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Protective immunity against *Nippostrongylus brasiliensis* requires antigen presentation by IL-4R α responsive B cells



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Abbreviations

APC	Antigen presenting cells
B220	B cell isoform of 220 kDa
B-cells; T-cells	B and T lymphocytes
CD19	Cluster of Differentiation 19
CD4 ⁺	Cluster Designation 4 positive
CD8 ⁺	Cluster Designation 8 positive
ELISA	Enzyme Linked Immunoabsorbent Assay
FACs	Flow cytometry
FCS	Foetal Calf Serum
IFN- γ	Interferon gamma
IgG/IgE	Immuno-globulin G/E etc
IL-4R α	Interleukin-4 receptor alpha
IL-x (x = 1-...)	Interleukin-x
IMDM	Iscove's Modified Dulbecco's Medium
JAK	Janus Tyrosine Kinase
KO	Knock out
MHC	Major Histocompatibility Complex
Myd88	Myeloid differentiation primary response gene (88)
<i>N. brasiliensis</i>	<i>Nippostrongylus brasiliensis</i>
NaCl	Sodium chloride
Ova	Ovalbumin
p.i.	Post infection
PAMP	Pathogen-associated molecular patterns
PAS	Periodic Acid Schiffs
PBS	Phosphate Buffered Saline (solution)
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
T _H 1/T _H 2	T-helper (cell) type 1 or 2
TLR	Toll Like Receptor
WT	Wild Type (Genetically unmodified)

Abstract

Nippostrongylus brasiliensis is a parasitic nematode infection that affects rodents. B-cells have been shown to play an important role in immunity to many different infections by antibody production and T-cell activation. But B-cell function in the protective T_H2 response against *N. brasiliensis* infection is an area of immunity that is currently not well defined. Recently, it has been shown that B-cells are essential to the resolution of a *Heligomosomoides polygyrus* infection, another parasitic helminth. Our aim in this study was to investigate any role that B-cells may play in response to a secondary *N. brasiliensis* infection by analysing the differences in immunity of wild-type and B-cell-specific IL-4R α knockout mice after a *N. brasiliensis* re-infection.

Protection against secondary infection depended on B-cell IL-4R α expression and IL-13 competency, but not IL-4 or antigen-specific IgG1 production. Re-infection of B-cell-specific IL-4R $\alpha^{-/-}$ mice resulted in increased worm burdens compared to control mice. Impaired protective immunity was related to reduced lymphocyte IL-13 production and B-cell MHCII and CD86 surface expression. Adoptive transfer into naïve BALB/c mice of *N. brasiliensis* primed IL-4R α -responsive B-cells, but not IL-4R α or IL-13 deficient B-cells, conferred protection against a primary *N. brasiliensis* infection. Protection was also B-cell MHC class II dependent as naïve mice receiving *N. brasiliensis* primed wild-type but not MHCII $^{-/-}$ or BALB/b B-cells were protected against primary infection. This B-cell driven protection was associated with cognate interactions driving CD4 $^{+}$ T-cell co-ordination of immunity. The rapid generation of protective ability by B-cells suggested a non-BCR mediated mechanism of antigen processing such as Toll Like Receptors. Transfer of CD4 $^{+}$ T-cells co-cultured with *N. brasiliensis* antigen pulsed Myd88 $^{-/-}$ B cells failed to confer protection. These data suggest TLR dependent antigen presenting IL-4R α -responsive B-cells that are able to produce IL-13

contribute significantly to CD4⁺ T-cell-mediated protective immunity against *N. brasiliensis* infection.

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1. Introduction

1.1 The immune system

The primary function of the immune system in living organisms is to recognise and eliminate a range of invasive pathogens, and to limit the damage that these can cause to the host [1]. These infectious pathogens can extend from miniature viruses and unicellular bacteria to fungi and considerably sized parasites. In order to provide efficient protection, a competent immune system includes several very general approaches that can deal with whole classes of pathogens, as well as some methods that give rise to defences that are specific to a particular species or sub-species of micro-organism. In the event that a foreign organism is detected in the host, a chain of events is set in motion which results in these components combining, co-ordinating and responding in a manner that will deal with the pathogen effectively. Ideally this will result in complete and rapid clearance of the pathogen from the body of the host.

The immune system is primarily maintained by leukocytes (immune cells, also known as white blood cells) but is also associated with physical and biochemical barriers such as skin, mucus, stomach acid and tears [2]. Leukocytes originate from hematopoietic stem cells found in the bone marrow. Some develop and mature there, while others mature elsewhere in the body in organs like the thymus or spleen. Mature immune cells migrate to peripheral tissues, and are found in blood, lymph fluid, mucosal surfaces and secondary lymphoid organs such as the lymph nodes and spleen, where they carry out protective activities [3].

Meticulous control of immune responses is essential. An immune system that is under-stimulated towards a pathogen responds poorly which allows the pathogen to thrive. But over or incorrect stimulation gives rise to immuno-pathology, and the resulting inflammatory response could be more dangerous than the original inciting stimulus [4]. Another factor that

has a significant impact on immune regulation is the recognition of self versus non-self. The immune system must be able to tolerate the hosts' own cells and tissues to prevent auto-immunity, while still recognising foreign organisms and proteins [5]. All of these issues make the control of the inflammatory and anti-inflammatory systems crucial. One of the most important ways of regulating an immune response is the production of cytokines and chemokines, low molecular weight glycoproteins or polypeptides that are secreted chemical messengers which allow a cell to communicate with the surrounding cells [6]. Activated leukocytes secrete the largest quantities of these proteins but they can be produced by a variety of cells including some un-stimulated leukocytes, neurons, epithelial cells and smooth muscle cells [7, 8]. Cytokines and chemokines can stimulate and draw other immune cells to a site of infection, thus causing inflammation in that area. However cytokines may also act in an anti-inflammatory manner by inhibiting cellular activation and modulating homeostasis [9].

1.2 Innate vs. Adaptive

There are two basic components to the immune defence, the innate system which provides the initial immediate non-specific response and the adaptive response which is slower to provide protection to a primary infection but retains specific memory cells that respond quickly and effectively to a secondary challenge.

The innate response is maintained by several cell types, including granulocytes, natural killer cells (NK), dendritic cells and phagocytes like macrophages and neutrophils. These cells can recognise pathogen associated molecular patterns (PAMPS), such as lipopolysaccharides (LPS), DNA, double stranded RNA and flagellin, by the surface receptors Pattern Recognition Receptors (PRRs) and Toll Like Receptors (TLRs) [10]. Since PAMPS are

found across whole families of pathogens and are highly conserved during pathogen reproduction, the response to an infection detected in this manner is rapid and non-specific.

Each cell type has a role to play in the overall construct of immunity. Phagocytic cells bind to foreign organisms or cell debris before engulfing and destroying them; granulocytes like eosinophils release enzymes e.g. eosinophil protease, which can neutralise pathogens that are too big to be phagocytosed [11]. NK cells can recognise and kill tumour cells or cells that are infected by internal pathogens, while dendritic cells are specialised for antigen presentation to adaptive cells [1]. The innate immune system is also associated with several secreted proteins contained in plasma, tissue fluid, tears and mucus. A group of serum proteins, known as 'acute phase proteins', assist in innate immunity by either directly killing pathogens or targeting them for phagocytosis [12]. These include C-reactive protein (CRP) and complement proteins.

One of the most important roles for the collective innate response is to collaborate with the adaptive immune response [11]. Antigen presentation is a common way of achieving this. Antigens take the form of a unique protein or other bio-molecular sequence which is obtained from the pathogen and is recognised as foreign [6]. This is especially likely to occur in phagocytic cells and dendritic cells which present peptide fragments from internalised organisms. The antigens are presented to certain adaptive cells on cell surface major histocompatibility complex (MHC) proteins [1]. Along with cytokine production by the innate cells, this helps to shape the adaptive immune response.

B-cells and T-cells mediate the adaptive immune response. Activation occurs when the cell is exposed to an antigen via their cell surface antigen receptor, the B-cell receptor (BCR) or T-

cell receptor (TCR). Both these receptors have a highly diverse range of antigen binding sites that are generated by DNA re-arrangement [10]. This allows these cells to recognise a wider range of antigens than innate cells, which increases the probability of recognising the pathogen, but it takes a longer time for the response to develop. The protection provided by activated adaptive immune cells is very specific to a particular type of pathogen because the stimulating antigen is commonly unique to the pathogen, or even to a sub-type of the pathogen. Activation leads the adaptive cell to differentiate, proliferate by clonal expansion and activate other immune cells and defence systems by releasing inflammatory cytokines; additionally B-cells also produce antibodies [6]. Once adaptive cells have gone through activation and clonal expansion a small proportion develop into relatively inactive long-lived central memory cells as opposed to the rest of the cells which become highly active shorter-lived effector cells. Memory cells can remain dormant in the host for many years but if the pathogen is ever encountered again they can rapidly respond by dividing to produce a new population of effector cells [11]. Thus a re-infection usually is usually weak and brief.

1.3 The adaptive response in detail

1.3.1 T-cells

The class of adaptive immune cells known as T-cells includes $CD4^+$ helper cells and $CD8^+$ cytotoxic cells. They are derived in the bone marrow and mature in the thymus. Activation of $CD4^+$ helper T-cells requires the display of antigen on MHCII proteins in order for the T-cell to recognise and bind to the antigen via their TCR in a receptor complex (**Figure 1.5**) [13]. MHCII receptors are only found on professional antigen presenting cells (APCs); these are dendritic cells, macrophages and B-cells. Following activation, helper T cells stimulate antibody production by B-cells and innate effector mechanisms such as increased killing by phagocytes [13]. $CD8^+$ cytotoxic cells are alerted to the presence of intracellular pathogens

by antigen presentation on MHC I receptors, which are found on all nucleated cells [1]. This causes the CD8⁺ cell to destroy the infected cell in order to contain the infection.

1.3.2 T helper 1 vs. T helper 2

A significant development in the research into the role of CD4⁺ T-cells shows that activated effector cells differentiate into separate, defined categories depending on the type of pathogen they are responding to and their cytokine profile [14]. Each subset activates different immune mechanisms and many varied factors play a part in influencing which type of response predominates and which is optimal. The regulatory mechanisms that cause polarisation of the T helper response one way or the other include the type of antigen presenting cell that activates the CD4⁺ response, the cytokine conditions and the co-stimulatory molecules at the time of antigen presentation [13, 15].

The two major classes of a T-cell response are the cellular T-helper cell 1 (T_{H1}) response and the humoral T-helper cell 2 (T_{H2}) response. Interleukin 12 (IL-12) is the predominant cytokine that induces T_{H1} differentiation while interleukin 4 (IL-4) promotes a T_{H2} response. The responses are characterised by the cytokines they produce (Figure 1.1). A T_{H1} response is defined by high levels of interferon gamma (IFN- γ), IL-2 and IL-12 production and protects against most intracellular pathogens [14], while T_{H2} cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and are associated with helminths and allergens [16]. Other features of a T_{H1} response are IgG2a antibody production, macrophage activation and CD8⁺ cytotoxicity [13]. The IL-4 that drives T_{H2} immunity is produced by conventional T and B-cells as well as several innate sources, including basophils, mast cells, $\gamma\delta$ T-cells and eosinophils [17]. An allergic reaction is an exaggerated T_{H2} reaction to an otherwise harmless antigen in individuals who are predisposed to do so genetically [18]. This can lead to anaphylactic

shock and even death. The details of the T_H2 response to helminths are discussed later in depth. These two types of effector cells inhibit the action of the other i.e. the IL-12 produced by a T_H1 response suppresses T_H2 polarisation and IL-10 blocks T_H1 cells developing [19].

Of course the T_H1/T_H2 paradigm does not cover all helper T-cell responses. Other categories of effector T-cells include T_H17 , stimulated by IL-6 to produce IL-17 in immunity to extracellular bacteria [20]. Also TGF- β 1 drives the generation of $CD4^+CD25^+FoxP3^+$ regulatory T-cells that cause immuno-suppression [20]. TGF- β also re-programs T_H2 T-helper cells to lose their characteristic IL-4 profile and switch to secretion of IL-9, making them T_H9 cells [21]. It is probable that there are other T-cell subsets that are yet to be researched but it is unclear how many or what their role may be.

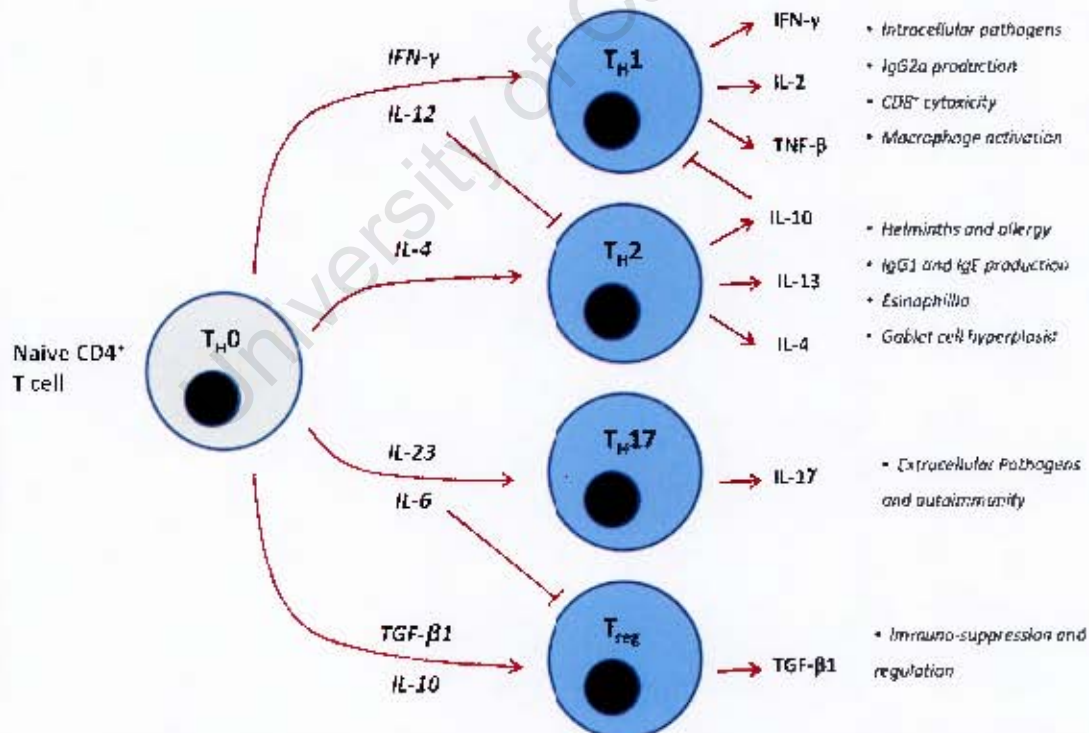


Figure 1.1 Naïve $CD4^+$ T-cells can polarise into different effector response classes. This depends on the stimulating cytokine environment. Each type of response produces a different set of cytokines and effector mechanisms.

1.3.3 B-cell development and maturation

B-cell progenitors originate in the bone marrow where they go through process referred to as primary or central B-cell development. This generates immature, surface immunoglobulin expressing B-cells which migrate to the spleen where they mature through the transitional phases 1 and 2 (T1 and T2) in the splenic follicles [22]. It is in the spleen that immature B-cells are directed toward a specific mature B-cell fate; this is known as secondary or peripheral development (**Figure 1.2**) [23]. The immature T2 B-cells can become mature follicular cells or marginal zone cells. Mature naive follicular cells may stay in the spleen or re-circulate in the bloodstream to follicles in other secondary lymphoid organs like lymph nodes where they wait until they encounter a stimulating agent. They are activated when they encounter antigen via the surface immunoglobulin, known as the B-cell receptor (BCR), or interact with an activating T-cell [24]. This turns them into germinal centre B-cells and results in rapid proliferation and somatic hyper-mutation of the cells. Activated germinal centre cells then develop into effector plasma cells or long-lived memory cells [25].

The almost infinite range of antigen specificities that a BCR can have is determined by partially random DNA re-arrangement of the genes that code for the BCR [6]. This takes place in the bone marrow. One of the major effector mechanisms of B-cells is the secreted form of the membrane bound BCR, called antibodies. Once genetic re-arrangement has successfully occurred, B-cells begin to secrete the Immuno-globulin M (IgM) antibody isotype of a naive mature B-cell [22]. Sometimes IgD is also co-expressed with IgM on mature cells. When the fully matured naive B-cell encounters antigen or is activated by a helper T-cell, the B-cell often goes through a process of isotype or class switching, whereby the cell progresses from producing IgM to producing IgG, IgA or IgE, although some cells continue to produce IgM [26]. The type of T-cell response and the stimulating antigen both

influence the eventual isotype of the immuno-globulin produced by the activated plasma B-cell.

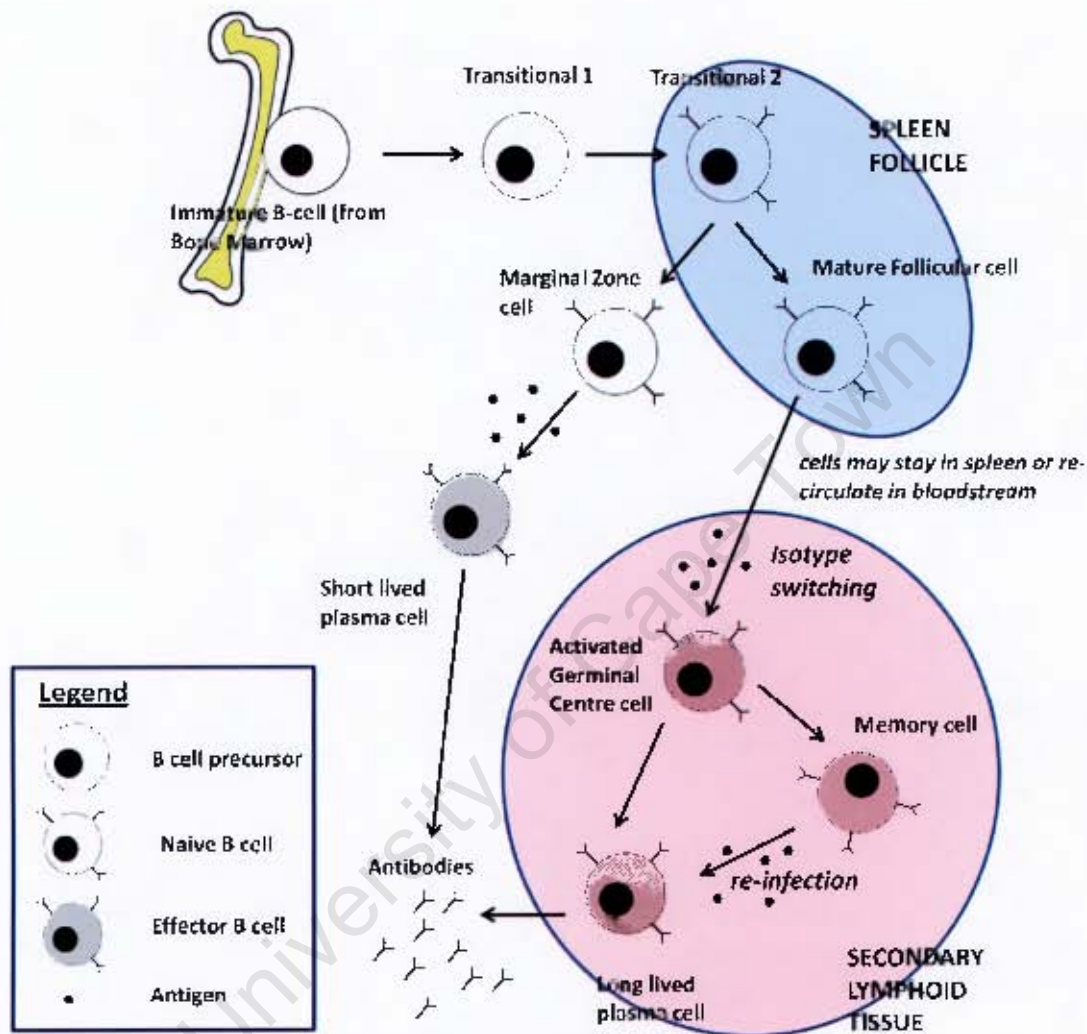


Figure 1.2 The progression of peripheral B-cell development. Naive B-cells go through several stages of development and maturation before they encounter antigen or a $CD4^+$ T-cell which activates them turning them into effector cells.

1.3.4 Follicular vs. Marginal Zone cells

The anatomical layout of spleen is highly organised. The lymphocytes are arranged in distinct locations in order to maximise the efficiency of development and cellular interaction with each other and with antigen [27]. The spleen mostly consists of red pulp, interspersed

with discrete areas of lymphoid white pulp which is arranged around central arterioles (Figure 1.3). The red pulp is a site of red blood cell destruction [28]. There are three subsets of mature B cells present in the spleen: Mature Follicular cells (FM), Marginal zone cells (MZ), and B-1 B cells [22]. FM B cells are located in the follicles of the splenic white pulp, while the MZ B cells exist in the Marginal Zone, a region lining the border between the white and red pulp [29]. The B-1 cells are a minor population in the spleen and are more likely to be found in the peritoneal cavity [30].

