D is an important component of host immunity to *N. brasiliensis* infection, offers a potentially new insight into how helminthes regulate and get regulated by host immunity. In our work, we have extended a role for SP-D in parasitic infections. Our data offers a possible mechanism by which the lung mediates a well-regulated protective response. It is known that SP-D can mediate immune-modulatory roles during allergen-induced airway inflammation. SP-D also controls inflammation within the lung by inhibiting IL-2 production from lymphocytes thereby preventing proliferation of T cells, diminishing lipid peroxidation and oxidative cellular lung injury (182, 184, 185). Our findings suggest SP-D to be a central regulator in the lung that induces sufficient protective responses and at the same time limits it to prevent excessive inflammation.

Complementing this is our observation that Th2 protective responses can be mediated by a lung-resident CD4$^+$ T cell population independent of T cell recruitment. This finding demonstrates a redundancy in the role of LN derived T cells and has important implications for understanding related Th2 pathologies, such as allergic airway disease and other chronic pulmonary diseases. It suggests that the pathology induced following respiratory infections are largely mediated by the T cells migrating from the LN while protective responses are largely mediated by lung resident T cells.

Whether SP-D can interact directly with these lung-resident T cells remains to be investigated. An intriguing possibility is the ability of SP-D to modulate lung Th2 effector responses by inducing a regulatory T cell population that is required to control the helminth associated pathology and at the same time induce a sufficient protective response. A recent study has shown the ability of SP-A to induce a Treg population by mechanisms that involves TGF-β production (282). Whether SPD uses similar mechanisms will need to be examined. Another appealing mechanism could be by direct binding to macrophages and DCs and modulating lung-resident T cell responses via these effector cells. Indeed it has been shown that lung-resident AMs can induce the generation of a FoxP3$^+$ regulatory T cell population via mechanism involving TGF-β (148).

All together, our findings represent an important expansion to the role of pulmonary innate and adaptive immune responses to helminth infections. It raises important questions that require further exploration. Gaining better understanding of these pulmonary immunological alterations that occur would address many gaps in our knowledge and serve to provide new targets for inducing vaccine-mediated immunity. It would also provide an important basis for understanding the rapid protective immunity that develops in the lung in response to challenge infections and suggests the ability of this tissue to provide a conducive environment for supporting the maintenance and functional properties of the Th2 immune response.

Future work:

The mechanism with which SP-D enhances Th2 immunity to nematode infection and interacts with the pulmonary immune environment specifically the lung-resident cells is yet to be understood. How nematode infections control and is controlled by SP-D levels needs to be further investigated. More importantly, how SP-D modulates the functions of its principle cellular source, ATII cells would be of particular interest. This is because ATII cells function as the first line of defense and its activation initiates a wide range of Th2 responses (47). Identifying where in hierarchy of Th2 immune response SP-D lies, will provide insight in to whether SP-D is involved in the initiation phase of the response or its maintenance and amplification. Furthermore, the use of SP-D KO mice would provide a more comprehensive understanding of the importance of SP-D in protective immunity to helminthes.

Having demonstrated the therapeutic potential of rfhSP-D in mouse models, we suggest that SP-D represents a potentially new therapeutic candidate for treatment of human nematode infections. In order to confirm this, translational studies are required to answer the following key questions:

1. Does human nematode infection increase SP-D titers
2. How does it influence the cellular source of SP-D
3. Does SP-D influence the immune cell responses to human nematode infection
4. Does it act as an interface between the parasite and host immune system

This will provide an important understanding on how SP-D modulation of host immunity may be used clinically to protect against a parasitic helminth infection. It will also identify how SP-D fundamentally alters cellular immunity in helminth-infected individuals.

It is hoped that these further studies will help to unravel specific targets for achieving vaccine-induced protective immunity.

6. References

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Appendix

A. General Buffer Recipes

Digestion Buffer:
0.002g DNase I (Roche Germany)
0.0200g Collagenase Type I (Gibco-Invitrogen)
Dissolve reagents in 150 ml DMEM (containing 100U/ml penicillin G, 100μg/ml streptomycin). Filter sterilize with a 0.22μM filter and store at 4oC for up to 7 days.