Lymphocytes (Naive mature T-cells and immature B T1 cells) and antigen-loaded dendritic cells enter the white pulp via the central arteriole into the peri-arteriolar lymphoid sheath (PALS). The PALS, which is mostly made up of T-cells, is a site of T-cell interaction with antigen-presenting dendritic cells [29]. T-cell independent antigen-mediated selection, BAFF and transient BCR stimulation all play a role in driving T1 development into T2 cells [31]. As the T1 B-cells develop into T2 cells they pass into the follicle which mainly consists of B-cells. The T2 cells may become mature follicular cells (FM) in the follicle or marginal zone cells (MZ) in the Marginal Zone. The regulatory factors that control T2 differentiation include BCR signalling [25], cognate interactions and co-stimulation [32] and antigen dependent selection [22]. Cells and antigen drain out of the white pulp in a trabecular vein [28]. The organization of the white pulp of the spleen is similar to that of a lymph node, but while the spleen regulates blood borne antigens, lymph nodes detect localised antigens in draining lymph fluid.

The most abundant type of mature B-cell in the spleen, FM cells, are conventional B-cells; they are stimulated by antigen and T-cells to become germinal centre cells, they switch antibody isotype classes to produce high affinity antibodies, present antigen and generate

plasma and memory cells [22]. FM cells develop in the spleen but re-circulate in the bloodstream around the body to peripheral secondary lymphoid tissue such as the spleen, lymph nodes or mucosal surfaces where they can screen large quantities of antigen. However in contrast to FM B-cells, MZ and B-1 cells respond rapidly to T-independent, blood-borne antigens and pathogens. They can differentiate into plasma cells in one or two days and produce secreted circulating low-affinity IgM auto-antibodies [33]. In addition MZ B-cells do not re-circulate like FM cells; they remain in the specialised marginal zone of the spleen for their entire life span. They exhibit little or no in vitro proliferative response to BCR engagement [34]. Thus conventional FM cells can be classed as 'adaptive' B-cells and MZ and B-1 cells as 'innate-like' B-cells. The strikingly different effector functions of these two lines of B-cell development distinguishes the MZ and FM cells as functionally discrete, mature B-cell populations [35].

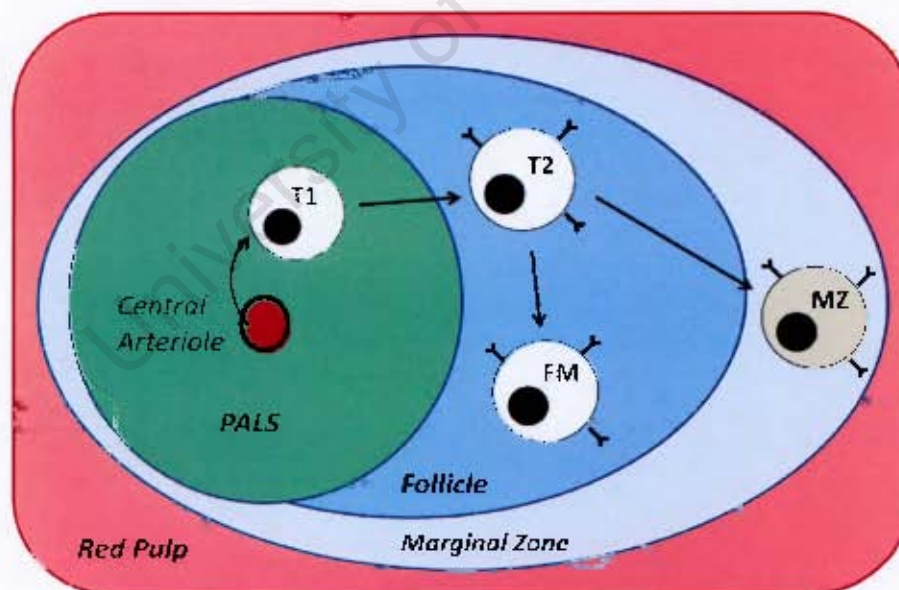


Figure 1.3 Transverse-section showing the organisation of the lymphoid tissue in the spleen. T1 cells enter the PALS from the blood and develop into T2 cells in the follicle. T2 cells can differentiate into FM or MZ cells.

1.3.5 Antibodies

B lymphocytes are commonly stereotyped to facilitate clearance of pathogens by producing and secreting antigen specific antibodies [36]. These are complex quaternary proteins that are made up of several polypeptide chains bound together [6]. Antibodies are structured so that they have a constant region that determines their effector function and two variable regions that possess the antigen binding sites of the molecule (**Figure 1.4**). It is these antigen binding sites that determine the specificity of the antibody and they can be composed of an almost infinite variety of amino acid sequences (which depends on the DNA rearrangement of the B-cell). The secreted antibodies produced by a B-cell have antigen binding sites that are identical to those on its BCR meaning that they bind the same type of antigen [24]. They are produced in large quantities in response to an infection and circulate throughout the body and tissues via plasma and the lymph, and can even be secreted across mucosal surfaces [1].

Antibodies function by binding to pathogens or their products via their antigen binding site. This can result in one of several protective effects. Generally antibodies help to mark the bound particles as foreign. Some antibodies cause neutralisation which prevents the pathogen or bacterial toxins from entering the host's cells or causing tissue/cell damage. Other antibodies cause opsonisation of pathogens which enhances leukocyte recognition of antibody-bound pathogens [37]. This marks the pathogen for ingestion and destruction by a phagocyte [38] or for destruction by a process called antibody-dependent cellular cytotoxicity (ADCC) [39]. During ADCC the release of lysis products from an immune effector cell (eosinophil or natural killer cell) destroys the antibody-bound pathogen or cell without the need for phagocytosis [40]. For instance, eosinophils are able to attack large antibody-bound parasites by releasing ionic proteins that disrupt the surface of the helminth or make the environment less hospitable to the helminth [41]. Another mechanism used by

antibodies is to activate complement proteins to attack an antibody-coated pathogen [42]. Complement proteins can cause osmotic cell lysis [43] or agglutination (binding together multiple units) [44] of the pathogen, or opsonisation of the pathogen in a similar manner to antibody [45].

Antibodies of different isotype classes differ in their constant region and therefore their functional activity [36]. IgM and IgD are the earliest secreted class of antibody. IgM forms pentamer polymers in the serum and strongly induces complement activation [13]. Polymerisation is important because this allows the antibody to bind to multiple antigens on the pathogen which increases binding affinity [11]. The exact function of IgD is currently unclear but there is evidence that it has a similar role to IgM [46]. IgG is the most common antibody class in the plasma and has several subclasses (IgG1, IgG2a, IgG2b etc.) [25]. It is multi-functional, fulfilling most roles described above, and can be transferred across the placenta from mother to child which gives the infant passive immunity [47]. IgA can form dimers, and is able to cross epithelial surfaces; it is sometimes found in mucous excretions. IgE is involved in activating mast cells and basophils to produce hypersensitivity in allergic type reactions [48].

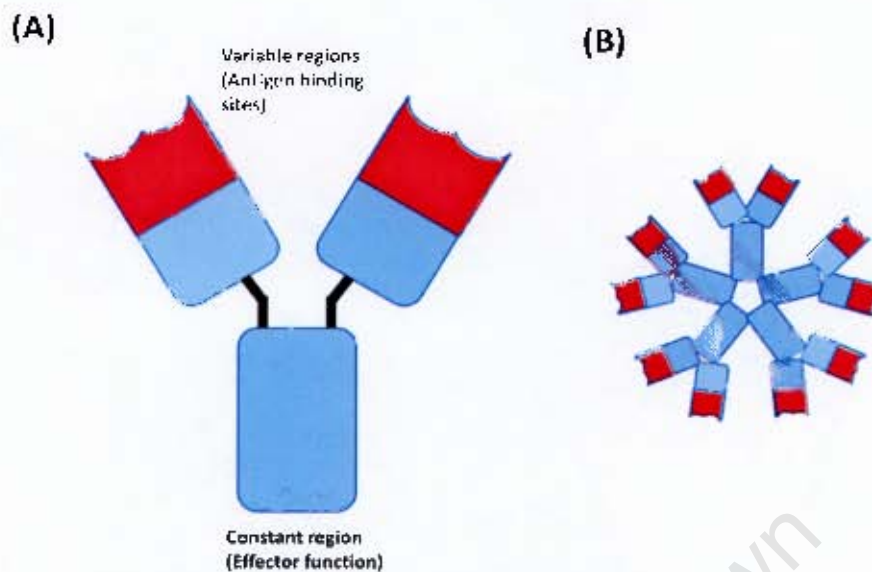


Figure 1.4 The structure of antibodies. (A) The general schematic structure of a typical antibody molecule. The two variable regions have identical antigen-binding sites. The constant region has a limited number of forms. (B) An IgM pentamer antibody.

1.3.6 B and T cell interactions

But B-cell function is not limited to antibody production; plasma B-cells actively regulate and participate in cellular immune responses by multiple antibody independent mechanisms as well [13]. B-cells have been shown to regulate T-cell mediated immune responses, even in mouse models which have B-cells that cannot secrete antibody. $IgM^{-/-}$ B-cells for instance can regulate T-cell responses via antibody independent mechanisms [49]. Other effector functions of B-cells include cytokine production, antigen presentation to $CD4^{+}$ T-cells and expression of co-stimulatory molecules [50]. These actions can direct the proliferation and effector functions of other responding cells and B-cells must carry out these functions successfully as well as produce antibodies, to efficiently protect the host against infection.

B-cells can internalise antigen selectively via the BCR; it is then processed in the endoplasmic reticulum and presented on MHCII receptors [13]. Co-stimulatory molecules

such as CD80 act together with the antigen presenting MHCII to trigger the T-cell to respond (Figure 1.5). Secondary lymphoid organs are a principle site of immune cell interaction. They are organised so that B-cells and helper T-cells can optimally interact with each other as well as innate APCs and antigens [25]. The co-operative interactions between B and T lymphocytes result in a positive feedback loop which causes both cells to become activated [51]. The activated B and T-cells begin to divide in a process called clonal expansion which produces large numbers of the antigen specific effector lymphocytes. The evidence showing the importance of this role is prevalent. Studies on B-cell deficient mice indicate that the absence of B-cells adversely affects both CD4⁺ and CD8⁺ responses to foreign or self antigen [52]. This shows that the magnitude and quality of the helper T-cell response in both primary and memory infections is significantly determined by interaction with B-cells [53].

B-cell chemokine and cytokine production is also an integral part of the immune system. B-cells can secrete cytokines constitutively or in response to a range of stimuli, including T-cell co-stimulation, antigen, TLR ligands and microbial products [54]. B-cells can be inflammatory or anti-inflammatory depending on which cytokines they release. For instance TNF- α secreting B-cells induce T-cells to generate IFN- γ , while IL-10 producing B-cells suppress T-cell mediated inflammation and play a vital protective role in several autoimmune diseases [55]. These cytokine-producing effector B-cells can modulate T-cell responses and can induce strong T_H1 or T_H2 immunity.

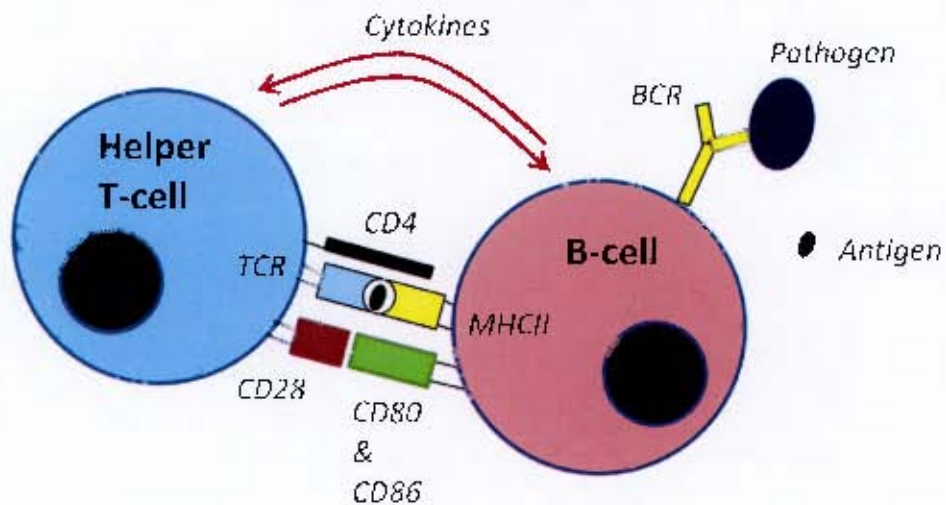


Figure 1.5 The interaction between B-cells and T-cells. B-cells present antigen to T-cells on MHCII. The T-cells form a cell surface receptor complex by binding to the MHCII with their TCR and CD4. Co-stimulation is provided by CD28 interaction with CD80/86, and cytokine production.

1.3.7 B effector 1 vs. B effector 2

It has been shown that activated effector B-cells (Be), like helper T-cells, can also be subdivided into discrete subsets, producing Be-1 [51], Be-2 [17] or B-regulatory [23] effector B-cells. Each group has different functions and produces a diverse array of cytokines. Recent findings suggest that a combination of cytokines, cognate T-cell interactions, pathogen-derived signals, and BCR signals is necessary to induce the differentiation of naive B cells [56]. Naive B-cells primed by interactions with T_{H1} cells and stimulated by $IFN-\gamma$ and $IL-12$ become Be-1 cells that produce the T_{H1} cytokines $IFN-\gamma$, $TNF-\alpha$ and $IL-10$ (Figure 1.6). B-cells that are primed by $IL-4$ and interact with T_{H2} cells differentiate into Be-2 cells and produce $IL-4$, $IL-13$, $IL-2$ and $IL-6$ [50]. Functionally Be-1 cells contribute significantly to immunity to bacterial infections, such as *Salmonella* [26]. Be-2 cells have been demonstrated to be important for immunity to helminth infections, including the humoral and cellular response to *H. polygyrus* [53, 57].

The B effector cells assist in establishing and amplifying the T helper responses in a cytokine driven positive feedback loop. Thus Be-1 cells drive the differentiation of naive T-cells into T_H1 and Be-2 into T_H2 [49, 54]. This is highly beneficial when a strong inflammatory immune response is required to combat a pathogenic infection. It has been shown that B-cell cytokines play a central role in T_H2 cell polarization and the number of IL-13 producing $CD4^+$ T-cells is greatly reduced during a type two response in B-cell deficient mice [53]. This indicates that the cytokines made by B effector cells control both the activity of the B-cells themselves and the proliferation and activation of effector and memory T-cells, regulating both cellular and humoral immune responses in vivo to pathogens. Therefore, B-cells represent an attractive target for future research related to vaccination and therapeutic strategies.

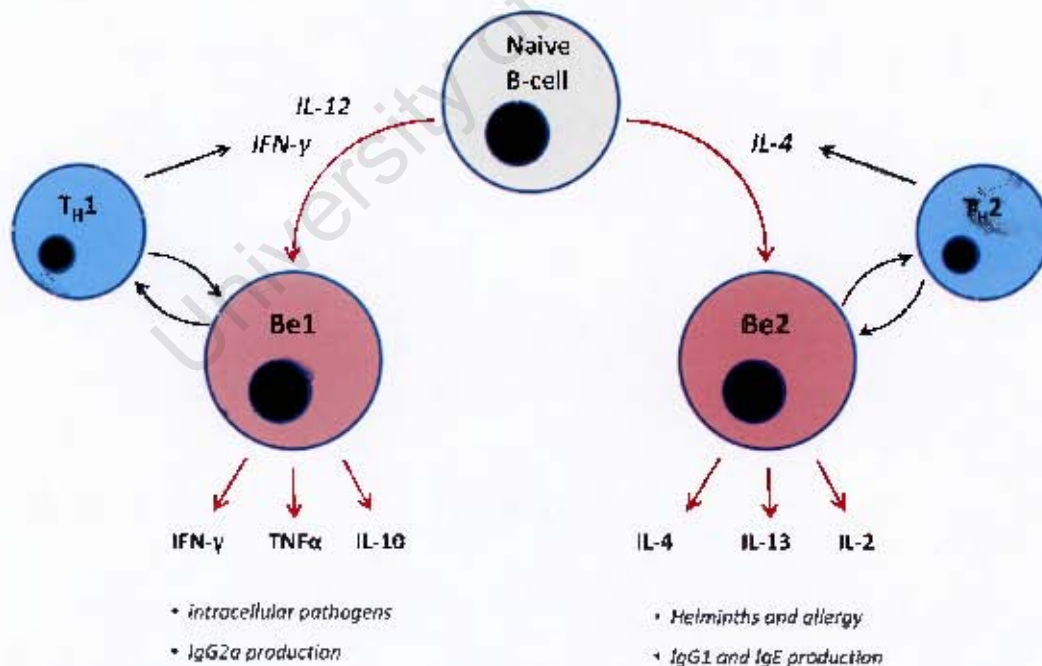


Figure 1.6 The development of effector B-cell populations. Naive B-cells can differentiate into discrete effector populations depending on the type of stimulation they get.

1.3.8 The Innate capabilities of lymphocytes

Re-arrangement of the BCR or TCR receptor gene to create receptor diversity is a defining characteristic of the adaptive immune system which enables B and T responses to specific antigen [1]. However a class of cells known as innate-like lymphocytes, including $\gamma\delta$ T-cells, B-1 B-cells and NK T-cells, shows less stringent antigen specific activation requirements [6]. The receptors found on these subsets are encoded by a few common genes and little rearrangement is necessary to make a functional receptor; thus they do not have the wide range of receptor variance found in the TCR or BCR of adaptive cells [13]. These lymphocyte subsets are limited in the range of their antigen specificities and the efficiency of their response, but they can respond rapidly to infections like innate immune cells, a quality not possible in lymphocyte populations which require clonal expansion to launch an effective and highly specific response [11].

Even classical mature adaptive cells can sometimes exhibit innate-like functions when they respond to antigen in a TCR or BCR independent manner [58, 59]. B-cells have been shown to express TLRs; currently 10 mammalian TLR's have been defined [50, 53]. These non-specific receptors allow the cells to recognise and respond to some types of antigens rapidly by cytokine and antibody production [60, 61] For example MZ B-cells are shown to respond to LPS rapidly via TLR signalling and innate response activator (IRA)-B-cells have been shown to protect against microbial sepsis in a TLR dependant manner [22, 62, 63]. FM B-cells and MZ B-cells have different cytokine profiles in response to TLR ligands [64]. T2 cell development toward FM B-cells has been shown to be enhanced upon simultaneous BCR and TLR activation; In B lymphocytes stimulated by antigen detected concurrently by B-cell antigen receptors and TLRs, antigen-dependent differentiation to adaptive FM B-cells is considerably improved and more rapid [22, 65].

Central to TLR signalling is MyD88, a key downstream adaptor molecule, for all TLRs except TLR-3. It has been shown that MyD88 competent pulmonary APC's are a vital innate component in the activation of TLR-dependent T_H2 responses to pulmonary antigen [5]. This paper suggests that there may be a subset of PAMPs uniquely capable of inducing a site-specific T_H2 response via TLR/MyD88 signalling. This shows a novel role for MyD88 in T_H2 immunity. Interestingly, analysis of B-cell populations in MyD88-deficient mice has revealed a decrease in splenic MZ and peritoneal B-1a B-cell numbers, but FM and T2 populations were not affected [65].

1.4 Helminths

1.4.1 Nematodes

Parasitic nematode infections are an extremely important global public health issue [66]. They are common to both humans and domestic livestock; Infections with *Ascaris lumbricoides*, *Trichuris trichuria* and the hookworms *Ancylostoma duodenale* and *Necator americanus* occur in a third of the world human population [67]. The silent but devastating effects of infection cause morbidity in health and economic damage. For example infections by hookworms or roundworms cause anaemia, poor immunity towards bacterial co-infection and widespread child development and growth problems, especially *in utero* [68]. Effects on cognitive development as a result of repeated childhood infections are widely reported [69]. Exacerbation of chronic lung pathologies associated with parasitic larval migrations in the host may also represent a further major health implication in endemic areas [70]. However mortality due to nematode infections is a rare occurrence.

Hookworms can sometimes be transmitted through improperly cooked meat and faecal contaminated water and food, but the more common method of infection is directly through the skin by walking barefoot through contaminated faecal matter [68]. Infection is chronic and may last 1-5 years for *Necator americanus* and about 6 months for *Ancylostoma duodenale* [16]. Although they are treatable with drugs, re-infections with these parasitic nematodes often occurs as humans do not develop protective immunity [71]. Ultimately the public health burden caused by these infections will be best controlled by the development of vaccines against nematode infections. To date no vaccination strategies exist against these parasites, although potential candidate vaccines against hookworms are being actively investigated [72, 73]. To accelerate and optimize the development of effective vaccines a detailed understanding of host immunity is essential.

1.4.2 *Nippostrongylus brasiliensis*

Nippostrongylus brasiliensis is a gastro-intestinal parasite of rodents, natural to rats, but it has been adapted for use in mice [13, 14]. *N. brasiliensis* is similar both physiologically and biochemically to the human hookworm parasites *N. americanus* and *A. duodenale*. Because of its similarity to human hookworms, *N. brasiliensis* has been developed for use in the laboratory as a murine model for human nematode infections for immunological experiments [16]. It is easy to work with, the life cycle is straightforward and simple to maintain [74] and it is not fatally harmful to rodents. In mice a primary infection is rapidly cleared, and the worms are expelled from the intestine around 8-9 days post-infection leaving behind sustained immunity to the pathogen against re-infection [13].

1.4.3 The life cycle of *Nippostrongylus brasiliensis*

No intermediate host is required for the maintenance of the life cycle of *N. brasiliensis*, or for the transmission from one host to another. Mature adult worms live in the lumen of the small intestine of the rodent where they produce eggs that pass out of the host with the faeces into the soil (Figure 1.7). The larvae hatch at the L1 stage, which is free living and motile [13, 74]. Further development carries them through to the infectious L3 larval phase. The L3 larvae can infect the host by oral ingestion and penetration of the mucosal epithelium of the upper intestinal system, or more likely they infiltrate the skin of the potential host [14, 70]. To mimic this in the laboratory, the L3 larvae are injected subcutaneously into the rodent's abdomen. Then the circulatory system carries the larvae to the lungs [75], where they get trapped in the capillaries, and by moulting to the L4 stage, breach the capillaries [13] and enter the pulmonary alveoli. Then they get coughed up into the oesophagus [14, 70] and are ingested when the animal swallows. In this way they migrate back to the gastro-intestinal system 4-5 day's post infection. They bind to the intestinal wall and there they develop and mature into adults and begin producing eggs about day 6 post infection [13]. In BALB/c mice the eggs stop about day 9 post infection when the worms are expelled due to the robust immune response against them [75, 76]. The *N. brasiliensis* induced IL-13 stimulation of intestinal cells is shown to be the most likely cause of expulsion of the adult worms from the intestine during a primary infection [77, 78]. *N. brasiliensis* is dioecious showing reproduction is sexual, with the males being the marginally smaller gender.

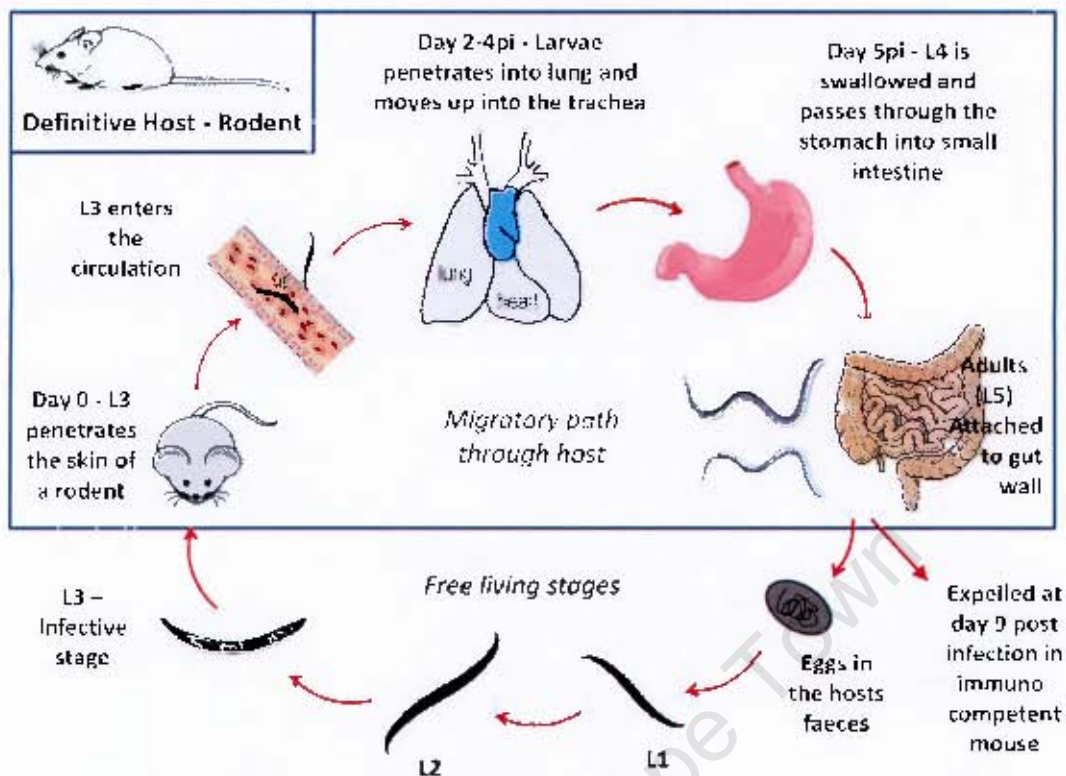


Figure 1.7 The life cycle of a primary *Nippostrongylus brasiliensis* infection. There are six developmental stages in the cycle, the egg, the four larval stages (L1-L4) and the adult stage (L5). There is no intermediate host. The eggs produced by the adults in the gut leave the infected rodent in the faeces. The larval stages L1-L3 are free-living in the soil and motile. The L3 larva is the infective stage.

1.4.4 The primary immune response to helminths

Use of experimental parasitic nematode models has established that T_H2 immune responses drive host resolution of primary helminth infections [79, 80] and these responses correlate with those found in humans [81]. An anti-helminth T_H2 response includes eosinophilia and basophilia (eosinophil and basophil recruitment into tissues), goblet cell hyperplasia (differentiation and growth) and mucous secretion, $CD4^+$ T-cell dependant generation of IgE and isotype switching of B-cells to produce IgG1 [13, 14, 16, 74]. However, while this immune response may be rapid and robust as a consequence of helminth infection, it is

sometimes not very effective at eliminating the parasite, as is the case for the parasite *Shistosoma mansoni* where the T_H2 response is merely able to control the infection [82].

IL-4 has been shown to be important in driving T_H2 mediated protection to helminths [83], but despite its importance IL-4 is not essential to protective responses against *N. brasiliensis* [80]. It is primarily IL-13 that drives the T_H2 response to *N. brasiliensis*, although it is aided by IL-4 and other T_H2 cytokines, and it has a wide range of T_H2 related functions on a variety of cell types. IL-13 can be produced by T-cells, B-cells, mast cells, basophils, dendritic cells and NK cells [84, 85]. Underlying effective expulsion of murine model parasites, such as *N. brasiliensis*, *Heligmosoimdes polygyrus* and *Trichuris muris*, is host expression of IL-4 receptor alpha (IL-4R α) [79].

1.4.5 The role of IL-4R α in T_H2

IL-4R α is found on many types of cells, including haematopoietic, muscle, epithelial and endothelial, fibroblast and neurons [86]. IL-4R α is an essential component of the cell surface heterodimeric receptors required for IL-4 and IL-13 signalling which ultimately drives host immune polarisation to T_H2 [85, 87]. Use of IL-4R α ^{-/-} mice has clearly demonstrated an absolute requirement for IL-4R α expression in resolving primary nematode infections [88]. It has been shown that IL-4/IL-13 signalling through IL-4R α activates the Signal Transducer and Activator of Transcription 6 (STAT6) and Janus Kinase 1 (JAK1) intracellular pathway [14, 89, 90]. This JAK1/STAT6 pathway promotes gene transcription that results in parasite clearance (**Figure 1.8**). Surprisingly it appears that clearance of a primary infection is not due to the effects of IL-13 on leukocytes (immune cells) but is associated with IL-13 stimulation of non-haemopoietic cells [14] including smooth muscle cells [90] and epithelial cells [91, 92]. It has been found that in modified chimeric mice that selectively express IL-4R α

normally on non-bone marrow derived cells, expulsion of worms is unaffected [77]. In mice that only display IL-4R α on immune cells, the worms are not expelled [77].

The question as to which non-immune cells are involved in clearance of a primary worm infection is complex, and one cannot highlight any single cell type to a direct STAT 6 “cause and effect relationship” [14]. The tissue that appears to be most involved is the small intestine. IL-4 and IL-13 induce several STAT 6 dependant changes there, including an increase in epithelial permeability and cell turnover, a heightened response to mediators and an increase in contractility of longitudinal smooth muscle as well as an increase in mucous production [14, 77, 90, 93]. IL-4R α dependant goblet cell hyperplasia and mucous production in the epithelial lining of the small intestine has been shown to play an important role in resolution of infections [14, 90]. Mice that had smooth muscle cell specific IL-4R α deletions showed a delay in goblet cell differentiation and reduced mucous production. They do not eliminate a *N. brasiliensis* infection as quickly as controls [90, 94]. Thus the answer to what causes the expulsion of the parasites is likely to be a combination of increased smooth muscle contractility and mucous production that creates a hostile environment to the adult worms in the gut and interferes with their ability to feed properly and bind securely to the intestinal wall [14, 77, 90].

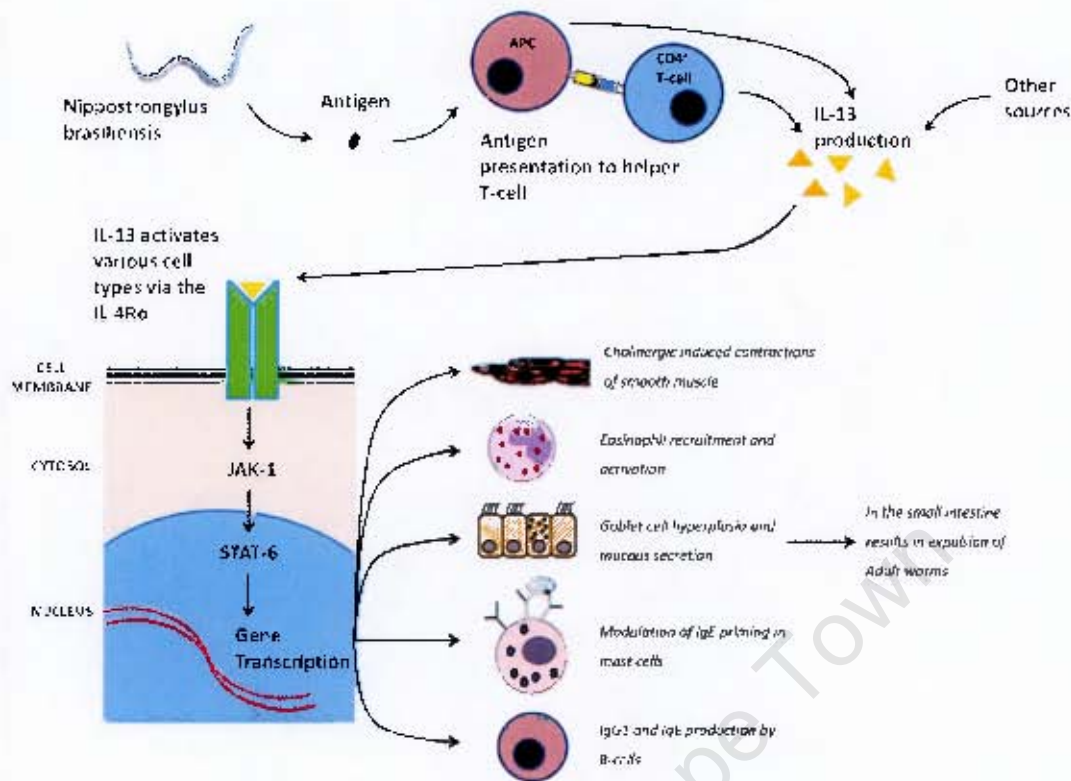


Figure 1.8 The development of the immune response to a primary *Nippostrongylus brasiliensis* infection. IL-13 is produced by several sources after infection. IL-13 signalling through the IL-4R α initiates a range of STAT6 dependant T_H2 responses in the host.

But *N. brasiliensis* and human infections with hookworms are not confined to the intestine. Larval migrations through the circulatory and pulmonary systems pre-empt establishment of the definitive intestinal infection. Such migrations cause significant pulmonary pathologies and are also potential sites of parasite killing [95]. The environment of the lung is significantly altered, immunologically, by the transient exposure to a primary *N. brasiliensis* infection, which lasts approximately 18-24 hours [75]. There is strong activation of alternative alveolar macrophages, structural damage, inflammation and other cellular and molecular changes that persist for days after the larvae have moved on [70, 75]. The disturbed lung tissue resembles emphysema-like pathology under a microscope and there is an impairment of lung function due to the destruction of the alveoli [70, 96]. Both IFN- γ and

IL-4/IL-13 levels are significantly elevated at day 8 post infection (p.i.), as well as other pro-inflammatory cytokines [75]. There is also an increase in eosinophil numbers and goblet cell differentiation in the lung and bronchioles [97]. And the airways are sensitised and remain hyper-responsive to a challenge with methacholine for more than 300 days after infection, indicating a “chronic inflammatory state” [70, 97].

1.4.6 The memory immune response to helminths

Our understanding of cellular mechanisms underlying protective immunity to helminth re-infection has, until very recently, been limited. In the case of the strictly intestinal parasitic nematode *H. polygyrus*, it has been well demonstrated that rapid resolution of re-infection requires alternatively activated macrophages [98], CD4⁺ T-cells [99], parasite specific type 2 antibody responses [57, 100] and B-cell cytokine production [53]. Studies with *N. brasiliensis* show host responses in the lung play a key role in the rapid resolution of re-infection [95, 101]. Furthermore, roles for eosinophils [42], basophils [102] and CD4⁺ T cells [103] in coordinating this immunity have also been demonstrated. No clear demonstration of IL-4R α expression in enhancing protection to a *N. brasiliensis* re-infection has yet been demonstrated.

1.4.7 The role of T-cells and B-cells

Even though IL-4R α expression on hematopoietic cells is apparently not essential to primary parasite clearance it does impact on the magnitude of the hosts T_H2 response to *N. brasiliensis*. For example, disruption of IL-4R α expression on CD4⁺ T-cells results in a significantly reduced T_H2 response to primary *N. brasiliensis* infection [96]. This implies an important role for adaptive immunity in the immune response to the parasite and also shows T-cells are essential to generate protective memory against a re-infection [103].

It has been suggested that B cells do not play a significant role in immunity to *N. brasiliensis* infection and re-infection [100]; here Liu et. al. demonstrate that, in the absence of B-cells, sufficient IL-13 to mediate protective immunity is provided by other sources. However, even if the immune system can compensate for the absence of B-cells, there is still a possibility that they can contribute to an immune response (Figure 1.9). Due to the multi-functional role of B-cells in the immune system and especially their influence on T-cell populations we felt this deserved another look. In a study on *H. polygurus*, the number of IL-4 producing T-cells was ten-fold lower in B-cell deficient mice than in wild type mice during a primary infection, and the B-cells were also required to maintain and reactivate T_H2 memory cells [53]. In the research presented here we demonstrate IL-13 signalling through B-cell IL-4R α and B-cell IL-13 production and antigen presentation to be essential for the development of effective immune memory against *N. brasiliensis*.

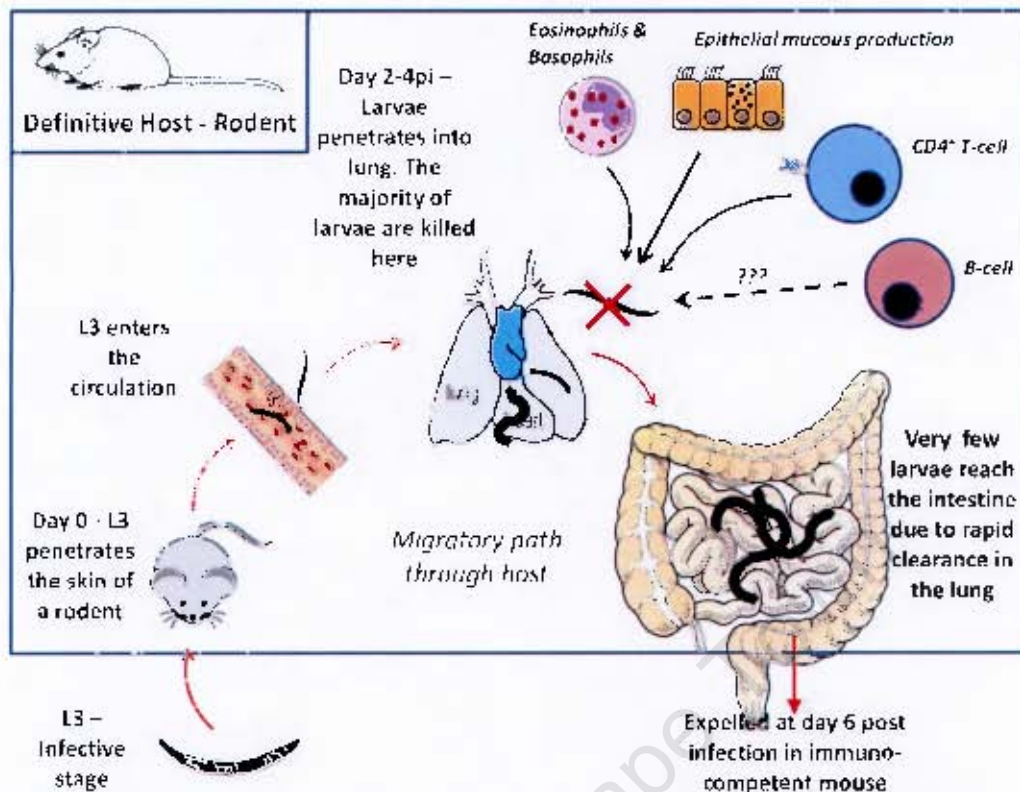


Figure 1.9 The life cycle of a memory *Nippostrongylus brasiliensis* infection. The lung is the main site of parasite clearance in immunocompetent mouse. This has been shown to be associated with lung eosinophil and T-cell functions. It is our aim to examine the role of B-cells in this response.

1.5 Objectives

As discussed above, B-cell function in the protective T_H2 response against *N. brasiliensis* infection is an area of immunity that is currently not well defined. However B-cell mediated protection has been shown in other helminth infections which indicated that B-cells may contribute to resistance to *N. brasiliensis*. Our aim in this study was to investigate any role that B-cells may play in response to a secondary *N. brasiliensis* infection. Related to this, we aimed to define the contribution of B-cells to the T-cell response against *N. brasiliensis* and how this was achieved. We did this by analysing the differences in immune function of wild-type and B-cell-specific IL-4R α knockout B-cells after a *N. brasiliensis* re-infection. Since

IL-4R α signalling has been shown to be essential to Be-2 differentiation [17], the absence of B-cell IL-4R α should disrupt an effective B-cell response which may affect the overall T_H2 anti-nematode response.

Memory experiments on IL-4^{-/-}, IL-13^{-/-}, IL-4R α ^{-/-} and B-cell specific IL-4R α knockout mice show that rapid resolution of a secondary infection is related to B-cells that express IL-4R α and are IL-13 competent, but not type 2 antibody responses or B-cell IL-4. This was confirmed by adoptive transfer of *N. brasiliensis* primed IL-4R α -responsive B cells into naïve BALB/c mice, which conferred protection against a primary *N. brasiliensis* infection.

University of Cape Town

2. Methods and Materials

2.1 Animal Work

2.1.1 Cell specific gene targeting of IL-4R α

In order to study the specific effects of IL-4 and IL-13 on certain cell types, one can genetically manipulate cells to specifically delete the gene for IL-4R α from that cell type. The gene for IL-4R α can be flanked by a pair of loxP sites (the gene is referred to as floxed), which are the binding sites for an enzyme known as cyclisation recombinase (Cre). Cre can delete the DNA sequence between the loxP sites and then join them together [104]. The gene for the Cre can be inserted into the genome of a cell downstream of a promoter, and then when transcription of the promoter occurs the Cre is expressed and removes the IL-4R α gene of that cell. If the promoter is only active in certain cell types then the Cre induced IL-4R α deletion is specific to that type of cell.

To identify IL-4R α -mediated effects on B-cell function during *N. brasiliensis* re-infection, B-cell specific IL-4R α knock outs were generated. B-cell-specific IL-4R α -deficient BALB/c mice were established by intercrossing floxed IL-4R α mice [97] with mice expressing Cre-recombinase (Cre) under control of the *MB1* promoter (*MB1*^{Cre} mice) [105]; The *MB1* gene encodes the Ig- α signalling subunit of the B cell antigen receptor [24]. Transgene-bearing hemizygous mice (*MB1*^{Cre}IL-4R α ^{-lox}) were identified by PCR genotyping and efficient B cell-specific depletion was demonstrated on a genomic, protein and functional level [106].

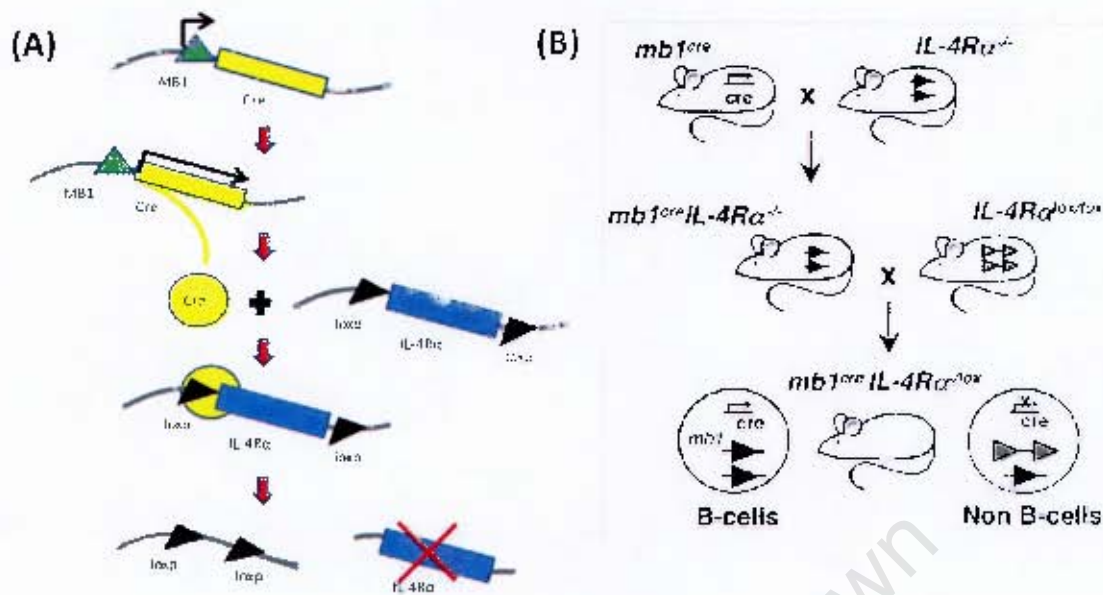


Figure 2.1: IL-4R α gene deletion under the influence of MBI. (A) When the MBI promoter is activated it initiates transcription of the gene for Cre. The Cre enzyme then targets and deletes the floxed IL-4R α gene. (B) A breeding scheme to produce MBI^{Cre}IL-4R α ^{-loxP} mice.