ELISA Blocking Buffer:
20g Powder Milk
0.2g NaN_3
Dissolve reagents in a final volume of 1000ml 1X PBS and store at 4°C.

ELISA Dilution Buffer:
10g BSA
0.2g NaN_3
Dissolve the above reagents in a final volume of 1000ml of 1X PBS and store at 4°C.

ELISA Washing Buffer:
20g KCL
20g $KH_2HPO_4.2H_2O$
800g NaCl
50ml Tween-20
100ml 10% NaN_3
Make up to 5L with ddH2O and store at room temperature. Dilute in 1:20 in ddH2O.

ELISA Substrate Buffer (For horseradish peroxidase conjugates):
Peroxidase Substrate Solution B (Roche Diagnostics GmbH, Mannheim, Germany)
TMB Peroxidase Substrate Solution A (Roche Diagnostics GmbH)
Just before use, mix equal volumes of TMB Peroxidase Substrate (Solution A) with Peroxidase Substrate Solution B.

MACS Buffer:
2mM EDTA
0.5% BSA
Dissolve the above in a final volume of 1000ml of 1X PBS and store at 4°C

Binding Buffer:
1XPBS
0.2% BSA
1mM$CaCl_2$

Red Cell Lysis Buffer:
8.34g NH_4Cl
0.037g EDTA
1.0g $NaHCO_3$
Dissolve reagents in 1000ml ddH2O. Filter sterilize (0.22μM) and store at 4 or 25°C.

Permeabilization Buffer:
0.5g Saponin
0.055g $CaCl_2$
0.0625g $MgSO_4$
0.25g NaN_3
0.5g BSA
10mM Hepes
Dissolve in a final volume of 500ml of 1 X PBS and store at 4°C

Tris Buffer:
1L of 200mM Tris base
1.5L of 154mM physiological NaCl
1.0L of 0.1M HCL
pH as desired (7.6 / 9.2)

Horseradish Peroxidase Substrate
Dissolve a single 3-3'-diaminobenzidine tetrahydrchloride (DAB) tablet (Sigma Aldrich) in 15ml Tris buffer pH 7.6. Filter to 10ml and add 1 drop of hydrogen peroxide (Sigma Aldrich). A single drop of HRP substrate was added to each section until the desired level of positivity was reached, whereupon slides were washed in Tris buffer pH 7.6.

Alkaline Phosphate Substrate:
Dissolve 8mg of Levamisole ((-)- Tetramisole hydrochloride; Sigma Aldrich) in 10ml Tris buffer (pH 9.2) to block endogenous phosphate activity. In a fume hood dissolve 4mg of napthol AS-MX phosphate (Sigma Aldrich) in 380ul dimethyl-formamide in a glass bottle. Add this to the middle of the levamisole solution, ensuring it does not touch the plastic of the tube. Add 10mg of the Fast Blue BB salt. Vortex and filter the solution and add a single drop to each section until the desired level of positivity is obtained.