2.1.2 The Mice Used

In this study the following BALB/c background mice were used: Wild type BALB/c controls, BALB/b, IL-4R α ^{-/-} {described as *Il4ra*^{tm1Fbb}/*Il4ra*^{tm1Fbb}}, IL-13^{-/-}, IL-4^{-/-} and μ MT. BALB/c background B-cell specific IL-4R α deficient MBI^{Cre}IL-4R α ^{-loxP} {described as *Il4ra*^{tm1Fbb}/*Il4ra*^{tm2Fbb}Tg (*Cd79a*^{tm1(cre)Reth})} were generated. The C57BL/6 background mice used included: MHCII^{-/-}, MyD88^{-/-} and their wild type C57BL/6 controls.

2.1.3 Ethics and animal unit

All studies were carried out in accordance with protocol 011/008 approved by the Faculty of Health Sciences Animal Ethics Committee from the University of Cape Town. Mice were bred and housed in specific pathogen-free conditions at the Animal Unit of the University of Cape Town, South Africa. All experimental mice were sex matched and used between 6–12

weeks of age with appropriate littermate controls of the same generation. Mice were killed by asphyxiation using pressurised CO₂.

2.2 Using *Nippostrongylus brasiliensis*

2.2.1 Cycling of the *N. brasiliensis*

The *Nippostrongylus brasiliensis* stock is maintained by the division of Immunology (Faculty of Health Science, UCT), by passage through Wistar rats. Every two weeks, a naive group of rats were infected subcutaneously with 5000 L3 larvae in 200µl of 0.9% NaCl. Rat faecal pellets were collected on day 6, 7 and 8 post infection and liquefied in 5µg/ml fungizone in H₂O, then incubated on moist filter paper in petri dishes at room temperature. After 7 days, the larvae hatched and migrated to the edge of the filter paper, where they can be harvested at the L3 stage for future infections.

2.2.2 Primary and secondary infections

To carry out infections L3 larvae were washed off the filter paper into 0.9% NaCl solution. The larvae were counted under a dissecting microscope to establish a concentration of 2500 L3/ml. The infections were carried out at the procedure room of the animal unit. Each mouse was injected sub-cutaneously with 500 x *N. brasiliensis* L3 larvae in 200 µl of 0.9%NaCl. For the primary infection the mice were killed on the relevant day after the infection (day 1, 5 or 9 post infection). The mice used for secondary infection experiments were treated with 10 mg/ml Ivermectin in their drinking water from day 7 to 14 post primary infection to clear the pathogen. Then they were re-infected approximately 6 week's post primary infection, and were killed on day 5 post secondary infection.

2.2.3 Generating NES (*N. brasiliensis* somatic antigen)

L3 larvae were washed from the edge of the filter paper into H₂O. Pen./Strep. (Penicillin + Streptomycin) at 50µg/ml was added to the H₂O to kill any contaminating bacteria. This mixture was left for 1 hour during which the larvae settle to the bottom of the container. Then the supernatant liquid can be aspirated off, and the larvae are washed twice in Pen./Strep. to ensure that they are completely sterile. The larvae are then concentrated into 2 ml of distilled H₂O and dipped into liquid nitrogen to snap freeze them and disrupt cellular walls and membrane in order to release the cells' contents. Following this they are homogenized before the whole solution is centrifuged to pellet out all the large insoluble cellular and tissue debris which is discarded. The supernatant contains the soluble fraction of the L3 larvae proteins and glycoproteins, and the protein concentration is measured and standardised using a BCA protein assay (Pierce; Chicago, IL). NES was added to the cells in solution at 10µg/ml for protocols which required the cells to be re-stimulated or pulsed with *N. brasiliensis* antigen.

2.2.4 Intestinal worm counts

The small intestines were removed from infected mice and cut longitudinally to expose the lumen, and incubated in 0.9% NaCl at 37°C for 4 hours to induce the worms to migrate out of the tissue into the supernatant. Then the supernatant containing the worms was poured out into a large graded Petri-dish and the total adult worms per intestine (or per mouse) in the counted under a dissecting microscope.

2.3 Cell and Tissue processing

The spleen or lymph nodes were removed from mice in sterile conditions and collected in IMDM cell media (GIBCO/Invitrogen; Carlsbad, CA). They were passed through a 40µm filter to create a single cell suspension. The red blood cells were removed by washing the

cells in red cell lysis buffer. The remaining lymphocyte cells were then counted under a microscope using a haemocytometer slide, and the cells were re-suspended at a concentration of 1×10^7 cells/ml in complete media with 10% Foetal Calf Serum (FCS) added. The cells are then ready for re-stimulation, FACS or to be sorted.

2.4 Flowcytometry

2.4.1 The basic method

Flow cytometry was used to analyse spleen and lymph node cells for cognate cell surface receptor expression in order to quantify the different populations of cells and their activation. Approximately 1×10^6 cells per FACS sample was used. The samples were stained with 25 μ l antibody master mix (FACS buffer containing 2% Rat serum, 2% anti-Fc γ II/III and the required antibodies diluted to the correct concentration) for 30min on ice, before the antibody was washed off in FACS buffer. The cells were re-suspended in FACS buffer and analysed on an appropriate flow cytometer. Antibodies were from BD Pharmingen (San Diego, CA). The samples were read by a 2 laser Bectin Dickinson FacsCalibur or a 4 laser Bectin Dickinson FacsFortessa machine and the data was collected by Cell Quest[©] or DIVA[©] BD (San Diego, CA).. FACS data was analysed by FlowJo[©] Treestar (Ashland, OR) (**Figure 2.2**). Appropriate isotype controls were run to ensure that the populations were accurately measured (to eliminate auto-fluorescence /non specific binding). All antibodies used are rat-anti-mouse.

Table 1. The monoclonal antibodies used for the flow cytometry, their Clone and Isotype controls. All antibodies used were made by Pharmingen/BioSciences (BD).

Antibody	Fluorochrome	Clone	Isotype control
CD4	PerCP	RM4-5	IgG2a
CD44	FITC	IM7	IgG2b
CD62L	APC	MEL-14	IgG2a
CD3	PE	500A2	IgG2
B220	FITC	RA3-6B2	IgG2a
CD19	PerCP	ID3	IgG2a
CD86	APC	16-10A1	IgG2
CD21	APC	7G6	IgG2b
CD23	PE	B3B4	IgG2a
MHCII	FITC	2G9	IgG2a

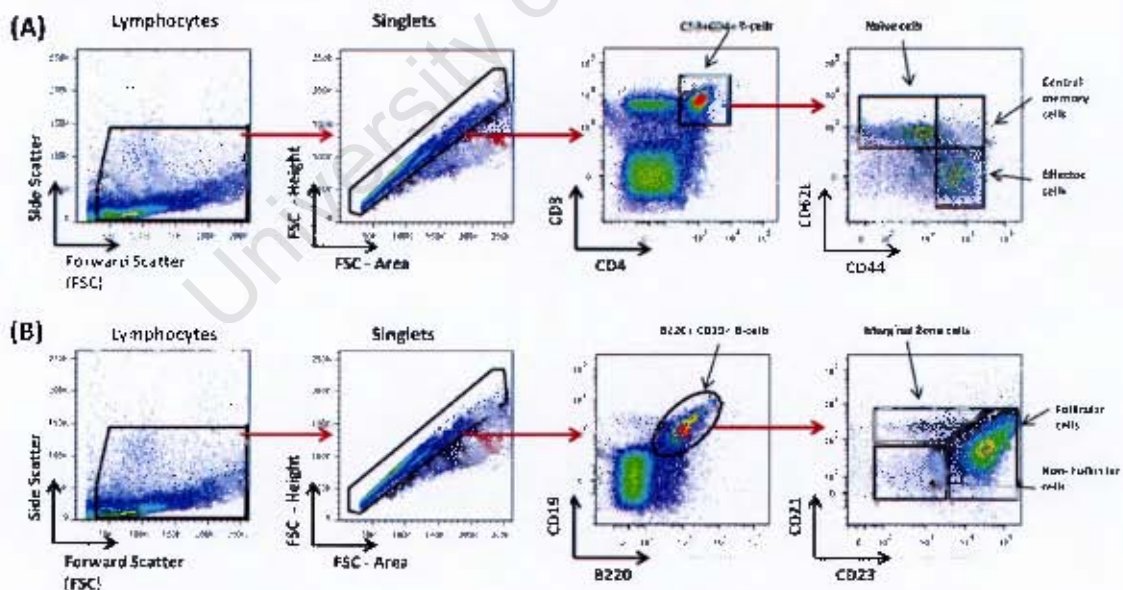


Figure 2.2 The FACS gating strategies used for data analysis. The Raw data is gated for lymphocytes, then singlet cells. Next the CD3⁺CD4⁺ T-cell (A) or B220⁺CD19⁺ B-cell (B) populations are determined and finally the activation markers of the cells are analysed.

2.4.2 Intra-cellular FACS

Intracellular cytokine staining was performed on cells re-suspended in complete media at $1 \times 10^7/\text{ml}$ and stimulated with $10 \mu\text{g}/\text{ml}$ of *N. brasiliensis* antigen + GolgiStop (BD Pharmingen) at 37°C for 4 hours. After re-stimulation, the cells were surface stained for CD3, CD4 and B220, then fixed and permeabilized with Cytofix/Cytoperm Plus (BD Pharmingen). Intracellular staining was performed by staining cells with IL-13-PE (Clone: ebio 13a) (eBioscience; San Diego, CA). or appropriate IgG1 isotype control

2.5 Cell sorting

The cells were stained for the required B or T cell markers with FACS antibodies as described above then re-suspended at $1 \times 10^7/\text{ml}$ in media. Then they were passed through a BD FACSARIA Flow cytometer which is able to sort and isolate the required populations (Figure 2.3 (A)). The purity of the isolated population was confirmed by flow cytometry, and samples showing $<95\%$ positive cells were discarded (Figure 2.3 (B)).

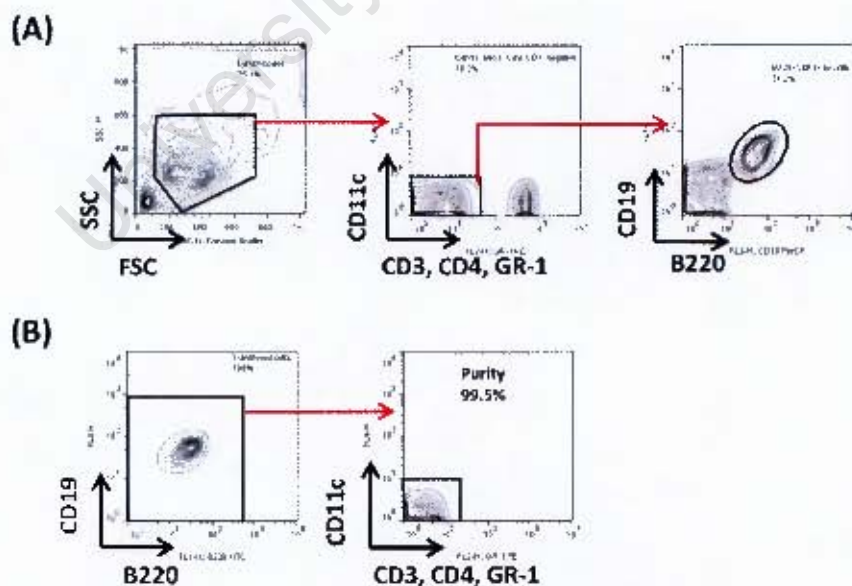


Figure 2.3 Isolation of specific cell populations by Flow cytometry. The gating strategy used for sorting $\text{B220}^+\text{CD19}^+$ B-cells on the BD FACSARIA is shown (A). The purity of the isolated population was confirmed by flow cytometry (B).

2.6 Culturing Cells and transfers

2.6.1 B-cell adoptive transfers

Spleen B cell populations were isolated. These were adoptively transferred to naive mice to assess their protective abilities. The cells were re-suspended at 2.5×10^6 cells/ml in media. Each mouse received 0.5×10^6 cells injected intravenously into the tail vein 24h prior to infection with *N. brasiliensis*.

2.6.2 B and T cell co-cultures

Previously isolated B and T cells were co-cultured at a ratio of 1:2 for 24 hours at 37°C. Cultures were then depleted of B cells and CD4+ T cell purity (>95%) confirmed by flow cytometry. Naïve BALB/c mice received 0.5×10^6 CD4+ T cells intravenously 24h prior to infection with *N. brasiliensis*.

2.7 Antigen Stimulation of B-cells (for Chapter 4)

2.7.1 24 hour infections

Naive mice were infected with *N. brasiliensis* as previously described. 24 hours after the infection they were killed and the B-cells were isolated as previously described and then re-suspended in media at 2.5×10^6 /ml. 0.5×10^6 cells were then transferred intravenously into naïve mice 24h prior to infection with *N. brasiliensis*, or the B and naive T cells were co-cultured as previously described, and the T-cells were transferred.

2.7.2 Culturing with NES

Cells were incubated (pulsed) with either 10µg/ml *N. brasiliensis* antigen, *Leishmania major* antigen or Ovalbumin in solution for 24h at 37°C. Cells were then washed 3 times in media and then re-suspended in media at 2.5×10^6 /ml. 0.5×10^6 cells were then transferred

intravenously into naïve mice 24h prior to infection with *N. brasiliensis*, or the B and naive T cells were co-cultured as previously described, and the T-cells were transferred.

2.8 Antibody ELISAs

A 96 well flat bottomed plate was coated with 10µg/ml of Somatic antigen (NES) in carbonate coating buffer overnight at 4°C before blocking buffer was added for 1hr at 37°C, after which the plate was washed in washing buffer. Then the blood serum in was added to the wells, starting with a 1/20 dilution and this was serially diluted at 1/5 down the plate using diluting buffer. The plate was incubated overnight at 4°C and washed the next day, before the biotinylated secondary antibody for IgG1 was added and the plate was again incubated overnight at 4°C. After a washing step, 4-P-Nitrophenol-Phosphate in substrate buffer (1mg/ml) added to develop the reaction and the plate was read at 405nm.

2.9 Histology slides

Periodic Acid Schiffs (PAS) slides were made of small intestine (duodenum) sections to examine mucous production. The tissue was stored in 4% phosphate buffered formalin to prevent autolysis, before it was embedded in paraffin wax and sectioned into 3µm thick slices. The process of staining required 1% aqueous periodic acid, H₂O, Schiff reagent, H₂O, haematoxylin, H₂O, alcohol and xylol in that order. Mucous producing goblet cells stain dark red and differences in size and number of goblet cells between mice can be analysed. The slides were made by the surgical research department of Groote Schuur hospital, Cape Town. The slides were examined on a light microscope with a Nikon camera for taking pictures.

2.9 Statistics

The results expressed below are either from individual mice or as group means \pm standard error. P values and significances were determined using either Mann-Whitney T-test or non-parametric one-way ANOVA (GraphPad Prism software; La Jolla, CA). Groups were judged to be significantly different if the P value was less than 0.05. [107]

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3. The role of B-cells in adaptive immunity to *N. brasiliensis*

3.1 Preliminary Results (Dr. William Horsnell & Dr. Jennifer Hoving)

3.1.1 Introduction

The preliminary results of this study showed that B-cells, which typically protect against infection by producing antibodies, protected against a hookworm like nematode infection by mechanisms other than antibody. The protective B-cell function indicated by these results depended on B-cells providing T cells with specific instruction. These B-cell driven T-cell responses resulted in a rapid resolution of the infection.

In the study presented here we demonstrate IL-13 signalling through IL-4R α to be essential for the development of effective immune memory against *N. brasiliensis*. Rapid resolution of secondary infection related to B cell IL-13 production and not type 2 antibody responses. Re-infected mice with cell specific disruption of B cell IL-4R α expression showed significantly increased intestinal worm burdens relating to reduced B-cell IL-13 production, CD86 and MHCII surface expression. Adoptive transfer studies demonstrated IL-4R α dependent B-cell IL-13 production and antigen presentation all contribute optimal immunity to *N. brasiliensis*.

3.1.2 IL-4R α is essential for immunity against re-infection with *N. brasiliensis*

To identify a possible role for IL-4R α in generating protective immunity, IL-4R α responsive IL-4R α ^{-lox} and deficient IL-4R α ^{-/-} mice were infected with *N. brasiliensis*, subsequently cleared by drug treatment and re-infected with 500 L3 larvae 8 weeks post primary infection. The intestinal worm burden was quantified at 5 days post secondary infection (**Figure 3.1A**). IL-4R α ^{-/-} mice consistently presented significantly higher intestinal worm burdens when compared to IL-4R α ^{-lox} mice at day 5 post secondary infection (**Figure 3.1B**). Mucosal goblet cell responses associated with T_H2 immunity are considered to be a potential

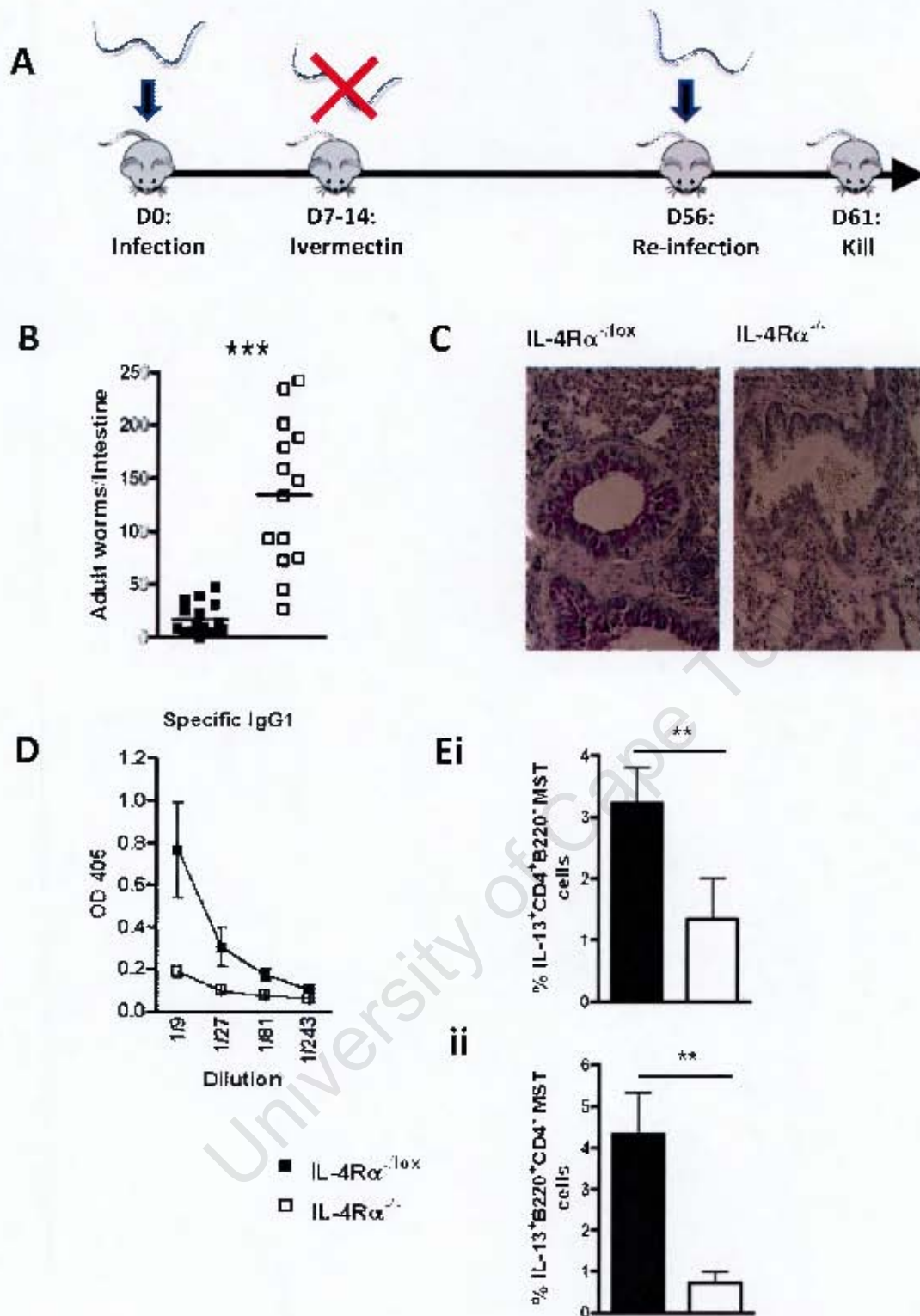


Figure 3.1: Protective immunity to *N. brasiliensis* re-infection requires IL-4R α . IL-4R α ^{-/-} and IL-4R α ^{-lox} mice were infected for 5 days post secondary *N. brasiliensis* infection (A). Intestinal worm burdens were then quantified (B). Pulmonary mucus production was established by PAS staining (C). Serum Antibody titers of *N. brasiliensis* specific IgG1 were determined by ELISA (D). Mediastinal lymph node IL-13 responses were established by intracellular FACS staining in CD4⁺ T-cell and B220⁺ B-cell populations (E). Data is representative of 3-4 independent experiments, n = 4-6 mice per group.

protective mechanism against parasitic nematodes [77]. Assessment of these responses showed reduced mucus production in the lung epithelium of IL-4R α ^{-/-} mice when compared to IL-4R α ^{/lox} mice (**Figure 3.1C**). Analysis of *N. brasiliensis* specific IgG1 (which is reflective of Type 2 immunity) antibody titers demonstrated a failure to develop effective type 2 antibody responses in IL-4R α ^{-/-} mice when compared to IL-4R α ^{/lox} mice (**Figure 3.1D**). Furthermore IL-4R α ^{-/-} mice showed reduced IL-13 production in both T and B cell populations in the mediastinal (lung draining)(MST) lymph nodes when compared to IL-4R α ^{/lox} mice (**Figure 3.1E**).

3.1.3 IL-4R α dependant immunity to *N. brasiliensis* re-infection is driven by IL-13

In order to investigate whether resolution of secondary *N. brasiliensis* infection was dependent on IL-4 and/or IL-13 signalling via IL-4R α , re-infection studies were carried out in IL-4^{-/-} and IL-13^{-/-} mice (**Figure 3.1A**). Here, the significantly higher intestinal worm burden in IL-13^{-/-} mice compared to IL-4^{-/-} mice (**Figure 3.2A**) demonstrated that IL-13 signalling through IL-4R α was essential for optimal immunity against re-infection with *N. brasiliensis*. This higher worm burden could also be associated with an absence in goblet cell mucus production in IL-13^{-/-} but not IL-4^{-/-} mice (**Figure 3.2B**). IL-13^{-/-} mice demonstrated equivalent *N. brasiliensis* specific IgG1 (type 2) antibody titers as IL-4R α ^{/lox} mice. Conversely, IL-4^{-/-} mice demonstrated reduced specific IgG1 responses (**Figure 3.2**). Taken together these data (**Figures 3.1 and 3.2**) indicate that antigen specific IgG1 antibody responses are not sufficient for optimal immunity to re-infection but that IL-4R α -mediated IL-13 signalling is required.

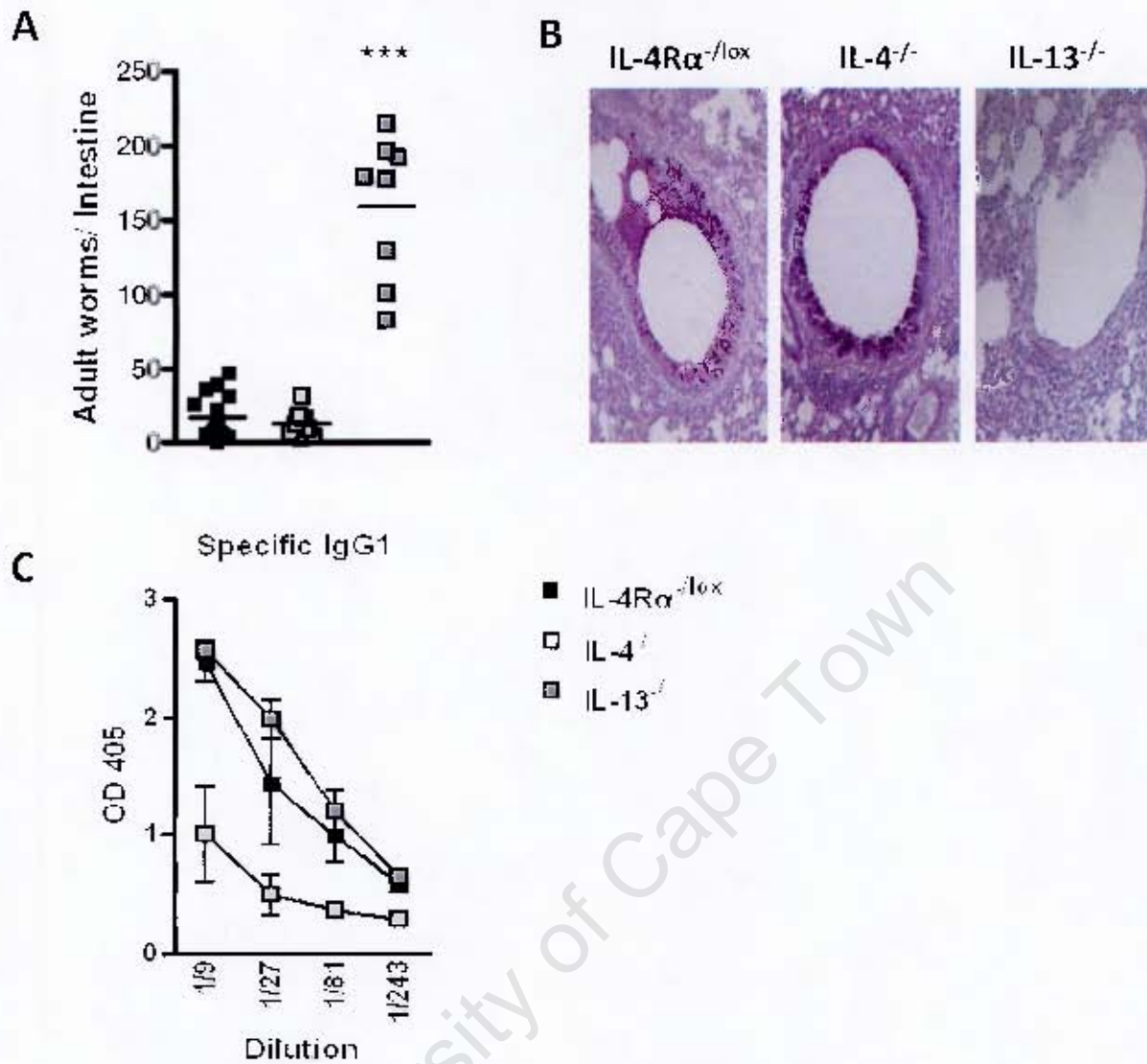


Figure 3.2: Protective immunity to *N. brasiliensis* re-infection is independent of antibody and IL-4 but dependent on IL-13. IL-4^{-/-}, IL-13^{-/-} and IL-4R α ^{-/lox} mice were infected for 5 days post secondary *N. brasiliensis* infection (**fig1A**). Intestinal worm burdens were then quantified (**A**). Pulmonary mucus production was established by PAS staining (**B**). Serum Antibody titers of *N. brasiliensis* specific IgG1 were determined by ELISA (**C**). Data is representative of 3 independent experiments, n = 4-6 mice per group.

3.2 Results

3.2.1 IL-4R α -responsive B-cells producing IL-13 are required for effective immunity to *N. brasiliensis* re-infection

It has been shown that IL-4R α expression by lymphocytes is not essential to resolution of a primary *N. brasiliensis* infection [14]. In contrast, analysis of worm burdens at day 5 post secondary infection (**Figure 3.1A**) showed a significantly higher intestinal worm burden in *MB1^{Cre}IL-4R α ^{-lox}* mice when compared to *IL-4R α ^{-lox}* mice (**Figure 3.3A**). Goblet cell hyperplasia in *MB1^{Cre}IL-4R α ^{-lox}* mice was equivalent to that seen in control *IL-4R α ^{-lox}* mice (**Figure 3.3B**). As expected from the cell-specific IL-4R α impairment, type 2 serum antibodies (antigen specific IgG1) were significantly reduced in infected *MB1^{Cre}IL-4R α ^{-lox}* mice when compared to *IL-4R α ^{-lox}* mice (**Figure 3.3C**). Interestingly, IL-13 cytokine production by MST T and B cells was consistently reduced in *MB1^{Cre}IL-4R α ^{-lox}* mice when compared to *IL-4R α ^{-lox}* mice (**Figure 3.3D**). Together, these studies demonstrate a requirement for IL-4R α responsive B-cells for optimal immunity to *N. brasiliensis* re-infection through the regulation of T and B lymphocyte IL-13 responses.

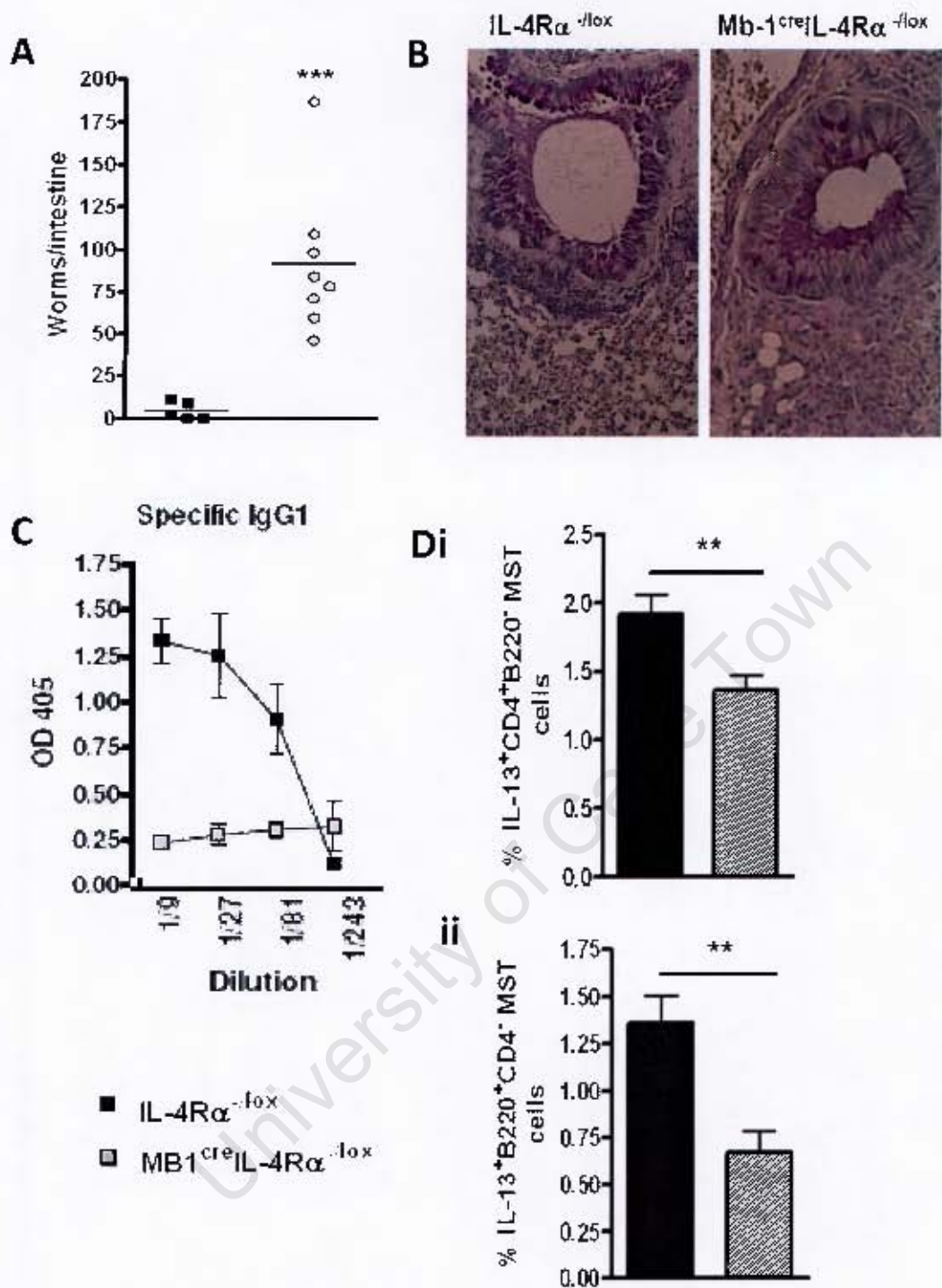


Figure 3.3: B-cell IL-4R α expression is required for optimal immunity to *N. brasiliensis* re-infection. Mb1^{Cre}IL-4R $\alpha^{-/-}$ and IL-4R $\alpha^{-/-}$ mice were infected for 5 days post secondary *N. brasiliensis* infection (fig3.1A) and intestinal worm burdens were then quantified (A). Pulmonary mucus production was established by PAS staining (B). Serum Antibody titers of *N. brasiliensis* specific IgG1 were determined by ELISA (C). Mediatstinal lymph node IL-13 responses were established by intracellular FACS staining in CD4⁺ T-cell and B220⁺ B cell populations (D). Data is representative of 3 independent experiments, n = 4-6 mice per group.

3.2.2 IL-4R α competent B-cells that can produce IL-13 activate *N. brasiliensis* protective responses.

In order to demonstrate if IL-4R α responsive and IL-13 competent B-cells can directly confer protection against *N. brasiliensis*, we adoptively transferred B-cells isolated from infected IL-4R α ^{-/lox}, IL-13^{-/-} or *MB1*^{Cre}IL-4R α ^{-/lox} mice into naïve BALB/c mice 24 hours before infecting them with 500 L3 *N. brasiliensis* larvae (**Figure 3.4A**). Transfer of primed IL-4R α -responsive B-cells into naïve BALB/c mice reduced intestinal worm burdens. In contrast, transfer of primed B-cells deficient for either IL-4R α or IL-13 did not reduce intestinal worm burden (**Figure 3.4B & C**). These results support our previous observations (**Figures 3.1, 3.2 and 3.3**) that IL-4R α -responsive and IL-13 competent B-cells contribute to protective immunity against *N. brasiliensis*.

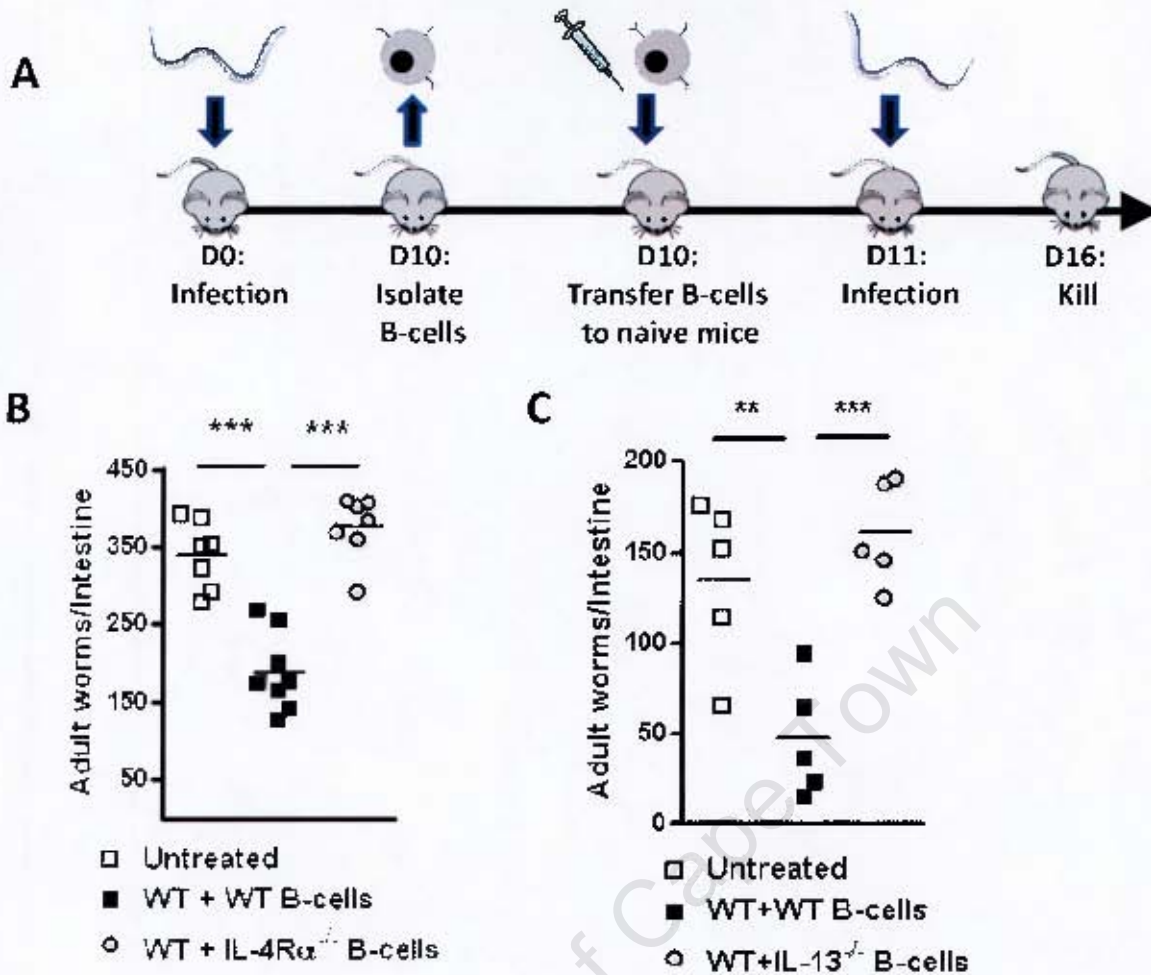


Figure 3.4: IL-4R α mediated B cell immunity to *N. brasiliensis* is IL-13 dependent. B cells were isolated from *N. brasiliensis* infected BALB/c, *MB1^{cre}IL-4R $\alpha^{-/-}$* and *IL-13 $^{-/-}$* mice and transferred into naïve BALB/c mice (A). Mice were then infected with 500 x L3 *N. brasiliensis* larvae and worm burdens were established at day 5 Post Infection (PI) (B & C). The results shown represent 2 independent experiments. n = 4-7 mice per group.

3.2.3 Transferred B-cells can provide protection in the absence of endogenous B-cell support

Based on these findings, we investigated if the protection seen in **Figure 3.4** requires support from endogenous B-cells. Previous studies have shown that in the absence of B-cells no change occurs in the kinetics of host control of *N. brasiliensis* re-infection [100]. In agreement with this, we also found host ability to control *N. brasiliensis* re-infection did not require B cells; μ MT mice showed equivalent worm burdens to those of BALB/c at day 5 post re-infection (**Figure 3.5A**). However, to test if IL-4R α expressing B cells can enhance host protection, irrespective of the presence of endogenous B cells, we transferred B cells from infected IL-4R $\alpha^{-/lox}$ and infected *MB1*^{Cre}IL-4R $\alpha^{-/lox}$ mice into naive μ MT mice 24 hours before infecting them with 500 L3 *N. brasiliensis* larvae (**Figure 3.5B**). Analysis of intestinal worm burdens showed transfer of *N. brasiliensis* primed IL-4R α responsive B-cells conferred protection in μ MT mice (**Figure 3.5Bi**), transferred B cells from infected *MB1*^{Cre}IL-4R $\alpha^{-/lox}$ mice did not. Endogenous MST CD4⁺ T-cell IL-13 production was increased in the protected group (**Figure 3.5Bii**). These results show that introduction of pathogen experienced IL-4R α -responsive B cells is sufficient to boost protective immunity against *N. brasiliensis* and that this relates to their ability to drive a CD4⁺ T-cell T_H2 response.

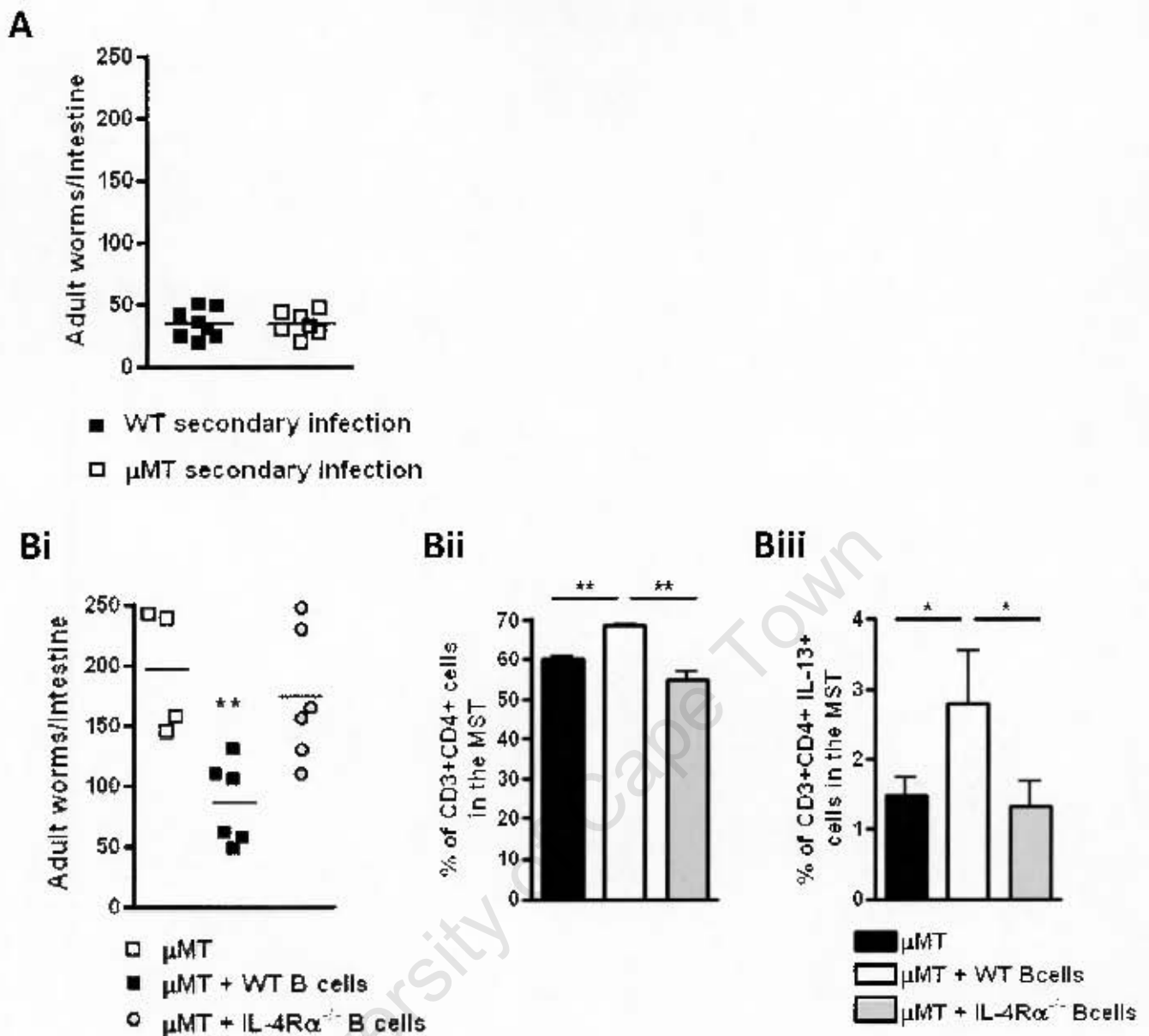


Figure 3.5: Transfer of *N. brasiliensis* experienced B cells enhances immunity to *N. brasiliensis* independently of endogenous B cell populations. μ MT and BALB/c mice were infected for 5 days post secondary *N. brasiliensis* infection and intestinal worm burdens were then quantified (**A**). Roles for IL-4R α expressing B cells in boosting immunity independently of endogenous B cells were established by transfer of B cells isolated from *N. brasiliensis* infected IL-4R α^{-lox} or *MB1^{Cre}*IL-4R α^{-lox} into naïve μ MT mice (As outlined in fig3.4A). These mice were then infected with 500 x L3 *N. brasiliensis* and worm burdens quantified at day 5 PI (**Bi**). Mediastinal lymph node CD3⁺CD4⁺ T-cell populations (**Bii**) and IL-13 responses (**Biii**) were established by FACS staining. Results shown represent 2 independent experiments. n = 4-7 mice per group.