B. Figures:

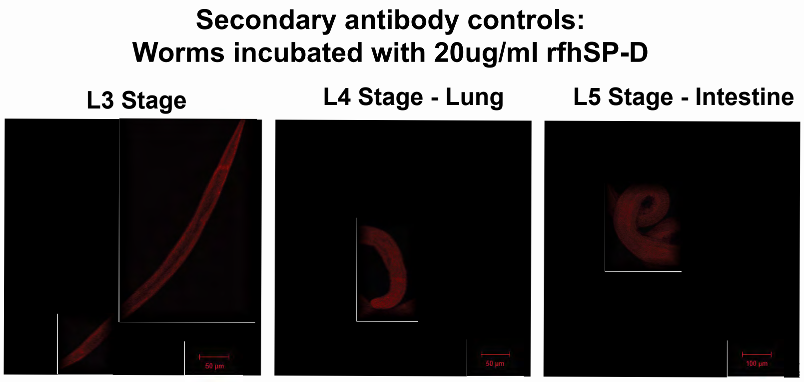
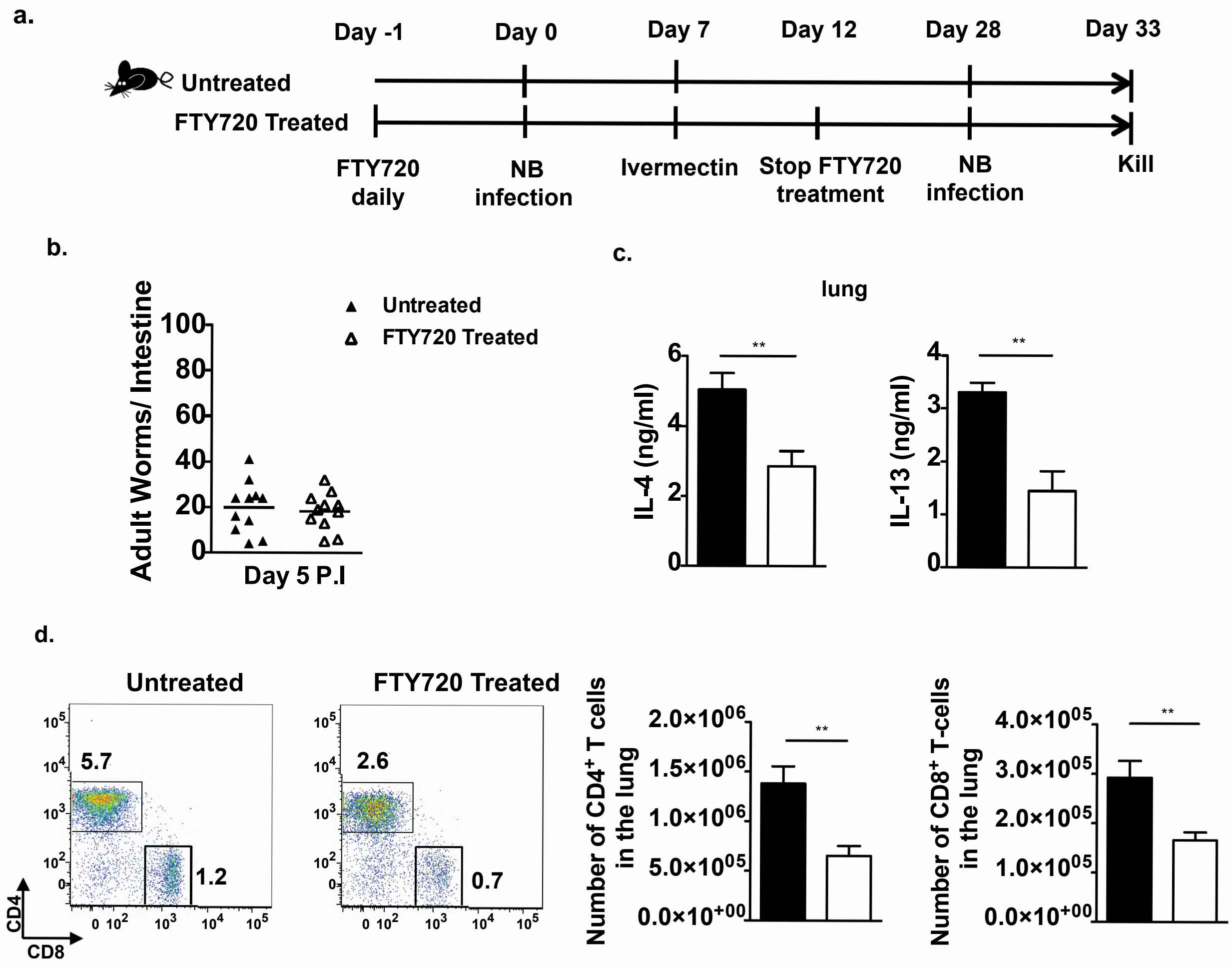


Fig 1: **rfhSP-D binds to L4 stage of *N. brasiliensis***. Confocal microscopic images of Secondary antibody only controls of L3, L4 and L5 stage of *N. brasiliensis* that were incubated with 20µg/ml of rfhSP-D. Data are representative of 2 individual experiment.