3.2.4 Transferred B-cells activate endogenous T-cell populations

In order to demonstrate a functional role for *N. brasiliensis* and IL-4R α dependent interactions between B-cells and T-cells we co-cultured B-cells isolated from naive or infected IL-4R $\alpha^{-/lox}$ or *MB1^{Cre}*IL-4R $\alpha^{-/lox}$ mice with naive BALB/c CD4⁺ T-cells (**Figure 3.6A**). Following co-culture the T-cells were adoptively transferred into naive wild type mice 24h prior to *N. brasiliensis* infection and protection against infection conferred by the transfer was assessed at day 5PI (**Figure 3.6Bi**). Quantification of intestinal worm burdens at day 5PI demonstrated a significant reduction in worm burden in mice which received CD4⁺ T-cells co-cultured with wild type B-cells isolated from infected mice when compared to mice which received CD4⁺ T-cells co-cultured with either naive wild type B-cells or IL-4R $\alpha^{-/-}$ B-cells isolated from *N. brasiliensis* infected mice. Associated with this protection was increased endogenous MST CD4⁺ T-cell and B220⁺ B-cell IL-13 production (**Figures 3.6Bii**).

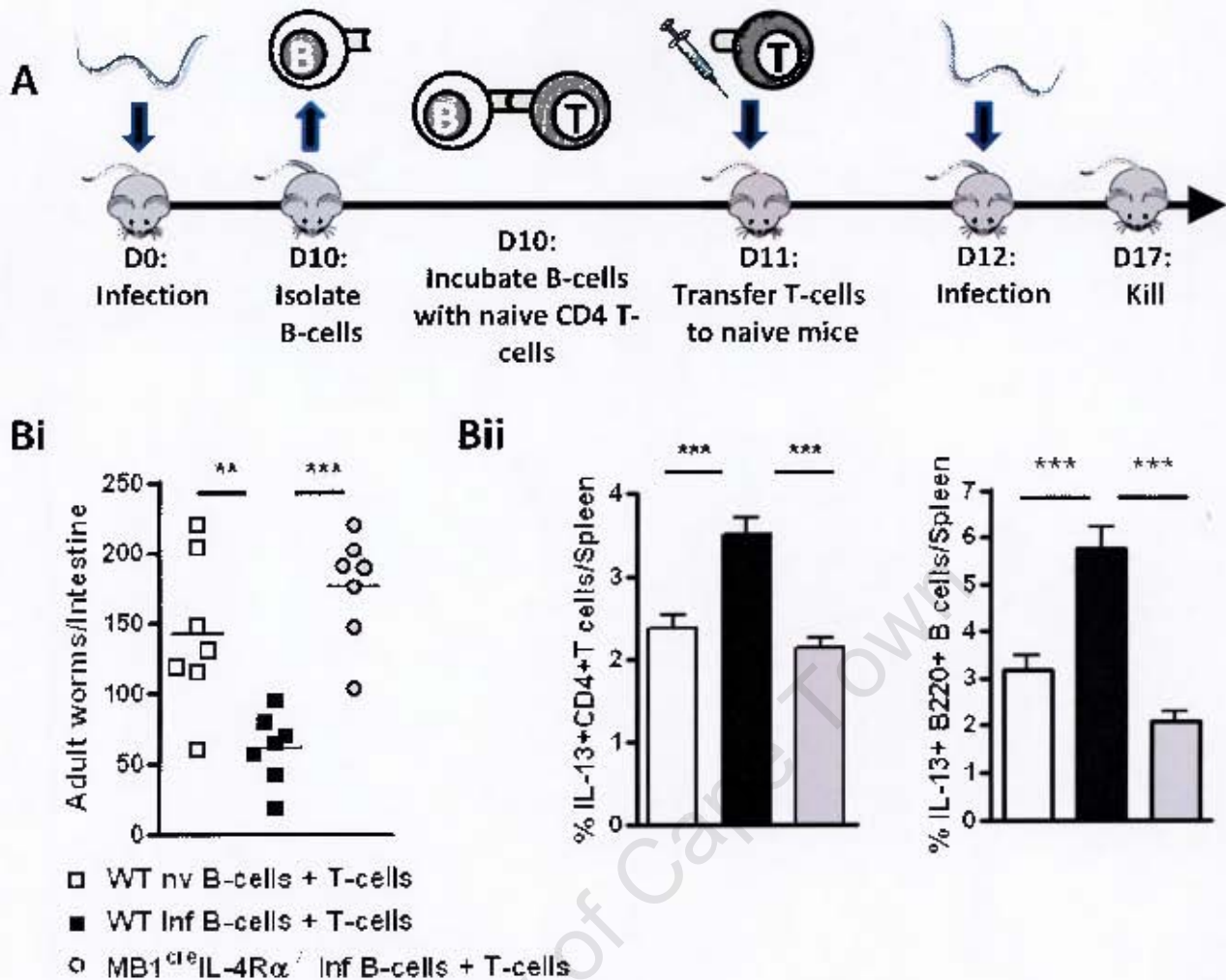


Figure 3.6: IL-4R α competent B-cells activate *N. brasiliensis* protective T-cell responses. The importance of T-cell interaction with B-cells was confirmed by isolating B-cells from naive or infected MB1^{Cre}IL-4R α ^{-lox} or IL-4R α ^{-lox} mice which were then co-cultured overnight with CD4⁺ T-cells isolated from naive BALB/c mice. Co-cultured CD4⁺ T-cells were then transferred into naive BALB/c mice 24h prior to infection (A). *N. brasiliensis* IL-4R α dependent B-cell driven activation of protective CD4⁺ T-cell population was demonstrated by intestinal worm counts at d5 PI (Bi). Protection was related to increased IL-13 production in endogenous MB1 CD4⁺ T-cell and B220⁺ B-cells (Bii). Data is representative of 2 independent experiments. n = 7 mice per group.

3.2.5 B-cell MHCII dependant interactions with T-helper cells are required for effective immunity to *N. brasiliensis* re-infection

B-cell activation and receptor expression is essential to an effective B-cell response and may underlie effector functions against *N. brasiliensis* re-infection. CD86 and MHCII interactions with CD28 and TCR are key mediators of B and T-cell functional interactions. We examined the surface expression of these common receptors in naive and re-infected IL-4R α ^{-lox} and MB1^{Cre}IL-4R α ^{-lox} mice. Here both CD86 and MHCII surface expression in MB1^{Cre}IL-4R α ^{-lox} re-infected mice were reduced when compared with IL-4R α ^{-lox} re-infected mice (Figure 3.7Aii & iii). CD86 and MHCII expression was equivalent between naive mice of the two groups (Figure 3.7Ai). CD4⁺ T cells showed no differences between the mouse strains in expression of CD28 and TCR. These data indicate that IL-4R α affects B-cell MHCII function and may directly influence the interactions between B cells and T cells which play an important role in host adaptive immunity to *N. brasiliensis*.

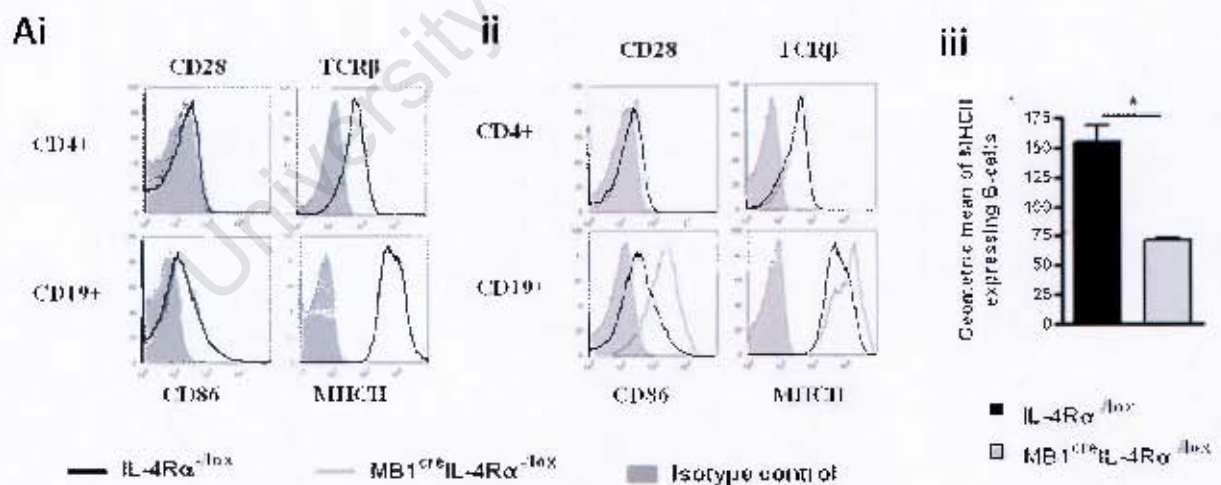


Figure 3.7: Protective immunity to *N. brasiliensis* re-infection is associated with increased B-cell MHCII expression. Surface expression of CD28 and TCR on CD4⁺ T-cells and CD86 and MHCII on B-cells in naive (Ai) and *N. brasiliensis* re-infected (Aii & Aiii) IL-4R α ^{-lox} mice and MB1^{Cre}IL-4R α ^{-lox} mice was established by FACS analysis. The results shown represent 2 independent experiments n = 4-7 mice per group.

B-effector cell MHCII expression is important because it mediates the presentation of antigen to T-cells. We have shown that B-cell MHCII expression is affected by B-cell IL-4R α competency. To demonstrate roles for antigen presentation we transferred MHCII^{-/-} B cells from *N. brasiliensis* infected mice into naive mice 24 hours prior to infection. Worm burdens at day 5PI in these mice were significantly higher when compared to mice which received WT B cells (**Figure 3.8Ai**). This may demonstrate MHCII dependent antigen presentation by B-cells is required for optimal immunity to *N. brasiliensis*. Protection is associated with increased expression of MHCII on endogenous MST B-cell populations (**Figure 3.8Aii**).

To identify if cognate interactions between B and T cells could contribute to this protection we co-cultured naive BALB/c CD4⁺ T cells with B cells isolated from *N. brasiliensis* infected BALB/b mice (**Figure 3.8Bi**). BALB/b APCs are unable to present antigen to BALB/c T-cells via MHCII because the receptors will not be able to bind to each other. Transfer of these T cells into naive mice demonstrated heightened intestinal worm burdens in recipients of CD4⁺ T-cells cultured with BALB/b B cells (**Figure 3.8Bii**). The protection was mediated by increased IL-13 production by B and T cell populations in the mediastinal lymph node (**Figure 3.8Biii**). This data demonstrates that MHCII antigen presentation to CD4⁺ T cells is an important component of the B-cell protective response to *N. brasiliensis* re-infection.

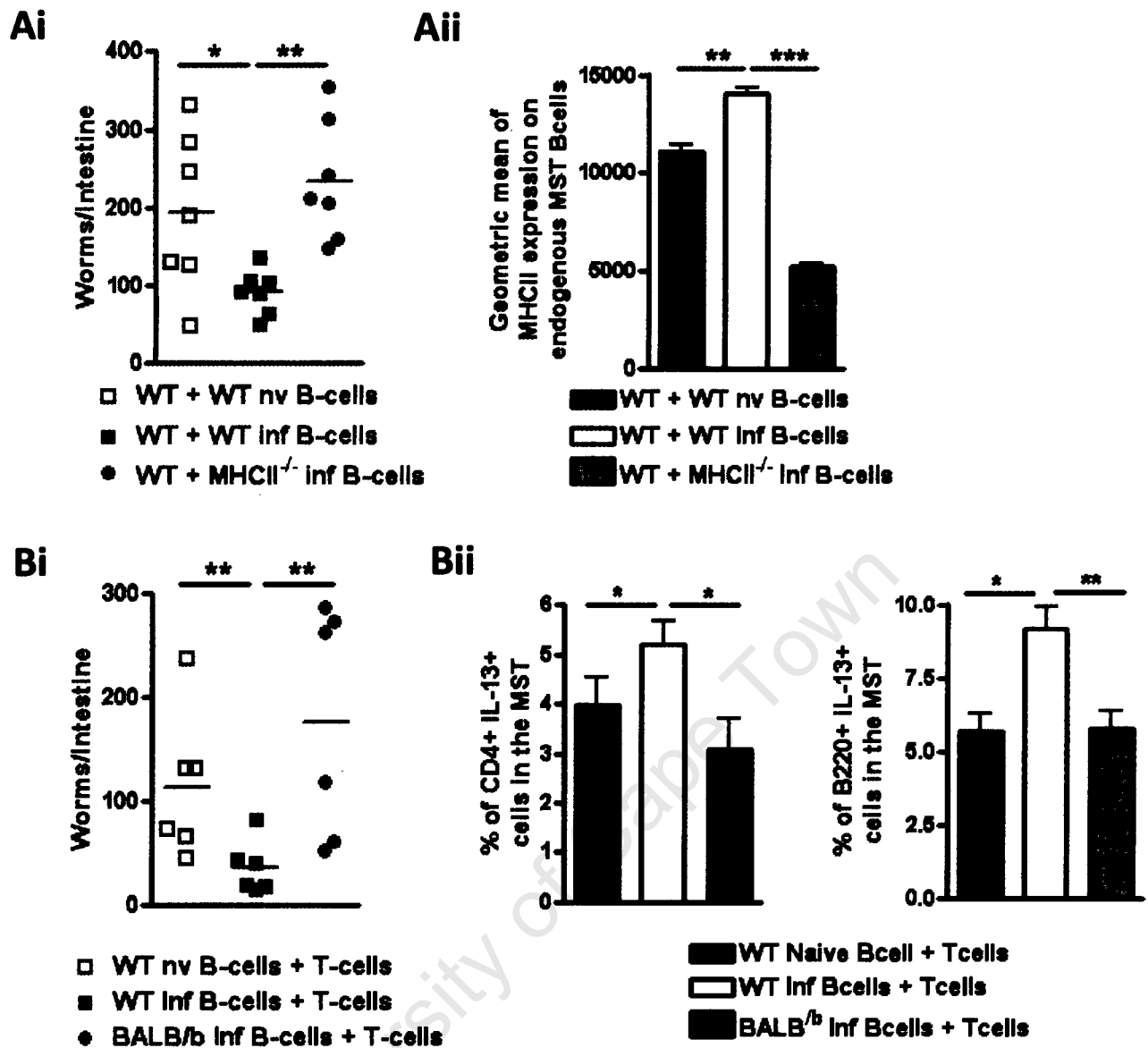


Figure 3.8: B-cell MHCII antigen presentation mediates optimal immunity to *N.*

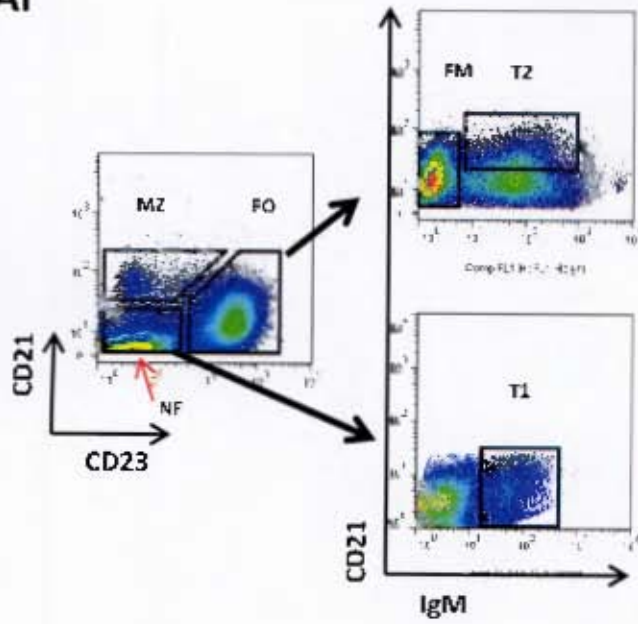
brasiliensis. Contributions by MHCII dependent antigen presentation were demonstrated by isolating WT or MHCII^{-/-} B cells from naive or infected mice then adoptively transferring into naive C57/BL-6 mice (As outlined in fig3.4A). Mice were then infected with 500xL3 *N. brasiliensis* larvae and worm burdens were established at day 5 PI (Ai). Endogenous MST B-cell MHCII expression was established by FACS (Aii). MHC dependent antigen presentation was confirmed by isolating WT and BALB/b B cells from naive or infected mice and co-culturing them with WT naive CD4⁺ T-cells (As outlined in fig.3.6A), which were then adoptively transferred into naive BALB/c mice. Mice were then infected with 500xL3 *N. brasiliensis* larvae and worm burdens were established at day 5PI (Bi). Mediastinal lymph node IL-13 responses were established by intracellular FACS staining in CD4⁺ T-cell and B220⁺ B cell populations (Bii). Data is representative of 2 independent experiments. n = 4-6 mice per group.

3.2.6 Protection to *N. brasiliensis* re-infection is associated with mature follicular cells

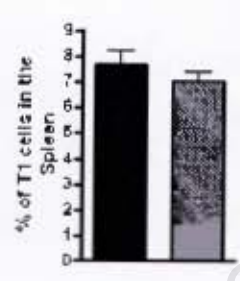
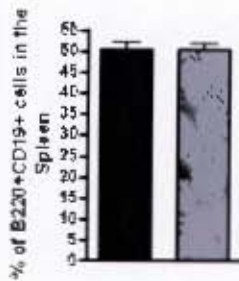
To address any IL-4R α associated alterations in developmental B-cell populations that may be associated with protective immunity to *N. brasiliensis* re-infection we analysed the B-cell populations in the spleens of naive or re-infected IL-4R α ^{-lox} or *MB1*^{Cre}IL-4R α ^{-lox} mice. The absence of IL-4R α did not appear to have any differential effect on B-cell developmental populations in the naive mice (Figure 3.9), but the protected IL-4R α ^{-lox} mice showed a significantly increased population of mature follicular cells (FM) and a lower population of marginal zone cells when compared to the *MB1*^{Cre}IL-4R α ^{-lox} group 5 day post re-infection (Figure 3.10).

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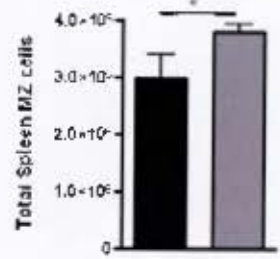
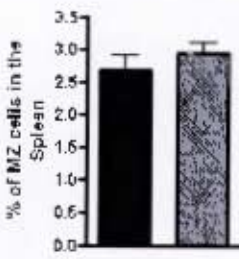
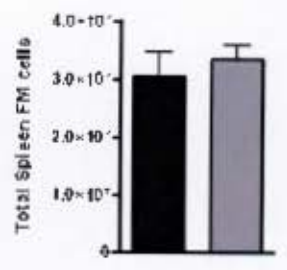
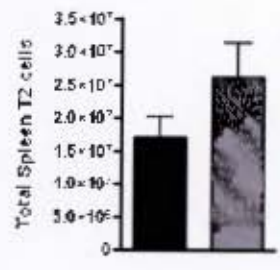
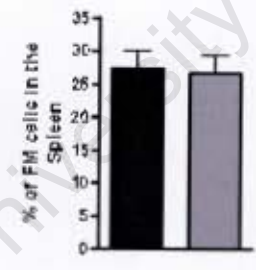
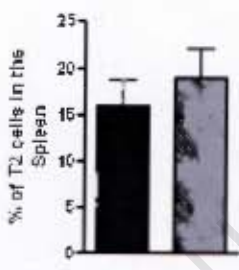
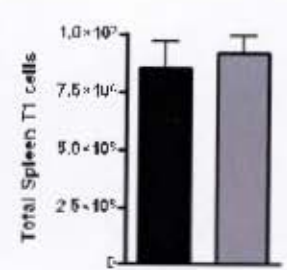
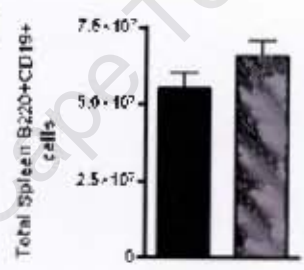
Ai



Aii



Aiii



Legend:
 ■ IL-4Rα^{-/-lox}
 ■ MB1^{Cre}IL-4Rα^{-/-lox}

Figure 3.9: B-cell IL-4Rα expression does not affect developmental populations in naive mice. B-cell developmental populations were established in naive MB1^{Cre}IL-4Rα^{-/-lox} and IL-4Rα^{-/-lox} mice by FACS (Ai). Relative size (Aii) of the populations and total cells were determined (Aiii). The results shown represent 2 independent experiments, n = 4-7 mice per group.