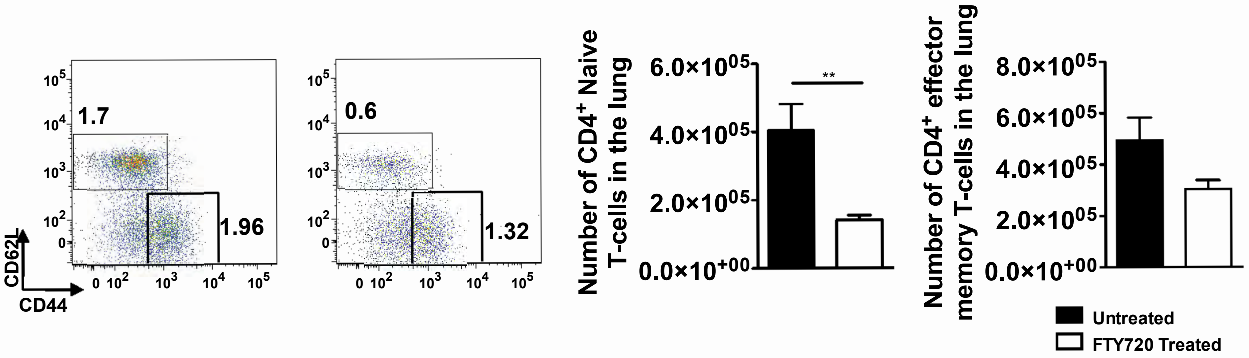
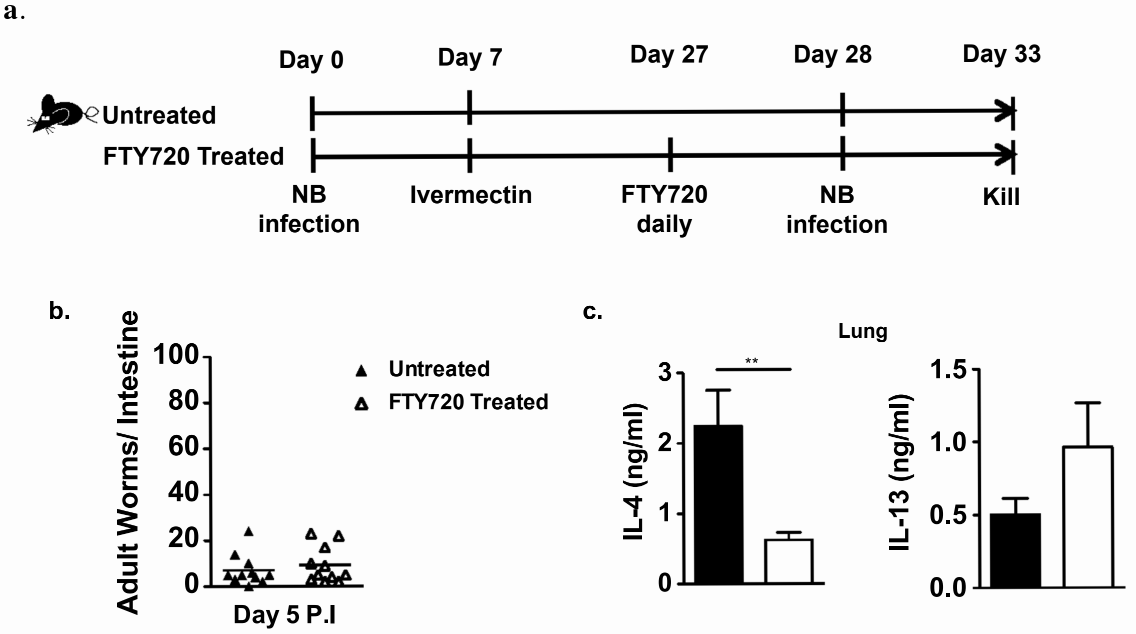


Fig 2: FTY720 treatment during primary *N. brasiliensis* infection does not affect ability to resolve re-challenge infection. Mice were treated daily with FTY720 beginning 1 day prior to primary infection. FTY720 treatment ceased on day 12 P.I. Primary infection was cleared at day 7 by ivermectin treatment and mice were given a secondary *N. brasiliensis* infection at day 28 before being killed at day 5 P.I (**a**). Adult worm burden from intestine was evaluated to examine ability of protection (**b**). Whole-cell preparations of lung were re-stimulated with NES proteins for 5 days, and cytokine secretion for IL-4 and IL-13 was detected by ELISA (**c**). Flow cytometric analysis of cell suspension of whole lung stained for $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD44^{lo}CD62L^{hi}$ naïve T cells and $CD4^+CD44^{hi}CD62L^{lo}$ effector memory T cells (**d**). Numbers in the flow cytograms indicate the percentage of T cells present relative to total cell numbers. Data are representative of 2 individual experiment. N=6 mice per group. ** P< 0.01.