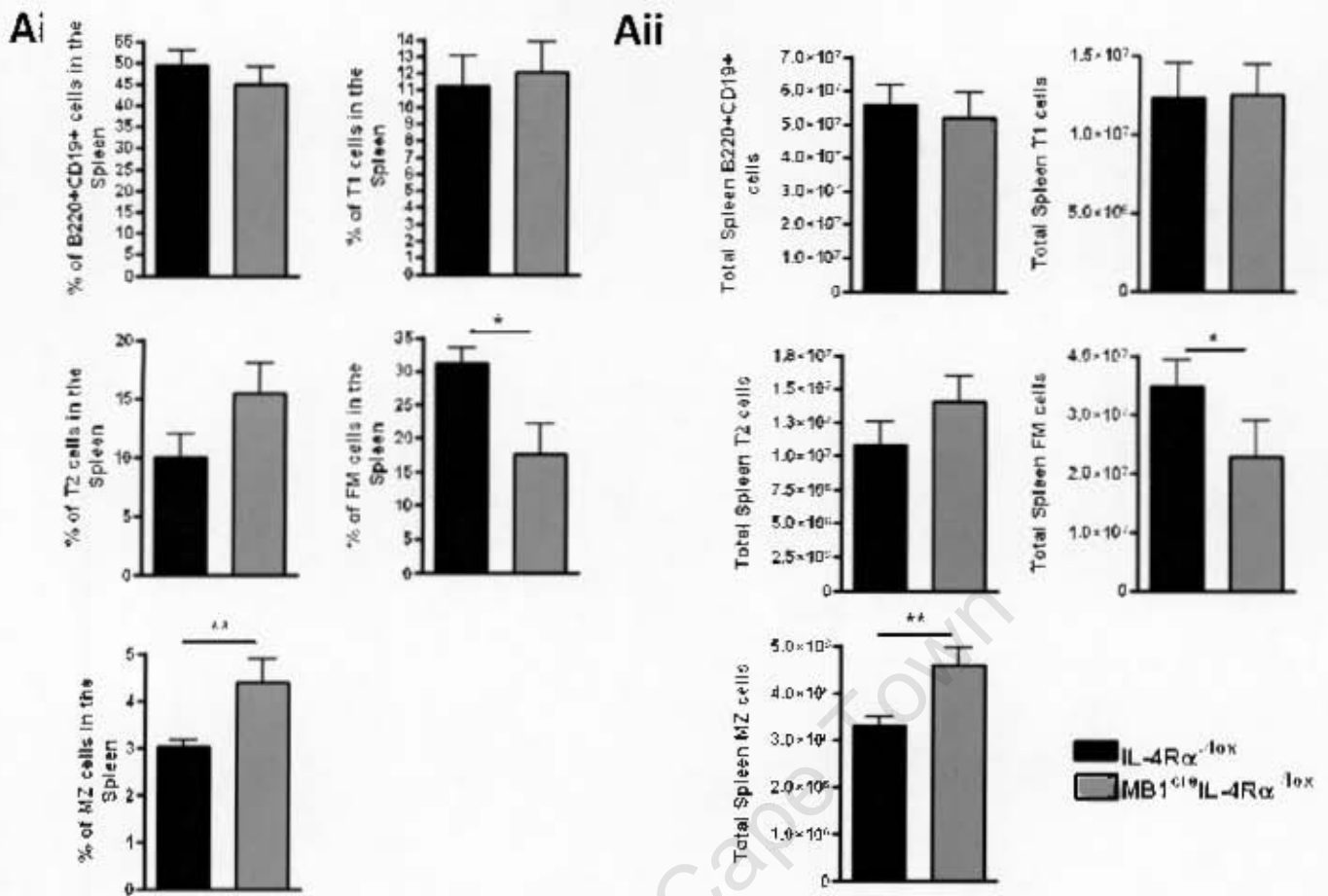


Figure 3.10: Protective immunity to *N. brasiliensis* re-infection is associated with mature follicular cells. B-cell developmental populations were established in re-infected MB1^{Cl}IL-4R α^{-lox} and IL-4R α^{-lox} mice by FACS. Relative size (Ai) of the populations and total cells were determined (Aii). The results shown represent 2 independent experiments. n = 4-7 mice per group.

3.3 Discussion

This study demonstrates that IL-4R α responsive B-cells co-ordinate optimal immunity to *N. brasiliensis*. This was related to IL-13 production but was not dependent on IL-4 production or IgG1 antibody secretion. B-cell IL-4R α mediated protection was associated with increased endogenous mediastinal B-cell and CD4⁺ T cell IL-13 production. There is also evidence that MHCII dependent B cell priming of T-cells may contribute to this effect. The antibody independent functions of Be B-cells are considered to be particularly important in controlling CD4⁺ T-cell driven immunity [50] via direct B and T cell interaction [51] as well as cytokine production [17].

B cell responses to *N. brasiliensis* have been suggested to be largely redundant [100]. Our study and Liu et. al. demonstrate humoral immunity to be superfluous for immunity to *N. brasiliensis*. Our work is also in agreement with Liu et al that an absence of B-cells *per se* does not necessarily alter host ability to control *N. brasiliensis* infection. However, we now show that a molecular change in B-cell function, such as cell specific disruption of IL-4R α expression on B-cells, significantly impairs host ability to resolve infection. Our data shows this effect to be related to B-cell IL-13 competency and antigen presentation. These findings suggest subtle yet important differences between B-cell dependent immunity to *N. brasiliensis* and *H. polygyrus*. B-cell Be2 immunity to *H. polygyrus* is independent of B-cell IL-4 production but dependent on B-cell IL-4R α expression and antigen presentation [53]. As with *H. polygyrus*, *N. brasiliensis* re-infection is also dependent on B-cell IL-4R α expression and antigen presentation, however, IL-13 production also appears to play a more prominent functional role.

In addition to antigen presentation we found IL-4R α -dependent increases in IL-13 production by endogenous B-cells to also be associated with control of secondary infection. This along with B-cell dependent induction of IL-13 production by CD4⁺ T-cell/ Be2 B-cells would provide an important source of IL-13 to activate potential effector cell populations, including epithelial [91], smooth muscle [88] and innate immune cells [102].

It is unclear whether the reduced resistance to *N. brasiliensis* infection in mice that receive transferred IL-13^{-/-} or MHCII^{-/-} B-cells when compared to mice that receive wild-type B-cells indicates that B-cell IL-13 production or MHCII expression is required to induce this resistance or whether expression of the gene within the donor is required to generate B-cells with a protective phenotype. In order to answer this question experiments will have to be carried out on mice that have B-cell-specific IL-13 or MHCII deletions.

Another novel finding was that IL-4R α expression affects T_H2 lineage commitment during a T_H2 response. Previously shown regulatory factors of T_H2 differentiation include BCR signalling capacity and strength [25], BCR ligand type [20], TLR signalling [64], cognate interactions and co-stimulation[6], antigen dependent selection and the internal signalling complexes that these factors generate [22] . However it has not yet been investigated whether IL-4/IL-13 and IL-4R α signalling could play a role in this development. Here we show that T_H2 cells which are IL-4R α competent are more likely to differentiate into Mature Follicular cells as opposed to marginal zone cell differentiation by cells which lack this signalling. This finding implies that protective immunity to *N. brasiliensis* is associated with mature follicular cells rather than marginal zone cells. If this is so, it confirms our previous findings, because the protective B-cell population which may activate T-cells via MHCII antigen presentation is likely to be FM B-cells, as marginal zone cells do not commonly interact with T-cells [22].

In summary this study demonstrates the principle role of IL-4R α -responsive B cells in resolving secondary *N. brasiliensis* infection is dependent on their ability to present antigen to T-helper cells and to produce and induce IL-13 production. This cognate interaction results in control of the infection by activating CD4⁺ T helper cell responses and driving IL-13 production. These are significant observations and of importance in understanding immunity to nematodes with analogous lifecycles, such as the hookworms.

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4. Rapid activation of B-cells independent of BCR

4.1 Introduction

Our demonstration that IL-4R α dependent B-cell responses are required for optimal immunity to *N. brasiliensis* re-infection via antibody independent mechanisms is novel for *N. brasiliensis* infection. However, other studies have shown that such antibody independent B-cell responses are required for recall immunity to *H. polygyrus* [53]. Our results agree with these previous observations, namely that B-cells contribute to recall immunity to *N. brasiliensis* T_H2 cytokine production and MHCII antigen presentation.

Previous studies have not investigated whether BCR plays a role in driving this response. BCR signalling is typically associated with the production of antigen specific antibodies [1]. Our data, namely the demonstration that B-cell driven immune function to *N. brasiliensis* infection was independent of antibody effector function (Figure 3.2), indicates that BCR dependent B-cell responses are unlikely to contribute to the protection we report. This suggests that the B-cells could be responding to *N. brasiliensis* antigen by other mechanisms that precede and are independent of BCR signalling. We hypothesized that this could be mediated through antigen interaction with receptors that have less stringent receptor-ligand requirements than BCR antigen interactions. It is established that B-cells can respond to antigen via a number of BCR independent mechanisms, including direct peptide loading of MHCII [36, 108] and TLR mediated responses [60, 61].

TLR mediated B-cells responses have been widely described and are diverse [58, 59]. Such responses include enhanced antibody production, up-regulation of activation markers and co-stimulatory surface receptors, increased cell proliferation and cytokine secretion [109, 110]. Importantly TLR mediated B-cell responses to antigen by polyfunctional B-cells can be rapid

and would not necessarily require the clonal expansion of B-cells [109]. For example, recent studies have elegantly demonstrated that TLR4 mediated activation of discrete B-cell subsets is a key requirement for protection against sepsis [63]. Importantly this protection can be mediated by B-cells exposed to LPS for a very short period of time, too short for BCR induced clonal expansion, showing effective antigen processing can occur quickly [22, 62, 63]. Recent work on DC control of *Salmonella typhimurium* has shown that 24 hours post infection DCs are capable of providing protective instruction to the host [111].

We wished to address how B cells responded to antigen, specifically looking at the role of BCR signalling and Toll like receptor mediated antigen internalisation and processing [60, 61, 110]. We investigated the capability of B-cells to determine their capability to provide protective instruction against *N. brasiliensis* infection rapidly and independently of BCR. Based on our findings, we hypothesized that TLR engagement with antigen by B-cells contributes to rapid effector functions before BCR mediated pathogen detection and activation [61]. We therefore tested the contribution of Myd88 signalling which is indicative of the importance of TLR signalling in this response.

4.2 Results

4.2.1 Rapid IL-4R α and Myd88 dependent B cell responses can protect against *Nippostrongylus brasiliensis* infection.

To test if B cells could rapidly develop a protective phenotype against *N. brasiliensis* infection we isolated splenic B-cells from $MB^{cre}IL-4R\alpha^{-/lox}$ and $IL-4R\alpha^{-/lox}$ mice 1 day post infection. These cells were then adoptively transferred into naive BALB/c mice. These mice were subsequently infected with *N. brasiliensis*. Assessment at day 5 post infection (**Figure 4.1A**) of recipient ability to control infection demonstrated an IL-4R α dependent decrease in worm burdens (**Figure 4.1B**). Having demonstrated that adoptive transfer of B-cells from 1 day infected mice could be protective we tested whether this protection was related to Myd88/TLR dependant B-cell activation. Here we isolated B-cells from $Myd88^{-/}$ and wild type mice 1 day post infection and transferred these into naive wild type recipients (**Figure 4.1A**). Again this was sufficient to induce Myd88 dependent decreases in worm burdens at 5 days post infection (**Figure 4.1Ci**). This protection was associated with increased IL-13 production by endogenous T-cells in the MST (**Figure 4.1Ci**).

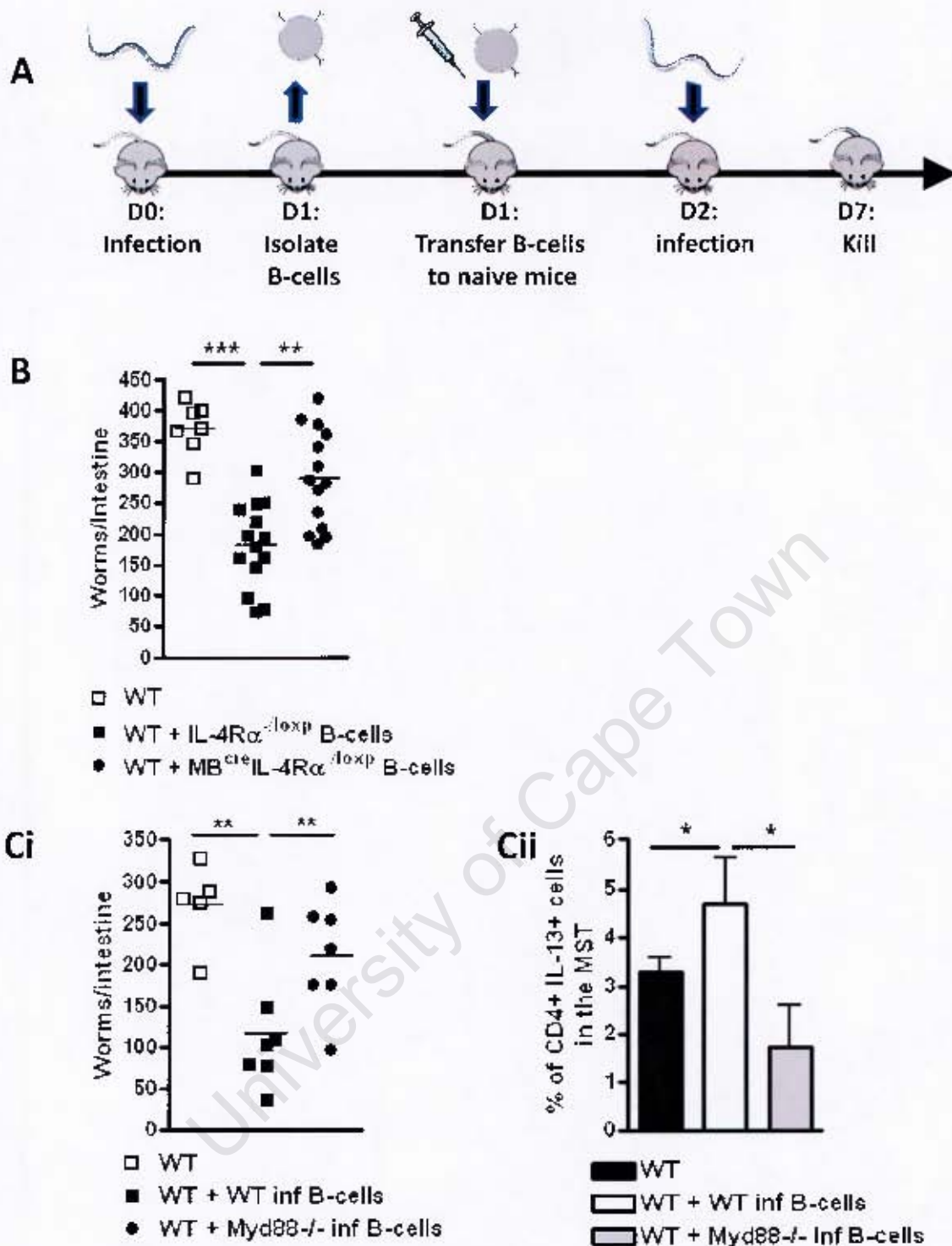


Figure 4.1: B-cells can provide rapid IL-4R α and Myd88 dependant protective immunity to *N. brasiliensis* infection. MB^{Cre}IL-4R α ^{-loxP} and IL-4R α ^{-loxP} mice were infected for 1 day with *N. brasiliensis* before spleen B-cells were isolated and transferred into naive wild type mice (A). These were infected with *N. brasiliensis* and intestinal worm burdens were quantified at day 5 post infection (B). Myd88^{-/-} and wild type mice were infected for 1 day with *N. brasiliensis* before spleen B-cells were isolated and transferred into naive wild type mice (A). Intestinal worm burdens were quantified at day 5 post infection (Ci). Protection was associated with MST T-cell IL-13 production (Cii). Data represents 2 experiments. n = 4-6 mice per group

As the B-cells were isolated from infected mice, there could possibly be antigen independent effects contributing to their activation. To test if B-cells could respond directly to *N. brasiliensis* antigen we isolated CD19⁺B220⁺ B-cells from the spleens of naive mice and treated them with 10µg/ml *N. brasiliensis* antigen overnight (Figure 4.2A). Then the cells were thoroughly washed to remove excess antigen before adoptive transfer into naive BALB/c recipients. 24 hours post transfer the mice were infected with *N. brasiliensis* and then killed 5 days post infection. Worm burdens were only reduced in the recipients of wild type *N. brasiliensis* pulsed B-cells while recipients of IL-4Rα^{-/-} or MyD88^{-/-} pulsed B-cells displayed worm burdens equivalent to recipients of naive B-cells (Figure 4.2Bi and Ci). This protection was associated with increased endogenous MST IL-13 production (Figure 4.2Bii and Cii). Together these results show that immunity to *N. brasiliensis* infection can be driven by naive B-cells responding directly and rapidly to antigen stimulation to drive systemic IL-13 responses, which would correlate with protective immunity to *N. brasiliensis*. This protection is related to B-cell IL-R4α and/or Myd88 responsiveness.

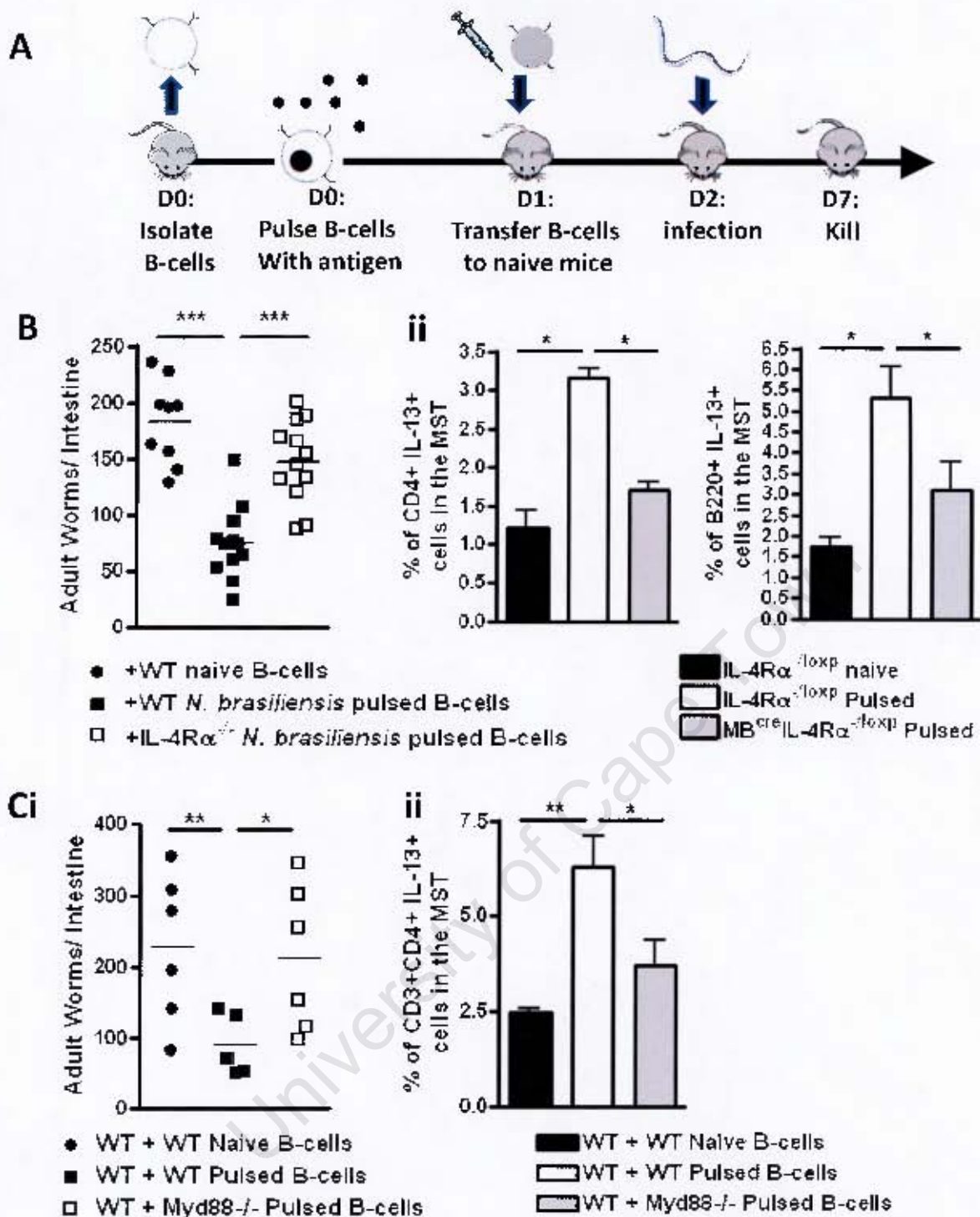


Figure 4.2: Pulsed B-cells provide IL-4R α and Myd88 dependant protective immunity to *N. brasiliensis* infection. Naive B-cells were isolated from MB^{cre}IL-4R α^{-lox} and IL-4R α^{-lox} mice and pulsed with antigen for 16 hours before being transferred into naive wild-type mice (A). After these were infected with *N. brasiliensis*, intestinal worm burdens were quantified at day 5 post infection (Bi). Protection was associated with MST T-cell and B-cell IL-13 production (Bii). Naive B-cells were isolated from Myd88^{-/-} and wild type mice and pulsed with antigen for 16 hours before being transferred into naive wild-type mice (A). Intestinal worm burdens were quantified at day 5 post infection (Ci). Protection was associated with MST T-cell IL-13 production (Cii). Data represents 2 experiments. n = 4-6 mice per group.

4.2.2 Rapid B-cell protection is mediated by MHCII presentation

In order to investigate any role played by MHCII antigen presentation we isolated MHCII^{-/-} or wild type B-cells which were pulsed with *N. brasiliensis* antigen overnight and transferred into naïve wild type mice before the mice were infected with *N. brasiliensis* (Figure 4.2A). Antigen experienced B-cells provided MHCII dependant reduced worm burdens at day 5 post infection (Figure 4.3Bi) associated with endogenous MST B-cell and T-cell IL-13 production (Figure 4.3Bii). In order to confirm that pulsed B cells were antigen specific, the protection against *N. brasiliensis* by *N. brasiliensis* antigen pulsed B cells was compared to unpulsed, *Leishmania major* antigen or Ovalbumin pulsed B cells. Only *N. brasiliensis* antigen pulsed B cells showed reduced worm burdens (Figure 4.3A). This implies an important role for B-cell MHCII antigen presentation in the rapid B-cell response to *N. brasiliensis*.

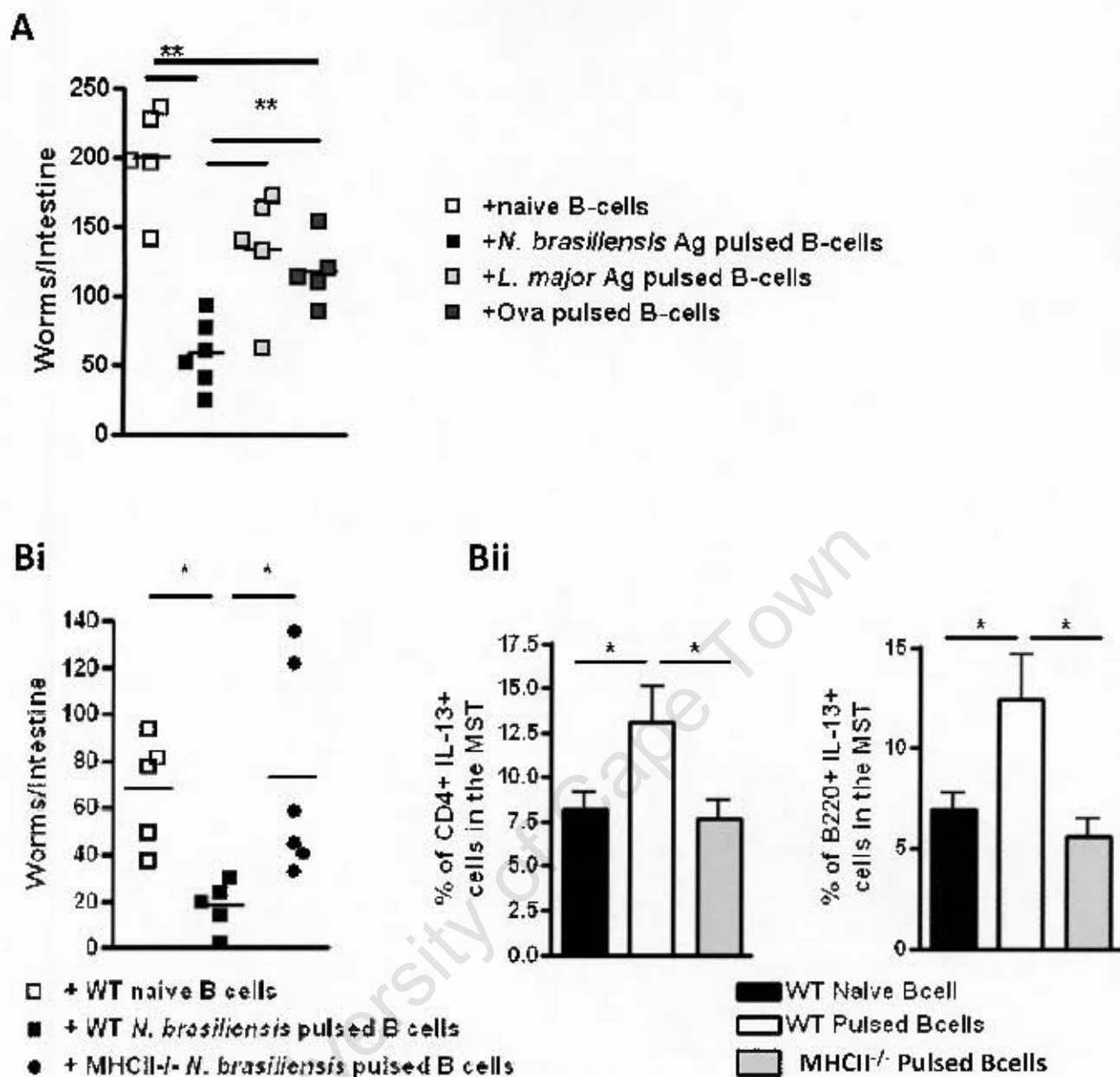


Figure 4.3: Pulsed B-cells provide antigen specific MHCII dependant protective immunity to *N. brasiliensis* infection. Naive wild-type B-cells were isolated and pulsed with antigen for 16 hours before being transferred into naive wild-type mice (**Fig4.2A**). These were then infected with *N. brasiliensis* and intestinal worm burdens were quantified at day 5 post infection (**A**). Naive B-cells were isolated from MHCII^{-/-} and wild type mice and pulsed with antigen for 16 hours before being transferred into naive wild-type mice (**Fig4.2A**). After these were infected with *N. brasiliensis*, intestinal worm burdens were quantified at day 5 post infection (**Bi**). Protection was associated with MST T-cell and B-cell IL-13 production (**Bii**). Data represents 2 experiments, n = 4-6 mice per group.

4.2.3 Rapid B-cell protection is related to cognate interactions with T-cells

We isolated B-cells from naive mice and exposed them overnight to *N. brasiliensis* antigen. We then washed the B-cells and co-cultured them overnight with isolated naïve wild type T-cells, before re-isolation of the T-cells, which were adoptively transferred into naïve wild type mice. These mice were then infected with *N. brasiliensis* (Figure 4.4A) T-cells co-cultured with antigen experienced B-cells mediated reduced worm burdens in the host associated with B-cell IL-4R α and/or Myd88 expression (Figure 4.4B and C). Mice that received T-cells co-cultured with antigen experienced IL-4R α ^{-/-} or Myd88^{-/-} B-cells showed similar worm burdens to mice that received T-cells co-cultured with naive B-cells.

Taken together these findings indicate that B-cells can be specifically activated rapidly in vivo or in-vitro by *N. brasiliensis* antigen, and this gives rise to rapid B-cell IL-4R α dependent protection which is in part at least due to TLR mediated antigen processing. These rapidly activated IL-4R α and Myd88 competent B-cells are able to initiate a T-cell mediated response which provides protection to *N. brasiliensis*.

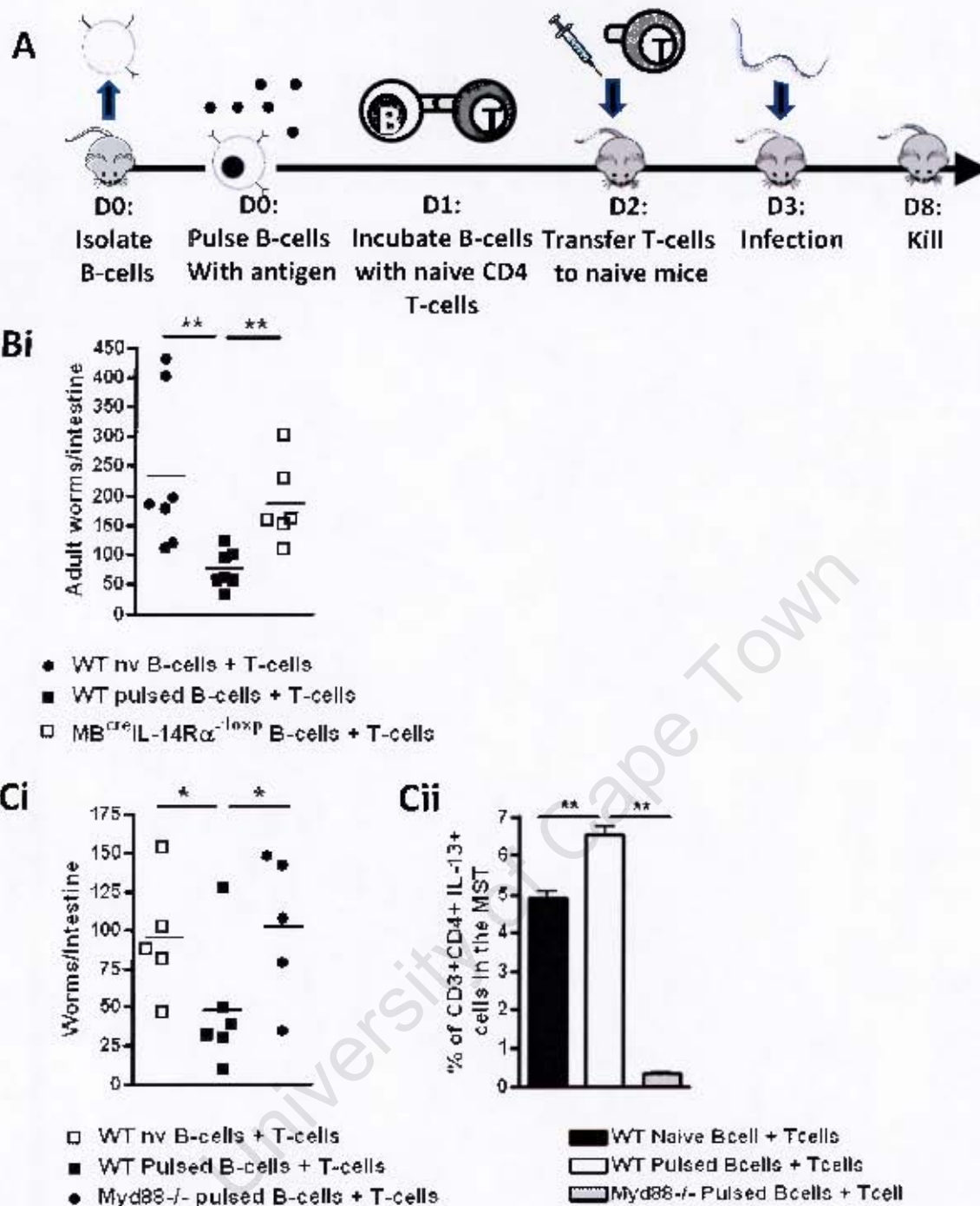


Figure 4.4: Pulsed IL-4R α and Myd88 competent B-cells give rise to T-cell dependant protective immunity to *N. brasiliensis* infection. Naive B-cells were isolated from MB^{cre}IL-4R α ^{-lox} and IL-4R α ^{-lox} mice and pulsed with antigen for 16 hours before co-culture with naive wild-type T-cells for 16 hours (A). The T-cells are then re-isolated and transferred into naive mice which are infected with *N. brasiliensis* the next day. Intestinal worm burdens were quantified at day 5 post infection (Bi). Naive B-cells were isolated from Myd88^{-/-} and wild type mice and pulsed with antigen for 16 hours before co-culture with naive wild-type T-cells for 16 hours (A). The T-cells are then re-isolated and transferred into naive mice. Intestinal worm burdens were quantified at day 5 post infection (Ci). Protection was associated with MST T-cell IL-13 production (Cii). Data represents 2

4.3 Discussion

We demonstrate in this study that B-cells can be rapidly activated by being briefly exposed to a *N. brasiliensis* infection. Transfer of B-cells isolated from mice 1 day post infection significantly reduced host worm burdens in an IL-4R α dependent manner. B-cells can recognise and respond to antigen via less stringent mechanisms than the BCR, such as direct peptide loading and Toll like receptors [108]. Antigen binding by TLRs in particular can play a key role in B-cell activation [61]. TLR signalling can increase both BCR dependent antigen presentation and cytokine production [60], while MyD88 dependent B-cell cytokine production has been demonstrated [26]. The novel findings we show here indicate that B-cells are rapidly activated by *N. brasiliensis* antigen and this is related to Myd88^{-/-} competency. The induction of this activation is so rapid it is likely to preclude BCR dependent antigen presentation and BCR dependent clonal expansion. Importantly we present data indicating rapid poly-functional antigen processing by B-cells, associated with Myd88 competency, was able to drive MHCII mediated protective immunity to *N. brasiliensis*.

Moreover, B-cell TLR activation has been shown to play a key role in B-cell cognate activation of T-cell responses, in *Helicobacter* for instance [110]. This confirms our finding that B-cell activation of a protective T-cell response to *N. brasiliensis* may be Myd88 as well as IL-4R α dependent. Our demonstration of *N. brasiliensis* pulsed Myd88^{-/-} B cells conferring a reduced protective ability in T cells against *N. brasiliensis* may indicate that TLR antigen processing by B cells underlies the phenotypes we report in the rest of this study.

5. Concluding remarks

5.1 Summary of results

This study demonstrates a significant role for IL-4R α expressing B-cells in controlling a *N. brasiliensis* infection. Although the presence of B-cells is not essential to the resolution of a primary or secondary *N. brasiliensis* infection, we have shown their ability to respond to the pathogen has important consequences for disease resolution. The results of our experiments indicate that they contribute to the immune response to *N. brasiliensis* by antibody independent mechanisms. However immunity was independent of production of antigen specific antibodies. We give evidence that B-cells are activated upon contact with *N. brasiliensis* antigen to a protective phenotype which activates CD4⁺ T-cells by cognate interactions, possibly via MHCII dependent antigen presentation. These functions are characteristic mechanisms of follicular mature B-cells. We show that IL-4R α expression is associated with a larger spleen follicular mature B-cell population. And we show that antigen experienced B-cells give rise to increased protective IL-13 production by a hosts endogenous lymphocytes. These B-cells functions lead to increased resistance to *N. brasiliensis* infection.

There is also evidence that B-cell activation by *N. brasiliensis* antigen is potentially very rapid. Rapid activation of B-cells was linked to Myd88 competency, indicating TLR signalling and activation. We see that IL-4R α or Myd88 competent B-cells are able to confer resistance to a *N. brasiliensis* infection after being exposed to *N. brasiliensis* antigen for a 24 hour period in vivo or 16 hours in-vitro. We give evidence that this rapid response is mediated by pathogen specific antigen presentation via MHCII to CD4⁺ T-cells. Again we show that antigen experienced B-cells give rise to increased protective IL-13 production by a hosts endogenous lymphocytes.

5.2 Future Work

However, several questions are raised by the *in vivo* protective effect driven by T-cells primed for only 24 hours by *in vitro* co-culture with B effector cells prior to transfer. Based on known priming mechanisms, this result is puzzling and it has not been achieved previously, with DCs, let alone B-cells. A 24 hour period is too short to allow differentiation of naive T-cells into effectors or clonal expansion of the T-cells. However we see that 24 hours is long enough for a measure of T-cell priming which confers a selective advantage on the T-cells when they are later transferred. After this there is a 5 day period for these cells to behave differently to cells which do not get this advantage and this contributes to resistance to the pathogen. We will have to confirm this by testing the quality of the T-cell response by taking the B cell-experienced T cells, labelling them CFSE and analysing their cell-division and IL-13 production after a 3 to 5 day *in-vitro* culture.

This leads to a question of mechanism: is this effect a result of B-cells activating the T-cell response by cognate delivery of cytokines or antigen presentation or both. Once again, the quality of the B-cell effector functions could be tested by *in-vitro* culture. B-cell proliferation could be analysed by CFSE, and their cytokine production measured by ELISA of the cell supernatant after a 24 hour culture. It is important to note that the cytokine that causes T_H2 differentiation is IL-4 as IL-13 has little stimulating effect on T-cells. Also it would be interesting to see if T-cells co-cultured with *N. brasiliensis* primed IL-4^{-/-} B-cells could provide a protective effect. While B-cell IL-13 should not have an effect on T-cells it does appear to play a part in the overall role of B-cells. This could be confirmed using mixed bone marrow chimeras using μ MT and IL-13^{-/-} bone marrow such that the resulting mice have B cells that specifically lack IL-13 production. The importance of MHCII has been demonstrated, as MHCII expression is higher on IL-4R α competent B-cells when compared

to B specific IL-4R α knockout cells and by transfer of MHCII^{-/-} B-cells. Again mixed bone marrow chimeras with MHCII^{-/-} and μ MT so the resulting mice would have B cells that specifically lack MHCII would confirm this. This would answer the question about whether B-cell IL-13 production/MHCII expression is required to induce resistance to *N. brasiliensis* or whether expression of the gene within the donor is required to generate B-cells with a protective phenotype.

It is also possible that there could be non-conventional lymphocytes in the sorted CD3+CD4+ T-cells. NK T-cells can be CD3+CD4+ and can rapidly respond to an infection by production of cytokines [112]. As mentioned before, they have less stringent antigen specific activation requirements than conventional T-cells [6], and they can be activated by CD1d⁺ APCs in conjunction with hydrophobic ligands like glycol-lipids and glycol-proteins [113]. NK T-cells have a substantial ability to produce IL-4 which indicates that they can play a significant (although not essential) role in a T_H2 response, and NKT-cell activation *in vivo* has been shown to promote T_H2-associated immunity [114, 115]. One CD1d ligand in particular, α -galactosylceramide, has been shown to be a potent activator of NK T-cells to produce IL-4 [116], which induces a T_H2 response by conventional T-lymphocytes [117] and other bystander cells including B-cells [118], and DC's [119]. NK T-cells can also be activated by B-cells, and CD1⁺ B-cells have been shown to interact with NK T-cells [120]. In order to analyse any role played by NK T-cells, we would have to exclude them from the sorted CD3+CD4+ T-cell preparation using another defining marker such as NK1.1. Alternatively the activated B-cells could be co-cultured with purified NK T-cells to analyse their protective effect. Finally the role of B-cell CD1d expression can be analysed by FACs and/or B-cell-specific CD1d deletion.

Our finding that B-cells are rapidly primed by *N. brasiliensis* antigen into cells that provide resistance to *N. brasiliensis* in a BCR independent manner also advances several questions about the mechanism that makes this possible. B-cell differentiation is usually believed to be a longer process due to the BCR mediated clonal expansion which accompanies a protective B-effector response. But rapid activation of B-cells has been demonstrated to be possible [60, 61, 63]. Our hypothesis is that the rapid B-cell activation seen in this case is TLR mediated, a generally more rapid route of immune cell stimulation. It has previously been established that both MHCII antigen presentation and TLR signalling play an important role in the repertoire of B-cell effector functions [49, 65]. But a direct link between TLR signalling and antigen presentation in B-cells has not yet been demonstrated. However Myd88 has been shown to enhance naive B-cell development to FM cells [5], as well as being linked to pulmonary T_H2 responses [22]. While our evidence indicates that Myd88 mediated TLR signalling is a probable mechanism which explains the rapid antigen-induced B-cell activation into effector cells, and these are able to prime protective T-cells, this requires significant further investigation. We will generate Myd88 or MHCII specific deficient B-cell mixed bone marrow chimera mice as previously described to confirm our finding that Myd88 competent B-cells act in an MHCII dependent manner. We will also carry out experiments where we transfer B-cells pulsed with antigen in different concentrations in order to see if the B-cells are stimulated by the antigen in a concentration dependant manner.

An interesting point to note it that *N. brasiliensis* antigen is not characterised, and neither are its properties and interactions with cells which are likely to be complex and multi-factorial. It is highly possible that the effect we see is due to the non-Ag specific uptake of some component of the *N. brasiliensis* antigen by B-cells. Also antigen preparations will most likely contain breakdown products (peptides) that might load directly onto MHC II. If the

effect we see is due to direct peptide loading then this result does not indicate any direct physiological role of B cells in priming T cell responses. However our data showing MyD88 dependent protection suggests antigen is being internalised and processed by the cells via TLRs and this contributes to the phenotype. The role of direct peptide loading if any will be examined in the future by analysis of the protective capabilities of B-cells which have been fixed in 0.025% glutamate prior to being pulsed with antigen; these will be unable to internalise antigen or produce cytokines. The antigen preparation could even contain α -galactosylceramide which could be rapidly taken up by the B-cells and presented to NK T-cells to induce IL-4 production and a T_H2 response. The makeup of NES could be checked by high-performance liquid chromatography to see if it contains any molecules of a similar molecular weight to α -galactosylceramide.

Thus while some of the conclusions we draw on the data presented in this study do contradict current scientific opinion, the results are reproducible and we hope to confirm them in future using the in-vitro techniques and chimeras discussed, and eventually fully explain the polyclonal activation of B cells and their effect on T-cells in response to an *N. brasiliensis* infection and re-infection.

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Appendix 1

Composition of complete media (Iscove's Modified Dulbecco's Medium (IMDM)):

1 tube IMDM (Gibco)

750 ml ddH₂O

81.7 ml NaHCO₃ (37g / L)

2 ml Penicillin/streptomycin (500X)

Adjust the pH to 7.2 – 7.4

Make up to 1 L with ddH₂O and filter sterilize

Composition of FACS Buffer:

0.1% BSA (Roche)

0.05% NaN₃ (Merck)

Made up in 1X PBS

University of Cape Town