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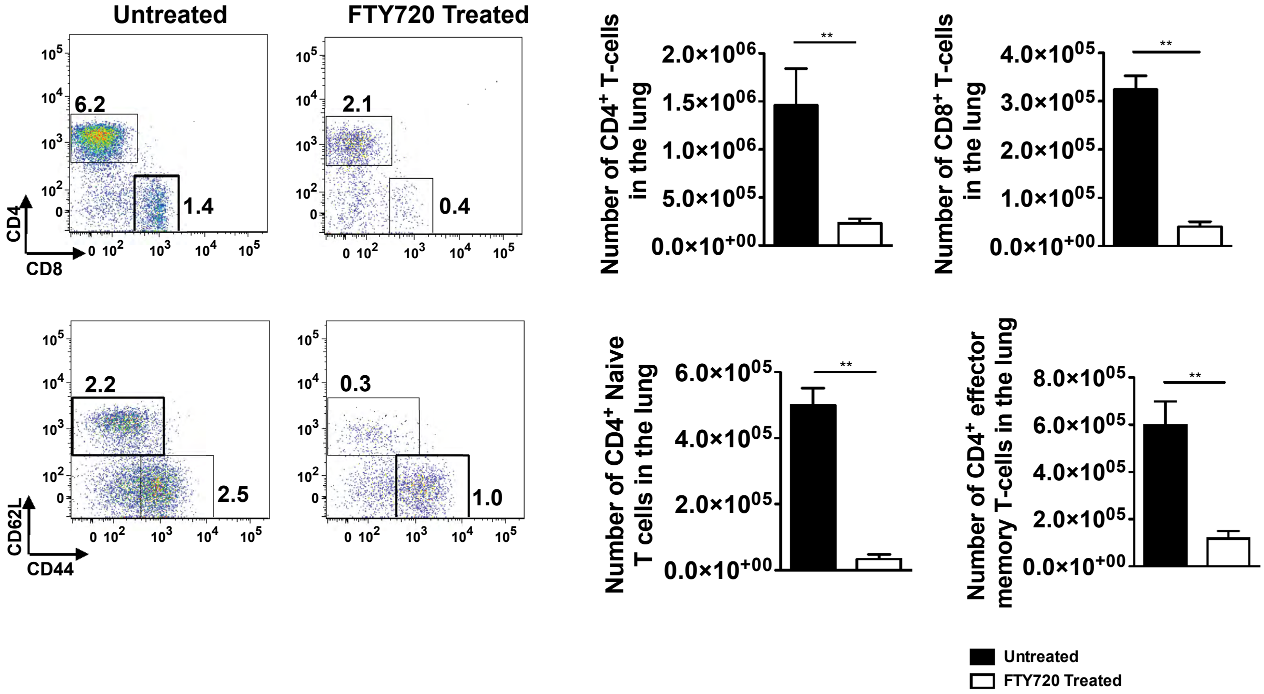

Fig 3: FTY720 treatment during secondary *N. brasiliensis* infection does not affect ability to resolve re-challenge infection. Primary infected mice were treated with ivermectin on day 7 to clear infection. Daily FTY720 treatment started on day 27, 1 day prior to a secondary infection and mice were killed at 5 days P.I (**a**). Adult worm burden from intestine was evaluated to examine expulsion kinetics (**b**). Whole-cell preparations of lung were re-stimulated with NES proteins for 5 days, and cytokine secretion for IL-4 and IL-13 was detected by ELISA (**c**). Flow cytometric analysis of cell suspension of whole lung stained for $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD44^{lo}CD62L^{hi}$ naïve T cells and $CD4^+CD44^{hi}CD62L^{lo}$ effector memory T cells (**d**). Numbers in the flow cytograms indicate the percentage of T cells present relative to total cell numbers. Data are representative of 2 individual experiment. N=6 mice per group. ** P< 0.01.